The role of *Chlamydia pneumoniae* infection and stress responses in vascular remodelling

By

Justin François Deniset

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

2013

Department of Physiology

Faculty of Medicine

University of Manitoba

Winnipeg

ABSTRACT

Strong clinical and experimental evidence has suggested the involvement of *Chlamydia pneumoniae* (*C. pneumoniae*) in the development of atherosclerosis. However, the direct role of *C. pneumoniae* infection in vascular remodelling processes in the absence of a host immune response remains undetermined.

To study the direct effect of this pathogen within the arterial wall, we developed a novel *ex vivo* porcine coronary artery model that supported bacterial growth for up to two weeks in culture. Employing this approach, we demonstrated that *C. pneumoniae* infection could alter vascular functions parameters, including endothelial-dependent relaxation responses. This impairment was associated with a decrease in eNOS expression and increased oxidative stress, changes that are also noted in atherosclerotic plaques. We further demonstrated that *C. pneumoniae* infection initiates medial thickening via vascular smooth muscle cell (VSMC) proliferation. This proliferative response was associated with an increase in expression of endogenous heat shock protein 60 (Hsp60) and alterations in nuclear protein import machinery. Additionally, *C. pneumoniae* infection and Hsp60 overexpression in primary VSMCs resulted in alteration in nuclear protein import parameters leading to the cell proliferation. Using a rabbit atherosclerotic model, we demonstrated that Hsp60 is induced during atherosclerotic lesion growth and correlated with both the proliferative status and the expression of protein involved in nuclear protein import within the atherosclerotic vessel.

In summary, our work has demonstrated the feasibility of studying the molecular mechanisms of infection-induced atherosclerosis using an *ex vivo* coronary culture system. Importantly, our data has provided the first direct evidence that an active *C. pneumoniae* infection alone, without contributions from a host immune system, can mediate endothelial dysfunction and stimulate arterial thickening, two key remodelling processes present during atherosclerotic progression. Our findings further suggest the involvement of Hsp60 as a key contributor in growth-based pathologies like *C. pneumoniae*-mediated atherosclerosis possibility through modulation of nuclear protein import.

ACKNOWLEDGEMENTS

I would like to recognize numerous individuals for their contributions to my graduate training. First, I would like to acknowledge the Pierce lab members, past and present, for creating such a knowledgeable, collaborative, and supportive working environment, which helped me navigate through the rigors of my graduate program. In particular, I would like to thank Dr. Paul Cheung, Thomas Hedley, Dr. Marketa Hlaváčková, and Alex Austria for their technical expertise, hard work and contributions to my research project. I also owe a great deal of gratitude to Dr. Elena Dibrov. Her knowledge and guidance were integral to the design, evolution and overall success of my research project. A special thanks to Andrea, Alex, Andrew, Chantal, Dave, Elena, Marketa, Melanie, Mirna, Renée, Reinhold, Richelle, Riya, Thane, and Thomas for all the great memories during my time in the lab. Dave, thank you for being such a great friend both in and out of the lab.

I would also like to thank my MSc. and Ph.D. graduate committee members, Dr. Ed Kroeger, Dr. Michael Czubryt, Dr. Elissavet Kardami, and Dr. Pavel Dibrov who provided valuable guidance and support throughout my graduate program. Thank you to Debbie Saurette, Karen Shearing, Gail McIndless and Judy Olfert for their outstanding administrative help.

I am grateful for scholarships from the following agencies: Canadian Institutes for Health Research (Master's and doctoral Canada Graduate Scholarship), Manitoba Health

Research Council (Graduate Studentship), St. Boniface Hospital and Research Foundation (Pawan K. Singal Graduate Scholarship in Cardiovascular Sciences), University of Manitoba (UofM) (Top-up Awards for Tri-Council Award Recipients). Thank you to the Canadian Institutes for Health Research and the St. Boniface Hospital and Research Foundation for supporting the research project.

Of course, none of this would have been possible without the tireless work of my supervisor and mentor, Dr. Grant Pierce. His advice, patience, insightful criticisms and encouragement aided the writing of this thesis and in my development as a researcher. He taught me valuable lessons of work ethic, integrity, perseverance, and team spirit. He showed me that research could be fun and exciting and that the nice guys can make it to the top. For all of these things and for the overall impact he has had on my life, I am forever grateful. Thank you!

Last, but certainly not least, this work could not have been achieved without the love and encouragement of my friends and family. Thank you to my brothers Shea, Paul, and Steph for helping to nurture my competitive side, which has and will continue to benefit me in my research career. Thank you to my parents Jean-Paul and Deanna for always supporting me and providing me the liberty to always follow my interests. Finally, thank you to my wife, Sylvie, for all of her love, patience, understanding and support. Merci!

À Sylvie,

Je t’aime infiniment

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF COPYRIGHTED MATERIAL

The following items were reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila* pneumoniae infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier:

LIST OF ABBREVIATIONS

Acyl-coenzyme A: cholesterol acyltransferase 1	ACAT-1
ATPase homolog-1	Aha1
Bone morphogenic protein	BMP
Ca^{2+}/calmodulin	CaM
CaM kinase II	CaMKII
Cardiovascular disease	CVD
Casein kinase II	CK2
Chlamydial protease-like activity factor	CPAF
Chlamydia pneumoniae	*C. pneumoniae*
Coronary artery disease	CAD
Cytomegalovirus	CMV
Dulbecco's modified eagle medium	DMEM
Elementary body	EB
Endothelial cell	EC
Endothelial nitric oxide synthase	eNOS
Extracellular matrix	ECM
Extracellular signal-regulated kinase	ERK
Fetal bovine serum	FBS
Glucose regulated protein 94	Grp94
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Glycogen synthase kinase 3β	GSK3β
Granulocyte-macrophage colony stimulating factor	GM-CSF

Heat shock factor	Hsf
Heat shock protein	Hsp
Helicobacter pylori	*H. pylori*
Helper T lymphocytes	Th
Herpes simplex virus	HSV
Hsp70-Hsp90 organizing protein	Hop
Human immunodeficiency virus	HIV
Human umbilical vascular endothelial cell	HUVEC
Hypoxia inducible transcription factor	HIF
Immunoglobulin G	IgG
Immunoglobulin M	IgM
Inclusion body	IB
Inducible nitric oxide synthase	iNOS
Infectious forming units	IFU
Inhibitor of κB	IκB
Inositol triphosphate receptor	IP3R
Interferon gamma	IFN-γ
Interleukin	IL
Intracellular cell adhesion molecule-1	ICAM-1
Kruppel-like factor	KLF
Lipopolysaccharide	LPS
Low-density lipoprotein	LDL
Major histocompatibility complex	MHC

Matrix metalloprotease	MMP
Mitogen-activated protein kinase	MAPK
Monocyte chemotactic protein-1	MCP-1
Myocardin-related transcription factor	MRTF
Myosin heavy chain	MHC
Myosin light chain	MLC
Multiplicity of infection	MOI
Nitric oxide	NO
Nuclear export signal	NES
Nuclear factor kappa-light-chain enhancer of activated B cells	NFκB
Nuclear localization signal	NLS
Nuclear pore complex	NPC
Nuclear protein import	NPI
Nuclear transport factor-2	NTF2
Nucleoporin	Nup
Nucleotide-binding oligomerization domain	NOD
Oxidized low-density lipoprotein	oxLDL
Pattern recognition receptor	PRR
Peroxisome proliferator-activated receptor alpha	PPARα
Peroxisome proliferator-activated receptor gamma	PPARγ
Phosphate-buffered saline	PBS
Phosphoinositide 3	PI3
Plasminogen-activating inhibitor-1	PAI-1

Platelet derived growth factor	PDGF
Platelet endothelial cell adhesion molecule-1	PECAM-1
Polymerase chain reaction	PCR
Proliferating cell nuclear antigen	PCNA
Reactive oxygen species	ROS
Regulatory T lymphocytes	Treg
Reticulate body	RB
Serum response factor	SRF
Signal transducer and activator of transcription	STAT
Sirtuin-1	SIRT1
Sodium nitroprusside	SNP
Sucrose-phosphate-glutamate medium	SPG
Terminal dUTP nick-end labelling	TUNEL
Tissue factor	TF
Toll-like receptor	TLR
Transforming growth factor beta	TGF-β
Translocated actin recruiting phosphoprotein	TARP
Tumor necrosis factor alpha	TNF-α
Tumor necrosis factor receptor associated protein-1	Trap1
Type II secretion	T2S
Type III secretion	T3S
Vascular cell adhesion molecule-1	VCAM-1
Vascular smooth muscle cell	VSMC

CHAPTER I: LITERATURE REVIEW

1. CARDIOVASCULAR DISEASE

Cardiovascular diseases are the leading cause of morbidity and mortality worldwide (1). Cardiovascular diseases can be defined as all diseases or injury that affect the cardiovascular system, including the heart and blood vessels which ensure the proper pumping and transport of the blood around the body. Prominent types of cardiovascular diseases include ischaemic heart disease, cerebrovascular disease, hypertensive heart disease, rheumatic heart disease, and cardiomyopathies.

Ischaemic heart disease is a condition that affects the blood vessels supplying blood to the heart. It has important clinical consequences, accounting for nearly half of all cardiovascular related deaths globally (1). The condition develops as a result of a build up of lipid rich plaques within the vessel wall, termed atherosclerosis. The growing plaque impedes blood flow leading to ischemic events that impair the function of the heart. As the atherosclerosis progresses, the plaques can become unstable and rupture, resulting in blood clot formation that will completely block the vessel. This event, known as a myocardial infarction, causes death to cardiac tissue leading to significant remodelling of the muscle wall and impairment in heart function. As the remodelling ensues it will eventually lead to congestive heart failure, a clinical syndrome characterized by the inability of the heart to pump enough blood to meet the demands of the organ system due to structural and/or functional abnormalities of the heart (2).

The causes responsible for the development of cardiovascular disease are numerous and can be classified as non-modifiable and modifiable risk factors. Non-modifiable risk factors such as age, gender and genetic background, all have a role to play in numerous aspects of cardiovascular disease. However, the development of cardiovascular disease depends more predominantly on the presence of modifiable risk factors such as hypertension, smoking, diabetes, physical inactivity, obesity and hypercholesterolemia (3). Therefore, strategies aimed to prevent and manage these risk factors will have an important impact on the global burden of disease.

2. PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disease leading to stiffening and blockage of the arteries. The development of the atherosclerotic plaque can be described as a response to injury mechanism (4), which evolves in many difference stages (Figure 1)(5). Initial injury to the endothelial layer caused by stress stimuli (eg. hypercholesterolemia, smoking), leads to increased infiltration of low density lipoprotein (LDL) molecules into the subendothelial space (5,6). The LDL molecules can subsequently be oxidatively modified and remain trapped with the vessel wall (6). The activated endothelium also recruits immune cells (eg.monocytes) via an increase in cell surface adhesion molecules and enhanced chemokine release (7,8). The recruited monocytes have the ability to participate in efflux of cholesterol out of the vessel, however, excessive uptake of modified LDL causes a dysfunctional monocyte/macrophage phenotype termed a foam cell (5,7). These foam cells make up the lipid core of the plaque and promote a pro-inflammatory environment (8).

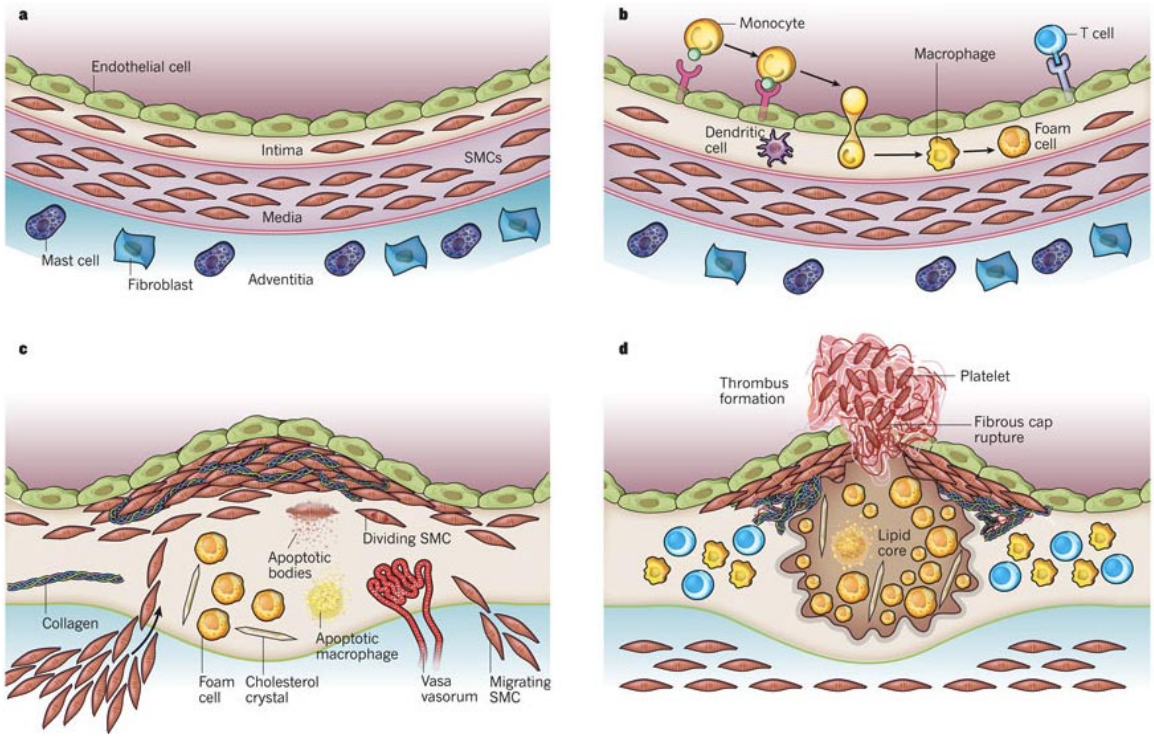

Figure 1. Stages in the development of atherosclerotic lesions
The normal muscular artery and the cell changes that occur during disease progression to thrombosis are shown. **a**, The normal artery contains three layers. The inner layer, the tunica intima, is lined by a monolayer of endothelial cells that is in contact with blood overlying a basement membrane. In contrast to many animal species used for atherosclerosis experiments, the human intima contains resident smooth muscle cells (SMCs). The middle layer, or tunica media, contains SMCs embedded in a complex extracellular matrix. Arteries affected by obstructive atherosclerosis generally have the structure of muscular arteries. The arteries often studied in experimental atherosclerosis are elastic arteries, which have clearly demarcated laminae in the tunica media, where layers of elastin lie between strata of SMCs. The adventitia, the outer layer of arteries, contains mast cells, nerve endings and microvessels. **b**, The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes (the most numerous of the leukocytes recruited) into macrophages, and their uptake of lipid, yielding foam cells. **c**, Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of resident intimal SMCs and media-derived SMCs, and the heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. Plaque macrophages and SMCs can die in advancing lesions, some by apoptosis. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid or necrotic core. Advancing plaques also contain cholesterol crystals and microvessels. **d**, Thrombosis, the ultimate complication of atherosclerosis, often complicates a physical disruption of the atherosclerotic plaque. Shown is a fracture of the plaque's fibrous cap, which has enabled blood coagulation components to come into contact with tissue factors in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow. Reprinted by permission from Macmillan Publishers Ltd: Nature, P Libby, PM Ridker, and GK Hansson. Progress and challenges in translating the biology of atherosclerosis (5), © 2011. http://www.nature.com/doi:10.1038/nature10146

As the plaque grows, vascular smooth muscle cells (VSMCs) proliferate and migrate to the luminal side of the plaque to form a fibrous cap (5,8). This results from newly secreted extracellular matrix (ECM) proteins (eg. collagen), which will help to stabilize the plaque (5,8). The inflammatory response will ensue with further recruitment of immune cell and formation of a necrotic core due to defective removal of apoptotic macrophages/foam cells (9). Both macrophages and the necrotic core can contribute to the breakdown of the fibrous cap by promoting VSMC apoptosis, release of proteolytic enzymes, thus leading to plaque rupture and thrombosis (7,10).

2.1. Endothelial dysfunction and atherosclerosis

The vascular endothelium represents an active paracrine, endocrine and autocrine organ responsible for regulating vascular tone and maintaining vascular homeostasis (11). Endothelial dysfunction is a pathophysiological state characterized by an impaired endothelial-dependent relaxation resulting primarily from a reduction in nitric oxide (NO) bioavailability (12) . This condition coincides with an activated endothelial state, described by an increase in cell surface adhesion molecules and secretion of pro-inflammatory chemokines and cytokines (13). Endothelial dysfunction is associated with all major cardiovascular risk factors including hypertension, hyperlipidemia, diabetes and smoking (14-16). Futhermore, it can be used as a predictive marker of future atherosclerotic disease progression (17-19) .

2.1.1. Regulation of NO synthesis and catabolism in the vasculature

NO plays an important homeostatic role within the vasculature, mediating vascular tone, inhibiting vascular smooth muscle proliferation, decreasing endothelial cell permeability, preventing immune cell recruitment and adhesion, and inhibiting platelet aggregation (20-30). NO production within the vessel wall is primarily mediated by endothlelial nitric oxide synthase (eNOS), a Ca^{2+}/calmodulin (CaM)-dependent enzyme (31). NO production is influenced by the level of eNOS expression and more acutely by regulation of its activity (32-34). Several different mechanisms are involved in eNOS activity: (1) protein-protein interactions of eNOS with the eNOS-binding proteins such as $Ca^{2+/}$CaM, heat-shock protein-90, caveolin-1, platelet endothelial cell adhesion molecule-1 (PECAM-1), (2) post-translational regulation by phosphorylation and acylation, (3) subcellular localization at the plasma membrane with caveolae, Golgi, mitochondria, and intercellular junctions and (4) availability of cofactors and substrates (33,35-40).

Shear stress and humoral factors such as vascular endothelial growth factor, bradykinin and estrogen stimulate NO production via eNOS (41-48). Areas of the vasculature with disturbed blood flow and lower shear stress near branches and bifurcations are more susceptible to endothelial dysfunction and subsequent plaque formation (49). Pro-atherogenic stimuli such as oxidized LDL (oxLDL) or tumor necrosis factor alpha (TNF-α) suppress eNOS expression (50,51). The expression of eNOS is depressed in advanced human atherosclerotic plaques but not in early atherosclerotic vessels (52-54). In atherosclerotic animal models, the level of eNOS expression has been variable despite the presence of endothelial dysfunction (55-57). The use of

pharmacological inhibitors of eNOS or eNOS gene deletion in atherosclerotic animal models, accelerates atherosclerotic progression (58-61). This would suggest that the regulation of eNOS activity may be the predominant mechanism of impaired NO production in early atherosclerosis.

An additional mechanism for endothelial dysfunction is mediated through NO catabolism. This is achieved with NO reacting with superoxide (O_2^-) leading to peroxynitrite ($ONOO^-$) formation. Peroxynitrite is produced in significant amounts in atherosclerotic lesions (33). This is likely a consequence of an increased superoxide production and due to the NO and superoxide reaction occurring several orders of magnitude faster than the removal of superoxide anions by superoxide dismutase. The main sources of vascular superoxide include NADPH oxidases (VSMC, endothelial cells, immune cells) and xanthine oxidase (endothelial cells) (62). NADPH activity and expression of the NADPH components (e.g. p22phox) are increased in human atherosclerotic plaques (63,64). Furthermore, eNOS itself can produce superoxide instead of NO, when found in an uncoupled form. The proposed mechanisms for this uncoupling process include tetrahydrobiopterin (BH4) and L-arginine deficiencies, an increase in the endogenous asymmetric dimethylarginine (ADMA), and oxidative stress (65). As such, eNOS has the ability to prevent and promote atherosclerotic development.

2.2. Vascular smooth muscle cells and atherosclerosis

VSMCs play an important physiological role as the mechanical component of the vessel that regulates vascular tone and blood pressure. In addition, VSMCs are known to

participate in healing processes within the vessel wall in response to injury. The versatility of this cell type is in part due to its plasticity and ability to de-differentiate into a synthetic state. However, dysregulation of this phenotypic conversion can contribute to a number of vascular pathologies such as atherosclerosis and restenosis (66,67).

2.2.1 VSMC phenotypes in the vasculature

Mature VSMCs are known to adopt two distinct phenotypes, contractile (differentiated) and synthetic (de-differentiated). Contractile VSMCs express a repertoire of SMC-specific proteins including contractile proteins (i.e. myosin heavy chain, smooth muscle actin,), ion channels (i.e. the L-type Ca^{2+} channel) and signaling molecules that allow these cells to regulate contractile function within the vessel wall (68). VSMC contraction is a Ca^{2+} -dependent process requiring the phosphorylation of myosin light chain (MLC) to facilitate the interaction between myosin and actin filaments necessary for cross-bridge cycling. The integration of vasoconstrictors (i.e. endothelin-1, angiotensin-II, thromboxane) and vasodilators (i.e. NO, prostaglandins) in the circulation or released from the neighbouring endothelium determines the level of SMC contraction by regulating intracellular Ca^{2+} levels, altering the plasma membrane potential, and directly modulating the level of MLC phosphorylation. Alternatively, under conditions of vascular injury, VSMCs undergo de-differentiation and adopt a synthetic phenotype. This state is characterized by a decrease in SMC markers, increased proliferation, migration, extracellular matrix production and matrix metalloprotease production and release. These characteristics confer the ability to engineer significant vascular remodelling.

SMC markers can be utilized to assess the level of differentiation (68). In addition, the relative expression of these markers correlates with the ability to proliferate in response to mitogens (69,70). Within a complex arterial environment, VSMCs display a broad phenotypic continuum between contractile and synthetic phenotypes. Interestingly, heterogeneity is even observed between adjacent VSMCs (71,72). This highlights the impact of the developmental origin of VSMCs on their responsiveness to environmental factors. In atherosclerotic plaques, heterogeneous patterns of SMC marker expression are also observed and are altered depending on the stage of atherosclerosis, the type of lesion and localization within the vessel wall (66). In particular, distinct differences between intimal and medial VSMCs are noted, with medial VSMCs displaying increased SMC marker expression compared to intimal VSMCs (73-75). These findings along with the cellular plasticity demonstrated in culture have lead to the belief that these intimal VSMCs are de-differentiated medial cells that have proliferated and migrated (66). Alternatively, work from Sata et al. had suggested hematopoietic progenitor cells contribute to VSMC populations in atherosclerotic lesions (76). However, multiple lineage tracing experiments in ApoE null mice have demonstrated very little contribution of hematopoietic cells to this population (77-79). The characteristics and expression/secretion profiles of synthetic VSMCs suggest that they may play both detrimental and protective roles during atherosclerotic development. Initial proliferation and migration of VSMCs contributes to important remodelling processes in the vessel wall (80). Alternatively, enhanced extracellular matrix production (eg. collagen) helps to stabilize advanced lesions and prevent them from rupturing (81).

Finally, matrix metalloproteases (MMPs), known to be secreted by synthetic VSMCs, can promote break down of the fibrous cap leading to atherothrombosis (82).

2.2.2. Regulation of VSMC phenotype and proliferation

The expression of SMC markers (eg. SM-α actin, SM-MHC, SM-22α) is mediated by the transcription factor serum response factor (SRF) in combination with co-factors myocardin and myocardin-related transcription factors (MRTF-A,MRTF-B)(83). These factors in complex bind CArG sequences within the promoter sequences of SMC genes to initiate transcription. A number of transcriptional regulators such as kruppel-like factor-4 and -5 (KLF4, KLF5) and ELK-1 are known to repress this process and may be involved in phenotype modulation during atherosclerosis. In an experimental atherosclerosis model, transcriptional repression of differentiation marker SM-22α is observed in VSMCs of the fibrous cap and intima of the atherosclerotic plaque (84). KLF4 expression is increased within lesions of ApoE null mice on a Western diet (85). In addition, microRNAs play a dynamic role in the regulation of SMC phenoconversion. miRs-143/145 and miR-1 play a central role in VSMC differentiation (67,86,87). miR-143/145 expression is down-regulated in the aorta of ApoE null mice (88). Alternatively, miR-221 has opposing effects, stimulating both de-differentiation and VSMC proliferation (67,89).

Regulation of the VSMC phenotype and its proliferative state is dependent on a number of environmental cues/factors such as humoral factors (ie. growth factors, contractile agonists), extracellular matrix (ECM) interactions, endothelial-SMC

interactions, mechanical force (ie. wall stress, hemodynamics), injury stimuli (ie. lipids, reactive oxygen species, inflammatory mediators), and cell-cell contact (66).

The best described biochemical pathways known to influence VSMC phenotype include platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-β) signaling. PDGF promotes a VSMC synthetic state and stimulates proliferation (90-93). In ApoE null mice, blockade of the PDGF-β receptor resulted in decreased lesion formation and VSMC content in the neointima (94). Alternatively, TGF-β and related bone morphogenetic protein (BMP) promoted VSMC differentiation by stimulating SMC marker gene transcription (95,96). In ApoE null mice, TGF-β receptor inhibition accelerated atherosclerotic plaque development (97).

A complex ECM, predominantly made of collagen (type I and III), elastin and proteoglycans, surrounds the medial VSMCs of the vessel wall (98). The ECM directly interacts and signals with the VSMCs via specific cell-surface receptors called integrins. Interactions with these components differentially influence their ability to respond to growth factors. In cell culture models, fibronectin has been demonstrated to promote a synthetic VSMC phenotype (99) , whereas laminin favours a contractile phenotype (100). Different forms and organization of the matrix components can also influence the proliferative response. For example, fibrillar and monomeric type I collagen inhibit and promote VSMC proliferation, respectively (101). Furthermore, SMCs cultured on a 3D collagen matrix are less proliferative compared to SMCs cultured on a 2D collagen matrix (102). In human atherosclerosis, collagen I, collagen III and fibronectin levels are

elevated in human atherosclerotic plaques (103). Furthermore, inducible fibronectin knockout in ApoE null mice caused a reduction in plaque size and fibrous cap formation (104). The production of ECM is primarily regulated by VSMCs in response to growth factors and cytokines (105). In addition, synthetic VSMCs are known to produce elevated levels of matrix metalloproteases (MMPs). MMPs are elevated in human atherosclerosis (106). MMPs can mediate remodelling of the ECM environment and can also liberate humoral factors attached to ECM components, both of which will influence VSMC phenotype.

The vessel wall is exposed to multiples varieties of physical and chemicals stresses that mediate VSMC proliferation. Regular shear stress exerted on the wall by the laminar flow of the blood inhibits VSMC proliferation via the release of endothelial derived mediators. NO has been postulated to be the key mediator of this response. However, recent data from Hergenreider and co-workers suggest that miRNAs may also be involved in a flow-mediated atheroprotective effect (107). Shear stress induced endothelial miR 143/145 cluster expression and their subsequent release via exosomes to regulate SMC phenotype (107). Furthermore, direct exposure of VSMCs to shear stress also inhibited proliferation in vitro (108). Alternatively, low shear stress using an EC-SMC co-culture system resulted in an increase in VSMC proliferation through endothelial release of PDGF-BB (109). During hypertension, an atherosclerotic risk factor, mechanical stretch is exerted on the vessel wall. Animal models of chronic hypertension demonstrate an increase in VSMC hyperplasia (110,111). Furthermore, mechanical stretch in vitro can induce VSMC proliferation (112,113). Finally, a number of chemical

stresses that are relevant to the atherosclerotic milieu, including oxidized LDL, hypoxia, reactive oxygen species (ROS) and heat shock proteins, all have mitogenic properties when in contact with VSMCs (114-119).

In summary, phenotypic switching leading to proliferation and migration of medial VSMCs is postulated as a key mechanism in plaque progression and fibrous cap formation. This process within the vascular wall is complex and will depend upon the integration of all of these environmental cues.

2.3. Involvement of the immune system and infection in atherosclerosis [1]

2.3.1. Innate immunity and atherosclerosis

The innate immune system, comprised of a network of specialized cells, is responsible for mediating the initial inflammatory actions in response to tissue damage and foreign particles within the body. Monocyte-derived macrophages were the first immune cells to be identified within atherosclerotic lesions (120) and represent a major component of the innate immune network during atherosclerotic development. Depletion of monocytes from the circulation using clodronate resulted in reduced plaque formation in rabbits (121) . Macrophages are involved in disease progression through their involvement in lipid metabolism and initial immune mediation (122). Pattern-recognition receptors, which include scavenger receptors (SRs) and toll-like receptors (TLRs) expressed on the cell surface are important components for both of these functions. SRs bind and mediate the uptake of modified LDL found within the vessel wall. During normal lipid loading, macrophages mediate reverse cholesterol transport by

[1] CMC Bassett et al ,*Pathophysiology of Coronary Artery Disease.* Functional Foods and Cardiovascular Disease. 1-29, ©2012, Reprinted,with permission from CRC Press.

expressing proteins involved in cholesterol efflux (123). However, under conditions of constant lipid loading this mechanism is overwhelmed and excessive lipid storing leads to foam cell formation and subsequent production and release of pro-inflammatory molecules such as cytokines and reactive oxygen species (124). TLRs recognize a broad range of targets that not only include pathogen-derived antigens such as lipopolysaccharide (LPS) but also endogenous molecules such as heat shock proteins (Hsps). Upon binding to TLRs, these molecules will stimulate downstream signaling cascades leading to activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) that are involved in the production of inflammatory mediators. TLRs appear to play an important role in atherosclerosis. Their expression is not limited to macrophages within the vessel wall and multiple members of its family are upregulated during human atherosclerosis (125). Furthermore, a deficiency in TLR2 and/or TLR4 signalling reduces atherosclerotic development in atherogenic-prone mice (126,127).

Other members of the innate immune system have also been localized within atherosclerotic lesions. Mast cells have been found in the adventitia and shoulder regions of atherosclerotic plaque (128). Once activated, mast cells may contribute to atherothrombosis through the release of pro-inflammatory cytokines and proteolytic enzymes. Atherogenic-prone mice deficient in mast cells display decreased atherosclerosis with a reduction in leukocyte population, apoptosis and necrosis within the plaque (129). In addition, there was an increase in fibrous cap formation and collagen production (129). These effects were dependent upon interferon (IFN)- γ and interleukin (IL)-6 release. Mast cells are also involved in lipid metabolism (130) and cholesterol

efflux (131). Dendritic cells, which can be localized in the intimal region of normal vessels, increase in number upon lesion progression (132) and their recruitment appears to be VCAM-1 dependent (133). They have the potential to prime naïve T lymphocytes through antigen presentation and thereby provide a link between the innate and adaptive immune system (132,134).

2.3.2. Adaptive immunity and atherosclerosis

The adaptive immune system offers the body a more refined network to fight pathogens and foreign molecules. It differs from the innate immune system because it is initially slower, is dependent on past exposure and provides much more specificity. T lymphocytes, the key mediators of the adaptive response, are recruited to the lesion during early and late atherosclerotic plaque development where they undergo activation (135). Additionally, the presence of oxLDL- (136), HSP60- (137) and *Chlamydia pneumoniae* (*C. pneumoniae*)-specific (138) T cells provide evidence of clonal expansion within the vessel wall. There exist two major subtypes of helper T (Th) cells involved in plaque development, Th1 and Th2. Th1 cells are the predominant form within the plaque (139) and promote atherogenesis by secreting pro-inflammatory cytokines, inducing macrophage activation and driving cell mediated responses (140). Th2 cells appear more anti-atherogenic by secreting cytokines such as IL-4,-5 and -10 that drive antibody production, and inhibit Th1 responses. Regulatory T (Treg) lymphocytes, mediators of immune tolerance within the body, play an important role in controlling Th1/Th2 switching (141). Induction of Treg cells in atherosclerotic-prone mice led to inhibition of Th1 responses and reduced atherosclerotic development. These changes were

accompanied by decreased IFN- γ levels and increased IL-10 levels (142). B cells, effector cells of the humoral immune response, produce antibodies that recognize specific antigens. Recent findings have suggested that natural antibodies against oxLDL (oxLDL IgM) may play a protective role in preventing atherosclerosis (143,144). These natural antibodies act by binding oxLDL molecules to prevent their uptake by macrophages, and block the subsequent inflammatory response and potential presentation of self-antigen to T lymphocytes (145).

2.3.3. Infectious disease and atherosclerosis

The important involvement of a host immune response in the development of atherosclerosis has prompted interest in infectious agents as potential triggers for the disease. Initial work in this field was pioneered by Fabricant et al. in the late 1970's when they showed that an avian herpes virus (Marek's disease virus) is able to promote atherosclerotic lesions in chickens(146). Since then, numerous investigations have established an association between coronary artery disease (CAD) and human pathogens such as *C. pneumoniae*, Cytomegalovirus (CMV), Herpes simplex virus (HSV), *Helicobacter pylori* (*H. pylori*), periodontal pathogens and more recently human immunodeficiency virus (HIV) (147-154).

Various mechanisms have been proposed to describe the involvement of these microorganisms in cardiovascular disease. First, invasion of vascular cells can stimulate cellular changes that can lead to vascular remodeling. *C. pneumoniae* and CMV have been recovered from human atheromas (155-158) and are proposed to be transported to these areas via monocytes from the lungs and bone marrow, respectively (159). Both can

reside in endothelial cells (ECs) and SMCs of the vessel wall and are able to initiate pro-atherogenic effects such as adhesion molecule expression, smooth muscle cell proliferation, cytokine production and matrix metalloprotease production (160-165). Pathogens have the ability to initiate inflammatory responses through activation of TLRs on macrophages, ECs and VSMCs. Their ligands include membrane components of bacteria and bacterial Hsps. Cellular components (ie. LPS, Hsp) of *C. pneumoniae*, *H. pylori* and *P. gingivalis* can stimulate atherogenic effects in these cell types via TLR binding (166-169). In addition to their ability to stimulate TLR signaling, Hsps may play a role in autoimmune responses during atherosclerosis. Hsps are highly conserved between species and as such, immune responses directed to bacterial Hsps may cross react with self-Hsp antigens (170). Antibodies against human Hsp60 correlate with the incidence and severity of atherosclerotic development (171) . Infectious agents may also play more of an indirect role by stimulating systemic responses. Periodontal pathogens have been shown to induce acute phase reactants (172).

There is considerable evidence to support the involvement of infectious disease in the development of atherosclerosis. However, it remains debatable whether pathogens are causal agents for the progression of the disease. As such, further research is needed to better understand the role of both the immune system and these pathogens.

3. *CHLAMYDIA PNEUMONIAE*

3.1. Clinical presentation, detection and treatment of *Chlamydia pneumoniae* infection

Chlamydia pneumoniae (C. pneumoniae) is a gram negative, obligate intracellular bacterium. *C. pneumoniae* is known to cause both upper and lower respiratory tract infections such as sinusitis, pneumonia and bronchitis (173,174). It is estimated that this pathogen accounts for roughly 10% of cases of community-acquired pneumonia and 5% of bronchitis and sinusitis cases (174). Persistent *C. pneumoniae* infections have also been associated with several chronic diseases such as asthma, chronic pulmonary disease and coronary heart disease (152,175-177). *C. pneumoniae* infection is most common in children aged 5-14 years old (174). Serological evidence of exposure is observed in 50% of the population by the age of 20 and 70–80% at age 60–70 years (178). Most patients with *C. pneumoniae* infection are asymptomatic, and the respiratory illness is typically mild (179). The clinical symptoms of *C. pneumoniae* infection are nonspecific and do not differ significantly from those caused by other atypical organisms (180).

A variety of methods including serology, culture and PCR are used to identify *C. pneumoniae* infections. Culture is regarded as the reference standard for *C. pneumoniae* infection (181). However, due to technical limitations, this method is not the primary technique for clinical diagnostic purposes (181). Serological testing is most commonly employed for assessment of *C. pneumoniae* infection, with microimmunofluorescence (MIF) being the method of choice (181,182). This approach, however, has a number of limitations such as paired sera samples requirement, inter-laboratory variability and variable correlations with other identification methods (182-184). PCR has also been employed for *C. pneumoniae* detection, however, no current standardized and validated

assay is available commercially (182). It is important to note, that there is no currently validated test for persistent infection (181).

Macrolides, tetracyclines, quinolones, and rifamycin all display antibiotic activity against *C. pneumoniae* infection in vitro (185). The microbial efficiency of these antibiotics in patients with respiratory infections varies from 70-86% (186-190). Treatment regimens for acute respiratory infections vary from 5 days to 3 weeks depending on the dosage and antibiotic given (191). In some cases, a second course of antibiotic treatment may be required (191).

3.2. *C. pneumoniae* infection

3.2.1. Chlamydial life cycle

Chlamydial species are characterized by a biphasic life cycle which lasts between 48-72 hours (192) and involves two morphologically distinct forms of the bacteria, the elementary body (EB) and the reticulate body (RB) (193) (Figure 2). EBs, the metabolically inactive and infective forms, will specifically bind cell surface receptors and mediate their uptake by rearrangement of the actin cytoskeleton (194). Upon endocytosis, EBs will differentiate into metabolically active RBs (by 2 hours) within the newly formed chamydial inclusion. RBs replicate asynchronously via binary fission (12-24 hours) before re-differentiation back into EBs (24-48 hours). EBs exit the cell through cytolysis or extrusion (48-78 hours) (193). Under certain environmental conditions (nutrient deprivation, inflammation, antibiotic treatment), *C. pneumoniae* can adopt a persistent form of infection (195,196). This aberrant but viable form of RBs will have

reduced metabolic activity, alterations in gene expression and is often refractory to antibiotic treatment (193,197,198).

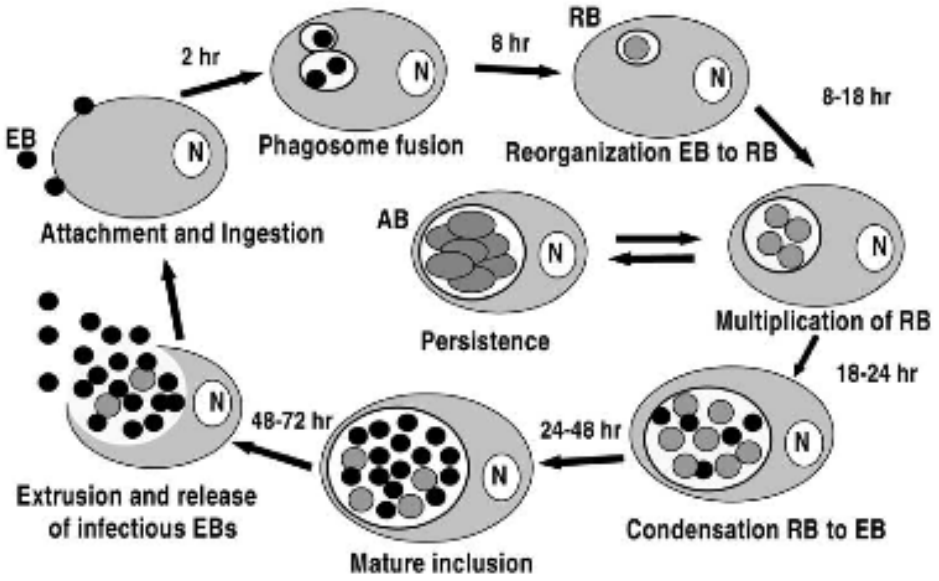

Figure 2. Life cycle of chlamydiae
EB= Elementary bodies, RB= Reticulate bodies, AB= Aberrant bodies
Reprinted from Seminars in Pediatric Infectious Diseases.13(4), MR Hammerschlag, The intracellular life of chlamydiae., 239-248, © 2002, with permission from Elsevier. (193)
http://www.sciencedirect.com/science/journal/doi:10.1053/spid.2002.127201

3.2.2. *C. pneumoniae* and host cell interactions

Chlamydial gene expression is temporally regulated throughout its life cycle and RNA transcripts are organized as early, mid- and late-cycle (199). These transcripts encode proteins that are directly involved in chlamydial replication processes, which include RNA, DNA and protein synthesis, nutrient translocation, metabolism and cytokinesis. In addition, *C. pneumoniae* infection is known to modulate host cell pathways and responses in order to facilitate replication. This ability is thought to be in large part regulated by chlamydial effector proteins that directly interact with host cell

machinery (200).

Effector proteins are produced by chlamydial RBs and they interact with host machinery involved in invasion, vesicle trafficking, immune/inflammatory response and cell survival pathways. The inability to perform genetic manipulation in Chlamydiae has presented problems in the identification and characterization of these proteins. However, the combinatory use of comparative genomics, DNA arrays and genome scale expression has allowed for the identification, localization and function of multiple chlamydial effector proteins. Secretion of these factors is thought to be mediated primarily through the use of a type III secretion (T3S) apparatus (201) , although type II secretion (T2S) has also been employed (202). Different chlamydial species including *C. pneumoniae* carry a complete set of genes for the T3S complex and genes encoding T3S system components have been shown to be actively expressed during replication and even persistent infection (203).

A number of secreted effector proteins, termed Inc proteins, have been demonstrated or are predicted to integrate into the inclusion membrane (204-206). This localization at the interface between the host cell and inclusion membrane suggests an important role in host-cell interactions. One member of the Inc family, *Cpn* 0585, has been shown to selectively recruit and interact with multiple host Rab GTPases (207). Rab GTPases are members of the Ras-like small GTPase family, which is involved in the generation, transport, docking and fusion of vesicles. This Inc protein was shown to be up-regulated during persistent infection (192). Interestingly, ectopic expression of *Cpn*

0585 in *C. pneumoniae*-infected cells has been shown recently to disrupt chlamydial replication (207).

While some effector molecules are destined for the inclusion membranes, others, which include translocated actin recruiting phosphoprotein (Tarp) and chlamydial protease-like activity factor (CPAF), are released into the host cytoplasm (206). Upon attachment of *C. pneumoniae* to the host cell membrane, Tarp is translocated into the cytoplasm to mediate nucleation of actin filaments which enables chlamydial invasion (208). The Chlamydial protease-like activity factor (CPAF) produced and secreted in the host cytoplasm during *C. pneumoniae* infection cleaves the eukaryotic transcription factor RFX5 responsible for expression of the major histocompatibility complex (MHC) (202). Inhibition of this action would improve host adaptive immunity towards *C. pneumoniae*. Other CPAF cleavage targets identified to date in *C. pneumoniae* infected hosts include various cytoskeleton proteins (209) and hypoxia inducible transcription factor (HIF-1) (210). Additionally, CPAF expressed during *Chlamydia trachomatis* infection was shown to cleave pro-apoptotic BH3-only proteins (211). *C. pneumoniae* infection has also been shown to inhibit apoptosis during both acute and persistent infection (212-214), however, alternative effector proteins may be involved.

Furthermore, secreted *C.pneumoniae* effector proteins may also regulate host cell signaling pathways. This could occur through direct phosphorylation/dephosphorylation of enzymes involved in a particular pathway, as Ser/Thr protein kinases and phosphatases are expressed during the *C. pneumoniae* replication cycle (192). *C. pneumoniae* infection

has been noted to rapidly induce phosphoinositide 3-kinase (PI3-kinase) and mitogen-activated protein kinase (MAPK) signaling during invasion (194). Chlamydial effector proteins may also activate these pathways via binding of extracellular and intracellular pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs), cell surface PRRs, have been shown to play an important role in the development of atherosclerosis. Chlamydial Hsp60 (cHsp60), a stress protein produced and released during both acute and chronic infection, has been shown to stimulate cell proliferation and inflammatory responses through TLR activation (168,215,216). Chlamydial virulence factors have also being regarded as potential stimulators of nucleotide-binding oligomerization domain (NOD) proteins, intracellular PRRs that lead to a stimulation of inflammatory responses (217).

3.3. *C. pneumoniae* and atherosclerosis [2]

3.3.1. Clinical evidence

Saikku et al. first established the link between *C. pneumoniae* infection and cardiovascular disease by demonstrating that high antibody titers against *C. pneumoniae* were associated with the incidence of chronic stable coronary artery disease or acute myocardial infarction (152). Since then, numerous cross-sectional and prospective sero-epidemiologic studies have provided varied findings (218). Despite these results, pathological evidence has been strongly supportive of a role for *C. pneumoniae* in heart disease. *C. pneumoniae* has been detected in atherosclerotic carotid and coronary arteries

[2] Reprinted from Fundamental & Clinical Pharmacology, 24(5), JF Deniset and GN Pierce, Possibilities for therapeutic interventions in disrupting *Chlamydophila pneumoniae* involvement in atherosclerosis, *607-617,* © 2010, with permission from John Wiley and Sons. www.onlinelibrary.wiley.com/doi/10.1111/j.1472-8206.2010.00863.x

but not in healthy tissues by electron microscopy, immunofluorescence and polymerase chain reaction (PCR) methods (219-221). *C. pneumoniae* strains retrieved from these plaques are still viable by evidence of their ability to be cultured (158,222). Furthermore, *C. pneumoniae*-specific T cells have also been detected in these atherosclerotic plaques demonstrating activation of the host immune response to *C. pneumoniae* replication (138).

3.3.2. Experimental studies

The mechanism whereby *C. pneumoniae* infection leads to atherosclerotic development has received considerable research attention. Multiple laboratories have established that *C.pneumoniae* has the ability to infect and actively replicate in cells relevant to atherosclerotic injury (monocyte/macrophages, endothelial cells and vascular smooth muscle cells (223-225) . Infection of these cell types stimulates cellular responses that support the involvement of *C. pneumoniae* in all stages of atherogenesis (Figure 3). The migration of *C. pneumoniae* from the lungs to the vessel wall is proposed to be through the infection of alveolar-derived monocytes which play a role as a vector for the bacteria (226) . Once at the vessel wall, *C. pneumoniae* can be released from the monocytes and subsequently re-infect the cells in the vicinity. The transfer process between peripheral mononuclear cells and endothelial cells is disturbed by shear stress and thus favors atherogenic prone areas (227) . This may explain why *C. pneumoniae* is not found in healthy tissues and provides potential evidence for the involvement of *C. pneumoniae* in the initiation of atherogenesis. To further support this hypothesis, *C. pneumoniae*-infected endothelial cells display an upregulation of adhesion molecules like

vascular cell adhesion molecule (VCAM)-1, intracellular cell adhesion molecule (ICAM)-1 and monocyte chemotactic protein (MCP) -1 (162,228-231). These changes resulted in increases in monocyte adhesion and transendothelial migration (229,230). cHsp60, which is secreted during *C. pneumoniae* infection, can additionally decrease endothelial nitric oxide synthase expression and activity (232). Foam cell formation is also induced by infection of macrophages (233-235). *C. pneumoniae* continues to promote the inflammatory environment by stimulating the production of pro-atherogenic cytokines and reactive oxygen species in macrophages, endothelial cells, and vascular smooth muscle cells (232,235-241). *C. pneumoniae* and *C. pneumoniae*- derived components can directly and indirectly stimulate smooth muscle cell proliferation through endothelial cell infection, a key step in plaque formation (161,168,242,243). *C. pneumoniae* may also be involved in atherothrombosis. *C. pneumoniae* can induce the production of degradative enzymes such as matrix metalloproteases (MMPs) and pro-coagulant factors such as tissue factor (TF) and plasminogen-activating inhibitor (PAI-1) in vascular cells (160,163,239,244).

Numerous animal studies have also produced supportive data for a cause-and-effect role of *C. pneumoniae* in atherosclerosis (Table 1). Rabbits fed with a normal diet or an atherogenic diet and then infected with *C. pneumoniae* display accelerated lesion development (245-247). Studies in mice also demonstrate the ability of *C. pneumoniae* infection to accelerate the process, however, this is dependent upon the initiation of the lesion through a cholesterol-enriched diet prior to infection (248-251).

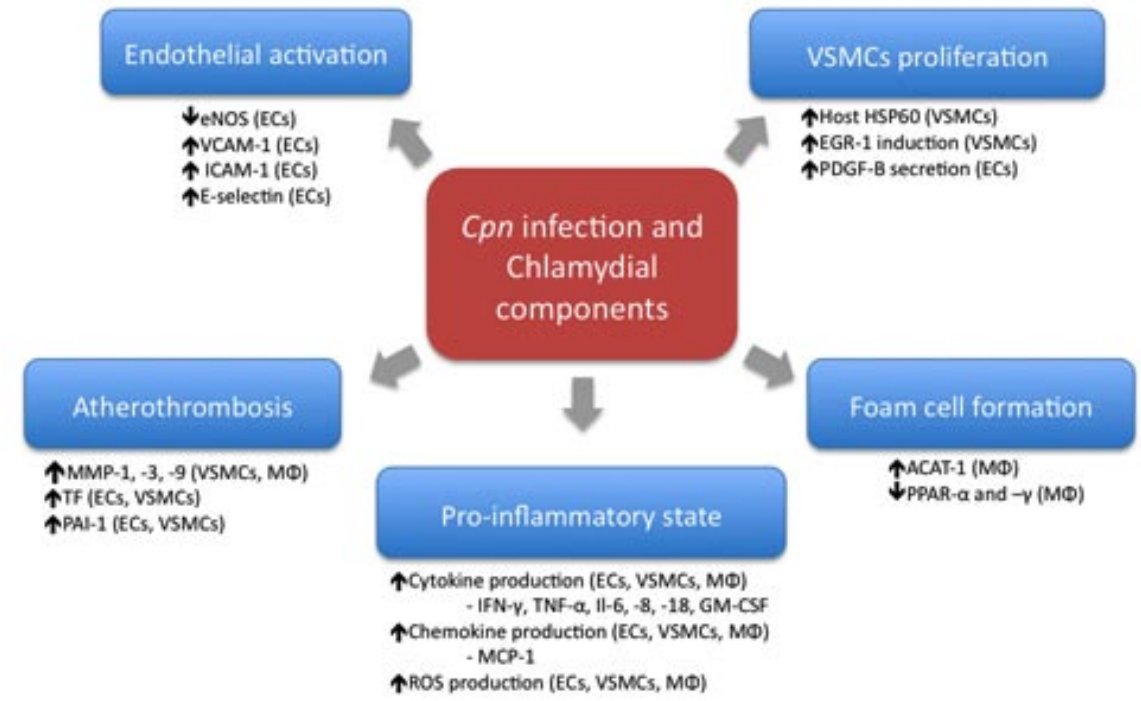

Figure 3. Pathophysiologic mechanisms of *C. pneumoniae* infection in atherosclerosis

C.pneumoniae (*Cpn*) and its components contribute to key steps of atherosclerotic development by eliciting specific cellular responses in endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and Macrophages(MØ). ACAT-1, acyl-coenzyme A: cholesterol acyltransferase 1; EGR-1, early growth response gene 1; eNOS, endothelial nitric oxide synthase; GM-CSF, granulocyte–macrophage colony-stimulating factor; HSP60, heat shock protein 60; ICAM-1, intracellular cell adhesion molecule 1; IFN-**c**, interferon gamma; Il-(6,8 and 18), interleukin (6,8 and 18); MCP-1, monocyte chemotactic protein 1; MMP- (1,3 and 9), matrix metalloprotease (1,3 and 9); PAI-1, plasminogen-activating inhibitor 1; PDGF-B, platelet-derived growth factor subunit B;PPAR-(**α** and –γ), peroxisome proliferator-activated receptor (alpha and gamma); ROS, reactive oxygen species; TF, tissue factor; TNF-**α**, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1. Reprinted from Fundamental & Clinical Pharmacology, 24(5), JF Deniset and GN Pierce, Possibilities for therapeutic interventions in disrupting *Chlamydophila pneumoniae* involvement in atherosclerosis, *607-617*, © 2010, with permission from John Wiley and Sons. (252) www.onlinelibrary.wiley.com/doi/10.1111/j.1472-8206.2010.00863.x

Regular mice fed with an atherogenic diet following infection did not exhibit any changes (253). Furthermore, these changes in both animal models appear to be specific to *C. pneumoniae*, as *Chlamydia trachomatis* and *Mycoplasma pneumoniae* infection in mice and rabbits, respectively, were not able to induce these changes (246,250,254). Further studies have also shown that *C. pneumoniae* infection leads to endothelial dysfunction and neointima formation (255-257).

3.3.3. Clinical antibiotic trials

Establishing a causative role for *C. pneumoniae* in atherogenesis opens a window of opportunity for the use of antibiotics as a treatment modality for cardiovascular disease. Both respiratory and vascular *C.pneumoniae* strains have displayed in vitro susceptibility to antibiotics like tetracycline, fluoroquinolones and macrolides (258,259). The newer generation macrolides, azithromycin, clarithromycin and roxithromycin have been the drugs of preference for use in cardiovascular interventions (218). Azithromycin can be effectively taken up by atherosclerotic plaques (260) and significantly inhibited *C.pneumoniae*-induced atherosclerotic development in rabbits (247,261). Retrospective studies in humans examining the incidence of acute myocardial infarction in patients who have been treated with antibiotics in the preceding 3–5 years have delivered conflicting conclusions (262,263).

Table 1. Animals studies with *C.pneumoniae* infection

References	Animal Model	Diet	*Cpn* strain	Inoculation(s)	Effect
(255)	Apo E -/- Mouse	Regular	IOL-207	3	Endothelial dysfunction
(264)	Apo E -/- Mouse	Regular	IOL-207	3	Endothelial dysfunction
(265)	Apo E -/- Mouse	Regular	AR-39	3	Accelerates atherosclerosis
(266)	Apo E -/- Mouse	Regular	?	2	Accelerates atherosclerosis
(267)	Apo E -/- Mouse	Regular	K6	2	No effect
(268)	Apo E-/- Mouse	Atherogenic	K7	3/4	No effect
(269)	Apo E /LDLr-/- Mouse	Regular	TWAR 2043	6	↓ Fibrous cap area
(251)	LDLr-/- Mouse	Atherogenic	AR-39	12	Accelerates atherosclerosis
(250)	LDLr-/- Mouse	Atherogenic	AR-39	9	Accelerates atherosclerosis
(250)	LDLr-/- Mouse	Regular	AR-39	9	No effect
(270)	BALB/C Mouse	Regular	TWAR	3	Inflammatory changes
(249)	C57BL/6J Mouse	Regular	AR-39	3	Inflammatory changes
(248)	C57BL/6J Mouse	Atherogenic	AR-39	3	Accelerates atherosclerosis
(253)	C57BL/6J Mouse	Atherogenic	AR-39	3	No effect
(245)	NZW Rabbit	Normal	VR1310	1	Lesion development
(246)	NZW Rabbit	Normal	VR1310/AR-39	1/3	Lesion development
(247)	NZW Rabbit	Atherogenic	VR1310	3	↑ Intimal Thickening
(271)	Swine	Atherogenic	AR-39	1	Endothelial dysfunction
(256)	Swine	Atherogenic	IOL-207	1	Endothelial dysfunction
(257)	Swine	?	AR-39	3	Neointima formation

Reprinted from Fundamental & Clinical Pharmacology, 24(5), JF Deniset and GN Pierce, Possibilities for therapeutic interventions in disrupting Chlamydophila pneumoniae involvement in atherosclerosis, 607-617, © 2010, with permission from John Wiley and Sons. (252)
www.onlinelibrary.wiley.com/doi/10.1111/j.1472-8206.2010.00863.x

Several randomized antibiotic trials have been completed (Table 2). Gupta et al. (272) performed the first small-scale antibiotic trial on patients with cardiovascular disease. They administered either azithromycin or a placebo to patients after they had suffered a myocardial infarction. The placebo group demonstrated a 4-fold increase in cardiovascular events compared to the azithromycin group and this effect correlated positively with *C.pneumoniae* IgG titers (272). This positive result justified pursuing additional trials. The Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with Chlamydia (ACADEMIC) study examined the effects of antibiotic treatment in patients with stable coronary artery disease (273). Despite extending the duration of the treatment from 3 or 6 days up to 3 months (compared with the previous study), no changes in cardiovascular events were observed (273). Three subsequent high-powered large-scale trials followed including the Weekly Intervention With Zithromax Against Atherosclerotic-Related Disorders (WIZARD), clarithromycin for patients with stable coronary heart disease (CLARICOR) and Azithromycin and Coronary Events Study (ACES). The WIZARD study demonstrated a 30% reduction in the incidence of death and myocardial infarction at 6 weeks but these effects could not be sustained throughout the follow-up period, perhaps because of an insufficiently long-treatment period (274). This question was answered by the ACES study in which a year long treatment period did not result in any reduction in cardiovascular events (275). The CLARICOR study confirmed these negative results (276).

Table 2. Antibiotic treatment trials with *C.pneumoniae* infection

References	Year	Trial	Patient population(n)	Antibiotic(s)	Treatment protocol	Follow-up (months)	Benefit
(272)	1997		Stable CAD (60)	Azithromycin	500mg/day (3 days) or 500mg/day (2x3 days, 3 mths apart)	18	Yes
(277)	1999	ROXIS	ACS (202)	Roxithromycin	300mg/d (30 days)	1 & 6	Yes
(273)	2000	ACADEMIC	Stable CAD (302)	Azithromycin	500mg/d (3 days) + 500mg/wk (3 months)	24	No
(278)	2002	CLARIFY	ACS (148)	Clarithromycin	500mg/d (85 days)	36	Yes
(279)	2002	STAMINA	ACS (325)	Azithromycin or amoxicillin	Azi : 500 mg/d, Amo : 2000mg/d (1 week)	12	Yes
(280)	2003	ANTI-BIO	ACS (872)	Roxithromycin	300mg/d (6 weeks)	12	No
(281)	2003	AZACS	ACS (1439)	Azythromycin	500mg (day 1) + 250mg (days 2-5)	6	No
(274)	2003	WIZARD	Stable CAD (7747)	Azithromycin	600mg/d (3 days, week 1) + 600mg/wk (11 weeks)	30	No
(282)	2005	PROVE-IT	ACS (4162)	Gatifloxacin	400mg/d (2 weeks) + 400mg/d (10 days /mth)	24	No
(275)	2005	ACES	Stable CAD (4012)	Azithromycin	600mg/wk (1 year)	46.8	No
(276)	2006	CLARICOR	Stable CAD (4373)	Clarithromycin	500mg/d (2 weeks)	36	No

ACS, Acute coronary syndrome; CAD, Coronary artery disease Reprinted from Fundamental & Clinical Pharmacology, 24(5), JF Deniset and GN Pierce, Possibilities for therapeutic interventions in disrupting Chlamydophila pneumoniae involvement in atherosclerosis, 607-617, © 2010, with permission from John Wiley and Sons. (252) www.onlinelibrary.wiley.com/doi/10.1111/j.1472-8206.2010.00863.x

A second series of clinical trials performed during this same period investigated the effect of antibiotics on patients with acute coronary syndromes. Initial pilot studies like the Roxithromycin in Non-Q Wave Coronary Syndromes (ROXIS), Clarithromycin in Acute Coronary Syndrome Patients in Finland (CLARIFY) and South Thames Trial Of Antibiotics in Myocardial Infarction And Unstable Angina Pectoris (STAMINA) all noted significant reductions in cardiovascular events with macrolide treatment (277-279). However, just as in the case with patients with stable coronary artery disease, larger scale trials like the Antibiotic Therapy in Acute Myocardial Infarction (ANTIBIO), Azythromycin in Acute Coronary Syndrome (AZACS), Pravastatin Or Atorvastatin Evaluation and Infection Therapy (PROVE-IT) all concluded that antibiotic treatments were not effective in preventing acute cardiovascular events (280-282).

Taken as a whole, it is possible to conclude from all of these studies that antibiotic treatment has no beneficial effect on cardiovascular outcomes in these disease populations. However, these antibiotics trials do not provide definitive information regarding the role of *C. pneumoniae* in the development of atherosclerosis. It is still not clear whether macrolides, the primary antibiotic used in the trials, are effective in fully eradicating *C. pneumoniae* infection within a vascular environment. Under unfavorable conditions such as nutrient deprivation, heat shock, inflammatory responses, or antibiotic treatment, Chlamydia species have the ability to transfer to a non-replicating, metabolically active form known as persistent bodies (283). This persistent form of infection is refractive to azithromycin treatment (284). Prolonged treatment with azithromycin, clarithromycin and levofloxacin in a continuous infection model could

reduce but not eliminate *C. pneumoniae (285)*. Furthermore, the high percentage of *C. pneumoniae* detection within the atherosclerotic plaque paired with the difficulty of isolating viable *C. pneumoniae* provides potential evidence for Chlamydial persistence within the diseased vessel wall (218). Despite the capacity for macrolides to accumulate to effective, relevant concentrations within atherosclerotic plaques, it remains ineffective against the persistent form of *C. pneumoniae* infection. To date, there is no reliable serologic marker for persistent *C. pneumoniae* infection(181). As such, it is impossible to determine whether these patients suffered from persistent infection and were, therefore, resistant to the anti-atherogenic effects expected of the drugs. If they did, this could explain the lack of positive results. It is also important to note that none of these trials actually addressed the potential causative nature of *C. pneumoniae* infection in atherosclerosis. Patients enrolled in the trials all displayed severe atherosclerotic plaque development and had already suffered an initial major cardiovascular event. This particular target group does not provide any insight into the ability of *C. pneumoniae* to initiate atherogenesis. Even if total eradication of *C. pneumoniae* could be achieved, the damage already inflicted may be too severe from which to recover. In the rabbit models where azithromycin treatment was able to inhibit *C. pneumoniae*-induced atherosclerosis, the antibiotic treatment was given shortly after *C. pneumoniae* inoculation (247,261) . However, this protective role was lost if the treatment regimen was given 6 weeks past inoculation (261). This suggests that there may be a critical window of time for the initiation of Chlamydial specific antibiotics. However, this becomes very difficult to examine in humans. Initial *C. pneumoniae* infections of the respiratory tract are often asymptomatic and thus are rarely treated unless severe. In addition, continuous long-term

antibiotic treatment may induce toxicity and antibiotic resistance. Finally, murine models of atherosclerosis have identified a high cholesterol environment as being critical to the atherogenic effects of *C. pneumoniae* infection[83]. It is possible therefore, that antibiotic treatment may be best utilized in a clinical population with high circulating cholesterol levels prior to the appearance of clinically-significant cardiovascular disease. Some clinical studies would lend support to this hypothesis (286) but further work is required to substantiate this conclusively.

4. **CELLULAR STRESS RESPONSES**

Over the course of evolution, organisms have developed a number of survival mechanisms such as the heat shock response, unfolded protein response, DNA damage response and the oxidative stress response to manage responses to the surrounding environment and maintain homeostasis (287) . Numerous key protein mediators in these stress pathways are conserved throughout human, yeast, eubacterial and archaeal proteomes, which highlights the importance of these survival pathways (288). The ability of a cell to withstand environmental stresses will depend on the integration of multiple cellular stress strategies. Dysfunction in any of these pathways may lead to pathological states.

4.1. Heat shock response

The heat shock response is characterized classically as a biochemical response to mildly elevated temperatures. Ritossa first described this phenomenon in 1969, noting a rapid new puffing (indicative of gene activation) pattern in salivary glands of Drosophila

in response to elevations in temperature (289). Follow-up work by Tissières et al. demonstrated a decade later that puffs in response to thermal stimuli correlated with the expression of a new set of proteins, now termed heat shock proteins (Hsps) (290). Numerous stress stimuli in addition to hyperthermia such as oxidative stress, heavy metals, bacterial and viral infections are now known to activate the heat shock response and expression of Hsps (291,292).

4.1.1. Heat shock proteins

Hsps are a family of evolutionary conserved proteins that traditionally function as molecular chaperones to ensure proper folding or refolding of proteins in order to avoid protein aggregation. In addition to their involvement during stress, Hsps can play important housekeeping roles in the cell such as regulation of protein degradation (293,294), cellular signalling (295,296), the translocation of protein into subcellular compartments (297,298), and regulation of cell death pathways (291,299-301). Hsps can be organized into the following subgroups based on their molecular weights, amino acid sequence and function: Hsp110, Hsp90, Hsp70, Hsp40, small Hsps and human chaperonins (Hsp60/Hsp10, TriC) (302).

The Hsp70 and Hsp90 families play prominent roles in cellular functions and share a number of similarities. Human genomes express both constitutive (Hsc70, Hsp90α) and inducible (for example Hsp72, Hsp90β) forms of Hsp70 and Hsp90 that are localized in the cytosol and nucleus (302-304). In addition, both families contain

mitochondrial (for example Hsp70-mtHSP75, Hsp90- tumor necrosis factor receptor associated protein 1 (Trap1)), and endoplasmic reticulum (for example Hsp70-Bib, Hsp90- glucose regulated protein 94 (Grp94)) specific isoforms (304-306). Cytosolic Hsp70 isoforms are involved in numerous cellular processes such as co-translational protein folding of nascent proteins, refolding of misfolded proteins, translocation of proteins across intracellular membranes, control of regulatory proteins, chaperone-mediated autophagy, and disassembly of clathrin-coated vesicles (297-299,304,307-311). Cytosolic Hsp90, estimated to account for 1-2% of all protein under basal conditions (312), is involved in cell cycle control, cellular signaling and apoptosis (295,296,313-315). The chaperone activity of Hsp70 and Hsp90 is ATP-dependent, which induces conformational changes in their protein structure. As such, these chaperones undergo constant cycling between ATP- and ADP- bound forms that confer different substrate binding capacities (316,317). The ATPase activity of both proteins is key for their functions and can be modulated by co-chaperones (like Hsp70-Hsp40, Hsp90- ATPase homolog 1 (Aha1), Hsp70–Hsp90 organizing protein (Hop), p23) and post-translation modifications (318,319). In addition, Hsp110 functions as the nucleotide exchange factor for Hsp70 thus re-establishing the low-affinity ATP-bound Hsp70 (320,321). In basal conditions, Hsp70 is involved in protein folding of nascent proteins whereas Hsp90 binds protein in a nearly native conformation (304,322,323). As such, Hsp70 and Hsp90 work in concert for the sequential folding of a number of proteins such as steroid hormone receptors (310).

Small Hsps (sHsps) represent a set of proteins ranging in molecular weight from 12 to 42kDa that contain a relatively conserved α-crystallin domain (324). A number of the sHsp family members are ubiquitously produced in most tissues, particularly in muscle tissues (325,326). Hsp27 is recognized as the primary inducible form (326). They are known to confer protection to proteins involved in signal transduction, metabolism, translation and transcription, and cytoskeleton organization (327-331). sHsps bind to partially unfolded proteins in an ATP-independent manner through the formation of dynamic oligomeric structures(332). The dimer subunits of sHsps can rapidly transfer from one oligomeric structure to another made of similar or different sHsps. Oligomerization correlates with chaperone function and is sensitive to phosphorylation status, temperature, pH and protein concentration (333-338). The chaperone function of sHsps is primarily to maintain a partially unfolded state of the client protein prior to transferring them to ATP dependent chaperone complexes such as Hsp70/40 to refold the protein (339,340) or alternatively, to sHsp for proteosomal degradation in protein catabolism (293,294).

The chaperonin family includes Hsp60/10 as well as the cytosolic-specific TriC protein family. Hsp60, the eukaryote homolog of GroEL, is ubiquitously expressed and is stress-inducible (161,341-343). Intracellular Hsp60 is primarily localized in both the mitochondria and cytosol(344). Hsp60 is an integral chaperone and aids in the folding and maintenance of approximately 15-30% of all cellular proteins(345). Hsp60 monomers form two stacked heptameric rings with a central channel containing hydrophobic residues to which unfolded proteins bind(346-348). The function of Hsp60

is ATP dependent and requires the interaction of heptameric Hsp10 (co-chaperone) structures forming a cap on the Hsp60 barrel-like structure(347,349,350). Alternatively, cytosolic Hsp60 appears to have both anti-apoptotic and pro-apoptotic functions depending on the cell type(351,352). During stress, Hsp60 can be secreted via exocytosis(353,354) and then activates cells via toll-like receptor signaling (355,356).

4.1.2. Regulation of heat shock protein expression

The expression of heat shock proteins is regulated by transcription factors termed heat shock factors (Hsf) that bind heat shock elements found within the promoter region of Hsp genes. To date, four Hsf members (Hsf1-4) have been cloned in vertebrates, all with different features and functions. In mice, Hsf1 and Hsf2 are constitutively expressed in all tissues, whereas Hsf4 is limited to lung and brain tissue (357). Hsf3 has been primarily characterized in avian species, although its expression has been recently noted in mouse cells (358). Under basal conditions, Hsf1 is monomeric, Hsf2 and Hsf3 are dimeric and Hsf4 is found in a DNA-binding competent trimeric form (357,359). Functionally, Hsf1 is regarded as the primary regulator of the heat shock response. An Hsf1 gene deletion in mice results in an inability to express Hsps in response to thermal injury (360). In addition to mediating the heat shock response, Hsf1 is thought to have other gene targets under non-stress conditions (361,362). Although the function of Hsf2 has traditionally been restricted to non-stress conditions (363), it appears to help modulate the expression of Hsps through interactions with Hsf1. Hsf2 binds the promoter region of multiple Hsps and modulates Hsp expression in response to heat stress and heme treatment (364-367). This function is dependent on active DNA-binding

of Hsf1 within the promoter (367). Furthermore, Hsf2 can bind the promoter region of inducible Hsp70 under basal conditions, preventing compaction of the DNA in this area leading to a faster stress response (366).

The activation of Hsf1 is a two-step process involving the transition from monomer to trimeric form in order to migrate to the nucleus to bind DNA, and subsequent post-translational modifications for trans-activation function (368). A number of mechanisms have been proposed for regulating Hsf1 activation. A well-described example is repression of Hsf1 activity through Hsf-Hsp interactions, with Hsp90 and Hsp70 being the primary regulators. Hsp90 interacts with Hsf1 under basal conditions, however, it dissociates in response to proteotoxic stress (369-371). Inhibition of Hsp90 via pharmacological inhibitors or antibodies, activates the DNA-binding ability of Hsf1 (369,372,373). Hsf1 also interacts with Hsp70 during a basal state and with trimeric Hsf1 under heat shock conditions (374-376). Hsp70 inhibits the trans-activating capacity of DNA-bound Hsf1 (377,378). Post-translation modifications such as phosphorylation, sumoylation and acetylation also play important roles in regulating Hsf1 activity. Although numerous modification sites have been identified within Hsf1, few have been functionally characterized. Phosphorylation at S216, S230, S326 or S419 stimulates activity(379-382). Alternatively, phosphorylation at T142, S303, S307, S363, or acetylation at K80 and sumoylation at K298 all have inhibitory effects on Hsf1 activity(383-393). Multiple signaling pathways have been involved in mediating this phosphorylation such as mitogen-activated protein kinases (MAPKs), glycogen synthase kinase 3β (GSK3β), CaM kinase II (CaMKII) and casein kinase II (CK2) (393-396).

Phosphorylation at S303/307 represents a constitutive repression mechanism that can be reversed during heat shock stress(383,393). Furthermore, phosphorylation at S303 is required for sumoylation-mediated repression(385,386). Acetylation at K80 decreases Hsf1 DNA-binding activity(387). The acetylation profile is dependent on sirtuin 1 (SIRT1) deacetylase activity. Modification of SIRT1 activity can influence the DNA-binding state of Hsf1 (387).

In addition to environmental stressors, cytokines are also known to induce Hsp expression independently of Hsf1 activation. IFN-γ stimulates Hsp70 and Hsp90 levels and can work synergistically with Hsf1 via stimulation of the signal transducer and activator sites of transcription (STAT) protein 1 (397) . Il-6 increases Hsp90 expression via a nuclear factor IL-6 (NF-IL6) and STAT3 mechanism (398,399). Tumor necrosis factor alpha (TNF-α) can induce Hsp60 expression via a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated mechanism (400).

4.2. Heat shock proteins and atherosclerosis

Due to the importance of the heat shock protein network in maintaining cell survival under both basal and stress conditions, Hsps have been associated with a number of clinical conditions such as autoimmune diseases, neurodegenerative disorders, cancer and cardiovascular disease. The involvement of Hsps in atherosclerosis is thought to be through a number of mechanisms and key Hsps appear to differentially influences atherosclerotic progression.

4.2.1. Intracellular mechanisms

The atherosclerotic plaque environment is a reservoir of stress factors that have the capacity to stimulate Hsp expression within vascular cells and immune cells. Numerous studies have noted various expression patterns of key chaperones such as Hsp27, Hsp60, Hsp70 and Hsp90 within human atherosclerotic plaques and with animal models of atherosclerosis (401-408). Current evidence indicates that these molecular chaperones may play different roles during the progression of atherosclerosis.

Hsp27 expression has been localized to the neointimal regions of both early lesions and fibrous plaques, and within VSMC-rich areas (409). It is thought to play anti-apoptotic and pro-survival roles during atherosclerosis. Overexpression of Hsp27 can attenuate atherosclerotic development by decreasing lesion size, arterial wall cholesterol content, apoptosis and macrophage recruitment (410-412). SMCs overexpressing Hsp27 are resistant to cell proliferation and cell death (413). Consistent with this action, the expression of Hsp27 in the atherosclerotic core is decreased compared to the border area adjacent to the plaque within the same patient (406). The expression of Hsp27 decreases as the disease progresses (407).

The phosphorylation status of Hsp27 can influence the cellular function and localization of this chaperone. Phosphorylation of Hsp27 is involved in actin re-arrangement and migration of VSMCs in response to angiotensin II and platelet derived growth factor-BB (PDGF-BB) (414). LDL, a major component of the atherosclerotic plaque, is able to block VSMC migration via stimulation of Hsp27 dephosphorylation

(409). In fact, staining of phosphorylated Hsp27 in atherosclerotic plaques is reduced in areas of the neointima that are positive for ApoB staining (409). Overall, these findings suggest an anti-atherogenic action for Hsp27, however, phosphorylation of Hsp27 (which opposes oligomerization and chaperone activity) may contribute to the pathogenesis.

Hsp70 expression in human atherosclerotic plaques is concentrated around necrotic and lipid accumulation areas, and is co-localized with macrophages but not VSMCs (402). Hsp70 staining is increased in necrotic plaques compared to fibrotic tissues (404) . Furthermore, VSMCs and endothelial cells located in and adjacent to the fibrous cap area lack Hsp70 expression (415). Hsp70 expression is also more prevalent in plaques with thicker fibrous caps (403). Hsp70 may represent a marker for the dysfunctional monocytes/macrophages/foam cells, which are known to secrete proteolytic enzymes that are associated with unstable plaques. A persistent state of dysfunction can eventually lead to cell necrosis, leading to the release of cytoplasmic Hsp70. Alternatively, this chaperone could play a role in mediating the proliferative response within the vessel wall. Thermal induction leading to Hsp72 expression can attenuate neointima formation and oxidative stress (416). Nitric oxide, a potent modulator of VSMC proliferation, can induce Hsp70 expression in VSMCs (417). It is possible that inducible Hsp70 is involved in eNOS mediated inhibition of VSMC proliferation.

Experimental and clinical findings suggest a pro-inflammatory role for Hsp90 in atherosclerotic pathogenesis. Hsp90 is a potent inducer of TNF-α and Il-1β and can activate monocytes/macrophages (418-420). The expression of Hsp90 within human

atherosclerotic plaques is increased within inflammatory regions (403). Furthermore, HSP90 inhibitor treatment decreases inflammation and oxidative stress within the atherosclerotic plaques of apoE$^{-/-}$ mice (403). There is evidence that Hsp90 plays an antagonist role to Hsp70 within atherosclerotic plaques. Treatment of human VSMCs and macrophages with Hsp90 inhibitor increases Hsp70 expression and decreases MCP-1 and Il-6 expression (403). In addition, Hsp90 expression is more prevalent in atherosclerotic plaques with a thin cap, whereas the opposite is true for Hsp70 expression (403).

Human atherosclerotic plaques display Hsp60 staining in endothelial cells, VSMCs and macrophages (421). Lesion-prone sites display strong endothelial Hsp60 expression in young apoE$^{-/-}$ mice (408). Fluid shear stress induces Hsp60 expression in endothelial cells in vitro and in vivo (422). SMC-rich areas underlying the necrotic core and fibrous cap regions exhibited increased expression of Hsp60 in apoE-/- mice (408). HSP60 may also be involved in VSMC proliferation. *C.pneumoniae* infection and mechanical stretch induce Hsp60 expression in VSMCs (161,343). This expression coincides with increased cell proliferation (161,343). The addition of oxLDL, an atherosclerotic risk factor, synergistically enhances the proliferative response in these cells while further increasing Hsp60 expression (343,423). Furthermore, Hsp60 overexpression alone stimulates VSMC proliferation (161).

4.2.2. Extracellular mechanisms

Under conditions of stress, Hsps can be released via exosomal pathways and are capable to signal via PRRs such as toll-like receptors and NOD receptors. Associations

of circulating HSP levels with CV again reveal both pro-atherogenic and anti-atherogenic effects of different Hsps.

Hsp27 secretion is decreased in complex atherosclerotic plaques and circulating levels are decreased in patients with atherosclerotic plaques (424). In addition, circulating Hsp70 levels are inversely correlated with atherosclerosis and CAD risk (425-427). The decrease in extracellular Hsp27 and Hsp70 levels may be in part due to proteolytic degradation. Plasmin, released from unstable plaques, efficiently degrades Hsp27 (428). Hsp70 can be degraded by elastase, which is released by an atherosclerotic plaque ex vivo (427). Furthermore, circulating elastase levels demonstrate an opposite trend to circulating Hsp70 levels (427). In contrast, numerous investigations have established positive associations between Hsp60 and atherosclerosis (429-431). Soluble Hsp90 levels are elevated in patients with atherosclerosis compared to healthy controls (405).

Hsp27 has been suggested to be a competitive inhibitor of acetylated LDL (acLDL) for the scavenger receptor, thus potentially reducing foam cell formation (410). Extracellular Hsp70 can contribute to plaque stabilization. Exogenous HSP70 can inhibit VSMC proliferation induced by cigarette extracts or H_2O_2 administration via a decrease in ERK phosphorylation(432). In vitro, Hsp70 increases ECM production by VSMCs by increasing TGF-β expression via TLR-4 signaling (433). Extracellular Hsp70 can also enhance BMP-4 signaling leading to endothelial proliferation and VSMC calcification by interacting with the matrix Gla protein, an inhibitor of BMP-4 (434). Soluble Hsp70 is

correlated with vascular calcification (435). As previously noted, Hsp70 staining is more prevalent in plaques with thicker caps. Hsp70 released from the necrotic core may be acting locally as a survival signal. Extracellular sources of both chlamydial and human Hsp60 can directly activate endothelial cells, VSMCs and macrophages (436). Treatment with chlamydial Hsp60 induced TLR-4 dependent decreases in both eNOS expression and NO mediated endothelial dependent relaxation in porcine coronary arteries(232). In addition, both chlamydial and endogenous Hsp60 stimulate VSMC proliferation via TLR 2 and 4 signaling (119,168).

4.2.3 Autoimmune mechanism

The potential involvement of infectious agents in the pathogenesis of atherosclerosis has been associated with the possibility of initiating an autoimmune response. Hsp60 represents an attractive immunogen for this type of response due to the conserved sequence homology and signaling profile shared between bacterial and human homologues.

Both human and chlamydial Hsp60 have been identified in atherosclerotic plaques, with greater expression in unstable plaques compared to stable plaques (437). The presence of these homologues correlates with antibody levels to Hsp65 (437). Levels of antibodies against Hsp60 and Hsp65 have been correlated with the risk and severity of atherosclerosis (431,438,439). Furthermore, serum antibodies against mycobacteria Hsp65 also react to endogenously produced human Hsp60 within atherosclerotic lesions, particularly within endothelial cells and macrophages (401). Hsp60 is also expressed on

the surface of endothelial cells under stressful conditions (440,441). Hsp65/60 specific antibodies can induce lysis of the cells in the presence of complement (440,442). Immunization with recombinant mycobacterial Hsp65 stimulates Hsp65 expression in the lesions, Hsp65 T-cell populations in the plaque and atherosclerosis in hyperlipidemic rabbits (443). In humans, Hsp60 reactive effector T-cells have been identified within early and late stage human atherosclerotic plaques (438). This study further demonstrated that anti-Hsp60 antibodies were observed in the circulation of patients with late stage plaques but not early stage plaques[155]. Cytotoxic Hsp60 specific T-cells populations are also increased in late lesions (438). These findings suggest that Hsp60 immunity may be initiated at the level of the plaque. The ability to modulate this response may represent an attractive treatment strategy for atherosclerosis. Immunization with Hsp60 or Hsp60 peptides but not Hsp70 peptides in atherogenic mouse models have resulted in a significant reduction in atherosclerotic plaque development via an increase in the regulatory T cell population (444,445).

5. NUCLEOCYTOPLASMIC TRAFFICKING

In eukaryotic cells, the genetic material is compartmentalized within the nucleus via the nuclear envelope, a double membrane that is continuous with the endoplasmic reticulum (446). As a result, the passage of proteins and RNAs in and out the nucleus requires transport across nuclear pore complexes (NPCs), multimeric protein structures that form channels in the nuclear envelope (447-449). Nuclear transport is a highly regulated process that is involved in cell differentiation, gene regulation and cell cycle control.

5.1 Structure of the nuclear pore complex

The nuclear pore complex (NPC) is a complex protein structure estimated to contain roughly 30 distinct proteins, called nucleoporins (Nups). The NPC has major spokes within it that are found in multiples of eight that form specific sub-structures within the complex (448-450) (Figure 4). It is characterized by a core structure imbedded in the nuclear envelope with extending structures on both the cytoplasmic (cytoplasmic filaments) and nucleoplasmic (nuclear basket) sides. Three major groups of Nups exist within the NPC which include pore membrane proteins (Poms), structural Nups and phenylalanine-glycine containing Nups (FG-Nups) (448,449,451). These three classes have distinct functions with Poms used to anchor the NPC to the nuclear membrane, structural Nups used to support the overall architecture and FG-Nups which mediate transport across the channel. FG-Nups are found on the periphery and innermost layer of the NPC channel with their FG rich repeats extending out to form a permeability barrier and to interact with nuclear transport receptors (448,450,451). In addition, FG-Nups (e.g. Nup358 (RanBP2)) also form the cytoplasmic filaments that extend out from the NPC core (448,452), which are required for efficient nuclear transport (453-456). The nuclear basket is made predominantly of the non-FG nucleoporin Tpr requiring FG-Nups (e.g. Nup153) for assembly (457,458) . Tpr has also been involved in export of mRNA out of the nucleus (459,460).

5.2. Receptor-mediated nuclear transport

The transport of ions, metabolites and small molecules usually occurs by passive diffusion. However, the transport of molecules larger than 40kDa usually

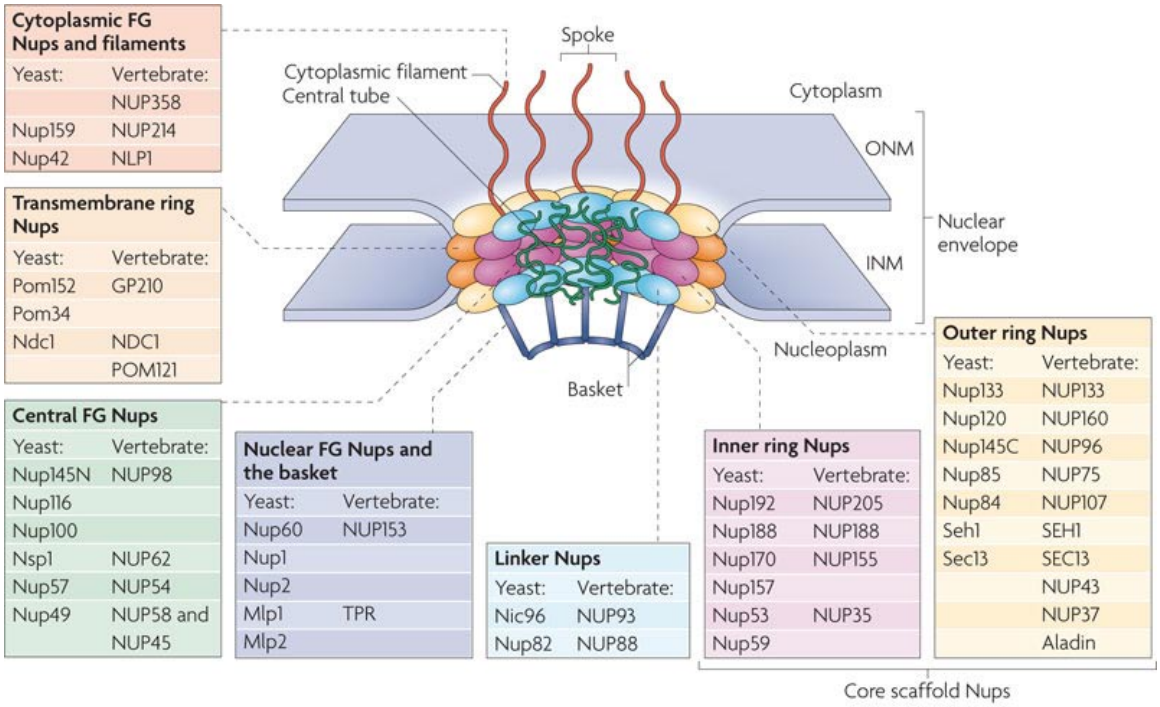

Figure 4. Nuclear pore complex structure

Each nuclear pore complex (NPC) is a cylindrical structure comprised of eight spokes surrounding a central tube that connects the nucleoplasm and cytoplasm. The outer and inner nuclear membranes (ONM and INM, respectively) of the nuclear envelope join to form grommets in which the NPC sits. The NPC is anchored to the nuclear envelope by a transmembrane ring structure that connects to the core scaffold and comprises inner ring and outer ring elements. Linker nucleoporins (Nups) help anchor the Phe-Gly (FG) Nups such that they line and fill the central tube. NPC-associated peripheral structures consist of cytoplasmic filaments, the basket and a distal ring. The Nups that are known to constitute each NPC substructure are listed, with yeast and vertebrate homologues indicated. Both inner and outer ring Nups are known to form biochemically stable NPC subcomplexes, which are thought to have a role in NPC biogenesis and nuclear envelope assembly. GP210, glycoprotein 210; Mlp, myosin-like protein; Ndc1, nuclear division cycle protein 1; Nic96, Nup-interacting component of 96 kDa; NLP1, Nup-like protein 1; Pom, pore membrane protein; Seh1, SEC13 homologue 1; TPR, translocated promoter region. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, C Strambio-De-Castillia, M Niepel and MP Rout The nuclear pore complex: bridging nuclear transport and gene regulation, © 2010. (461)
http://www.nature.com/doi:10.1038/nrm2928

requires nuclear transport receptors to efficiently mediate this dynamic process (462,463). The bidirectional transport of macromolecules across the NPC is facilitated by nuclear transport receptors that will bind cargo molecules via either a nuclear localization signal (NLS) or a nuclear export signal (NES) and mediate translocation of the cargoes across the NPC. The β-karyopherin protein family, which include importins and exportins, represent a well characterized subset of 20 (in humans) nuclear transport receptors (464). Importin-β isoforms will bind the cargo protein directly or via adapter proteins. Importin β1 (Impβ), along with the adapter protein importin α (Impα), mediate the classical nuclear import pathway. Impα binds classical NLSs (cNLSs) characterized by one (monopartite) or two (bipartite) clusters of basic residues (465-467). Prototypical examples include the SV40 large T antigen NLS (PKKKRKV) and the nucleoplasmin NLS (KRPAATKKAGQAKKKK) for monopartite and bipartite NLSs, respectively (466,468,469). Importin β2 (transportin) mediates the import of mRNA processing proteins via direct binding of PY-NLSs (470). Export of many proteins, pre-microRNA and tRNA are also mediated via the importin-β homologues Crm1, exportin-5 and exportin-t, respectively (471). However, additional karyopherin-independent pathways have been identified including the transport of mRNA which is mediated via the NXF1:NXT1 heterodimer receptor (472) . β-catenin, a Wnt family signaling molecule, translocates across the NPC by directly interacting with the FG-Nups (473,474).

Karyopherin-mediated cargo transport is an energy-dependent process. However, the energy required is not for the translocation but rather the dissociation of the transport complex. The Ras-related GTPase , Ran, which cycles between both GDP- and GTP-

bound states governs this process by directly interacting with karyopherin receptors (446). In a GTP-bound form (RanGTP), Ran will strongly associate with karyopherins causing dissociation of the cargo:importin complex and the formation of new cargo:exportin complexes (446,471,475). Alternatively, Ran in the GDP bound form (RanGDP) will weakly associate with karyopherins. Proteins found on opposite sides of the nuclear membrane regulate the RanGDP/RanGTP levels. Ran guanine nucleotide exchange factor (RanGEF) is found within the nucleus and ensures the conversion back into a GTP bound Ran (476). Alternatively, Ran GTPase activating protein (RanGAP) and Ran-binding protein 1 (RanBP1) found on the cytoplasmic side facilitate the hydrolysis of RanGTP into RanGDP (477,478). RanGDP is subsequently shuttled back into the nucleus using the alternative transport receptor nuclear transport factor-2 (NTF2) and re-converted into RanGTP via RanGEF (479). The compartmentalization of these accessory proteins along with an elevated cellular GTP/GDP ratio ensures the establishment of a concentration gradient of RanGTP, with elevated levels in the nucleus (480). As such, RanGTP levels dictate the directionality of the transport system (480).

5.2.1. Classical nuclear protein import pathway

The cycle of classical NPI includes four steps: 1) Transport complex formation, 2) Translocation through the NPC , 3) Impα/Impβ dissociation, and 4) Recycling of importins (Figure 5) (471) . The process begins with formation of the cargo:Impα:Impβ complex. Impα mediates the recognition of the cNLS in the cytoplasm (481) and interacts with Impβ via an importin-β binding (IBB) domain (482). The IBB domain also functions as an autoinhibitory domain for the NLS docking sites when unbound to Impβ

(483). Consequently, the binding affinity of Impα for cNLS is much greater when bound to Impβ (484). As such, the sequence of assembly may subsequently favor initial Impα:Impβ prior to cNLS interaction. Following binding to cargo protein via cNLS, Impβ mediates docking and transport of the complex across the NPC via sequential interactions with FG-Nups. Once on the nuclear side, RanGTP will bind Impβ to initiate dissociation from the complex (485). Nup50 will bind the cargo bound Impα and along with the autoinhibitory domain will facilitate the release of the cargo protein (483,486). For the final step, the receptors are then recycled back to the cytoplasm. Impβ is able to move back across in complex with RanGTP, whereas Impα requires an export receptor, CAS, which is thought to initiate dissociation from Nup50 and then mediates the translocation of a Impα:CAS:RanGTP complex (486-488). Once on the cytoplasmic side, the interaction with RanGAP initiates the GTPase activity of Ran, thus leading to subsequent release of Impα and Impβ, which are ready to participate in another cycle.

5.2.2 Regulation of nuclear protein import

Nuclear protein import can be regulated at the level of the NPCs, the transport receptors and the cargo proteins themselves. An NLS is required for karyopherin mediated binding and transport. Masking the NLS via protein:protein interaction represents an effective strategy to prevent recognition by the import receptors. For example, NFκB is bound to IκB under basal conditions, thus masking the NLS (489). Upon activation, IκB is phosphorylated and degraded, leaving the NLS exposed on p65 for the import receptor to bind and translocate into the nucleus (490). Post-translational modifications, such as

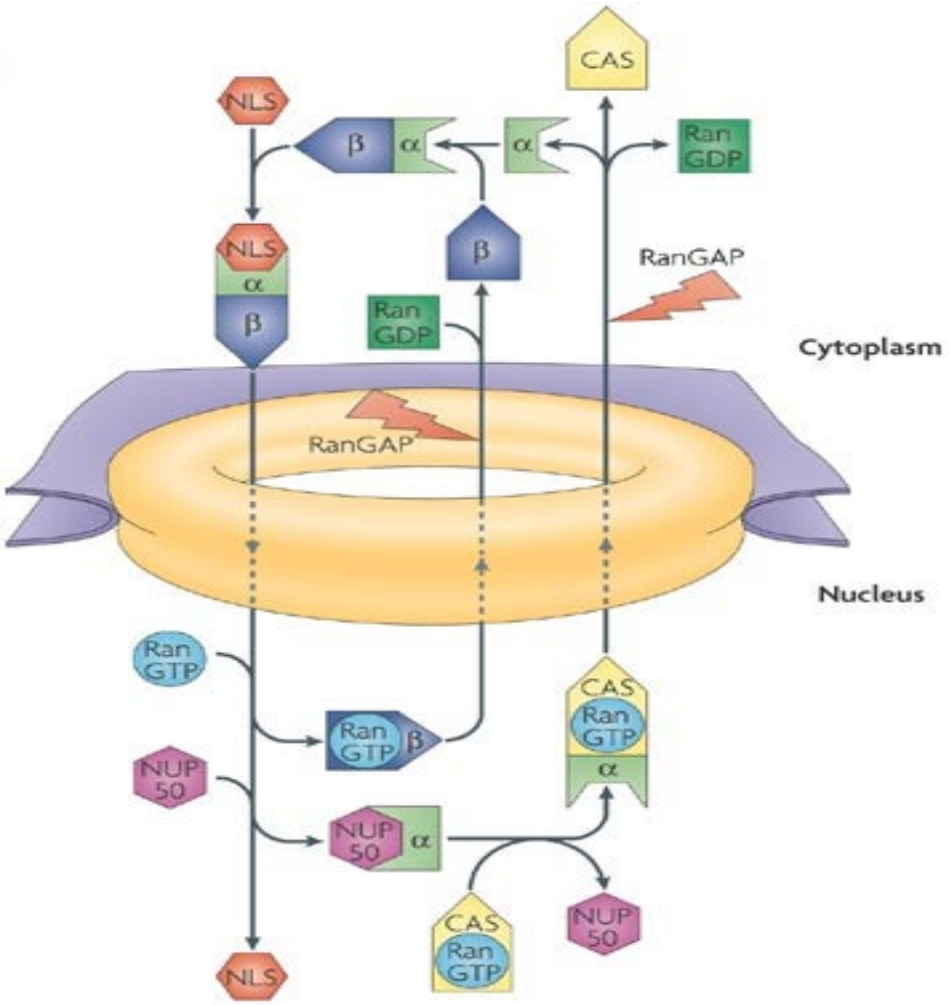

Figure 5. Nuclear protein import
An import complex is formed in the cytoplasm between cargoes bearing nuclear localization signals (NLSs), importin-α and importin-β. After passing through the nuclear pore complex (NPCs), the binding of RanGTP to importin-β dissociates importin-β from importin-α. The NLS-containing cargo is then displaced from importin-α and the importin-α is recycled to the cytoplasm by its nuclear export factor, CAS, complexed with RanGTP. In the cytoplasm, RanGAP stimulates GTP hydrolysis, releasing the importins for another import cycle. Nucleoporins such as NUP50 catalyse cargo dissociation and function as molecular ratchets to prevent futile cycles Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, M Stewart, Molecular mechanism of the nuclear protein import cycle, © 2007. (471) http://www.nature.com/doi:10.1038/nrm2114

phosphorylation, within or near the NLS can enhance receptor binding and affinity. Phosphorylation upstream of the SV40 large T antigen NLS by protein kinase CK2 enhances import 50-fold, possibly by increasing access for importin α1 receptor (491,492). In addition, phosphorylation of a serine residue in the Epstein-Barr nuclear antigen 1 NLS increases the binding affinity for Impα5, which in turn enhances nuclear import (493).

The availability of transport receptors (e.g. Impα/Impβ) as well as accessory proteins involved in NPI can influence transport parameters. Increasing levels of Impβ and other karyopherins enhances transport efficiency and rate of NPI (494,495). In addition, Impα, Ran and NTF2 all have the ability to play rate-limiting roles in NPI experimentally (496,497). Inhibition of NTF2 function with the use of NTF2 antibodies inhibited NPI (498). Alternatively, sequestration of Impα in the nucleus during stress conditions prevents recycling back to the cytoplasm and inhibits NPI(499-501).

NPCs are dynamic in nature as their density and size can be altered during metabolic, ionic and proliferative changes in the cell (113,502-506). They are also modulated throughout the cell cycle, with de novo formation during interphase and reversible disassembly during mitosis (507-509). During proliferation, the expression of integral FG-Nups, such as Nup62 and Nup153, is up-regulated indicating an increase in NPC number (113,115,343). The expression of these nucleoporins positively correlated with the rate of NPI (113,115,343). As such, an increase in NPCs directly impacts NPI potentially by increasing docking sites for transport receptors. Post-translational

modifications of the NPC are known to modulate pore function. Phosphorylation and glycosylation of multiple nucleoporins, including Nup50, Nup62, Nup88, Nup98, Nup153, and Nup214 have been noted under a variety of cellular conditions (510-512). These modifications alter the binding affinity of these nucleoporins for karyopherin β receptors and can regulate both nuclear import and export (510,512). Mitogen-activated protein kinases are suggested to play key roles in this process. MAPK activation has been involved in both enhancing and inhibiting NPI (113,115,513,514). Furthermore, MAPK family members associate with the NPC and directly mediate phosphorylation of Nup30, Nup 50, Nup153 and Nup214 (512,515). The NPC can also adopt different conformations in response to metabolic conditions that dictate pore permeability. Ca^{2+} depletion results in NPC closure and inhibition of import via passive diffusion and a receptor-mediated mechanism (504,506). In addition, ATP/GTP depletion also leads to NPC closure but selectively only inhibits receptor-mediated nuclear import (506).

5.3. Nuclear protein import in cardiovascular pathologies

Nuclear protein transport represents an integral regulator of cellular responses. Alterations of nuclear protein transport can result in pathological consequences. These mechanisms are well defined for viral infection and cancer, however, their impact on cardiovascular disease development is not as fully understood.

Experimentally, NPI can be altered in both VSMCs and cardiomyocytes contributing to pathological responses. Treatment of VSMCs with oxLDL, a major cardiovascular risk factor, results in a biphasic response in the rate of NPI with an

increase during short exposure and a decreased rate with prolonged exposure (115). The biphasic states coincide with cell proliferation (short exposure) and cell apoptosis (long exposure) (115). Mechanically stretching VSMCs, mimicking hypertension, can also induce an increase in rate of NPI and proliferation (113). This response is further enhanced with the addition of low dose oxLDL (343). The increases in the rate of NPI in these studies correlated with an up-regulation in Nup62 expression (113,115,343). Alternatively, factors relevant to atherosclerosis like ceramide and H_2O_2 inhibit NPI partly via a relocalization of the nuclear transport accessory proteins in the cytoplasm (eg. Impα, CAS, Ran) (514,516). Both VSMC proliferation and apoptosis are involved in the pathogenesis of atherosclerosis. We can postulate that NPI regulates cell fate decisions of VSMCs during atherosclerotic development. However, to date, no information is available for NPI status within atherosclerotic plaques.

Alterations in proteins associated with nuclear transport have also been reported for the heart. A mutation in the gene encoding Nup155 leads to atrial fibrillation and sudden death (517). This mutation or reduction in Nup155 resulted in impaired Hsp70 import and Hsp70 mRNA export (517). Experimental hypertrophy in isolated cardiomyocytes induced a reversible inhibition of nuclear import of histone H1 proteins by relocalizing Ran and closing the cytoplasmic side of the NPC, thus favouring nuclear export (518). Furthermore, inhibition of export resulted in restored import (518). Direct alterations in the expression and localization of nuclear transport components have been reported for human heart failure (519-521). The expression of transport receptors, Impβ3, Impα2, Crm1, and exportin-4 are elevated in heart samples from patients

suffering from ischaemic cardiomyopathy (ICM) and diabetic cardiomyopathy (DCM) (519). RanGAP expression is also elevated in failing hearts (519). Interestingly, in failing hearts Impα2 localizes in the nucleus, which could indicate increased import but may also reveal impaired recycling via nuclear sequestration (519). Furthermore, heart failure (ICM or DCM) also induced differential induction of structural and functional nucleoporin expression, with up-regulation in Nup62, Nup160, Nup153, Nup93 (DCM only), and NDC1 but without an effect on Nup155 or TPR expression (519-521). Despite increases in nucleoporins, heart failure does not increase NPC density (519) most likely due to a concurrent increase in nuclear size (521). The collective results from this human pathological data would indicate that the pore and nuclear transport is capable of adaptive changes during disease conditions and that there is an increased capacity for nuclear protein transport during heart failure. However, the direct effects on the import/export of proteins remains unclear and warrant further investigation.

CHAPTER II : RATIONALE AND HYPOTHESES

1. *C. pneumoniae* infection can stimulate VSMC proliferation *in vitro* and accelerate atherosclerorosis *in vivo*, thus we hypothesize that *C. pneunomiae* infection will directly induce structural remodelling via stimulation of VSMC proliferation in porcine coronary arteries.

2. *C. pneumoniae* infection can stimulate endothelial activation *in vitro* and stimulate endothelial dysfunction *in vivo*, thus we hypothesize that *C. pneumoniae* infection will directly impair vascular contractile function parameters in porcine coronary arteries.

3. Stress mediated VSMCs proliferation coincides with the induction of endogenous Hsp60 and alterations in NPI, thus we hypothesize that *C. pneumoniae* infection and atherosclerotic development will alter NPI parameters via an Hsp60 mediated mechanism

CHAPTER III: OBJECTIVES

The following objectives aim to address all three hypotheses:

1. To develop an ex vivo model for *C. pneumoniae* infection of the arterial wall
2. To evaluate the migration and growth of *C. pneumoniae* within the arterial wall of porcine coronary arteries

The following objectives aim to address the first hypothesis:

3. To determine the effect *C. pneumoniae* infection on the structural morphology of porcine coronary arteries
4. To evaluate the mechanisms responsible for structural alterations induced by *C. pneumoniae* infection in porcine coronary arteries

The following objectives aim to address the second hypothesis:

5. To determine the effect of *C. pneumoniae* infection on the vascular contractile function in porcine coronary arteries
6. To identify the underlying mechanisms for *C. pneumoniae* mediated alterations in vascular contractile performance

The following objectives aim to address the third hypothesis:

7. To determine the effect of Hsp60 overexpression on nuclear protein import in VSMCs
8. To identify mechanisms for Hsp60 mediated alterations in nuclear protein import
9. To determine the expression of NPI related components in response to *C. pneumoniae* infection
10. To determine the expression of NPI related components and heat shock proteins during atherosclerotic development

CHAPTER IV: MATERIALS AND METHODS

Reagents

Product	Source
9,11-Dideoxy-11α, 9α-epoxy-methanoprostaglandin $F_{2\alpha}$ (u46619)	Sigma-Aldrich Canada Co.(Oakville, ON)
Acetic acid	Fisher Scientific (Nepean, ON)
Acetone	Sigma-Aldrich Canada Co.(Oakville, ON)
Amido black staining solution	Sigma-Aldrich Canada Co.(Oakville, ON)
Ammonium persulfate	Sigma-Aldrich Canada Co.(Oakville, ON)
Amphotericin B	Life Technologies (Canada) Corp. (Burlington, ON)
Antigen unmasking solution	Vector Laboratories (Canada) Inc. (Burlington, ON)
Ascorbate	Mallinckrodt Inc. (St. Louis, MO)
ATP	Sigma-Aldrich Canada Co.(Oakville, ON)
BCA protein assay kit	Pierce (Rockford,Il)
Benzamidine	Sigma-Aldrich Canada Co.(Oakville, ON)
Bis-acrylamide	BioRad Laboratories (Canada) Ltd. (Mississauga, ON)
BODIFY FL-conjugated BSA	Life Technologies (Canada) Corp. (Burlington, ON)
Bovine Serum Albumin	Sigma-Aldrich (St-Louis, MO)
Bradykinin acetate	Sigma-Aldrich Canada Co.(Oakville, ON)
Calcium chloride	Sigma-Aldrich Canada Co.(Oakville, ON)
CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay	Promega (Madison, WI)
CellTiter-Glo Luminescent Cell Viability Assay	Promega, Madison, WI
Cycloheximide	Sigma-Aldrich Canada Co.(Oakville, ON)
DAPI	Sigma-Aldrich Canada Co.(Oakville, ON)
Dextrose	Mallinckrodt Inc. (St. Louis, MO)
Dihydroethidium	Life Technologies (Canada) Corp. (Burlington, ON)
DMEM	Life Technologies (Canada) Corp. (Burlington, ON)
DNase I	Worthington Biochemicals, (Freehold, NJ)
Dithiothreitol (DTT)	Sigma-Aldrich Canada Co.(Oakville, ON)
EDTA	Sigma-Aldrich Canada Co.(Oakville, ON)
EGM-2	Lonza, Walkersville, MD

Product	Source
EGTA	Sigma-Aldrich Canada Co.(Oakville, ON)
Elastic Staining Kit	Sigma-Aldrich St-Louis, MO
Eosin	Ricca Chemical Company (Arlington, TX)
Ethanol	Fisher Scientific (Nepean, ON)
FBS	Life Technologies (Canada) Corp. (Burlington, ON)
FluorSave reagent	EMD Millipore (Billerica, MA)
Formalin	Sigma-Aldrich Canada Co.(Oakville, ON)
FragEL ™ DNA fragmentation detection kit	EMD Millipore, Billerica, MA
Fungizone	Life Technologies (Canada) Corp. (Burlington, ON)
Glycine	Fisher Scientific (Nepean, ON)
Glutamic acid	Sigma-Aldrich Canada Co.(Oakville, ON)
Glycerol	Fisher Scientific (Nepean, ON)
Hematoxylin	Sigma-Aldrich Canada Co.(Oakville, ON)
HEPES	Life Technologies (Canada) Corp. (Burlington, ON)
Hoescht 33342	Sigma-Aldrich Canada Co.(Oakville, ON)
Hydrochloric acid	Fisher Scientific (Nepean, ON)
Insulin	Sigma-Aldrich Canada Co.(Oakville, ON)
KCl	Sigma-Aldrich Canada Co.(Oakville, ON)
Leupeptin	Sigma-Aldrich Canada Co.(Oakville, ON)
Luminata Forte Western HRP Substrate	Millipore (Billerica, MA)
Magnesium chloride	Fisher Scientific (Nepean, ON)
Magnesium sulfate	Sigma-Aldrich Canada Co.(Oakville, ON)
β-Mercaptoethanol	Sigma-Aldrich Canada Co.(Oakville, ON)
Methanol	EMD Millipore (Billerica, MA)
Mitochondrial isolation kit	Pierce (Rockford,Il)
MitoTracker Red CMXRos	Life Technologies (Canada) Corp. (Burlington, ON)
Nitrocellulose membranes	Pall (Canada) Ltd. (Mississauga, ON)
Optimal cutting temperature (OCT) compound	Sakura Finetek (The Netherlands)
Paraformaldehyde	Alfa Aesar (Ward Hill, MA)
Penicillin	Life Technologies (Canada) Corp. (Burlington, ON)
Phosphate saline buffer	Life Technologies (Canada) Corp. (Burlington, ON)
PMSF	Sigma-Aldrich Canada Co.(Oakville, ON)
Poncean S solution	Sigma-Aldrich Canada Co.(Oakville, ON)

Product	Source
Potassium chloride	Fisher Scientific (Nepean, ON)
Potassium phospate monobasic	Sigma-Aldrich Canada Co.(Oakville, ON)
Prolong Gold antifade reagent	Life Technologies (Canada) Corp. (Burlington, ON)
Selenium	Sigma-Aldrich Canada Co.(Oakville, ON)
Serum cholesterol kit	Genzyme Canada (Mississauga, ON)
SimplyBlue™ SafeStain	Life Technologies (Canada) Corp. (Burlington, ON)
Sodium bicarbonate	Fisher Scientific (Nepean, ON)
Sodium chloride	Alfa Aesar (Ward Hill, MA)
Sodium dodecyl sulfate (SDS)	Fisher Scientific (Nepean, ON)
Sodium deoxycholate	Sigma-Aldrich Canada Co.(Oakville, ON)
Sodium hydroxide	Sigma-Aldrich Canada Co.(Oakville, ON)
Sodium nitroprusside	Sigma-Aldrich Canada Co.(Oakville, ON)
Sodium phosphate dibasic	Sigma-Aldrich Canada Co.(Oakville, ON)
Sodium phosphate monobasic	Fisher Scientific (Nepean, ON)
Streptomycin	Life Technologies (Canada) Corp. (Burlington, ON)
Sucrose	Fisher Scientific (Nepean, ON)
Supersignal West Femto Chemiluminescent Substrate	Pierce (Rockford,Il)
Supersignal West Pico Chemiluminescent Substrate	Pierce (Rockford,Il)
TEMED	Sigma-Aldrich Canada Co.(Oakville, ON)
Transferrin	Life Technologies (Canada) Corp. (Burlington, ON)
Trichloroacetic acid	Sigma-Aldrich Canada Co.(Oakville, ON)
Tris-HCL	Sigma-Aldrich Canada Co.(Oakville, ON)
Tris-OH	Sigma-Aldrich Canada Co.(Oakville, ON)
Triton X-100	Fisher Scientific (Nepean, ON)
TrypLE™ Express	Life Technologies (Canada) Corp. (Burlington, ON)
Trypsin/EDTA	Life Technologies (Canada) Corp. (Burlington, ON)
Tween-20	Fisher Scientific (Nepean, ON)

Antibodies

Primary Antibody	Type	Host	Source
α-Actin (smooth muscle)	Monoclonal	Mouse	Sigma-Aldrich Canada Co.(Oakville, ON)
Actin (total)	Polyclonal	Rabbit	Sigma-Aldrich Canada Co.(Oakville, ON)
Chlamydia genus	Monoclonal	Mouse	Argene (France).
COX IV	Monoclonal	Mouse	Santa Cruz Biotechnology (Dallas, TX)
eNOS	Monoclonal	Mouse	BD Biosciences (Mississauga, ON)
GAPDH	Monoclonal	Mouse	Abcam (Cambridge, MA)
Hsp10	Monoclonal	Mouse	Enzo (Farmingdale, NY)
Hsp27	Monoclonal	Mouse	Enzo (Farmingdale, NY)
Hsp60 (chlamydial)	Monoclonal	Mouse	Affinity BioReagents (Golden, CO).
Hsp60 (mammalian)	Monoclonal	Mouse	Stressgen (Ann Arbor, MI).
Hsp70			Stressgen (Ann Arbor, MI).
Hsp90	Monoclonal	Mouse	Enzo (Farmingdale, NY)
Hemagglutinin (HA)	Monoclonal	Mouse	Abcam (Cambridge, MA)
IκB-α	Polyclonal	Rabbit	Cell Signaling (Danvers, MA)
Importin-α	Monoclonal	Mouse	BD Biosciences (Mississauga, ON)
Importin-β	Monoclonal	Mouse	Abcam (Cambridge, MA)
iNOS	Polyclonal	Rabbit	Abcam (Cambridge, MA)
IP3-1 receptor	Polyclonal	Rabbit	Alomone Labs (Israel)
L-type voltage-gated Ca^{2+} channel	Polyclonal	Rabbit	Alomone Labs (Israel)
Myosin heavy chain (smooth muscle)	Monoclonal	Mouse	Sigma-Aldrich Canada Co.(Oakville, ON)
MAb414 (Nup62)	Monoclonal	Mouse	Covance, Princeton, NJ
NFκB	Monoclonal	Mouse	EMD Millipore (Billerica, MA)
Nup153	Monoclonal	Mouse	Covance (Princeton, NJ)
NTF2	Monoclonal	Mouse	BD Biosciences (Mississauga, ON)
p22-phox	Polyclonal	Rabbit	Santa Cruz Biotechnology (Dallas, TX)
p38 MAPK	Polyclonal	Rabbit	Cell Signaling (Danvers, MA)
p44/42 MAPK	Polyclonal	Rabbit	Cell Signaling (Danvers, MA)
PCNA	Monoclonal	Mouse	Zymed (Carlsbad, Ca)

Primary Antibody	Type	Host	Source
Phospho-p38 MAPK	Monoclonal	Mouse	Cell Signaling (Danvers, MA)
Phospho-p44/42 MAPK	Monoclonal	Mouse	Cell Signaling (Danvers, MA)
Ran	Monoclonal	Mouse	BD Biosciences (Mississauga, ON)
α-Tubulin	Monoclonal	Mouse	Abcam (Cambridge, MA)

Secondary Antibody	Type	Host	Conjugate	Source
Anti-mouse IgG	Monoclonal	Goat	HRP	EMD Millipore (Billerica, MA)
Anti-mouse IgG	Monoclonal	Goat	Alexa488	Life Technologies (Canada) Corp. (Burlington, ON)
Anti-mouse IgG	Monoclonal	Donkey	Texas Red	Jackson Laboratory (Bar Harbor, ME)
Anti-rabbit IgG	Monoclonal	Goat	HRP	Life Technologies (Canada) Corp. (Burlington, ON)
Anti-rabbit IgG	Monoclonal	Goat	Alexa488	EMD Millipore (Billerica, MA)

Methods common to all studies

Propagation of C.pneumoniae

C. *pneumoniae* AR39 strain was obtained from the University of Washington, Seattle, Wash. The organism was propagated in HL cells as described previously (522). The purified organism was resuspended in chlamydial sucrose-phosphate-glutamate medium (SPG) and stored at -80°C until use. The titer of C. *pneumoniae* was determined in cycloheximide-treated HL cells, and concentrations used were expressed as inclusion-forming units (IFU) per ml (523).

Porcine coronary explant and organ culture

The coronary artery explant and organ culture method has been adapted from Saward et.al (524). Whole hearts from 10-month old neutered male swine were obtained from a local abattoir. The left anterior descending coronary artery was flushed with standard phosphate-buffered saline (PBS) supplemented with an antibiotic mixture (penicillin G, ,1500 units/ml; streptomycin sulfate, 1500 µg/ml; amphotericin B, 2.5 µg/ml) and subsequently dissected out of the heart and cleaned of adhering fat and connective tissue. The explanted artery was cut into segments of 5mm in length and incubated at 37°C and 5% CO_2 in organ culture medium (DMEM supplemented with 20% FBS) supplemented with antibiotics (penicillin G ,1500 units/ml; streptomycin sulfate, 1500 µg/ml; amphotericin B, 2.5 µg/ml). Every 24 hours, explants were placed in fresh organ culture medium with gradually decreasing amount of antibiotics. At 72 hrs, the coronary segments were washed with PBS and incubated in the organ culture medium for 3 hours. The segments were then infected with *C. pneumoniae.* No antibiotics were used for the duration of the experiment.

Infection of coronary segments with C. pneumoniae

To perform the infection, each coronary segment was placed into an isolated well of a 96-well culture plate. The segments were oriented upright within the well so that *C. pneumoniae* (5x 10^6 IFU in 100ml) could be applied directly into the lumen of each coronary vessel. After three hours of incubation at 37°C and 5% CO_2 , each coronary segment was transferred to an isolated well of a 24-well plate containing 1.5ml of organ

culture medium. The vessels were incubated for a maximum of 10 days. The incubation medium was changed every 48 hrs. Coronary segments were collected for analysis immediately after infection and from day 2 to day 10 post-infection (pi.). Heat inactivated *C. pneumoniae* (mock-infection) was used for controls in each experiment.

Statistical analysis

Data are presented as mean ± SEM unless otherwise stated. Differences between treatment groups were assessed by one-way ANOVA using the Student-Newman-Keuls post-hoc test. A probability of $p < 0.05$ was considered statistically significant. In addition for Project 3, Pearson correlation and linear regression were used to assess the relationship between HSP60 expression levels and PCNA, Nup62, and serum cholesterol.

1. Effects of *C.pneumoniae* infection on structural remodelling in porcine coronary arteries [3]

Histological staining

To determine the extent of media thickening, coronary segments were collected at four time points pi: day-4, day-6, day-8 and day-10. The extent of media thickening in *C. pneumoniae* infected segments was compared with corresponding mock-infected control segments. The segments were fixed in 10% formalin and mounted in *Tissue-tek* O.C.T compound prior to being cut into 6 μm cross-sections. Hematoxylin and eosin (H/E) staining as well as elastic staining were applied to these cross-sections. Images of the

[3] Reprinted from Am J Path 176(2), JF Deniset et al., *Chlamydophila pneumoniae* infection leads to smooth muscle cell proliferation and thickening in the coronary artery without contributions from a host immune response. 1028-1037, © 2010, with permission from Elsevier. http://www.sciencedirect.com/science/journal/

cross-sections were obtained at 4x magnification (*Nikon TE-2000s*). Imaging software (*Adobe photoshop CS3 extended*) was used to quantify the pixel area of the lumen, media and total vessel for each coronary cross-section.

Western blot analysis

Coronary segments were collected at four time points: day-0 (prior to infection), days 2-4, days 5-6, and days 8-10 pi. These collection intervals were chosen to coincide with the C. *pneumoniae* replication cycle. Three independent experiments were performed. In each experiment, two coronary segments per time point per treatment were collected, rinsed with PBS, flash-frozen in liquid nitrogen and subsequently stored at -80°C. Paired coronary segments were ground and resuspended in RIPA buffer (50mM TrisHCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 0.1% SDS, 0.5% Na Deoxycholate, 1ug/ml Leupeptin, 1mM PMSF, 1mM protease inhibitor cocktail, 1mM DTT and 1mM Benzamidine). Proteins were separated using a 10% denaturing polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane. Membranes were incubated with anti-chlamydial Hsp60, anti-mammalian Hsp60 or anti -PCNA primary antibodies. HRP-conjugated anti-mouse IgG was used as a secondary antibody. GAPDH was used as a loading control. Bands were visualized with the Supersignal West Pico Chemiluminescent Substrate (*Pierce*) and subsequently quantified by densitometry (Software: Quantity One, Bio-Rad). Densitometry values for targeted proteins were normalized by GAPDH band values within the same lane.

Immunofluorescent microscopy

Infected and mock-infected coronary segments were collected at various time points pi: day-2, day-4, day-6, day-8 and day-10. The segments were fixed in ethanol, rehydrated and subsequently equilibrated in 30% sucrose solution prior to mounting in *Tissue-tek* OCT compound. These OCT mounted segments were slowly frozen to -80°C, cut (HM 500 OM Cryostat, Microm) into 8-µm sections and placed on positively coated glass slides (Fisher). OCT was washed off the glass slides with PBS and antigen retrieval was performed with the Unmasking solution (*Vector Laboratories*) at 65°C for 25 minutes. To detect C. *pneumoniae* in the specimen, anti–Chlamydia genus antibody (1:100) was applied according to manufacturer's protocol. Alexa-488-conjugated anti-mouse IgG (1:800) was used as a secondary antibody. To localize PCNA expression within the vessel, anti-smooth muscle actin (1:500) and anti-PCNA (1:100) were used. Texas Red-conjugated anti-mouse IgG (1:1000) and Alexa-488-conjugated anti-rabbit IgG (1:1000) were used as secondary antibodies for the respective primary antibodies. Hoescht staining solution (5ng/ml) was added to the slides to identify nuclei. Slides were then fixed with FluorSave™ reagent to preserve fluorescence. The location and number of *C. pneumoniae* inclusion bodies (IB) in the coronary cross-sections were visualized as green fluorescent signals at 40x magnification on an inverted microscope (*Nikon, TE-2000s*). In each field of view, the number of fluorescent inclusion bodies (IB) was calculated using imaging software (*Adobe photoshop CS3 extended*). To identify apoptotic cells within the vessel, terminal dUTP nick-end labeling (TUNEL) assays using

the FragEL DNA fragmentation detection kit were performed on coronary cross-sections. Cross-sections pre-treated with DNase I were used as positive controls.

Vascular function assessment

To determine tissue viability over the duration of the experiment, untreated coronary segments were collected at days 0, 5 and 10 pi to coincide with the start, mid-point and end of the infection timeline. These tissues were used for vascular function experiments. The 5mm coronary rings were fastened in an organ bath with surgical wire, perfused with Krebs-Henseleit solution, aerated with 95% O_2 and 5% CO_2, and equilibrated at 37°C and pH 7.4. Vascular function was measured with a force transducer (FSG-01/20, Experimentia Ltd, Budapest, Hungary) as mechanograms of tension. The coronary segments were brought to a basal tension of 5g and then contracted three times with 47mM KCl, with washout periods using Krebs solution between each contraction. To assess relaxation responses, rings were pre-contracted with 30nM u46619 and allowed to reach a steady state of contraction. Bradykinin was then administered without washout at concentrations of 10^{-10} to 10^{-6} M to develop an endothelial dependent response curve. After washout and a second pre-contraction with 30nM u46619, sodium nitroprusside (SNP) was administered at concentrations of 10^{-7} to 10^{-4} to develop an endothelial independent response curve. Following a washout, a dose-response curve to u46619 was constructed with concentrations of 0.3-300 nM.

2. Effects of *C. pneumoniae* infection on vascular contractile function in porcine coronary arteries [4]

Vascular function assessment

Coronary segments were collected at days 4-5 and 9-10 post-infection to assess vascular contractile function prior to and following *C. pneumoniae* mediated vascular hypertrophic responses. Both vascular contractile and relaxation responses were evaluated using the experimental protocol as previously described (525). All functional experiments were completed within a laminar flow biosafety cabinet to prevent spread of infection.

Treatment of HUVECs with conditioned medium

HUVECs were obtained from Lonza (Walkersville, MD) and maintained in culture conditions with EGM-2. Cells from P3-P6 were used for experiments. HUVECs were plated at $2x10^6$ cells per well in a 6-well plate. Organ culture medium was collected every 48 hours during media changes. Media from similar treatments were combined in pairs, a portion (2 ml) of the medium was placed directly over HUVECs plated in 6 well plates. HUVECs were collected at 24 hours following incubation with conditioned medium. The additional medium (1ml) was collected and spun at 1105 rpm for 5 min. The supernatant was collected and stored at -80C for protein analysis.

[4] Reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila pneumoniae* infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. http://www.sciencedirect.com/science/journal/

Western Blot Analysis

To assess protein expression levels within treated vessels, coronary segments were collected at days 4-5 and 9-10 post infection and prepared as described above. HUVECs collected following conditioned medium treatment were re-suspended in RIPA buffer, sonicated, centrifuged at 14000 rpm for 10 minutes at 4 °C and the supernatant was collected for western blotting. Protein assays were performed to determine concentration within tissue and HUVEC lysates. Lanes were loaded with 5 µg tissue lysate for detection of smooth muscle actin and myosin and 30 µg protein for the other targeted proteins. Tissue samples were run in duplicate, with half of the blot used to probe with antibodies and the other was stained with amido black for loading control purposes. For HUVEC lysates, lanes were loaded with 30 µg protein. Proteins were separated using a 4-12% gradient denaturing polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane. Membranes were incubated with anti-smooth muscle actin (1:20000), anti- myosin heavy chain (MHC-smooth muscle) (1:1000), anti-IP3 receptor-1(1:500), anti-L-type voltage-gated Ca^{2+} channel (1:500), anti-eNOS (1:200), anti-iNOS (1:500), anti-p22phox (1:500), anti-NFκB (1:1000), anti-IκB (1:1000), or anti-GAPDH (1:5000) antibodies. HRP conjugated anti-mouse IgG and anti–rabbit IgG were used as secondary antibodies. Bands were visualized with the Supersignal West Pico or Femto Chemiluminescent Substrates (Pierce). Bands and Amido Black staining were quantified by densitometry (Software: Quantity One, Bio-Rad). Protein expression for tissue and HUVEC lysates were normalized by amido black staining and GAPDH, respectively.

Protein concentrations of cHSP60 and hHSP60 within the conditioned medium were also assessed using western blot methodology. A trichloroacetic acid (TCA) precipitation protocol was performed to concentrate protein in conditioned medium. In short, 250 µl conditioned medium was combined with ½ volume of 50% TCA, precipitate was spun down, washed with acetone, spun and pellet was reconstituted in 40 µl 0.5M NaOH and 40 µl 2x SDS-PAGE loading buffer. 40 µl concentrated protein lysate from each treatment at days 4, 6, 8 and 10 pi were loaded alongside varying amounts of recombinant cHsp60 or hHsp60 (1- 0.01 µg protein) employed to construct a standard curve. Proteins were separated using a 9% denaturing polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane. Membranes were incubated with either anti-cHSP60 (1:1000) or anti-hHSP60 (1:1000) antibodies and HRP-conjugated anti-mouse IgG was used as secondary antibody. Bands were visualized with the Supersignal West Pico or Femto Chemiluminescent Substrates (Pierce). Bands and Amido Black staining were quantified by densitometry (Software: Quantity One, Bio-Rad).

In situ Superoxide Detection and Immunofluorescence Staining

Coronary segments were collected at days 9-10 post-infection, fixed in ethanol, rehydrated and subsequently equilibrated in 30% sucrose solution prior to mounting in *Tissue-tek* OCT compound . These OCT mounted segments were slowly frozen to -80°C, cut (HM 500 OM Cryostat, Microm) into 8 µm sections and placed on positively coated glass slides. To evaluate protein expression within coronary sections, antigen retrieval was first performed as described above. Subsequently, sections were incubated

with anti-eNOS(1:25), anti-p22phox (1:50), anti-TLR4 (1:10), anti-cHsp60 (1:50) and anti-hHsp60 antibodies. Texas Red-conjugated anti-mouse IgG (1:750), Alexa-488-conjugated anti-mouse IgG (1:1000) and Alexa-488-conjugated anti-rabbit IgG (1:1000) were used as secondary antibodies for the respective primary antibodies. Hoescht staining solution (5ng/ml) was added to the slides to identify nuclei. To determine the levels of superoxide anions within the vessels, sections were incubated with dihydroethidium as previously described (526). Slides were fixed with FluorSave™ reagent to preserve fluorescence. Staining was visualized using a fluorescent inverted microscope (*Nikon, TE-2000s*) and mean intensity was quantified using imaging software (Nikon, NIS Elements).

3. Role of Hsp60 in modulating nuclear protein import during VSMC proliferation

Vascular smooth muscle cell isolation and culture

Primary VSMCS were isolated from the aorta of New Zealand White rabbits using an explant technique as previously described (113). The animal use was according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Approval was obtained for the conduct of this study from the Animal Care Protocol and Review Committee of the University of Manitoba. To induce synchronization, VSMCs were placed in serum-free DMEM supplemented with transferrin (5μg/ml), selenium (1nmol/L), ascorbate (200μmol/L), and insulin (10nmol/L) for 72 hours prior to the start of experiments.

Adenoviral vectors

Recombinant adenovirus-encoding human Hsp60 (AdHsp60) or human Hsp60 lacking the mitochondria-targeting sequence (AdHsp60^{mito-}) were constructed using the AdEasy Vector System (Qbiogen) as previously described (161). A hemaglutinin (HA) tag was fused in frame to the Hsp60 sequence. The recombinant adenoviral constructs were then transfected into QBI-293A cells to produce viral particles. Titer of the viral stock was determined using the tissue culture infectious dose 50 ($TCID_{50}$) method. A successful transfer of a reporter gene into 100% of VSMCs was achieved with a MOI (multiplicity of infection) of 100 to 200 viral particles/cell after 48 hours of incubation in 0.5% FBS supplemented DMEM. An empty virus (AdQBI) was utilized as an infection control.

Infection protocol

VSMCs were seeded at $1.5x10^5$ cells/well in 6-well plates for western blot analysis, cellular fractionation and the determination of cellular ATP levels. For the nuclear protein import assay and immunocytochemistry, VSMCs were seeded at $4x10^4$ cells/well on glass coverslips in 6-well plates. For cell number determination VSMCs were seeded at $5x10^3$ cell/well in 96 well plates. Two separate sets of experiments were performed. First, cells were inoculated with *C. pneumoniae* ($1x10^5$ IFU/ml) in 1% FBS supplemented DMEM for up to 48 hours as previously described (161). Control cells were incubated for the same duration in a similar medium either free of *C. pneumoniae* (Control) or containing heat-inactivated *C. pneumoniae* (HI *Cpn*). For overexpression experiments, cells were infected with AdHsp60, AdHsp60^{mito-} or AdQBI (infection

control) at an MOI of 100-150 in 0.5% FBS-supplemented DMEM for 48 hours as previously described (161).

Measurement of nuclear protein import by confocal microscopy

Following 48 hours, coverslips were removed from the plate and placed in a Leyden dish containing 1 ml pre-warmed perfusate buffer (6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM dextrose, 6 mM HEPES, pH 7.4). Cells were maintained at 37 °C using a microperfusion chamber. An Alexa-BSA nuclear localization signal (NLS) fluorescent substrate was prepared in our laboratory by conjugating BODIPY-BSA to the SV40 large T antigen NLS (CGGGPKKKRKVED) (514). Thin walled glass capillary tubes (1.0 mm, 3 inch) were used to fashion micropipettes for cell injection. The capillary tubes were made using a Flaming/Brown micropipette puller (Sutter Instruments, Model p-97). The substrate was added to a micropipette, which was then inserted into the cell cytoplasm using an MS314 micromanipulator (Fine Science Tools). Cells were injected using a PV830 Pneumatic PicoPump (World Precision Instruments) and the pipette was slowly removed. Images of the cell after microinjection were acquired on a Bio-Rad MRC600 CLSM. Images were taken of the pre- and post-injected cells to observe the rate of nuclear import of the Alexa-BSA NLS fluorescent protein for each cell over time (up to 30 minutes). There is no movement of the fluorescent marker into the cell nucleus over this time frame when it does not contain an NLS. As previously discussed (514,527), the use of this marker protein is advantageous because, instead of measuring the import of one specific protein, it identifies regulatory factors and pathways applicable

to the nuclear import of all proteins. The ratio of nuclear fluorescence to cytoplasmic fluorescence was assessed using ImageJ software.

Atherosclerotic rabbit model

Male New Zealand White (NZW) rabbits weighing between 2.7 and 3.0 kg (Spilak Farms) upon arrival were housed in individual cages in rooms with controlled temperatures, humidity, and a 12-hour light cycle. The animal use was according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). The experimental protocols were reviewed and approved by the University of Manitoba Protocol Management Review committee. Rabbits were fed a 1% cholesterol-supplemented diet (Ren's Pet Depot) for 4 weeks and then placed on a regular rabbit chow (Nutrena, Nature Wise Performance Rabbit Formula), for an additional 0, 2, 4, 8, or 22 weeks. Control rabbits were fed a regular rabbit chow for 12 weeks. Prior to death, blood samples were taken from the jugular vein while the animal was anesthetized with isoflurane gas (5% with O_2 per minute). Samples were collected into EDTA vacutainer tubes (Becton Dickinson, Mississauga, ON, Canada) and centrifuged at 1,800x *g* for 15 min (4°C). Blood serum was removed and stored at -80°C. Serum cholesterol was measured enzymatically using a commercial kit. Following termination, aortas were immediately removed from the proximal aspect of the aortic arch to the base of the diaphragm. The extent of plaque accumulation on the vessel surface was determined by en face analysis, as previously described (528). In brief, the aorta was opened longitudinally and digitally photographed. Luminal images were analyzed using Nikon imaging software (Elements). The percent

lesion area was tabulated from the fraction of area covered with lesions relative to the total area of the aorta. Aortas were subsequently stored at -80°C for western blot analysis.

Western Blot analysis

VSMCs were harvested at 24 or 48 hours by trypsinization, then washed and lysed in sample buffer ($5x10^4$ cells/lane). Porcine coronary arteries and rabbit aortic arch segments were ground and re-suspended in RIPA buffer (50mM TrisHCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 0.1% SDS, 0.5% Na Deoxycholate, 1ug/ml Leupeptin, 1mM PMSF, 1mM protease inhibitor cocktail, 1mM DTT and 1mM Benzimidine). Samples were further processed by alternating freeze-thaw cycles, followed by sonication. After a spin at 14,000 rpm, the sample supernatant was collected, and protein concentrations were determined using a BCA protein assay kit. Proteins were separated on 9% or 12% polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane. Membranes were incubated with anti-mammalian Hsp60, anti-α-tubulin, anti-Hsp70, anti-Hsp90, anti-Hsp27, anti-Hsp10, anti-PCNA, anti-Mab414, anti-Nup153, anti-Importin-α, anti-Importin-β, anti-RAN, anti-NTF2, or anti-total actin primary antibodies. HRP-conjugated anti-mouse and anti-rabbit IgGs were used as secondary antibodies. Bands were visualized with Supersignal West Pico Chemiluminescent Substrate or Luminata Forte Western HRP Substrate and subsequently quantified by densitometry using Quantity One software (Bio-Rad). Bands and Amido Black staining were quantified by densitometry (Software: Quantity One, Bio-Rad).

Protein expression for porcine coronary tissue, rabbit aorta or VSMC lysate tissue were normalized by amido black staining, total actin and α-tubulin, respectively.

Immunocytochemistry

At 48hrs, cells on coverslips were washed 2x with 1xPBS and incubated with a MitoTracker Red CMXRos (80nM) for 20min at 37°C. Cells were then fixed with 3.7% paraformaldehyde for 15 min and permeabilized with 0.1% Triton-X100. Cells were blocked with 5% milk in PBS 0.1% Triton-X for 2 hours at room temperature and incubated overnight at 4°C with either anti-HSP60 or anti-HA tag primary antibodies in 1% skim milk in PBS 0.1% Triton-X100. Cells were then washed and incubated with Alexa 488-conjugated goat anti-mouse IgG in 1% skim milk in PBS 0.1Triton-X100 for 1 h in the dark. The cells were rinsed, treated with DAPI for 5 min, and inverted on a microscope slide using 10µl Prolong Gold antifade reagent. Stained cells were visualized using a 100x oil immersion objective on a Nikon TE 2000s microscope and images captured using NIS Elements software (Nikon).

Cellular fractionation

Cellular fractionation was performed on treated cells using a mitochondria isolation kit. In brief, cells from a 6-well plate were pooled, spun down and resuspended in PBS. Following Dounce homogenization and subsequent differential centrifugation, both the cytoplasmic and mitochondrial fractions were obtained. The mitochondrial pellet was directly resuspended in sample buffer. Cytosolic fractions were TCA precipitated first and then resuspended in sample buffer. Proteins were separated on a

15% polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane. Membranes were incubated with anti-Hsp60, anti-α-tubulin or anti-COX IV primary antibodies. HRP conjugated anti-Mouse IgG was used as the secondary antibody. Bands were visualized with Supersignal West Pico Chemiluminescent Substrate or Luminata Forte Western HRP Substrate and subsequently quantified by densitometry using Quantity One software (Bio-Rad). The levels of α-tubulin and COX-4 expression were used as loading controls for the cytosolic and mitochondrial fractions, respectively.

Determination of cellular proliferation and cellular ATP Levels

The increase in cell number in response to Hsp60 overexpression was determined by a colorimetric enzyme assay (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay) based on a cytoplasmic enzyme activity present in viable cells. To determine cellular ATP levels a CellTiter-Glo Luminescent Cell Viability Assay was employed. In brief, cells from 3 identical treatment wells were pooled, counted and redistributed in an opaque 96-well plate at $5x10^3$ cells/well in DMEM. ATP standards (10^{-4}-10^{-12}M) were made up in DMEM and distributed in duplicate. CellTiter-Glo reagent was added to each well and following an incubation period, luminescence was assessed using the GLOMAX Multi+ detection system (Promega).

CHAPTER V : RESULTS

1. Effects of *C. pneumoniae* infection on structural remodelling in porcine coronary arteries [5]

Isolated coronary segments maintain functional viability in culture

Vascular function was determined in untreated coronary vessels at days 0, 5 and 10 pi. There were no significant changes in maximal contractile force development to KCl or to u46619 at any of the time points (Figure 6A and 6B). Furthermore, both endothelial-dependent and -independent relaxation response curves to bradykinin and SNP, respectively, were maintained for the duration of the experiment (Figure 6C and 6D).

C. pneumoniae propagates in the coronary arterial environment

Chlamydial inclusion bodies (IB) are represented as green fluorescent signals within the coronary cross-sections (Figure 7). Magnified views of both the endothelial layer (bottom right insert) and the medial layer (top left insert) are provided within each figure panel for improved visualization of inclusion body distribution. *C. pneumonia* infected tissues displayed the presence of IBs at all time points, with variations in their distribution, number and intensity. On day 2 pi, intense green fluorescence was localized in the endothelial layer but not in the smooth muscle cells (Figure 7A). On day-4 pi, *C. pneumoniae* IBs were still detected in the endothelium but were now also visible in the smooth muscle layer (Figure 7B). This distribution trend continued through days 6, 8,

---

[5] Reprinted from Am J Path 176(2), JF Deniset et al., *Chlamydophila pneumoniae* infection leads to smooth muscle cell proliferation and thickening in the coronary artery without contributions from a host immune response. 1028-1037, © 2010, with permission from Elsevier. http://www.sciencedirect.com/science/journal/

and 10 pi. At these time points, IBs were primarily localized in the media and only occasionally in the endothelium (Figures 7C, 7D, 7E). The number of IBs were quantified (Figure 8A), with the exception of IBs at day-2 pi, when it was too difficult to resolve the continuum of green fluorescence in the endothelial layer into individual IB units. From days 4 to 10 pi, a significantly ($p<0.0003$) increasing number of IB was observed. *C.pneumoniae* IBs at days 8 and 10 pi were also noticeably larger in size than IBs detected at earlier day points. There were no *C. pneumoniae* IBs detected in mock-infected controls. To further confirm the metabolic activity of the *C. pneumoniae* infection, chlamydial Hsp60 expression was quantified by western blot analysis (Figure 8B). From days 4 to 10 pi, expression of chlamydial Hsp60 significantly increased from 1.67 ± 0.07 to 2.13 ± 0.33 fold ($p< 0.01$) over baseline values (the amount of chlamydial Hsp60 detected at 3 hrs). Chlamydial Hsp60 was not observed in mock-infected control. These results demonstrate that *C. pneumoniae* was able to infect isolated coronary arteries and actively replicate within the vessel over a period of 10 days in culture.

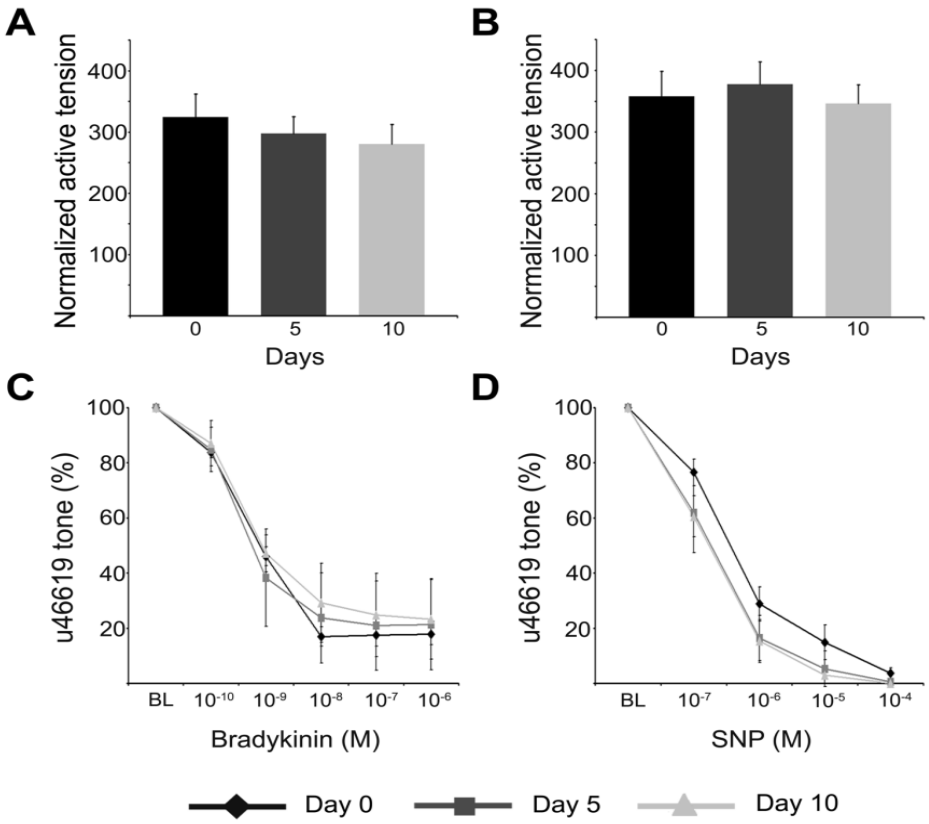

Figure 6. Vascular contractile function of cultured pig coronary arteries
Maximal contractile response to (A) 47mM KCl or (B) 300nM u46619. Relaxation of rings to (C) 10^{-10}-10^{-6} bradykinin or (D) 10^{-7}-10^{-4} SNP after pre-contraction with 30 nM u46619. All values were the mean ± SEM for n = 4-6; P> 0.05 vs 0 time point. Reprinted from Am J Path 176(2), JF Deniset et al, Chlamydophila pneumoniae infection leads to smooth muscle cell proliferation and thickening in the coronary artery without contributions from a host immune response. 1028-1037, © 2010, with permission from Elsevier.(525)
http://www.sciencedirect.com/science/journal/

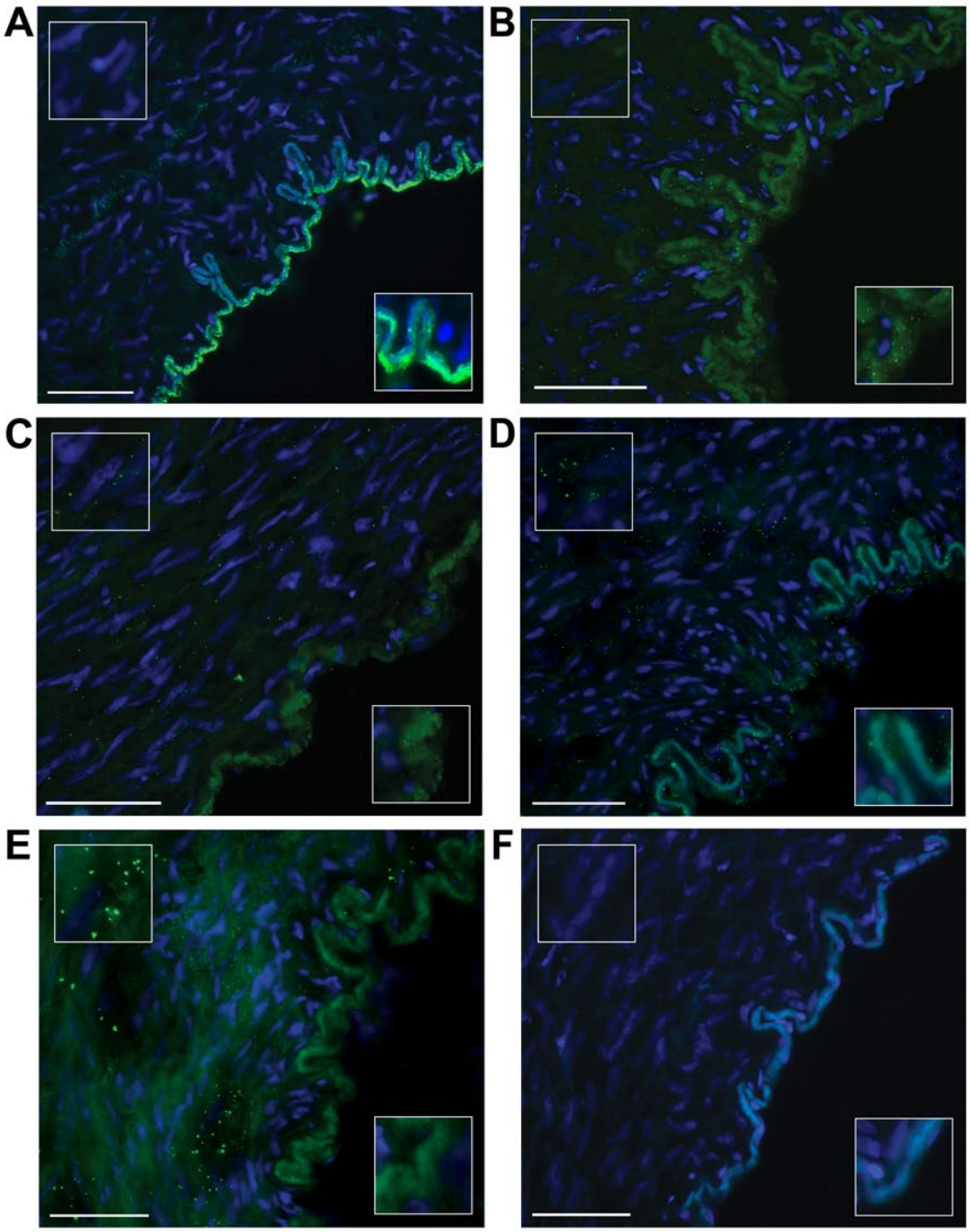

Figure 7. Immunofluorescent detection of C. *pneumoniae* inclusion bodies within infected coronary rings

(A) Day 2 pi; (B) Day 4 pi; (C) Day 6 pi; (D) Day 8 pi; (E) Day 10 pi; (F) Mock infection. C. *pneumoniae* MOMP is represented as fluorescent green signals and nuclear staining is observed as blue. Magnified images of both the medial layer and endothelium are found in the top left and bottom right corners of each panel respectively. Scale bar = 50µm. Reprinted from Am J Path 176(2), JF Deniset et al, Chlamydophila pneumoniae infection leads to smooth muscle cell proliferation and thickening in the coronary artery without contributions from a host immune response. 1028-1037, © 2010, with permission from Elsevier.(525)
http://www.sciencedirect.com/science/journal/

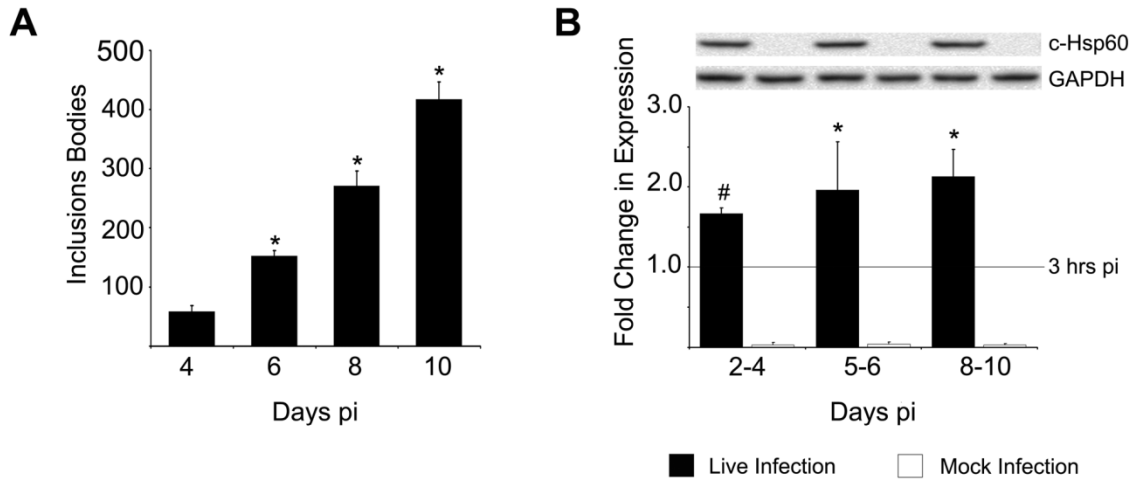

Figure 8. C. *pneumoniae* replication and metabolic activity within isolated coronary sections. (A) The number of *C. pneumoniae* inclusion bodies at days 4-10 pi (mean ± SEM, n=3, * P<0.001). (B) Western Blot analysis of chlamydial HSP60 (c-Hsp60) expression in the coronary segments over the course of infection. Representative western blots of c-Hsp60 and GAPDH (loading control) are displayed above graph. Normalized protein expression levels were represented as fold increase of the basal level. Basal level of c-Hsp60 was assessed by vessels infected by the bacteria for 3 hrs. (* P<0.05 vs basal level. # P<0.01 vs mock-infected control, n=3).Reprinted from Am J Path 176(2), JF Deniset et al, *Chlamydophila pneumoniae* infection leads to smooth muscle cell proliferation and thickening in the coronary artery without contributions from a host immune response. 1028-1037, © 2010, with permission from Elsevier.(525) http://www.sciencedirect.com/science/journal/

C. pneumoniae infection stimulates mammalian Hsp60 and PCNA expression

Previous work from our lab has shown that host Hsp60 expression (hHsp60) was induced during the *C. pneumoniae* infection and this stimulated SMC proliferation. PCNA was used as a reliable marker of cell proliferation (161). Expression levels of h-Hsp60 and PCNA from infected and mock-infected coronary segments were determined by western blot analysis (Figure 9). Live *C. pneumoniae* infection strongly induced expression of h-HSP60 (2.94±0.74 ($p< 0.005$), 2.17±0.65, and 2.64±0.27 ($p< 0.005$) fold over basal levels (day 0) at days 3-4, 5-6, and 8-10 pi, respectively. These increases were

all statistically significant when compared to h-Hsp60 expression levels in mock-infected coronary segments, all of which remained at basal levels (day 0) (Figure 9A). Similarly, expression levels of PCNA in infected coronary segments gradually increased throughout all three time points, up to a maximum fold increase of 3.20 ± 0.52 ($p<0.005$) over baseline on days 8-10 pi (Figure 9B) This increase was statistically significant compared to expression levels in mock-infected vessels. Mock-infected segments did not exhibit a statistically significant increase in PCNA expression over the baseline. The expression pattern of PCNA within the vessel wall was assessed in infected vessels collected at day 10 pi via immunohistochemistry techniques (Figure 9C). A marked difference in PCNA expression between treatments was again observed. Furthermore, PCNA expression in the C.pneumoniae infected vessels was primarily localized within the nucleus of smooth muscle cells in the medial layer.

We have previously shown that *C.pneumoniae* infection in culture induces cell death in endothelial cells (161). However, there was no evidence of apoptosis in the present conditions as detected by TUNEL labeling as a function of *C.pneumoniae* infection (data not shown).

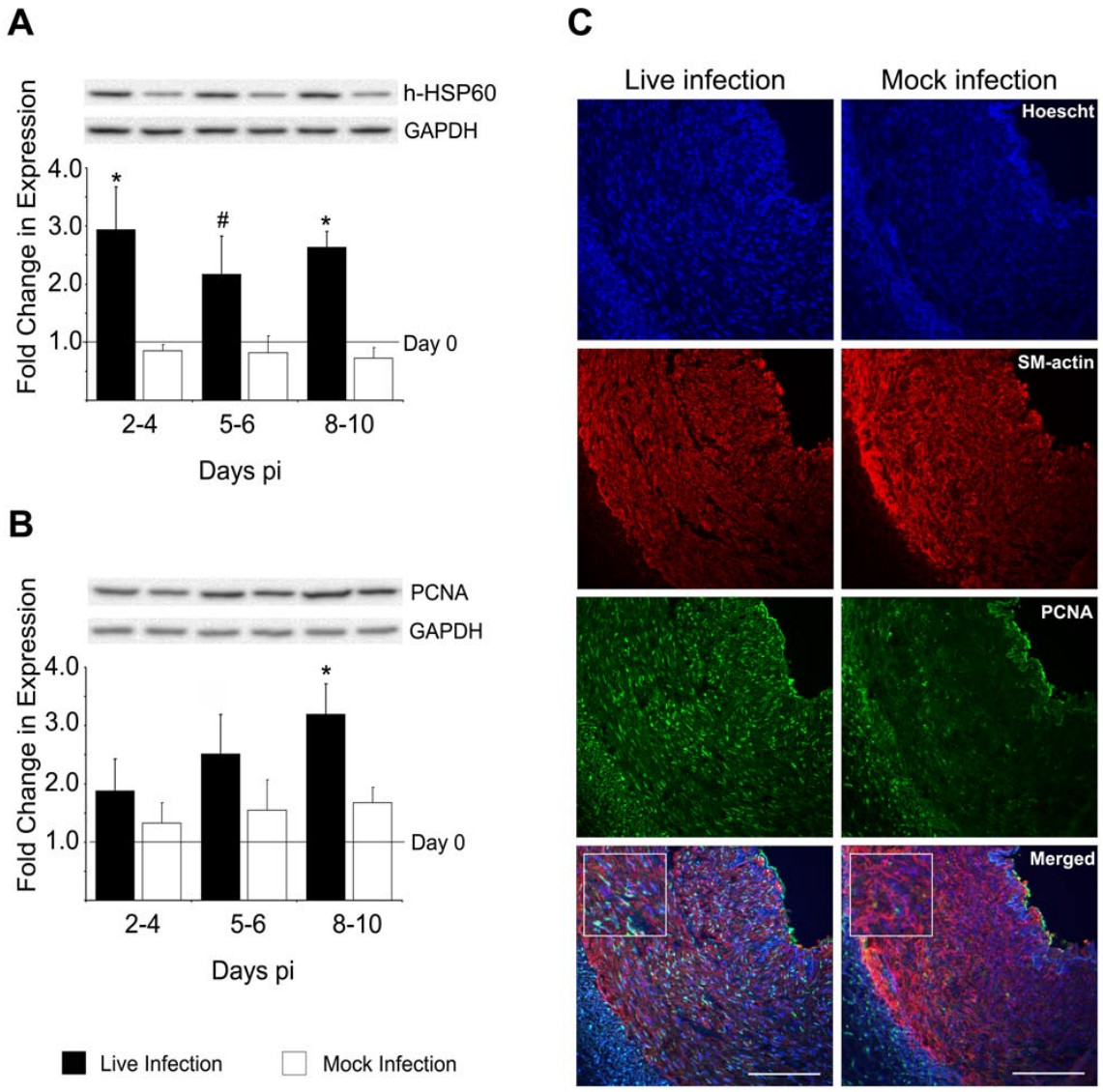

Figure 9. Host-Hsp60 and PCNA expression in coronary segments during *C. pneumoniae* infection

Representative western blots for h-Hsp60 (A) and PCNA (B) along with GAPDH are displayed above their corresponding graphs. Normalized protein expression levels were represented as fold increase of the basal level (Day 0, vessels collected just prior to infection). (* $P<0.05$ vs basal level and mock infected control. # $P<0.05$ vs mock-infected control, n=3). (C) Immunohistochemistry staining of vessels collected at day 10 pi. Left column - Mock infection; right column – Live (Cpn) infection. Hoescht-blue; α-smooth muscle actin-red; α-PCNA – green. Scale bar = 200 μm. Reprinted from Am J Path 176(2), JF Deniset et al, *Chlamydophila pneumoniae* infection leads to smooth muscle cell proliferation and thickening in the coronary artery without contributions from a host immune response. 1028-1037, © 2010, with permission from Elsevier.(525) http://www.sciencedirect.com/science/journal/

C. pneumoniae infection of explanted vessels induces medial thickening

The PCNA data above would suggest that smooth muscle cells are entering the cell cycle in the *C. pneumoniae*-infected vessels but it is not clear if this proliferative process is continuing to termination. Tissue thickening would provide clear evidence of this response. Representative H/E and elastic staining at days 4, 6, 8 and 10 pi. of both *C. pneumoniae* and mock-infected coronary segments are shown in Figure 10. The extent of media thickening was determined by the calculation of two separate ratios from specific arterial areas. The ratio of luminal area to total vessel area (Figure 11B) represents the degree of arterial thickening, whereas the ratio of medial area to luminal area (Figure 11B) shows the primary site for the thickening. The *C. pneumoniae*-infected segments displayed a progressive regression in lumen to total vessel area: 48 ± 3% (day 4 pi), 36 ± 2% (day 6 pi), 26 ± 2% (day 8 pi) and 23 ± 3% (day 10 pi). Furthermore, the ratio of media to luminal area was significantly increased from 113±16% on day 4 pi to a maximum of 365±66% on day 10 pi ($p < 0.02$). In contrast, ratios for mock-infected tissues remained unchanged from days 4 to 10 pi (Figure 11A and 11B). These data demonstrate that *C.pneumoniae* infection has the ability to induce media thickening in isolated coronary arteries.

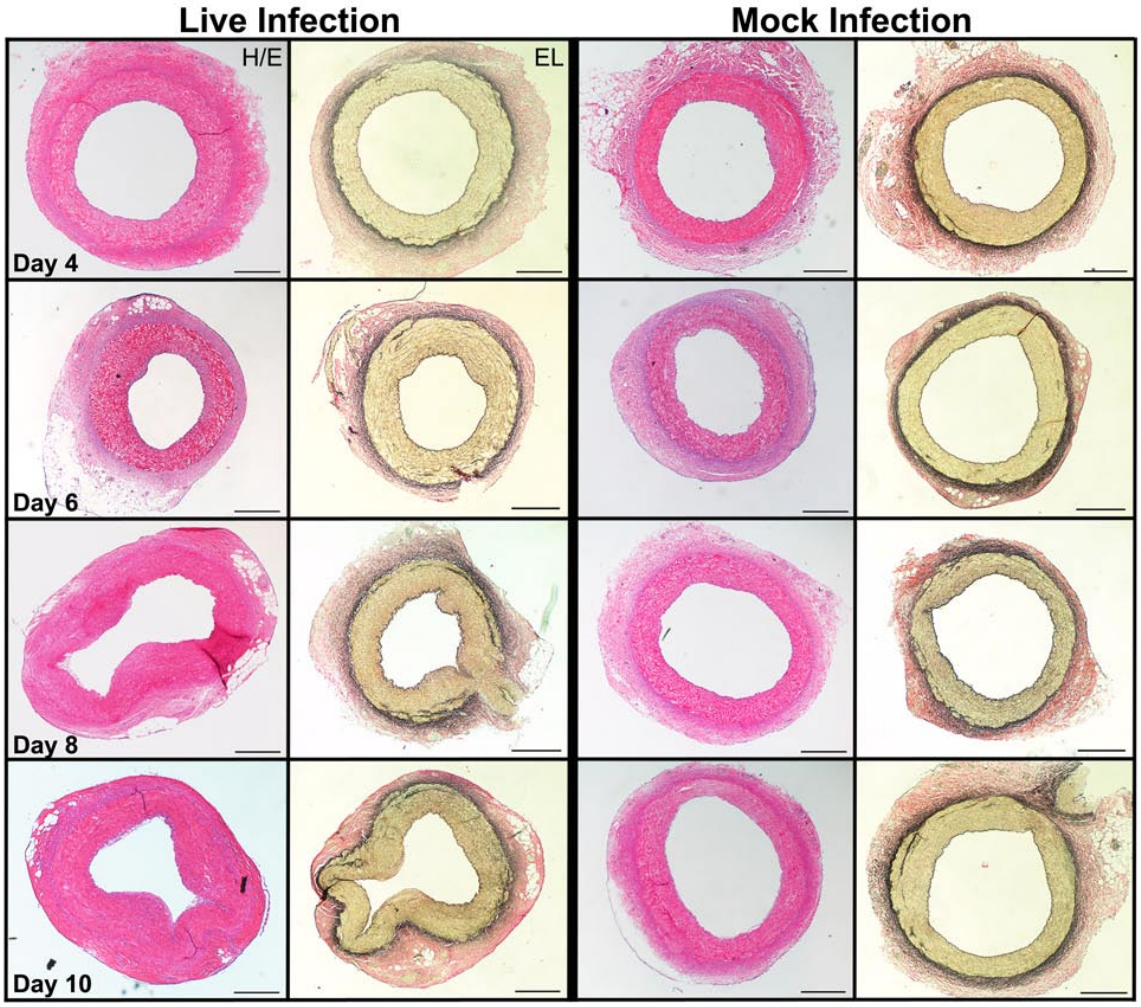

Figure 10. Histological staining of *C. pneumoniae* infected coronary arteries
Representative hematoxylin/eosin (H/E, left panel) and elastic (EL, right panel) of the infected coronary segments at day 4, 6, 8 and 10 pi. Left columns – Live (Cpn) infection; right columns - Mock infection. Scale bar = 500 µm. Reprinted from Am J Path 176(2), JF Deniset et al, *Chlamydophila pneumoniae* infection leads to smooth muscle cell proliferation and thickening in the coronary artery without contributions from a host immune response. 1028-1037, © 2010, with permission from Elsevier.(525) http://www.sciencedirect.com/science/journal/

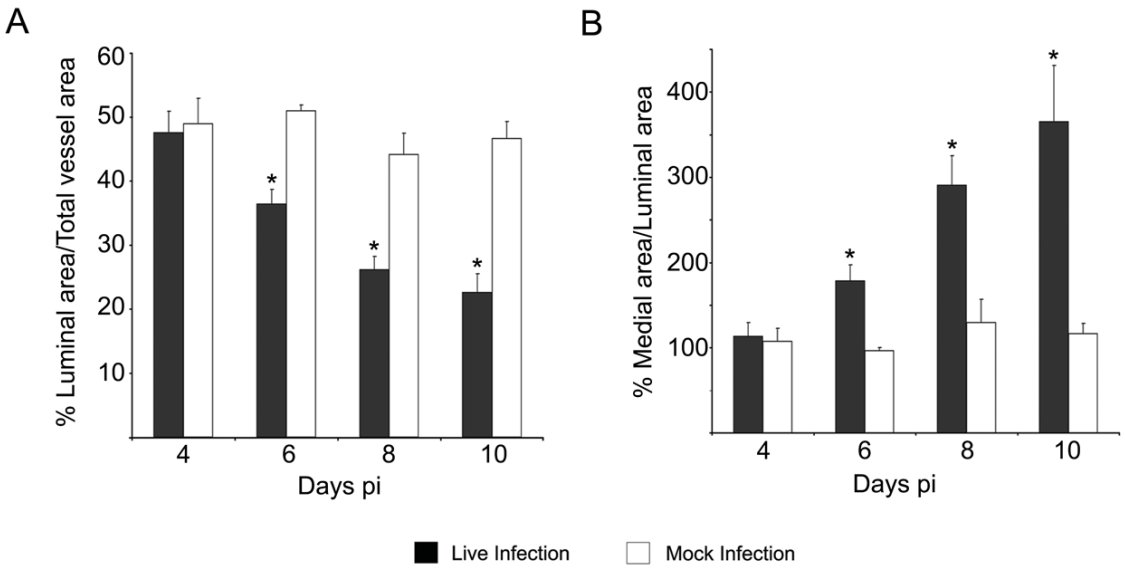

Figure 11. Quantitative representation of arterial wall thickening during *C .pneumoniae* infection. Thickening was quantified and represented as ratios of (A) lumen to total vessel area, and (B) media to luminal area (mean ±SEM). *P<0.02 vs. Day 4 pi. Reprinted from Am J Path 176(2), JF Deniset et al, *Chlamydophila pneumoniae* infection leads to smooth muscle cell proliferation and thickening in the coronary artery without contributions from a host immune response. 1028-1037, © 2010, with permission from Elsevier.(525) http://www.sciencedirect.com/science/journal/

2. Effects of *C. pneumoniae* infection on vascular contractile function in porcine coronary arteries [6]

C. pneumoniae induces alterations in vascular contractile function

Isolated coronary vessels infected with *C. pneumoniae* displayed significant decreases in maximal contractile response to KCl when compared to control and mock infected vessels at both days 5 (Figure 12A) and 10 post-infection (Figure 12B). Similar qualitative responses were found in response to receptor-mediated contraction with u46619 (Figure 12C and 12D). At day 5 post-infection, the contractile response to both

[6] Reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila pneumoniae* infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. http://www.sciencedirect.com/science/journal/

150 and 300 nM u46619 in *C. pneumoniae* infected segments was decreased compared to control and mock-infected vessels. At day 10 post-infection, the contractile response to u46619 in infected segments was decreased only compared to control.

C. pneumoniae infection alters expression of calcium handling proteins but not contractile proteins

The expression of the smooth muscle contractile proteins actin and myosin heavy chain was assessed in vessels at days 5 and 10 post infection (Figure 13A and 13B). Expression levels appeared to decrease with *C. pneumoniae* infection, however these changes were not significantly different compared to other treatment at both day 5 (SM-actin: P= 0.087; Myosin: P= .172) and 10 (SM-actin: P= 0.145; Myosin: P=0.143). The expression of calcium handling proteins involved in smooth muscle contraction, L-type voltage gated Ca^{2+} channel and IP_3 receptor, was also determined at days 5 and 10 post infection (Figure 14A and 14B). The expression of the L-type Ca^{2+} channel was significantly decreased in *C. pneumoniae* infected vessels compared to control and mock-infected vessels at day 5 post-infection and at day 10 (Figure 14A). The expression of IP_3 receptor was unchanged between groups at day 5 post infection but was significantly decreased in infected vessels compared to control at day 10 post-infection (Figure 14B).

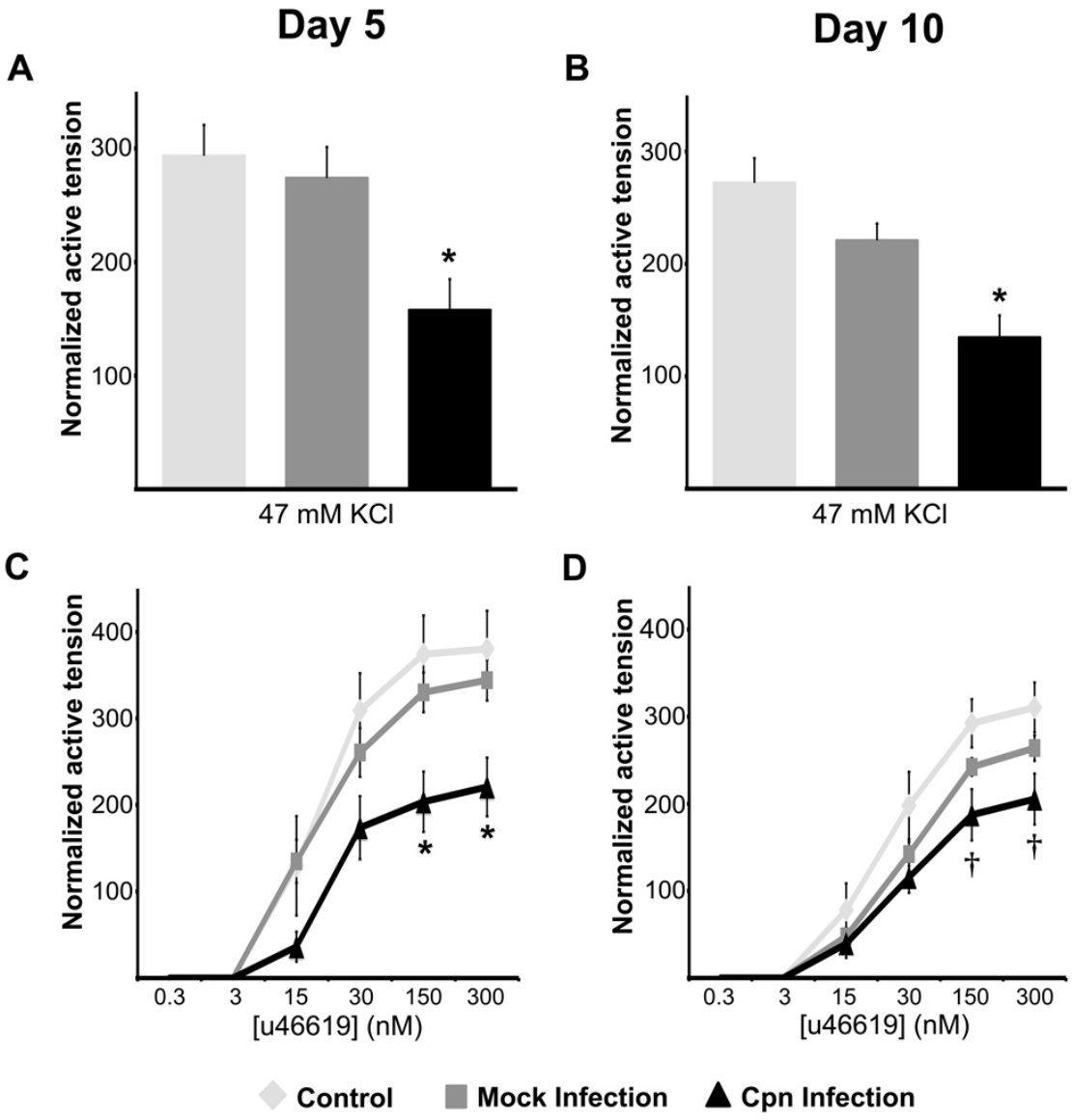

Figure 12. Vascular contractile response of *C. pneumoniae*-infected coronary rings
Maximal contractile response to 47mM KCl (A) Day 5 post-infection (B) Day 10 post-infection and 0.3-300nM u46619 (C) Day 5 post-infection (D) Day 10 post-infection. All values are represented as the mean ± SEM, n = 7-11; *= P < 0.05 vs Control and Mock infection, †= P < 0.05 vs Control. Reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila pneumoniae* infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. (529) http://www.sciencedirect.com/science/journal/

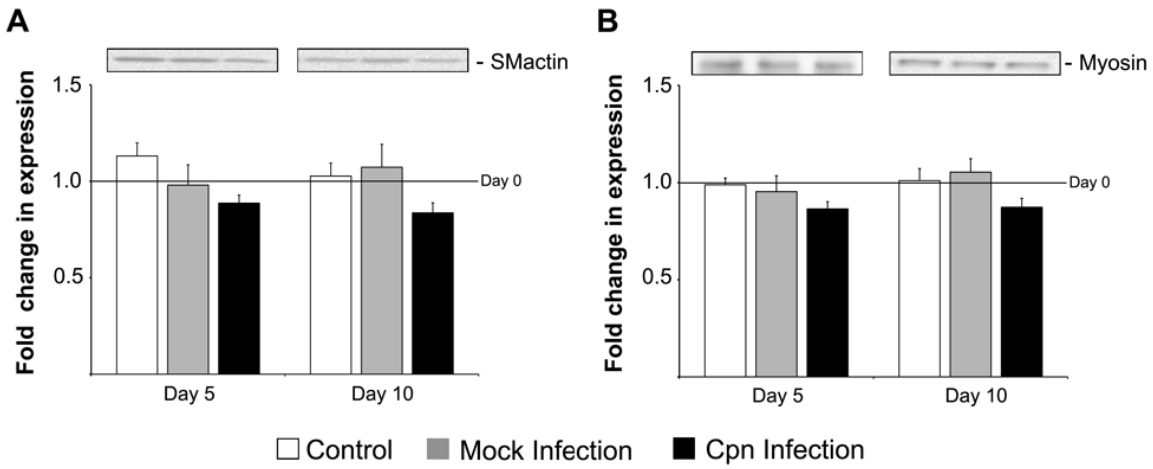

Figure 13. Western blot analysis of contractile proteins with *C. pneumoniae* infection
(A) SM-actin and (B) SM-myosin expression at Day 5 and Day 10 post-infection in infected coronary segments. Representative western blots are displayed above their corresponding graphs. Expression levels were represented as fold change over Day 0 tissue ± SEM, n = 6**.** Reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila pneumoniae* infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. (529) http://www.sciencedirect.com/science/journal/

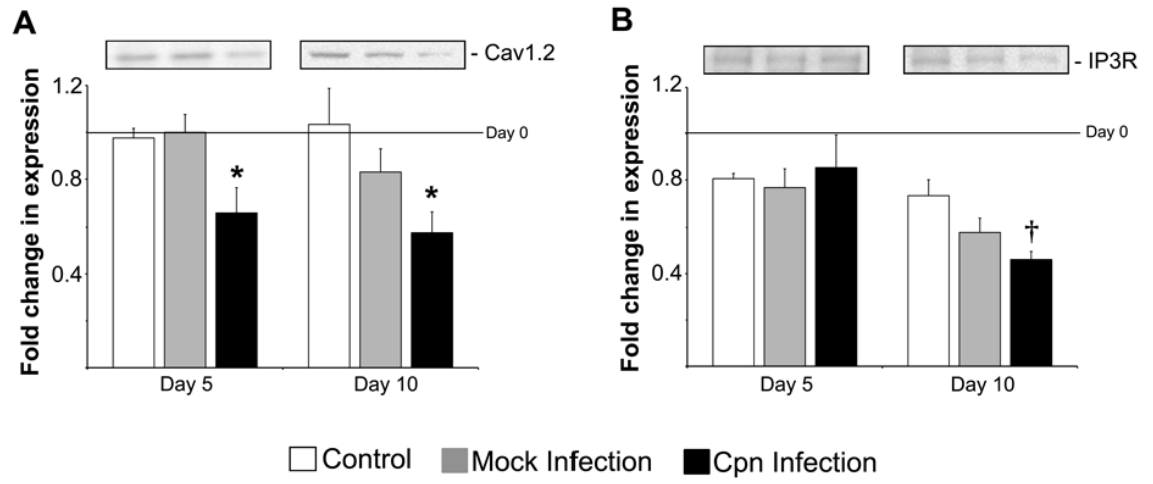

Figure 14. Western blot analysis of Ca^{2+} handling proteins with *C. pneumoniae* infection
(A) L-type Ca^{2+} channel and (B) IP3R expression at Day 5 and Day 10 post-infection in infected coronary segments. Representative western blots are displayed above their corresponding graphs. Expression levels were represented as fold change over Day 0 tissue ± SEM, n = 4-6; *=$P < 0.05$ vs control and mock infection, †= $P < 0.05$ vs Control. Reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila pneumoniae* infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. (529) http://www.sciencedirect.com/science/journal/

C. pneumoniae infection attenuates endothelial dependent relaxation in isolated coronary vessels

Pre-contraction levels in response to 30nM u46619 remained unchanged between treatment groups at both days 5 and 10 post-infection (data not shown). Endothelial-dependent relaxation responses to bradykinin remained unaltered between groups at day 5 post infection (Figure 15B) but at day 10, it was significantly reduced in infected vessels compared to control at 10^{-9}-10^{-6} M and at 10^{-7}-10^{-6} M in comparison to mock-infected vessels (Figure 15B). No change in endothelial-independent relaxation responses to SNP was observed amongst groups at either time-point (Figure 15C and 15D).

C. pneumoniae infection induces a decrease in eNOS expression

Expression of eNOS and iNOS, the nitric oxide producing enzymes within the vessel was determined via western blot analysis. eNOS expression was unchanged amongst treatment groups at day 5 post-infection (Figure 16A). At day 10 post-infection, eNOS was down-regulated in infected vessels compared to control and mock-infected vessels (Figure 16A). Further analysis via immunostaining of coronary vessels at day 10 post infection revealed a significant decrease in mean fluorescence of infected cross-sections compared to control and mock-infected tissues (Figure 16B and 16C). iNOS expression remained unchanged between treatment groups at both time-points (data not shown).

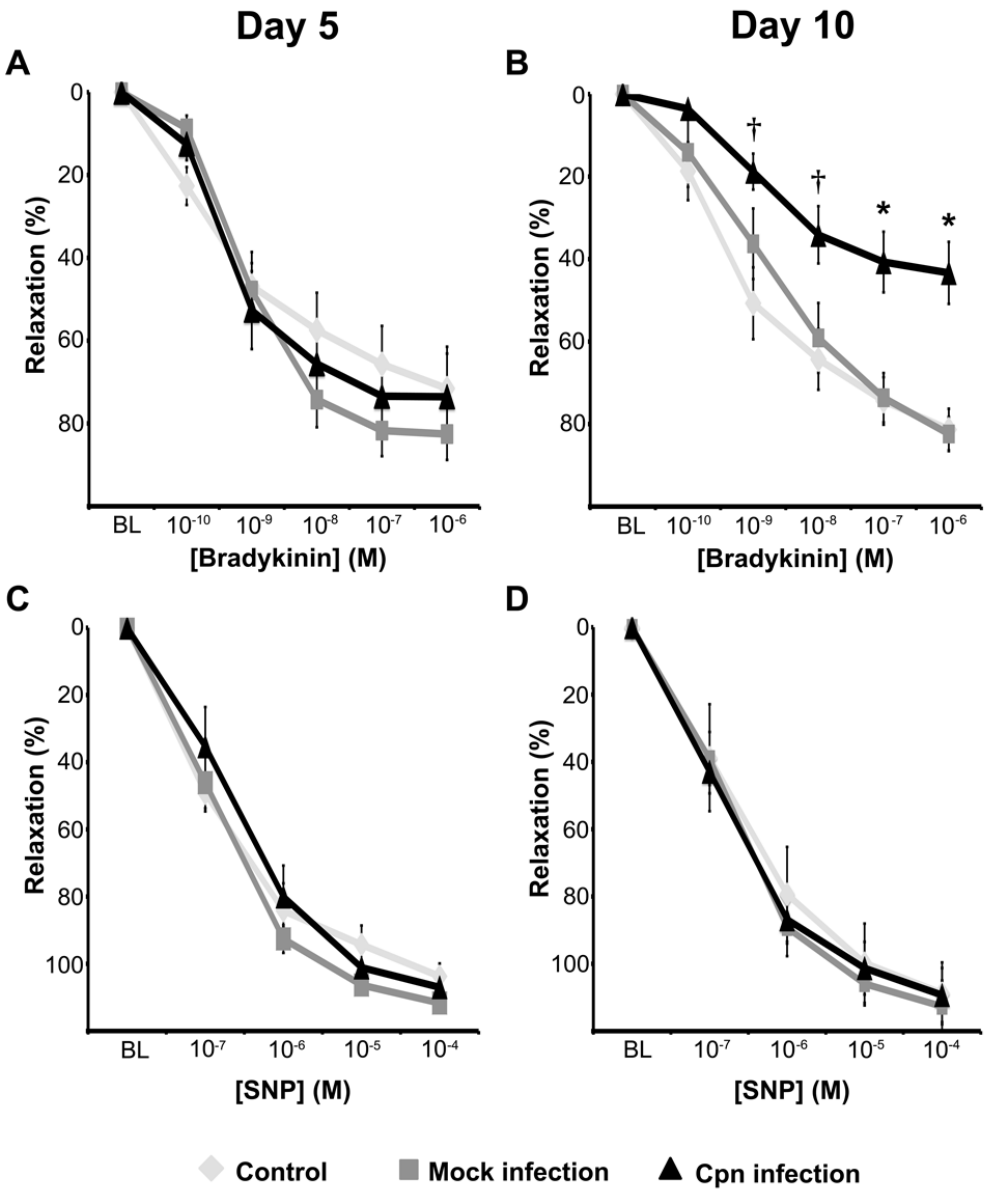

Figure 15. Relaxation responses of *C. pneumoniae*-infected coronary rings
Vascular preparations were relaxed to 10^{-10}-10^{-6} bradykinin (A) Day 5 post-infection and (B) Day 10 post-infection or 10^{-7}-10^{-4} SNP (C) Day 5 post-infection (D) Day 10 post-infection after pre-contraction with 30 nM u46619. All values are represented as the mean ± SEM, n = 7-11; *=P < 0.05 vs control and mock infection, †= P < 0.05 vs control. Reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila pneumoniae* infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. (529)
http://www.sciencedirect.com/science/journal/

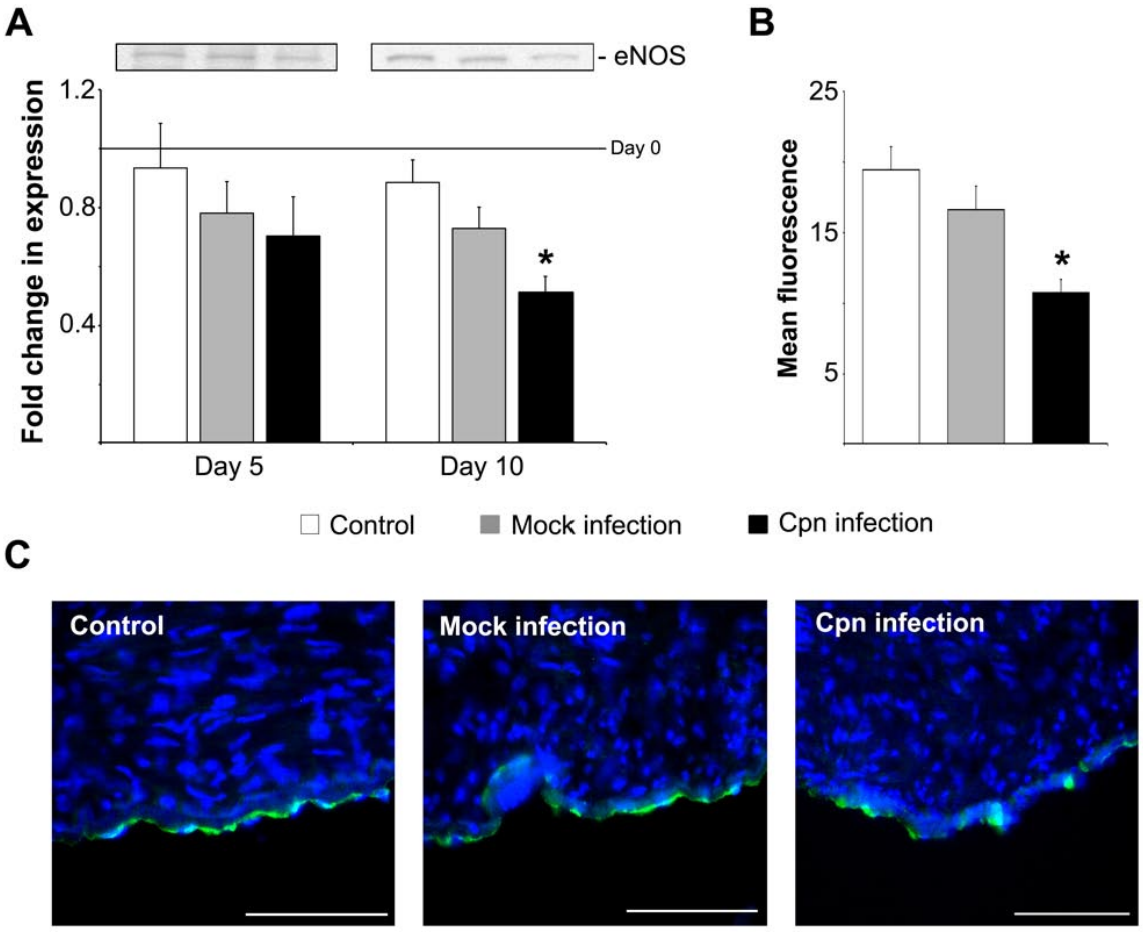

Figure 16. eNOS expression in *C. pneumoniae*-infected isolated coronary arteries
(A) Western blot analysis of whole tissue lysates at Day 5 and Day 10 post-infection. Representative western blots are displayed above graph. Expression levels were represented as fold change over Day 0 tissue ± SEM, n =4-6. Representative immunohistochemistry staining (B) quantification and (C) images of vessels collected at day 10 post-infection. Fluorescence intensity (B) is represented as mean ± SEM, n = 4. Hoescht-blue; eNOS–green. Scale bar = 100 µm. *=P < 0.05 vs control and mock infection. Reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila pneumoniae* infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. (529)
http://www.sciencedirect.com/science/journal/

C. pneumoniae components stimulate superoxide anion production and p22phox expression

Expression of p22phox, a major subunit of NADPH oxidase, was determined via western blot and immunostaining (Figure 17). p22phox expression as detected by western blots was unchanged amongst groups at day 5 post-infection but was up-regulated in both mock-infected and *C. pneumoniae*-infected vessels compared to control vessels at day 10 post-infection (Figure 17A). p22phox immunostaining at day 10 post-infection confirmed these results with higher fluorescence levels in mock and *C. pneumoniae* infected vessels compared to control (Figure 17B and 17C).

Superoxide anion production at day 10 post-infection was visualized using dihydroethidium staining on infected coronary cross-sections (Figure 18). Quantification of this staining, revealed significant increases in mean fluorescence in both mock-infected and *C. pneumoniae*-infected vessels compared to control (a 99% ($p<0.05$) and 207% ($p<0.01$) change in comparison to control, respectively).

NFκB activation is not altered in infected coronary arteries

The NFκB pathway is known to be involved in the inflammatory process within the vessel wall, as such both NFκB and IκB protein expression was assessed. Expression of both proteins did not significantly change between treatments at days 5 and 10 post-infection (data not shown). The ratios of IκB to NFκB in both *C. pneumoniae*- and mock- infected vessels varied from control vessels but not enough to be statistically significant.

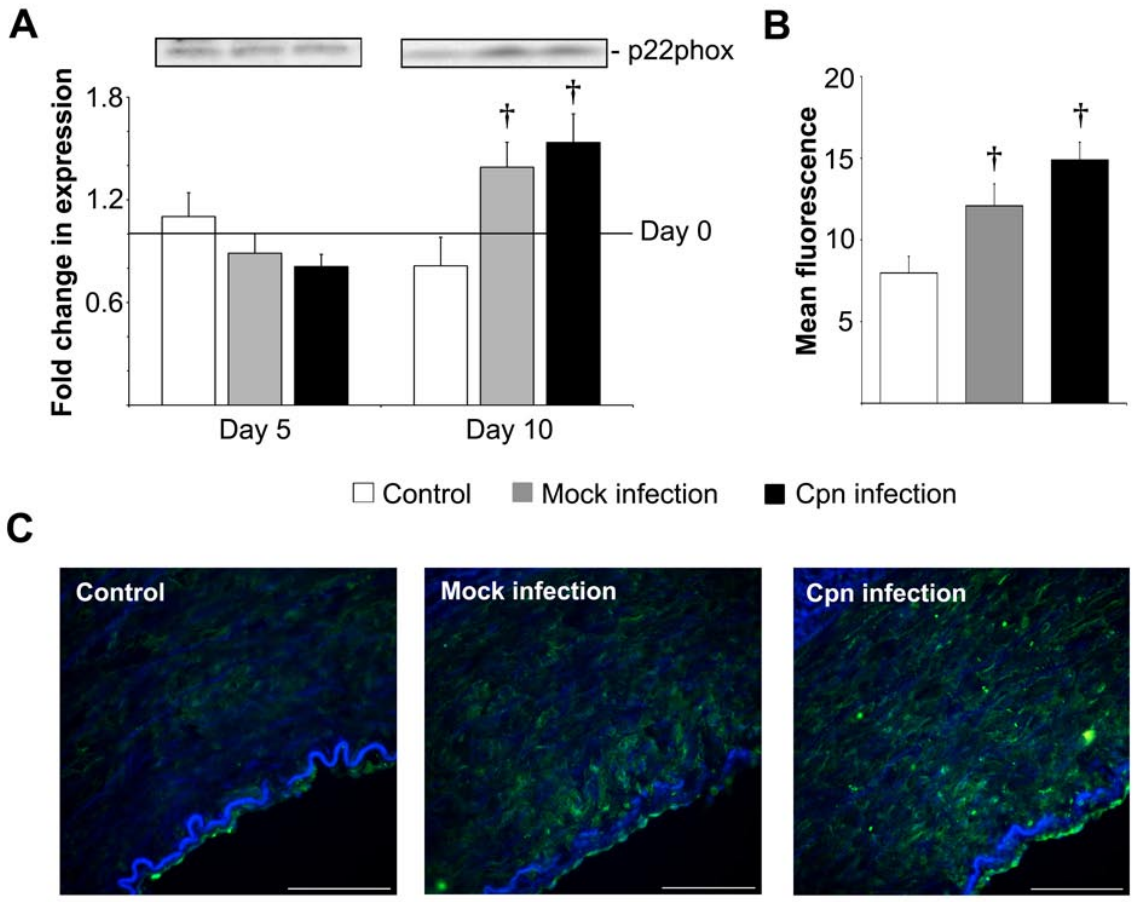

Figure 17. p22phox expression in *C. pneumoniae*-infected isolated coronary arteries
(A) Western blot analysis of whole tissue lysates at Day 5 and Day 10 post-infection. Representative western blots are displayed above the graph. Expression levels were represented as fold change over Day 0 tissue ± SEM, n =4-6. Representative immunohistochemistry staining (B) quantification and (C) images of vessels collected at day 10 post-infection. Fluorescence intensity (B) is represented as mean ± SEM, n = 4. Hoescht-blue; p22phox–green. Scale bar = 100 µm. †= P < 0.05 vs Control. Reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila pneumoniae* infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. (529) http://www.sciencedirect.com/science/journal/

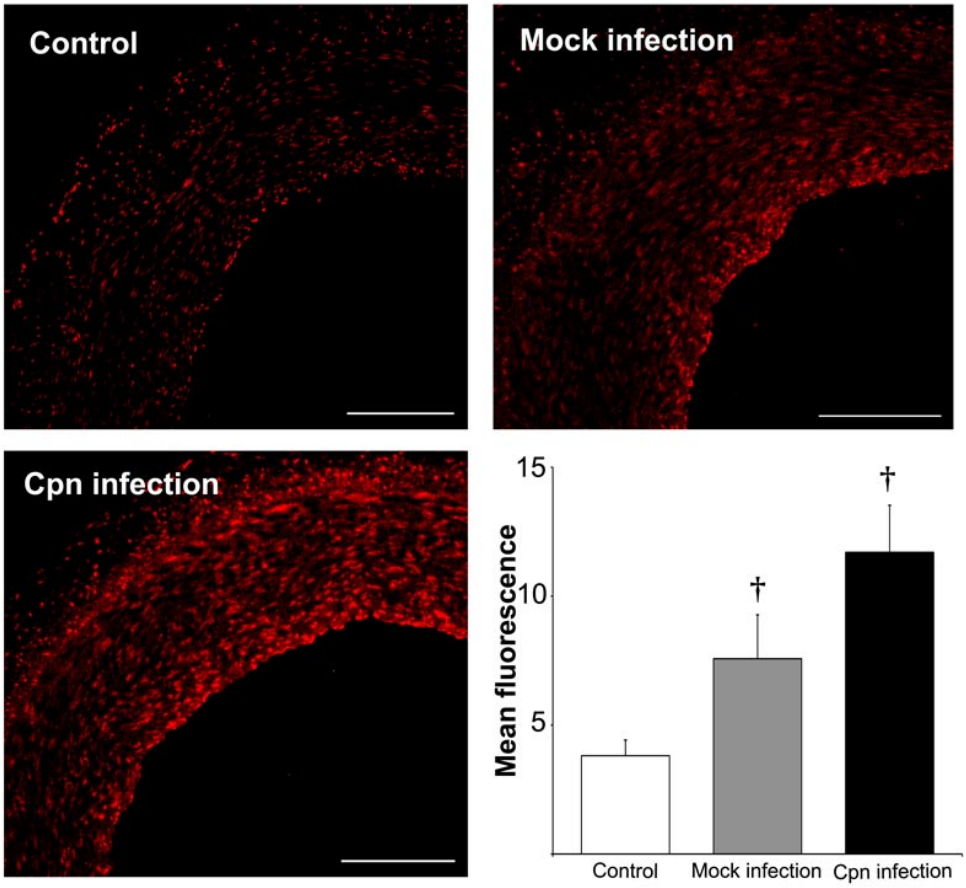

Figure 18. Superoxide detection in *C. pneumoniae* infected isolated coronary arteries
Representative staining of vessels was performed at day 10 post-infection. Dihydroethidium staining indicated in red. Fluorescence intensity (bottom right panel) is represented as mean ± SEM, n = 4; †= P < 0.05 vs control. Reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila pneumoniae* infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. (529) http://www.sciencedirect.com/science/journal/

C. pneumoniae increases endothelial TLR4, hHsp60 and cHsp60 expression

TLR4, cHsp60 and hHsp60 localization along the endothelial layer was determined via immunostaining (Figure 19). Both *C. pneumonia*- and mock-infected vessels displayed an increase in TLR4 and hHsp60 expression when compared to non-treated vessels (Figure 19D,19E, 19G and 19H). In addition, cHsp60 expression could be localized along the endothelial layer in *C. pneumonia*-infected vessels (Figure 19I) but was absent in both control and mock-infected coronary vessels.

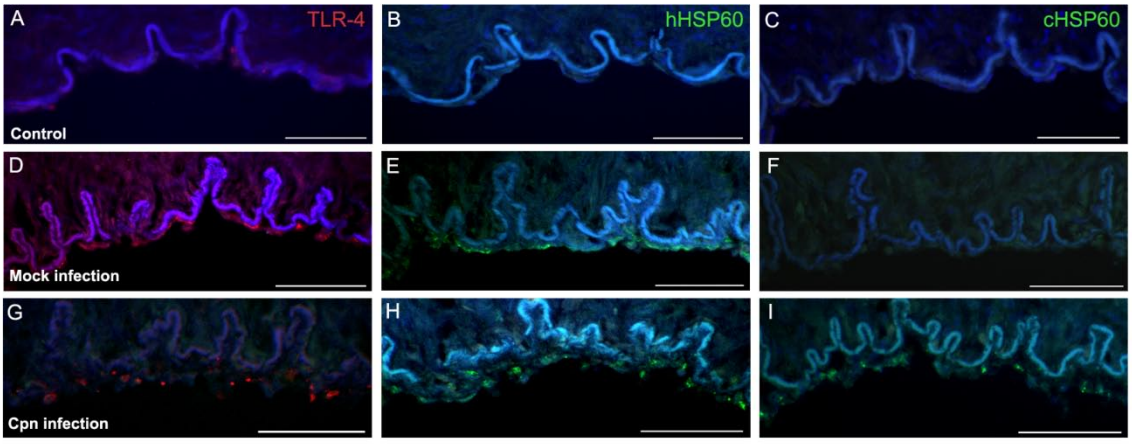

Figure 19. TLR-4, hHsp60 and cHsp60 localization within infected coronary arteries
Representative staining of control (A,B,C), mock infected (D,E,F) and *Cpn* infected (G,H,I) vessels collected at day 10 post-infection. All panels: Hoescht-blue; First column panels: TLR-4-red; Second column panels:hHSP60–green; Third column panels: cHSP60-green. Scale bar = 100 µm. Reprinted from Am J Path 180(3), JF Deniset et al., *Chlamydophila pneumoniae* infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. (529) http://www.sciencedirect.com/science/journal/

Conditioned medium does not alter eNOS and p22phox expression in HUVECs

eNOS and p22phox protein expression was evaluated in HUVECs treated with conditioned medium from days 4,6,8 and 10 pi via western blot. Expression of both eNOS and p22phox in HUVECs treated with conditioned medium from all four timepoints were not significantly different between treatments (data not shown). cHsp60 and hHsp60 levels within conditioned medium from the different treatments were analyzed via western blot. All conditioned medium displayed cHsp60 and hHsp60 levels below the bottom level of our standard curve of 0.01 µg of protein, equivalent to 0.08 µg/ml from the non-concentrated conditioned medium (data not shown). cHsp60 could not be detected in culture medium from control and non-infected coronary vessels.

3. Role of Hsp60 in modulating nuclear protein import during VSMC proliferation

Effect of HSP60 overexpression on heat shock protein expression

Previous results have shown Hsp60 can induce VSMC proliferation (161). Although the mechanism is unclear, it is possible that it may occur through a modulation of nuclear protein import. To test this hypothesis of the relationship between Hsp60, VSMC proliferation and nuclear protein import, an adenovirus-mediated model of Hsp60 overexpression in VSMC was employed. Adenoviral mediated overexpression in VSMCs resulted in a ~2 fold increase in Hsp60 levels in comparison to control and AdQBI treatments, respectively (Figure 20A). It also induced a significant increase in cell number (Figure 20B).

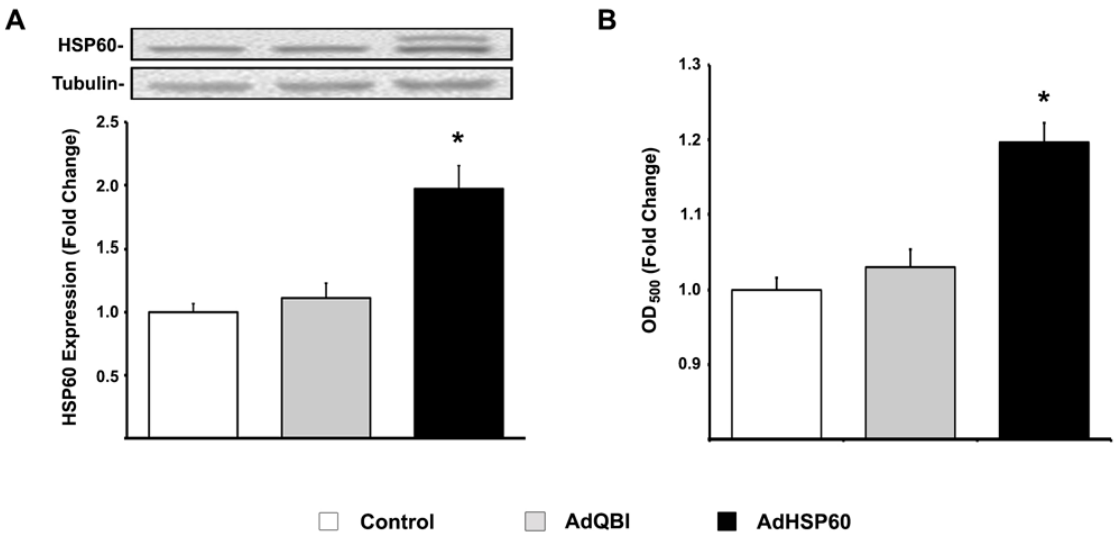

Figure 20. Hsp60 overexpression and VSMC proliferation
Western Blot analysis of HSP60 (A) overexpression. Normalized protein expression levels were represented as fold change to control samples (*n*=4-5). Cell number measurement(B) in response to HSP60 overexpression as determined by OD at 500nm. Numbers were represented as fold change to control samples (*n*=8).*P<0.05 versus control and AdQBI; values are means± SEM.

Cellular localization of Hsp60 overexpression

Cellular fractionation experiments and co-localization staining were performed to determine the location of the adenovirally-derived Hsp60 (full length-AdHsp60 and truncated mitochondria targeting sequence-AdHsp60^{mito-}) (Figures 21 and 22). Fluorescent labeling of Hsp60 and HA along with a mitochondrial stain (MitoTracker) was used to detect cellular localization (Figure 21). Staining in the AdQBI group reflected endogenous Hsp60 localization in the VSMC (Figure 21A). Hsp60 staining was elevated in AdHsp60 and AdHsp60^{mito-} treated VSMCs compared to the AdQBI group (Figure 21A). Staining within the AdQBI cells was limited to the mitochondria whereas both overexpression groups demonstrated staining in both the mitochondria and cytoplasmic areas. The AdHsp60^{mito-} treated VSMCs also showed distinctive staining of

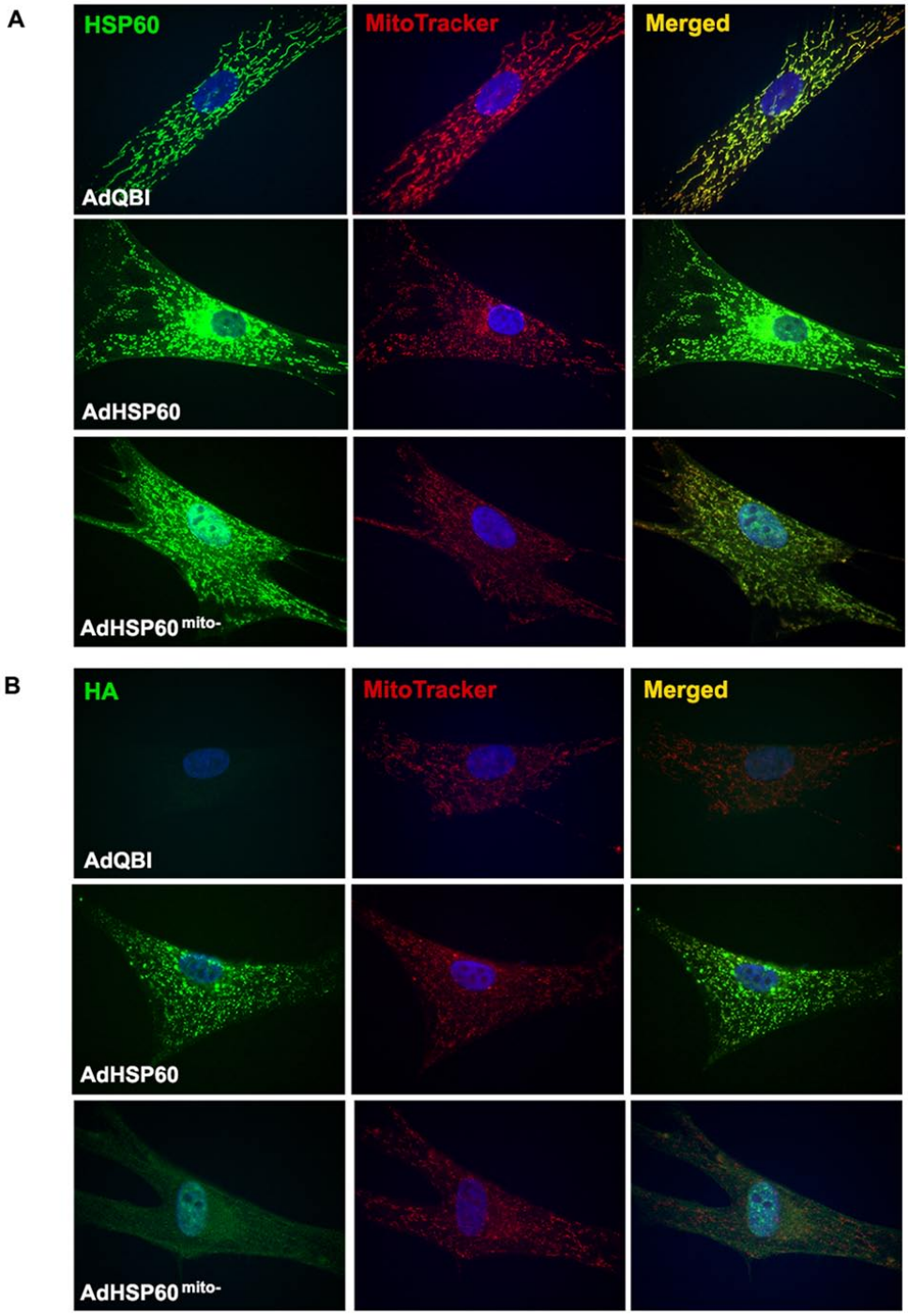

Figure 21. Cellular Hsp60 staining
Representative immunocytochemistry images of Hsp60 (A) and HA (B) co-localization with mitochondrial and nuclear staining. **left column**: Hsp60 (A) or HA (B) expression, green; **middle column:** MitoTracker, red; **right column:** Merged staining. DAPI, blue.

Hsp60 associated with the cell nucleus. Adenovirally derived Hsp60 was also tracked within the cell by labeling with an anti-HA antibody (Figure 21B). As expected, no HA staining was observed in the AdQBI group. The HA staining in AdHsp60 treated cells demonstrated a similar expression pattern with localization within the mitochondria and the cytoplasm. Alternatively, HA staining in AdHsp60^{mito-} treated cells was distributed across the entire area of the cell. The localization of Hsp60 was further quantified by western blotting of isolated cell fractions. AdHSP60 treatment resulted in increased Hsp60 levels in both cytoplasmic and mitochondrial compartments (Figure 22). Conversely, overexpression of Hsp60 with a truncated mitochondrial targeting sequence (AdHsp60^{mito-}) resulted in elevated Hsp60 levels only within the cytosolic fraction (Figure 22). The expression of other heat shock proteins such as Hsp70, Hsp90, Hsp27 and Hsp10 remained unaffected by the overexpression of Hsp60 levels (Figure 23).

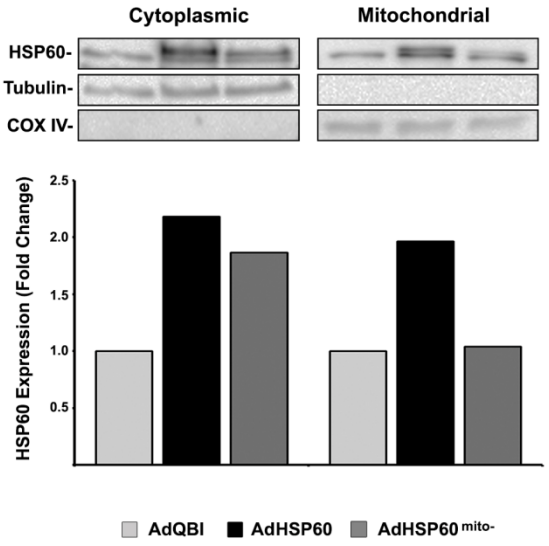

Figure 22. Cellular localization of Hsp60 overexpression
Western blot analysis of cytosolic and mitochondrial Hsp60 expression in response to overexpression. Representative Western blot for Hsp60 along with α-tubulin (cytosolic loading control) and COX IV (mitochondrial loading control) are displayed above their corresponding portions of the graph. Normalized protein expression levels were represented as fold change to AdQBI samples (*n*=2); values are means.

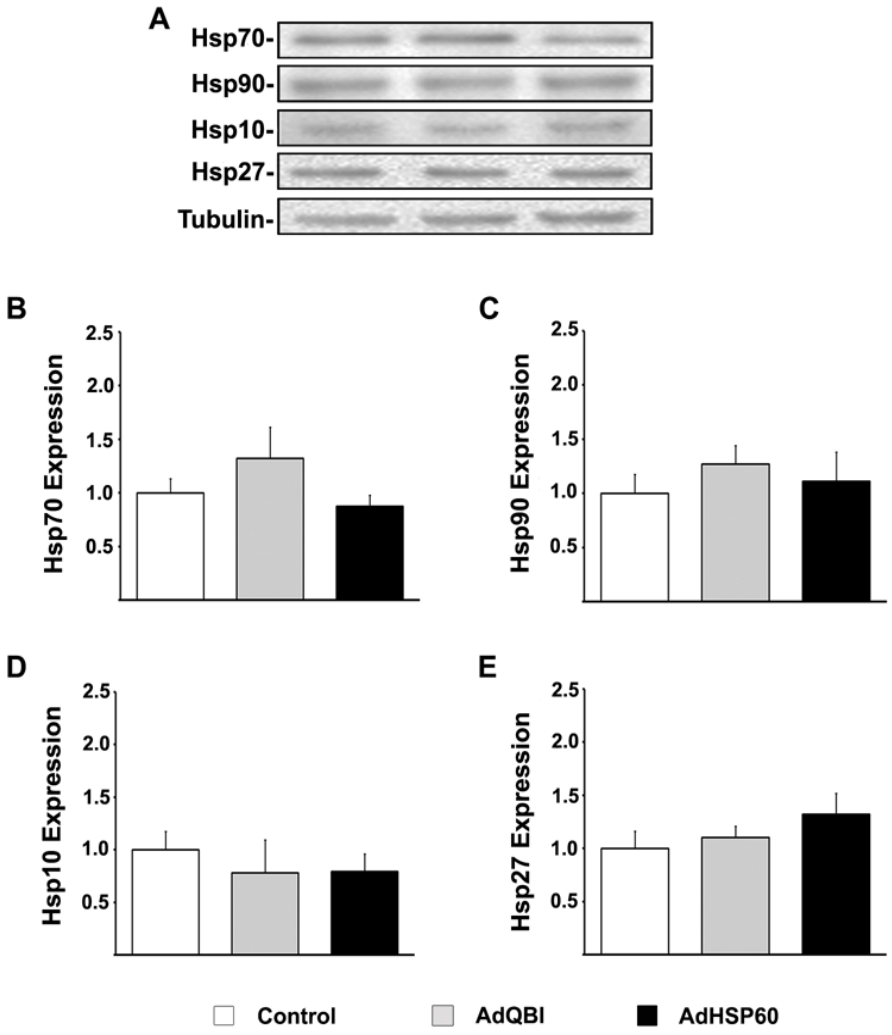

Figure 23. Effect of Hsp60 overexpression on Hsps
Representative Western Blot images (A) and analysis of Hsp70 (B), Hsp90 (C), Hsp10 (D), Hsp27 (E) in response to Hsp60 overexpression. Protein expression levels are normalized to α-tubulin and are represented as fold change to control samples (*n*=4-5). Values are means± SEM.

Hsp60 overexpression stimulates increase in NPI rate

The possibility that Hsp60 could induce VSMC proliferation via a modulation of nuclear protein import was examined. The rate of NPI was assessed by tracking nuclear/cytoplasmic fluorescence for up to 30 minutes post-injection of a nuclear protein import substrate as used previously (Figure 24) (113,516). Representative confocal images demonstrate the migration of the fluorescently labelled import substrate from the

cytoplasm into the nucleus (Figure 24A). The rate of NPI was significantly elevated with both AdHsp60 and AdHsp60^{mito-} treatments compared to control groups (Figure 24B).

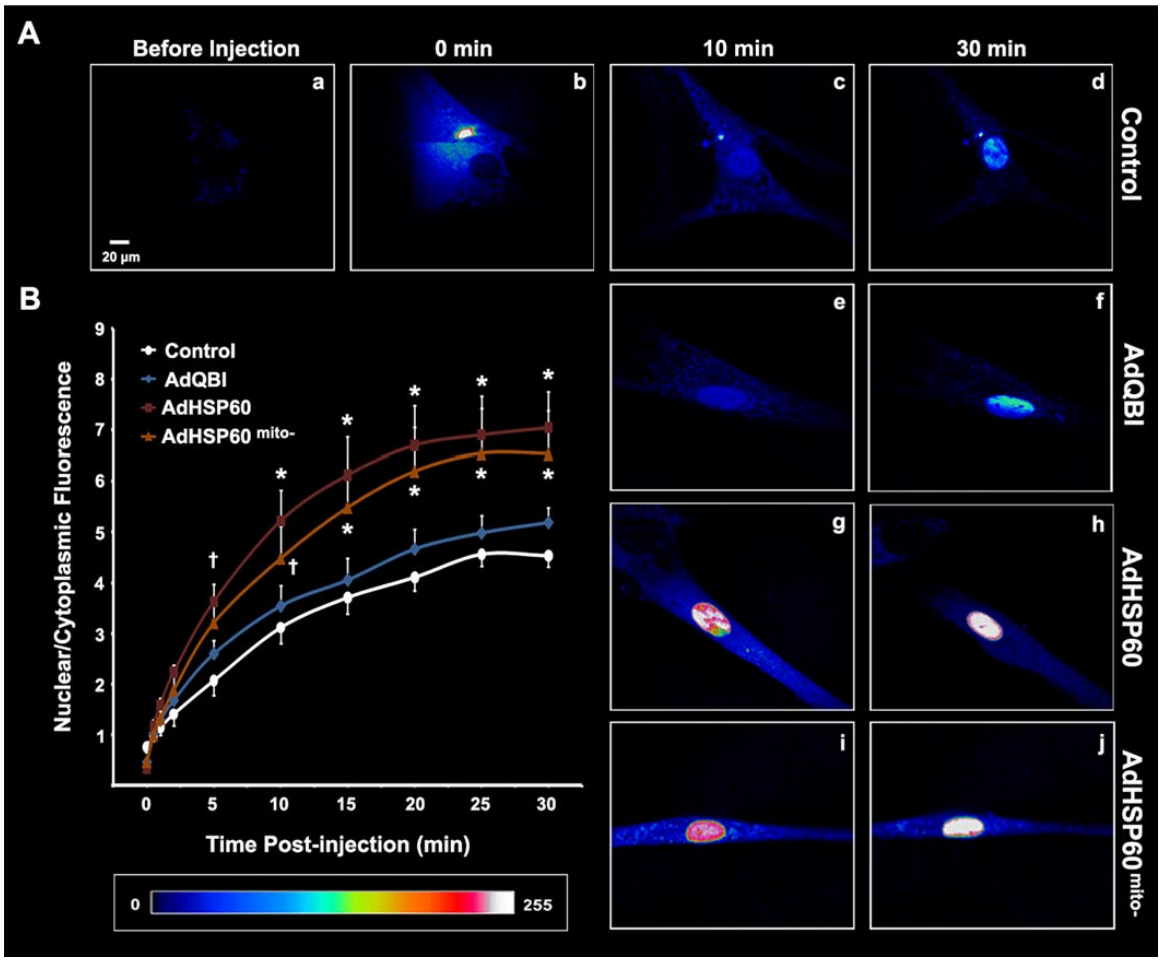

Figure 24. Effect of Hsp60 overexpression on nuclear protein import in VSMCs
(A) Representative images of VSMCs microinjected with ALEXA488-BSA-NLS substrate following 48h infection with adenoviral constructs: AdQBI (*e–f*), AdHsp60 (*g–h*), AdHsp60 mito- (*i–j*), or the control cells (*a–d*). Images were taken (*a*) before injection, (*b*) 0 min pre-injection, (*c,e,g,i*), 10 min post-injection, and (*d,f,h,j*) 30 min post-injection. The pseudo-color scale represents fluorescence intensity levels from 0 (black) to 255 arbitrary units (white). The white scale bar represents 20 µm. Laser scanning settings are identical for all cells and experiments. (B) The curve summarizes the nuclear protein import in control cells (white) or VSMCs infected with AdQBI (blue), or AdHsp60 (red), or AdHsp60^{mito-} (orange) over time. *P < 0.05 vs. Control and AdQBI; †P < 0.05 vs. Control; n = 5–8. For cytosolic fluorescence, an area that surrounded the nucleus and equivalent to the nuclear area was used. The ratio of nuclear/cytoplasmic fluorescence was calculated from these values. Values are means ± SEM.

Effect of Hsp60 overexpression on NPI machinery

The expression of cellular components associated with NPI was evaluated to identify the mechanism responsible for the alteration in the rate of NPI that was observed in Figure 24. As shown in Figure 25B and 25C, AdHsp60 stimulated an upregulation in nuclear pore complex components, Nup62 and Nup153, compared to all other treatment groups. Importin-α, importin-β, and Ran, key players in the classical import pathway (446), all increased their expression levels significantly in response to AdHsp60 and AdHsp60^{mito-} treatment versus both control groups (Figure 25D-F). NTF2 protein expression remained similar across all treatment groups (Figure 25G).

Effect of Hsp60 overexpression on proliferative and metabolic status of the cell

Both AdHsp60 and AdHsp60^{mito-} treatments induced a proliferative response as shown by a significant upregulation in PCNA expression levels compared to both control and AdQBI groups (Figure 26A). The metabolic state of the cells was assessed via cellular ATP levels (Figure 26B). Adenoviral infection alone induced an increase in ATP levels compared to control. The AdHsp60 treatment further enhanced this response compared to both AdQBI and AdHsp60^{mito-} groups.

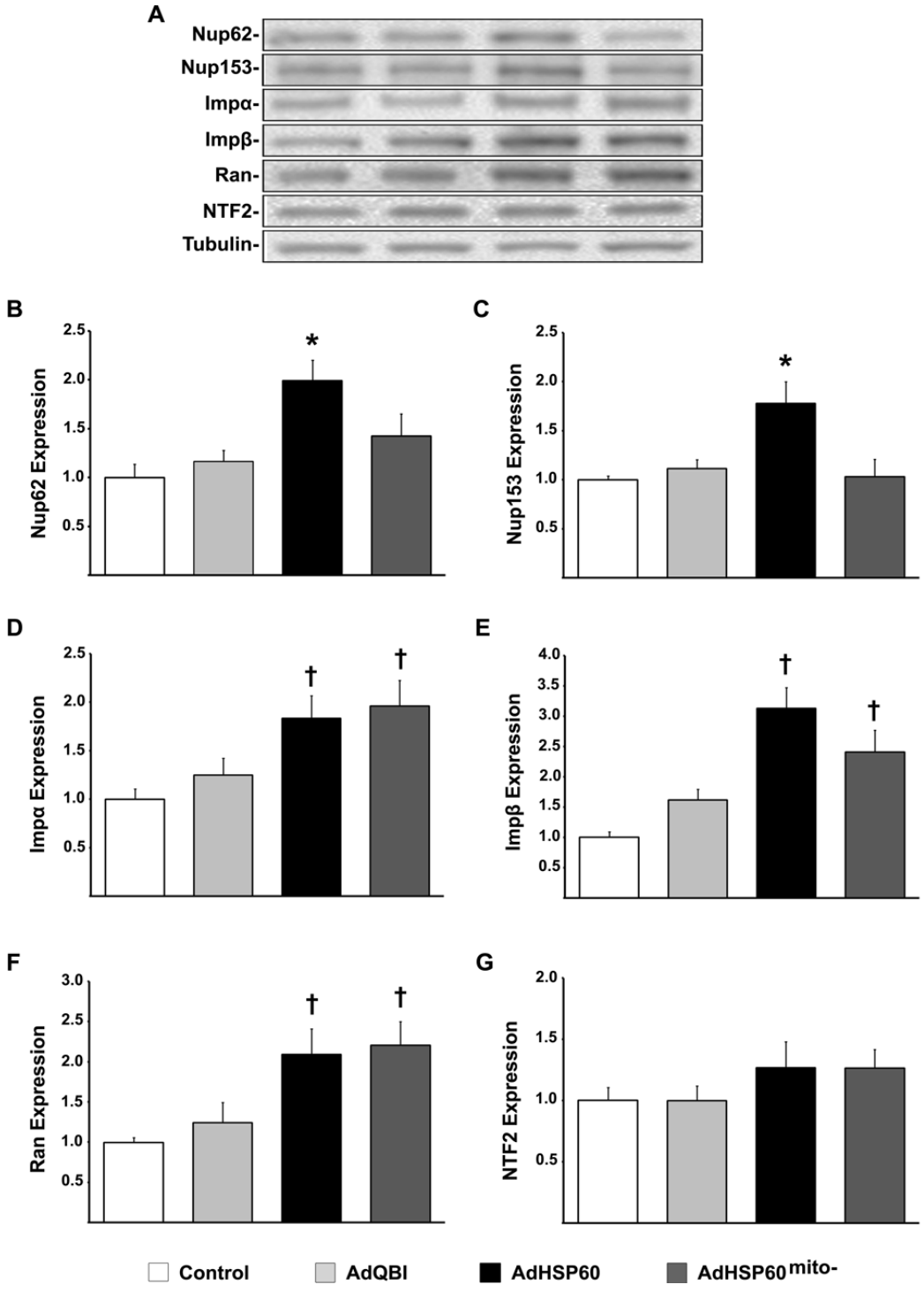

Figure 25. Effect of Hsp60 overexpression on NPI machinery
Representative Western blot images (A) and analysis of Nup62 (B), Nup153 (C), Impα (D), Impβ (E), Ran (F), and NTF2 (G) expression in response to Hsp60 overexpression. Protein expression levels are normalized to α-tubulin and are represented as fold change to control samples (*n*=6-10). *P<0.05 versus Control, AdQBI and AdHsp60^{mito-};†P<0.05 vs Control and AdQBI ; values are means± SEM.

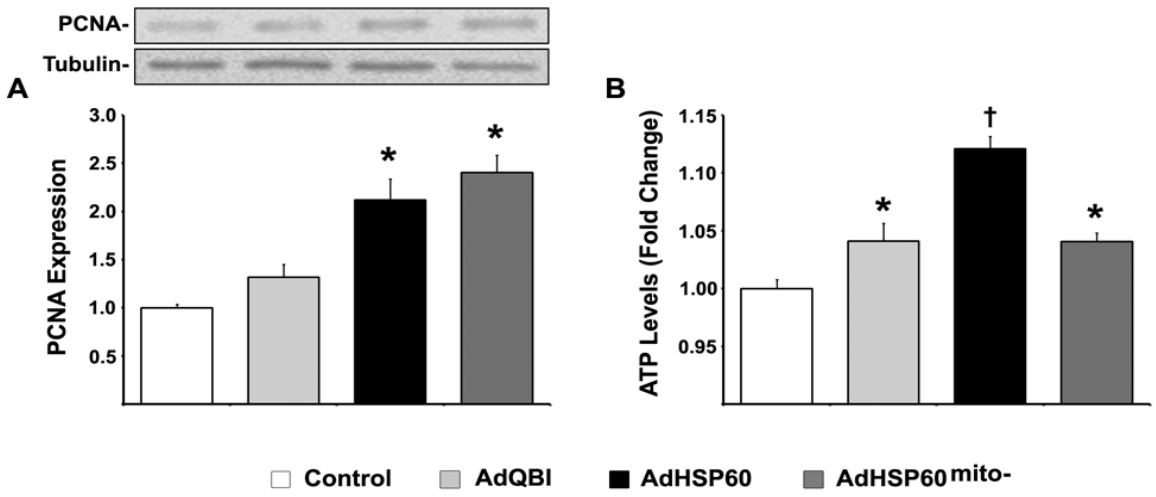

Figure 26. Effect of Hsp60 overexpression on proliferative and metabolic status of the cell
Western Blot analysis of PCNA (A) expression in response to Hsp60 overexpression. Representative Western Blot for PCNA and Tubulin (loading control) are displayed above their corresponding graphs. Normalized protein expression levels were represented as fold change to control samples (*n*=8-10). *P<0.05 versus Control and AdBI. Cellular ATP levels analysis (B) in response to HSP60 overexpression. ATP levels were represented as fold change to control samples (*n*=8). *P<0.05 vs. Control; †P<0.05 vs. Control, AdQBI and AdHsp60^{mito-}; values are means ± SEM.

MAPK activation has been associated with induction of nuclear protein import (113,115) and, therefore, was assessed in response to Hsp60 overexpression. AdQBI, AdHSP60 and AdHsp60^{mito-} treatments all induced modest but not statistically significant increases in p44/42 activation (1.37±0.25, 1.61±0.22 and 1.32±0.13 fold change over control, respectively). p38 activation also remained unchanged in response to AdQBI, AdHsp60 and AdHsp60^{mito-} treatments (1.16±0.15, 0.90±0.14, 0.73±0.09 % of control, respectively).

C.pneumoniae induces proliferation and Hsp60 expression

The data above suggests that Hsp60 overexpression can induce VSMC proliferation through a modulation of the expression levels of cellular import proteins that in turn modulate nuclear protein import. To determine if this modulation of import by Hsp60 over-expression has relevance to VSMC proliferation under physiological/pathological conditions, we chose to examine these responses in VSMCs exposed to *C. pneumonia.* Based on our previous results (161), *C. pneumonia* can induce VSMC proliferation and the expression of PCNA in VSMCs. VSMCs exposed to *C. pneumonia* responded with a moderate increase in PCNA expression at 24 hours that reached statistical significance at 48 hours compared to control and mock infection with heat-inactivated *C. pneumoniae* (Figure 27B). This coincided with a 1.6 fold increase in Hsp60 protein expression at 24 hours compared to the other treatments and this response was sustained at 48 hours ($P < 0.05$) (Figure 27C).

C.pneumoniae alters NPI machinery expression

Both Nup62 and Nup153 expression was up-regulated at 24 hours compared to control and mock infection (Figure 28B and 28C). Impα and Impβ were similarly increased by >2 fold at 24 hours versus control groups ($P < 0.05$) (Figure 28D and 28E). All four import components returned to control levels at 48 hours (Figure 9). In *C.pneumoniae* infected porcine coronary arteries, Nup62 expression was significantly induced at day 5 post-infection compared to both control and mock-infected vessels (Figure 29). At day 10 post-infection, both *C. pneumoniae* and mock-infected coronary arteries showed a depression in Nup62 expression (Figure 29).

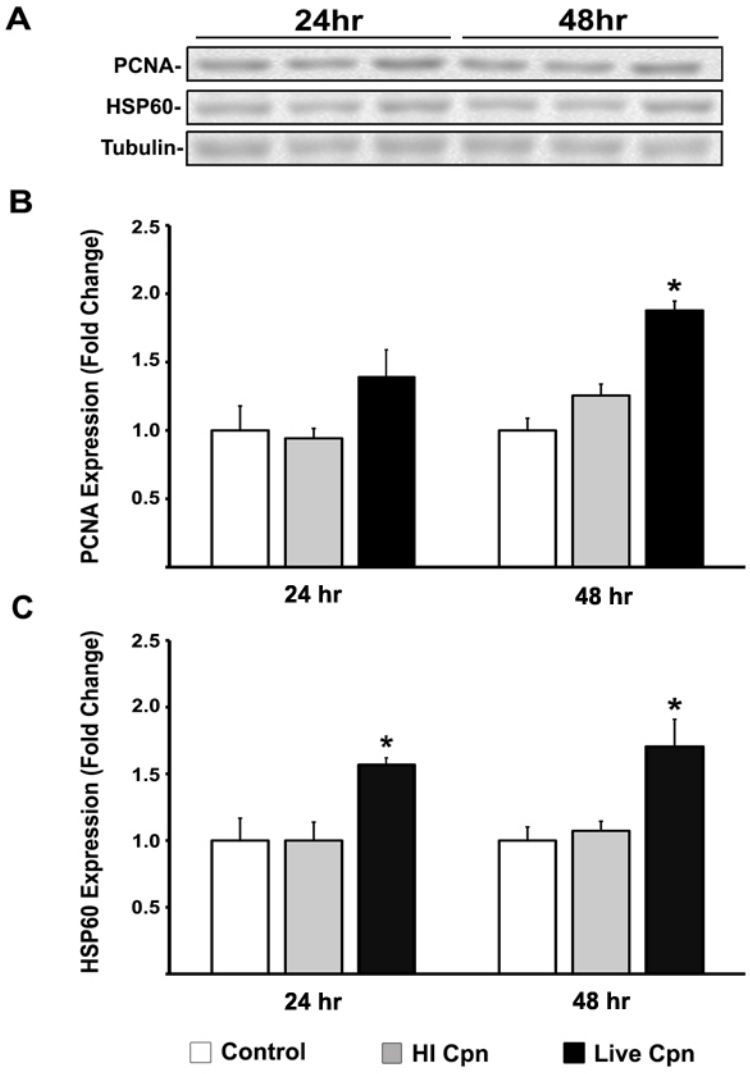

Figure 27. *C. pneumoniae* induces proliferation and Hsp60 expression in VSMCs
Representative Western Blot images (A) and analysis of PCNA (B) and Hsp60 (C) expression in response to *C. pneumoniae* infection at 24 and 48 hours. Protein expression levels are normalized to α-tubulin and are represented as fold change to control samples (n=3-4). *P<0.05 versus Control and AdQBI; values are means ± SEM.

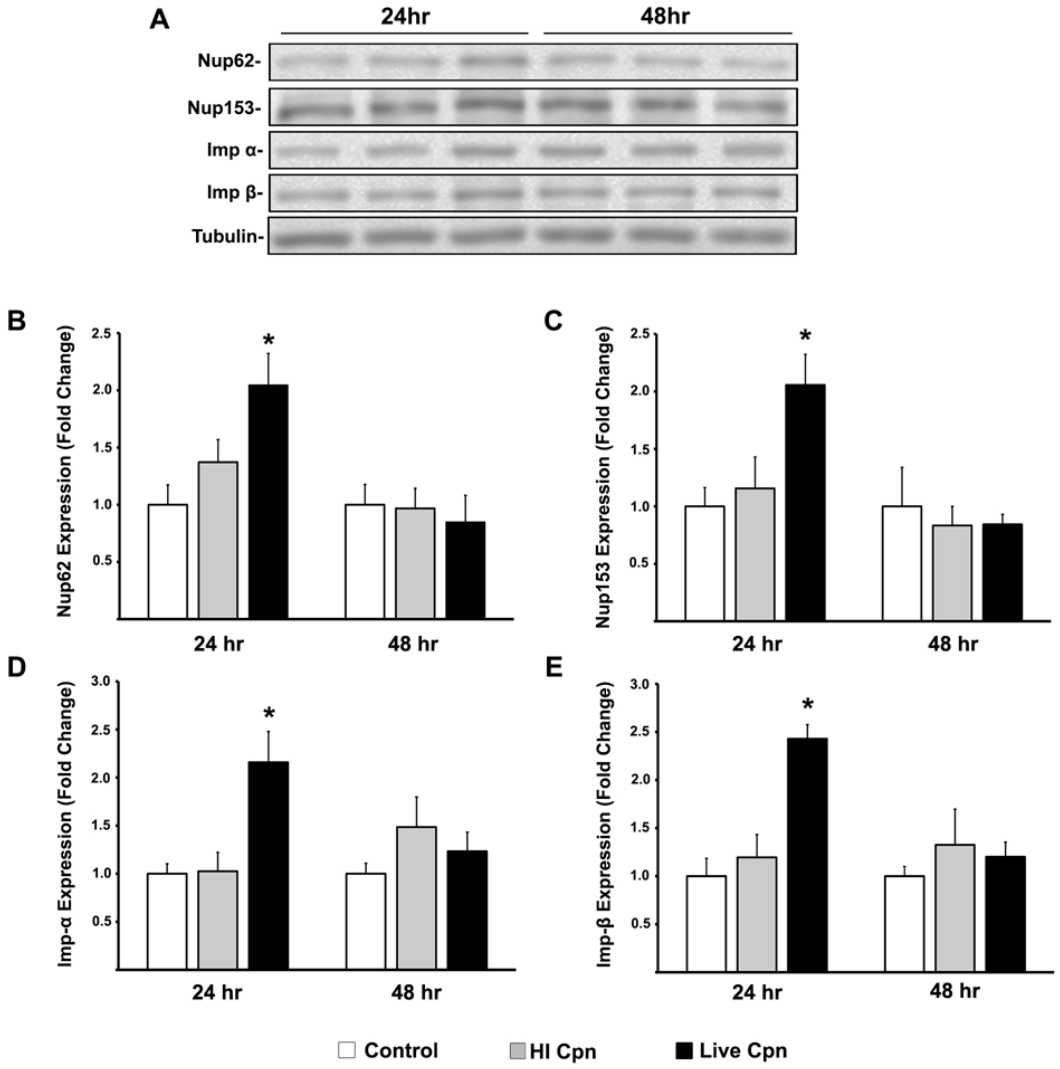

Figure 28. *C. pneumoniae* induces NPI machinery expression in VSMCs
Representative Western blot images (A) and analysis of Nup62 (B), Nup153 (C), Importin-α (D), Importin-β (E) in response to *C. pneumoniae* infection at 24 and 48 hours. Protein expression levels are normalized to α-tubulin and are represented as fold change to control samples (*n*=3-4). *P<0.05 versus Control and AdBI; values are means ± SEM.

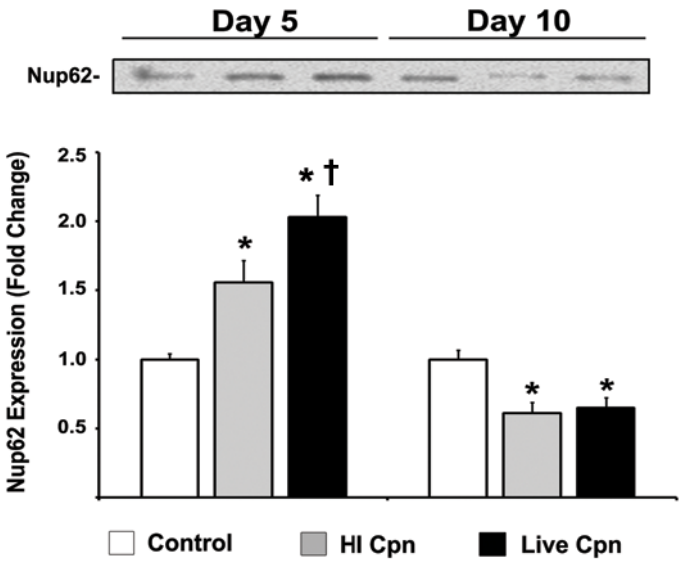

Figure 29. Nup62 expression in *C. pneumoniae* infected isolated coronary arteries
Western blot analysis of whole tissue lysates at Day 5 (left) and Day 10 (right) post-infection. Representative western blots are displayed above graph. Expression levels were represented as fold change over control ± SEM, n =5-6. *P<0.05 vs. Control; †P<0.05 vs. HI *Cpn*

Cholesterol diet withdrawal induces a biphasic atherosclerotic plaque development

We have previously demonstrated with this atherosclerosis model a biphasic atherosclerotic plaque development pattern (Figure 30) (530). Atherosclerotic development is observed between 0-8 weeks of cholesterol withdrawal followed by a stabilization period between 8-22 weeks of withdrawal (Figure 30A). Serum cholesterol levels inversely correlated with atherosclerosis, with elevated levels at 0 weeks of withdrawal and sequential decreases until reaching control levels at 22 weeks of cholesterol withdrawal (Figure 30B).

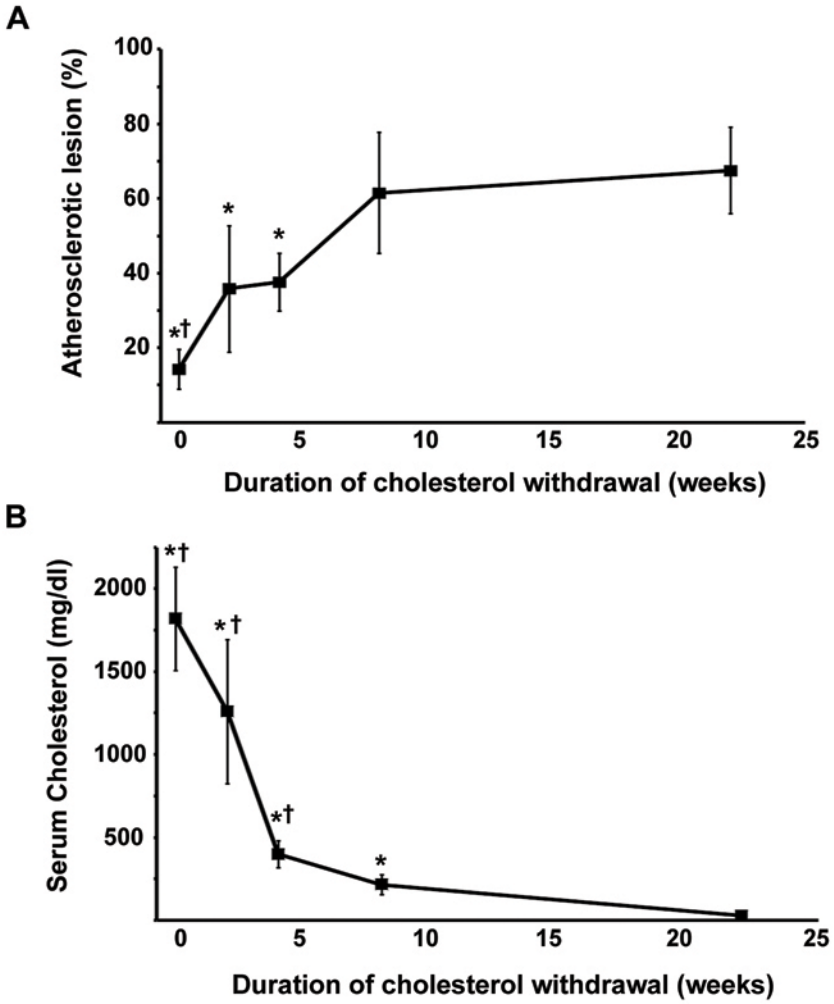

Figure 30. Atherosclerotic lesion development and serum cholesterol following cholesterol withdrawal

Aortic atherosclerotic lesion **(A)** and serum cholesterol (B) after 0, 2, 4, 8, and 22 weeks of withdrawal from cholesterol feeding. Values are means±SEM, n=4. *P<0.05 vs. 22 wks of diet withdrawal; †P<0.05 vs. 8 wks of diet withdrawal. This figure has been adapted from Francis et al. (530).

Atherosclerotic plaque development induces alterations in Hsps expression

To evaluate stress responses during both the growth and stabilization phases within this model, Hsp expression within atherosclerotic tissues was evaluated. Hsp60 expression was induced during the growth phase at both 2 and 4 weeks following cholesterol withdrawal when compared to control and longer withdrawal periods (8 and

22 weeks) (Fig 31b). A depression in both Hsp70 and Hsp90 expression was observed during the growth phase of plaque development (Figure 31C and 31D). This decrease was followed by a subsequent return to baseline or an increase during the stabilisation phase for Hsp70 and Hsp90 expression, respectively (Figure 31D). Significant decreases in Hsp70 were noted at 4 weeks of withdrawal versus both control and 22 weeks of withdrawal (Figure 31C). Prolonged removal of cholesterol (8 and 22 weeks) significantly increased Hsp90 expression compared to early withdrawal periods at 2 and 4 weeks (Figure 31D).

Atherosclerotic plaque development coincides with alterations in proliferative status and NPI machinery expression

PCNA expression was upregulated at 0, 2,4 and 8 weeks prior to a return to baseline at 22 weeks after cholesterol withdrawal from the diet (Figure 32B). Nup62 expression was significantly induced at 2 and 4 weeks of withdrawal compared to 22 weeks of withdrawal and control (Figure 32C). Additionally, Nup62 expression at 4 weeks of withdrawal was significantly increased compared to 8 weeks of cholesterol withdrawal (Figure 32). Importinβ expression did not change throughout the entire cholesterol withdrawal period (Figure 32D). Eight weeks of cholesterol withdrawal coincided with an increase Ran expression compared to both early withdrawal periods (0,2,4 weeks) and control (Figure 32E).

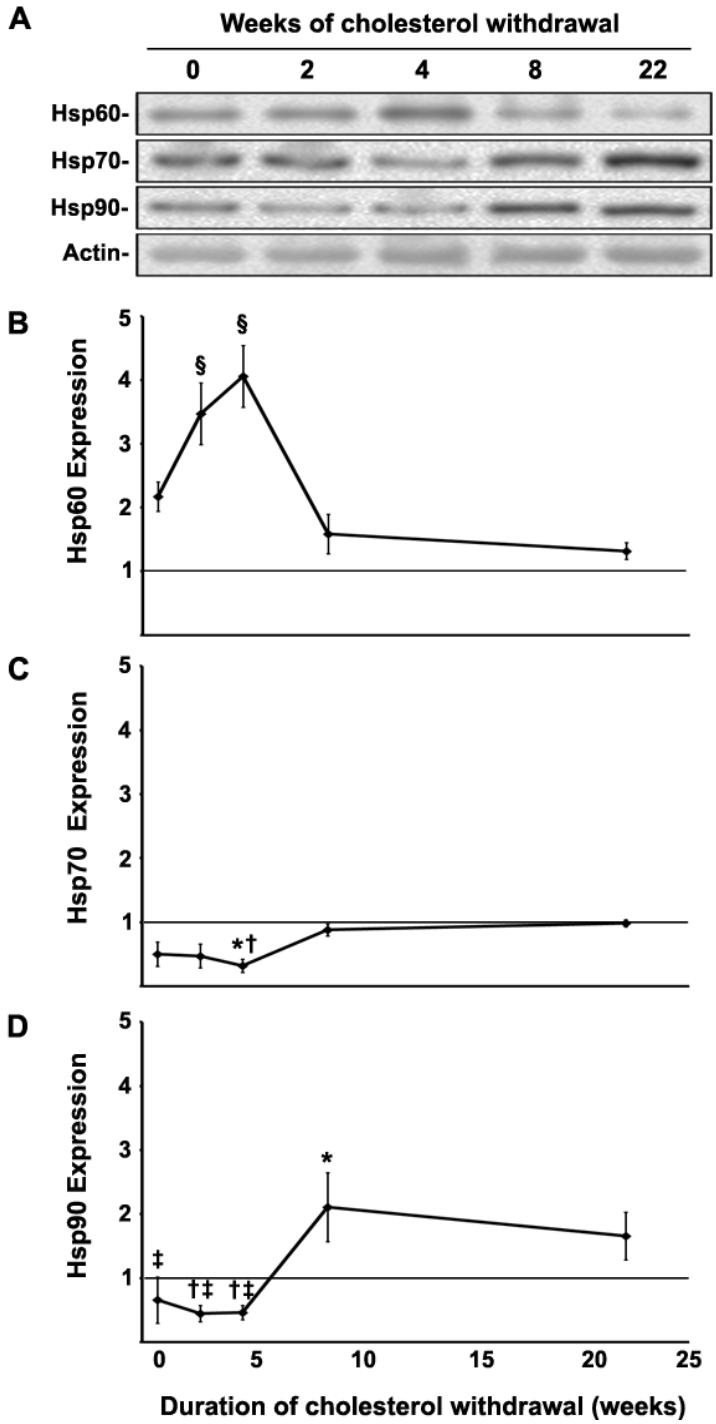

Figure 31. Hsps expression during atherosclerotic development
Representative Western blot images (A) and analysis of Hsp60 (B), Hsp70 (C), Hsp90 (D) after 0,2,4,8, and 22 weeks of withdrawal from cholesterol feeding. Protein expression levels are normalized to total actin and are represented as fold change to control samples. Values are means±SEM, *n*=3-4. **P*<0.05 vs. control; †P<0.05 vs 22 wks of cholesterol withdrawal; ‡ *P*<0.05 vs. 8 wks of cholesterol withdrawal; §*P*< 0.05 vs. control, 0, 8, and 22 wks of cholesterol withdrawal.

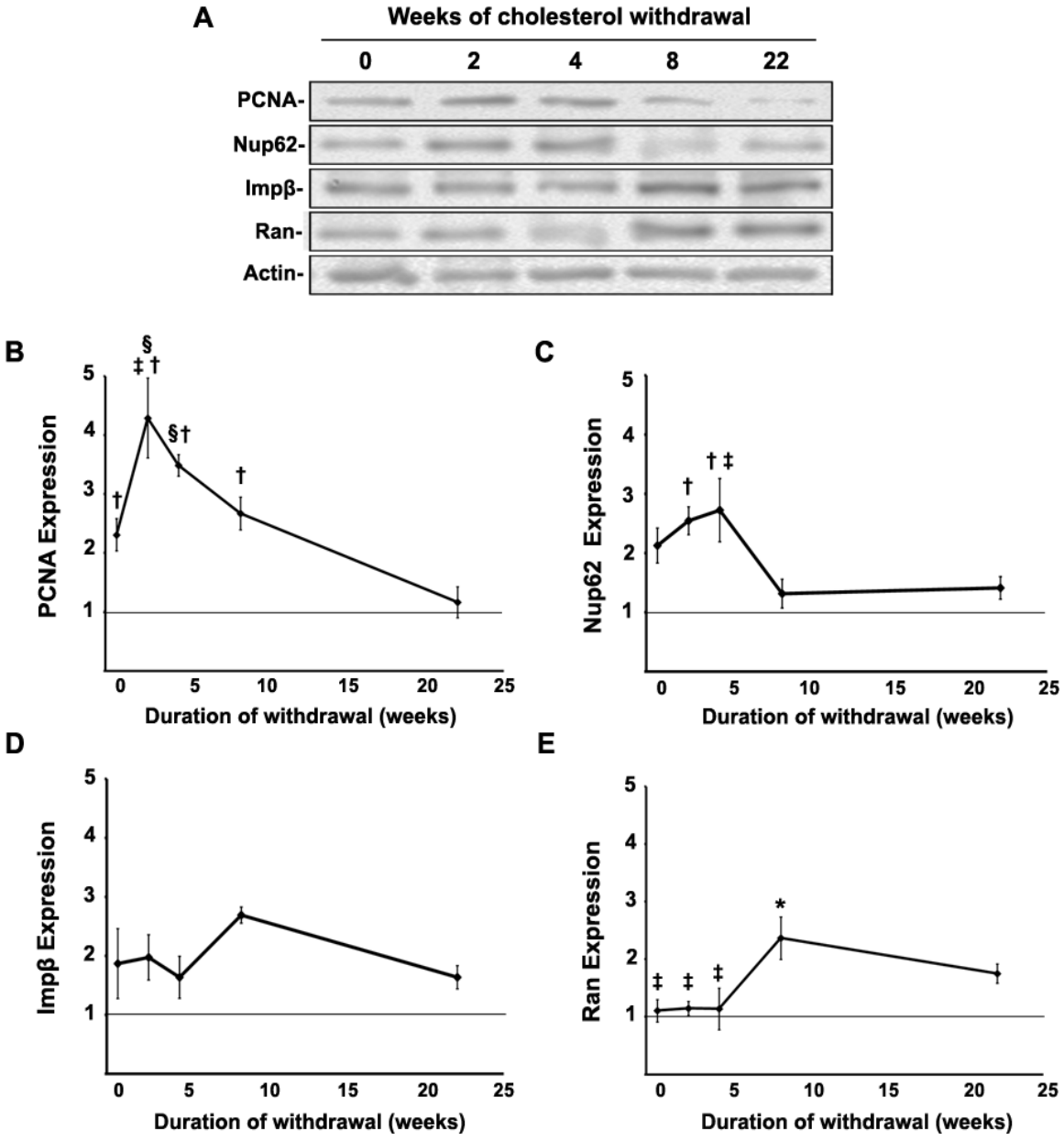

Figure 32. PCNA and NPI machinery expression during atherosclerotic development
Representative Western blot images (A) and analysis of PCNA (B), Nup62 (C), Impβ (D), and Ran (E) after 0,2,4,8, and 22 weeks of withdrawal from cholesterol feeding. Protein expression levels are normalized to total actin and are represented as fold change to control samples (Regular diet for 12 weeks). Values are means±SEM, *n*=3-4. **P*<0.05 vs. control; †P<0.05 vs control and 22 wks of cholesterol withdrawal; ‡ *P*<0.05 vs. 8 wks of cholesterol withdrawal; §*P*< 0.05 vs. 0 wks of cholesterol withdrawal.

Aortic Hsp60 levels correlate with PCNA, Nup62 and plasma cholesterol

To evaluate the relationship between Hsp60 and other parameters during atherosclerosis development, correlation analysis was performed (Figure 33). Hsp60 expression strongly correlated with both PCNA and Nup62 expression ($r^2 = 0.3027$ and $r^2 = 0.3164$, $p<0.01$) (Figure 33A and 33B). In addition, a moderate correlation between aortic Hsp60 expression and plasma cholesterol was also observed ($r^2 = 0.1682$, $p<0.05$) (Figure 33C).

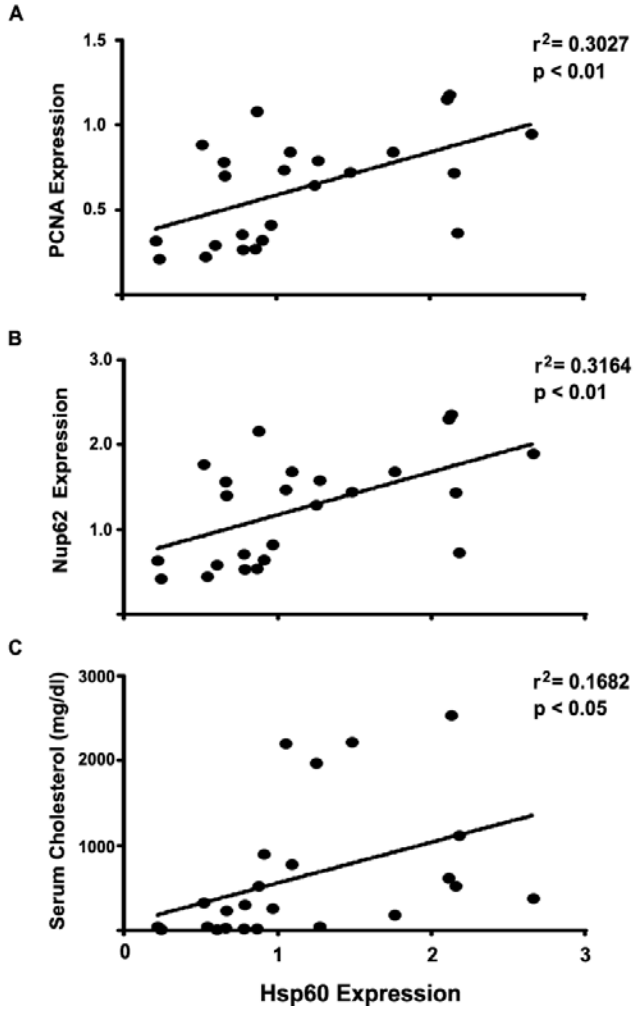

Figure 33. Correlations of aortic Hsp60 levels with PCNA, Nup62 and plasma cholesterol Relationship between aortic Hsp60 and aortic PCNA (A), aortic Nup62 (B), and serum cholesterol (C) during atherosclerotic development.

CHAPTER VI: DISCUSSION

1. Effects of *C. pneumoniae* infection on structural remodelling in porcine coronary arteries [7]

Strong clinical correlations between *C. pneumoniae* infection and coronary artery disease have been reported (531-534). Consistent with these findings, in the present study we have shown that *C. pneumoniae* can infect the coronary artery in culture and this infection promotes a stimulation of cell proliferation and intimal thickening. *C. pneumoniae* can infect and persist through multiple life cycles (which is typically 48-72hrs) (161,535) in a coronary artery. At day 2 pi, *C. pneumoniae* IB distribution was found mostly in the endothelial layer (Figure 7a) which is expected since only the endothelium was exposed to the pathogen during the initial infection. On subsequent days, the *C. pneumoniae* IBs distribution was shifted and it was found almost exclusively in the smooth muscle cell media (Figure 7b-e). Our data is consistent with previously published findings, [161,536,537] that the smooth muscle cell media is the preferred location of the *C. pneumoniae* propagation. *C.pneumoniae* infection did not induce endothelial cell apoptosis (data not shown), therefore, the inability of the endothelial layer to support stable *C. pneumoniae* replication is probably due to the initial damage of the endothelial cells caused by the infection (161).

Increasing levels of chlamydial Hsp60 in the infected coronary segments (Figure 8b) further supported the assertion that *C. pneumoniae* was metabolically active in the

[7] Reprinted from Am J Path 176(2), JF Deniset et al., *Chlamydophila pneumoniae* infection leads to smooth muscle cell proliferation and thickening in the coronary artery without contributions from a host immune response. 1028-1037, © 2010, with permission from Elsevier. http://www.sciencedirect.com/science/journal/

coronary segments. Chlamydial Hsp60 is synthesized only by metabolically active *C. pneumoniae*-RB and not the progeny *C. pneumoniae*-EB (538), and a significant amount of chlamydial Hsp60 is released into the circulation during infection (539,540). As such, the observed increase of chlamydial Hsp60 protein (~2.5x) in the protein lysate of coronary segments may only represent a conservative estimate of the total *C. pneumoniae* in the infected vessels. According to this data, *C. pneumoniae* replicates and remains metabolically active in our novel ex-*vivo* coronary artery model. Another important conclusion from these findings is that *in vivo C. pneumoniae* infection of the endothelial cells layer is sufficient to initiate propagation of the infection into the arterial media.

The infection of the isolated coronary artery with *C. pneumoniae* had structural implications for the arterial wall. The induction of PCNA would suggest that cell proliferation is stimulated in the artery after *C. pneumoniae* infection. Consistent with our previous work (161), the mechanism by which infection with *C. pneumoniae* augments the proliferation of VSMC may involve stimulating the expression of endogenous Hsp60. In the present study, the expression of h-Hsp60 was increased in the tissue during *C. pneumoniae* infection (Fig. 9a). Increasing PCNA expression confirmed the proliferative nature in the infected vessel. However, it remains uncertain from *in vitro* cell culture studies if the proliferative responses ultimately result in structural thickening. Our data here now clearly shows that *C. pneumoniae* infection can directly induce thickening of the arterial wall in the intimal region in as little as a few days after exposure. Overall, we have observed a significant increase in thickening of the media starting at day-6 pi. Also, we observed that a vast majority of infected vessels demonstrated a collapse in the

circular geometry, most notably from day-8 and onward. We did not collect vessels beyond day-10 because most infected vessels were so severely affected that they exhibited a completely collapsed lumen beyond that time point. Such narrowing would be expected to be associated with clinically significant effects. Our data is in agreement with observations that *C. pneumoniae* seropositivity is associated with arterial thickening,(541) and that infection by *C. pneumoniae* without other atherosclerotic factors can induce atherosclerotic events (246).

One of the most important findings from this study is the demonstration that an active *C. pneumoniae* infection alone can lead to arterial thickening in the absence of host immune responses. Previous *in vivo* studies have not been able to distinguish the contributions of the host immune responses at the infected location from the direct molecular consequences of bacterial replication in the infected media. Our study was designed to insure that the coronary arteries were isolated from host immune responses (including macrophages, T-cells) and other biological molecules (such as elevated cholesterol) that may contribute to lesion formation. The use of heat-inactivated *C. pneumoniae* as a control is appropriate to reduce the potential contribution to the thickening effect that occurs as a result of bacterial LPS or mechanical stress induced by our handling. Further, comparisons were undertaken using paired coronary segments from the same heart (Figure 10) to strengthen the reliability of our observations. Thus, our h-Hsp60 and PCNA expression data suggest *C. pneumoniae* infection can directly lead to the proliferation of the smooth muscle cells within the intima strongly enough to ultimately produce arterial thickening, without contribution from the host immune

system. Despite the absence of an active immune response, however, immune mediators such as pro-inflammatory cytokines and chemokines could be involved in this proliferative response. *C. pneumoniae* infection of vascular smooth muscle cells has been shown to stimulate production and release of interleukin (IL)-6 (542) and monocyte chemoattractant protein (MCP)-1 (231) two mediators known to have proliferative effects on vascular smooth muscle cells (543-545). Thus, both IL-6 and MCP-1 through autocrine and/or paracrine actions may additionally stimulate the *C.pneumoniae* effects beyond that which were observed in the present set of experiments. The addition of monocytes to the system could further enhance this effect, as co-culturing of monocytes and vascular smooth muscle cells alone can not only enhance the growth of *C. pneumoniae* (546) but also stimulate the production of IL-6 and MCP-1 (547). These data do not dispute the importance of a host immune response as an essential contributor to the overall atherosclerotic plaque development process. Rather, the present work has provided important evidence that *C. pneumoniae* on its own can induce a significant component of atherosclerosis (cell proliferation and intimal thickening).

In conclusion, our data has provided the first direct evidence that an active *C. pneumoniae* infection alone, without contributions from a host immune system, can stimulate arterial thickening. This effect is associated with the up-regulation of h-Hsp60 and PCNA expression. Our work has also demonstrated the feasibility of studying the molecular mechanisms of infection-induced atherosclerosis using an *ex vivo* coronary culture system.

2. Effects of *C. pneumoniae* infection on vascular contractile function in porcine coronary arteries [8]

We hypothesized that *C. pneumoniae* infection may participate in phenotype switching in VSMCs and as such may interfere with the ability of these cells to properly mediate the contractile and relaxation responses within an arterial environment. Our findings clearly demonstrate that *C. pneumoniae* infection leads to an impairment of both receptor (u46619) and depolarization (KCl)- mediated contractile responses (Figure 12). The involvement of the L-type Ca^{2+} channel and IP3R in this impairment was evaluated in the present study. In VSMCs, the L-type Ca^{2+} channel is the most important ion channel for the influx of extracellular Ca^{2+} (548) leading to downstream activation of myosin light chain kinase (MLCK). The channel can be opened directly through membrane depolarization by high concentrations of K^+ or indirectly through some agonists (549). The contractile responses elicited by u46619 are also dependent significantly upon activation of the L-type Ca^{2+} channel. Inhibition of the L-type Ca^{2+} channel resulted in a 50% reduction of maximal force production generated by u46619 in porcine coronary arteries (550). In the present study, expression of the L-type Ca^{2+} channel was down-regulated at both time points when compared to control and mock infected vessels (Figure 14A and 14B). This is similar to the contractile changes exhibited in response to KCl and u46619. IP3R, found on the sarcoplasmic reticulum (SR), are also involved in MLCK activation through the release of SR Ca^{2+} stores in response to agonist mediated G-protein coupled signaling.(549) IP3R expression in infected coronary vessels remained unaffected at day 5 pi but was significantly

[8] Reprinted from Am J Path 180(3), JF Deniset et al., Chlamydophila pneumoniae infection induces alterations in vascular contractile responses, 1264-72, © 2012, with permission from Elsevier. http://www.sciencedirect.com/science/journal/

downregulated at day 10 pi (Figure 14C and 14D). Because a decrease in contraction at day 5 post infection was observed without a change in IP3R expression and a decrease in IP3R expression at day 10 post infection does not result in a further reduction in contractile performance of infected tissue, the contribution of IP3R in the defects exhibited in response to u46619 is questionable. The most likely mechanism for the alterations in contractile responses to both high K^+ and u46619 is through the reduction in L-type Ca^{2+} channel expression induced by *C. pneumoniae* infection. A decrease in the L-type Ca^{2+} channel expression occurring in synthetic VSMCs is accompanied with an attenuation in SM-actin and SM-MHC expression, two key components of the contractile apparatus. In the current study, although not significant, a decreasing trend in SM-actin and SM-MHC expression in *C. pneumoniae* infected vessels can be observed. Considering the mixed population of VSMCs (contractile and synthetic), it is possible the abundant expression of these markers in the contractile VSMC population may be dampening the decreases in expression within the synthetic VSMC population, thus contributing to the overall expression observed within whole vessel lysates. As such, the potential contribution of both these contractile proteins in the altered contractile responses should not be completely dismissed.

Other mechanisms beyond simple changes in contractile machinery expression could be consider for the observed contractile responses. Structural changes in *C. pneumoniae* infected vessels have previously been noted using this same model system (525). Synthetic VSMCs demonstrate an increase in extracellular matrix production (551). As such, an increase in medial thickening via VSMCs proliferation could in

theory confer changes in vessel stiffness through increased collagen production. However, it is important to note that these potential alterations in vessel stiffness would likely occur at later time-points as medial thickening only starts to occur from day 6 post-infection onward (525). It is then unlikely that the changes in vascular contractile function observed at day 5 post-infection are due to this phenomenon. Additionally, the attenuation in contractile responses in *C. pneumoniae*-infected vessels is not further aggravated at day 10 post-infection, which suggests vessel stiffness is likely not a major contributor.

C. pneumoniae infection also induced a significant attenuation in bradykinin-induced relaxation of pre-contracted tissue (Figure 11B). Bradykinin elicits relaxation of vascular tissue through an endothelial-dependent mechanism (552). The impairment in vasorelaxation was limited to the endothelial layer in the present study as *C. pneumoniae* infection did not affect relaxation induced by the NO donor SNP. *C. pneumoniae* is known to induce endothelial damage (161). However in this model, *C. pneumoniae* infection does not induce endothelial cell death (525). Therefore, the attenuated relaxation response is more likely due to a cellular mechanism and not a deterioration of the endothelial layer.

Alterations to the (eNOS)/nitric oxide (NO) pathway, leading to a reduction in NO bioavailability, represent the most important mechanism for impaired endothelial-dependant relaxation (553). The relaxation responses to bradykinin in porcine coronary arteries is endothelial NO-dependent (554). Bouwman et al. have previously noted that *C.*

pneumoniae infection in HUVECs results in a decrease in eNOS and downstream cGMP downregulation (555). Our data further supports this mechanism as *C. pneumoniae* infection is also capable of inducing decreased eNOS expression in this organ culture system. However, it is unclear whether these changes are directly as a result of live *C. pneumoniae* within the endothelial layer. As previously described with this model, there is a significant migration of *C. pneumoniae* from the endothelium into the medial area of coronary arteries from day 6 post infection and onward. It is difficult to determine whether the *C. pneumoniae* remaining population in the endothelium at day 9-10 post-infection is large enough to alone account for these changes in eNOS expression. Alternatively, secretory factors released by the host cells or the bacteria itself within the neighboring medial layer may play a role in the changes observed. cHSP60 represents a potential candidate for such a role. It is highly expressed in *C. pneumoniae*-infected coronary arteries (525) and Chen et al. established its ability to down-regulate eNOS expression and activity in human coronary artery endothelial cells (HCAECs) and porcine coronary arteries via TLR-4 signalling (232). We have demonstrated that cHsp60 expression is localized with TLR-4 along the endothelial layer in *C. pneumonia* infected vessels (Figure 19I and 19G). However, conditioned medium retrieved from *C. pneumoniae* infected vessels was unable to elicit changes in eNOS and cHsp60 levels were well below the concentrations used by Chen et al. (232) This data would suggest that cHsp60 secreted from either infected VSMCs and/or endothelial cells is quickly binding TLR-4 along the endothelium and eliciting changes in eNOS.

In addition to a reduction in NO production, NO breakdown by superoxide radicals represents another mechanism for the *C. pneumoniae* derived impairment in endothelial-dependent relaxation. Dihydroethidium staining of coronary vessels revealed an increase in superoxide production in both mock-infected and *C. pneumoniae*-infected vessels (Figure 18). Among the many sources of reactive oxygen species, NADPH oxidase is a major producer of superoxide within the vessel wall (556). p22phox, a major component of NAPDH oxidase, is expressed in ECs, VSMCs, adventitial fibroblasts and macrophages within human atherosclerotic plaques (64). p22phox expression was upregulated in both mock-infected and *C. pneumoniae*-infected vessels at day 10 pi (Figure 17B). Furthermore, its expression was identified in both endothelial and medial layers (Figure 17C). The ability for the heat-inactivated *C. pneumoniae* to also elicit this response suggests that this effect is lipopolysaccharide (LPS)-mediated. Wick et al. described that *in vivo* LPS administration increases hHsp60 expression on the surface of vascular endothelial cells (557). Our results indicate that *C. pneumoniae* and mock-infection result in an increase in hHsp60 expression along the endothelium. In addition to downregulating eNOS, Chen et al. (232) also described cHsp60's ability to upregulate oxidative stress machinery including p22phox. Due to conserved sequence homology for Hsp60 between species, it is possible that hHsp60 may also induce similar downstream cellular changes through TLR-4 signalling.

The maintenance of isolated coronary vessels in a tissue culture environment for up to 10 days does not significantly alter the functional integrity of these arteries (525). Thus, the alterations observed in the present study are directly dependent on exposure to

C. pneumoniae and/or *C. pneumoniae* components. The findings of the current study clearly demonstrate that *C. pneumoniae* infection is able to directly induce a significant impairment to the functional capacity of arteries. These defects are observed prior to (at 5 days) and after (10 days) significant thickening (hypertrophy) of the aorta is observed (525). These changes may have physiological consequences with regards to regulating normal blood flow and may play an important role in the pathophysiology of hypertension, restenosis and atherosclerosis.

3. Role of Hsp60 in modulating nuclear protein import during VSMC proliferation

The current study establishes a novel intracellular signaling role for Hsp60 in VSMC through a modulation of NPI. Furthermore, the induction of cytoplasmic Hsp60 alone was sufficient to influence NPI. This is the first report of a function for the cytoplasmic pool of Hsp60 within VSMCs. In contrast to mitochondrial Hsp60, which forms a multimeric structure with its co-chaperone Hsp10, cytoplasmic Hsp60 is monomeric and independently interacts with different proteins (558). Cytoplasmic Hsp60 can sequester pro-apoptotic factors like Bcl-2-associated X protein (Bax) and Bcl-2 antagonist/killer-1 (Bak) by binding to them and thereby preventing mitochondrial pore formation and induction of apoptosis (344,559). This type of interaction may be occurring within our VSMCs which in turn may indirectly influence NPI by altering the growth/death signal balance. Alternatively, we have shown that cytoplasmic Hsp60 was also directly affecting proteins involved in NPI. Importin-α, importin-β and Ran expression were all increased in response to Hsp60 overexpression. All of these proteins

play critical roles in the import of cargo protein into the nucleus (446). The localization of cytosolic Hsp60 at the nucleus (Hsp60 and HA, Figure 21) implies binding of Hsp60 to the nuclear envelope or an active shuttling of Hsp60 across the nuclear envelope.

Precedents have been observed for a role for heat shock proteins in nuclear trafficking. Heat shock stress has been shown to suppress the classical NPI pathway through nuclear retention of importin-α and –β (499). The heat shock stress response is regulated by heat shock factors (Hsfs) leading to an upregulation of heat shock proteins (560). Hsp70 can play an important role in NPI by regulating the export of importin-β back to the cytoplasm (561). In addition, both Hsp70 and Hsp90 have been implicated in the nucleocytoplasmic transport of specific cargo proteins (562,563). In the present study, none of these other heat shock proteins could have affected our results since their expression levels were not altered by an overexpression of Hsp60 (Figure 23).

Although cytoplasmic Hsp60 could induce changes in NPI on its own, mitochondrial Hsp60 induced specific changes in nucleoporin (Nup62, Nup153) expression. Physiological and pathological conditions can also induce changes in Nup62 and Nup153 expression (113,115). This induction in nucleoporins may result from an enhancement of the metabolic state in the cells as indicated by an increase in cellular ATP levels (Figure 26B).

Our current findings have important pathological implications. VSMC proliferation is a key mediator in vascular pathologies such as atherosclerosis (564).

Human Hsp60 expression has been detected in VSMCs within atherosclerotic lesions (421). Furthermore, human Hsp60 co-localizes with chlamydial Hsp60 within the atherosclerotic plaques (239). *C. pneumoniae,* including viable forms of the bacterium, have also been localized within human atherosclerotic plaques (221). Importantly, *C. pneumoniae* infection can initiate medial thickening and VSMC proliferation *in situ* independently of a host immune response (525). Furthermore, this proliferative response in the aortic tissue coincided with an increase in vascular Hsp60 expression (525). In the present study, *C. pneumonia* also stimulated VSMC proliferation and Hsp60 expression. It was not possible for us to test the direct effects of *C. pneumoniae* in NPI experiments on the confocal microscope due to the danger of using live *C. pneumoniae* outside of a sterile hood. However, as shown in Figure 28 and 29, *C. pneumonia* infection did alter the expression of nucleoporins and the importins. This would suggest a contribution from both cytosolic and mitochondrial forms of Hsp60 in the *C. pneumonia*-mediated response. The biphasic effect of *C. pneumoniae* on the expression of NPI machinery *in vitro* and within a vascular environment is consistent with previous data that indicated that stress-mediated changes in NPI precede the proliferative response in VSMCs (115).

In addition, we also demonstrate the contribution of Hsp60 to alterations in components involved in NPI during atherosclerotic development. Induction of Hsp60 in this model occurred during the growth phase of atherosclerotic plaque and correlated with the proliferative status within the atherosclerotic vessel wall, as indicated by PCNA expression. Nup62 was similarly induced during the growth phase and its expression correlated with Hsp60 throughout atherosclerotic development, whereas both Impβ or

Ran did not. We have previously demonstrated that oxLDL can induce Hsp60 expression and alterations in Nup62 expression as well as NPI (115,343,423). Although not addressed, we can postulate that the elevated circulating cholesterol levels contributed to the increases in oxLDL within the atherosclerotic lesion, and may, therefore, be involved in the alterations observed. Interestingly, differential expression patterns for Hsp60 compared to Hsp70 and Hsp90 were observed during atherosclerosis. This would suggest that Hsp60 induction might result from an Hsf1-independent mechanism. Alternative pathways such as NFκB signalling, are known to influence Hsp60 expression in this manner (400).

Three distinctive stress agents relevant to the vascular remodelling processes in hypertension and atherosclerosis –oxLDL, mechanical stretch and now *C. pneumoniae* infection, have demonstrated an ability to induce both Hsp60 expression along with alterations in NPI leading to VSMC proliferation. This supports the contention that Hsp60 may work as a common downstream mediator of VSMC proliferation during atherosclerotic development. Targeting Hsp60, therefore, may represent an effective alternative therapeutic strategy. By targeting a common cellular process as opposed to individual stressors involved in cellular dysfunction, one may be able to prevent these deleterious cardiovascular side effects of *C. pneumoniae* infection, mechanical stretch and exposure to oxLDL. Additionally, our findings also carry with them important considerations with regards to cancer treatment. Hsp60 expression is upregulated in a number of different cancers (565). In addition to its anti-apoptotic effects within these cells, it is possible that Hsp60 may also modulate changes in NPI thus further

accelerating tumor growth. Targeting nuclear protein import and/or Hsp60 may, therefore, represent a fruitful strategy to regulate pathological cell growth in many chronic diseases (566).

CHAPTER VII : CONCLUSIONS AND FUTURE DIRECTIONS

Through these studies, we have identified that:

1) *C. pneumoniae* infection represents a causative agent for vascular remodelling processes via induction of medial thickening, endothelial dysfunction and impairment of vascular contractile parameters.

2) Impairment of vascular contraction and relaxation induced by *C. pneumoniae* infection may occur through changes in the oxidative status of the tissue, ion transport and nitric oxide synthesis in the vessel wall.

3) *C. pneumoniae* infection can induce medial thickening and cell proliferation within the vascular wall independent of contributions from a host immune response.

4) *C. pneumoniae* infection can induce Hsp60 expression and these changes in Hsp60 expression correlate with proliferative responses in the vessel wall and during atherosclerotic development.

5) Hsp60 directly modulates nuclear protein import leading to VSMC proliferation.

6) Vascular remodelling in response to *C. pneumoniae* infection or a high cholesterol diet is associated with alterations in the expression of accessory proteins involved in nuclear protein import.

Future studies that would complement these findings and further advance the field:

1) Evaluating the direct impact of persistent *C. pneumoniae* infection on vascular remodelling processes employing our *ex vivo* coronary artery infection model.

2) Investigating the additive and/or synergistic effects of *C. pneumoniae* infection in combination with other atherosclerotic risk factors (e.g. oxLDL) on vascular remodelling processes employing our *ex vivo* coronary artery infection model.

3) Determining the direct contribution of *C. pneumoniae* infection in VSMC proliferation during restenosis following balloon injury employing our *ex vivo* coronary artery infection model.

4) Evaluating the ability of other pathogens (e.g. *H. pylori*, CMV, *P. gingivalis*) to stimulate vascular remodelling in the absence of a host immune response employing our *ex vivo* coronary artery infection model.

5) Confirming the direct involvement of Hsp60 in stress-mediated VSMC proliferation via alterations in NPI using loss-of-function approaches (e.g. siRNA knockdown) in isolated VSMCs.

6) Determining the contribution of Hsp10 (Hsp60 co-chaperone) to VSMC proliferation and modulation of NPI using overexpression approaches (Hsp10 alone and/or Hsp10/Hsp60 co-overexpresion) in isolated VSMCs.

7) Investigating the role of Hsf1 in stress mediated VSMC proliferation using overexpression (e.g. heat shock, adenoviral-mediated overexpression) and loss-of-function approaches (e.g. siRNA knockdown) in isolated VSMCs.

8) Evaluating the cell specific roles of Hsp60 during atherosclerotic development using an EC- or VSMC-specific conditional knockout approach in atherosclerotic mouse models.

9) Evaluating NPI parameters in cells directly derived from atherosclerotic plaques (mouse and human).

LITERATURE CITED

1. Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., Abraham, J., Adair, T., Aggarwal, R., Ahn, S. Y., Alvarado, M., Anderson, H. R., Anderson, L. M., Andrews, K. G., Atkinson, C., Baddour, L. M., Barker-Collo, S., Bartels, D. H., Bell, M. L., Benjamin, E. J., Bennett, D., Bhalla, K., Bikbov, B., Bin Abdulhak, A., Birbeck, G., Blyth, F., Bolliger, I., Boufous, S., Bucello, C., Burch, M., Burney, P., Carapetis, J., Chen, H., Chou, D., Chugh, S. S., Coffeng, L. E., Colan, S. D., Colquhoun, S., Colson, K. E., Condon, J., Connor, M. D., Cooper, L. T., Corriere, M., Cortinovis, M., de Vaccaro, K. C., Couser, W., Cowie, B. C., Criqui, M. H., Cross, M., Dabhadkar, K. C., Dahodwala, N., De Leo, D., Degenhardt, L., Delossantos, A., Denenberg, J., Des Jarlais, D. C., Dharmaratne, S. D., Dorsey, E. R., Driscoll, T., Duber, H., Ebel, B., Erwin, P. J., Espindola, P., Ezzati, M., Feigin, V., Flaxman, A. D., Forouzanfar, M. H., Fowkes, F. G., Franklin, R., Fransen, M., Freeman, M. K., Gabriel, S. E., Gakidou, E., Gaspari, F., Gillum, R. F., Gonzalez-Medina, D., Halasa, Y. A., Haring, D., Harrison, J. E., Havmoeller, R., Hay, R. J., Hoen, B., Hotez, P. J., Hoy, D., Jacobsen, K. H., James, S. L., Jasrasaria, R., Jayaraman, S., Johns, N., Karthikeyan, G., Kassebaum, N., Keren, A., Khoo, J. P., Knowlton, L. M., Kobusingye, O., Koranteng, A., Krishnamurthi, R., Lipnick, M., Lipshultz, S. E., Ohno, S. L., Mabweijano, J., MacIntyre, M. F., Mallinger, L., March, L., Marks, G. B., Marks, R., Matsumori, A., Matzopoulos, R., Mayosi, B. M., McAnulty, J. H., McDermott, M. M., McGrath, J., Mensah, G. A., Merriman, T. R., Michaud,

C., Miller, M., Miller, T. R., Mock, C., Mocumbi, A. O., Mokdad, A. A., Moran, A., Mulholland, K., Nair, M. N., Naldi, L., Narayan, K. M., Nasseri, K., Norman, P., O'Donnell, M., Omer, S. B., Ortblad, K., Osborne, R., Ozgediz, D., Pahari, B., Pandian, J. D., Rivero, A. P., Padilla, R. P., Perez-Ruiz, F., Perico, N., Phillips, D., Pierce, K., Pope, C. A., 3rd, Porrini, E., Pourmalek, F., Raju, M., Ranganathan, D., Rehm, J. T., Rein, D. B., Remuzzi, G., Rivara, F. P., Roberts, T., De Leon, F. R., Rosenfeld, L. C., Rushton, L., Sacco, R. L., Salomon, J. A., Sampson, U., Sanman, E., Schwebel, D. C., Segui-Gomez, M., Shepard, D. S., Singh, D., Singleton, J., Sliwa, K., Smith, E., Steer, A., Taylor, J. A., Thomas, B., Tleyjeh, I. M., Towbin, J. A., Truelsen, T., Undurraga, E. A., Venketasubramanian, N., Vijayakumar, L., Vos, T., Wagner, G. R., Wang, M., Wang, W., Watt, K., Weinstock, M. A., Weintraub, R., Wilkinson, J. D., Woolf, A. D., Wulf, S., Yeh, P. H., Yip, P., Zabetian, A., Zheng, Z. J., Lopez, A. D., Murray, C. J., AlMazroa, M. A., and Memish, Z. A. (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **380**, 2095-2128

2. Francis, G. S., and Tang, W. H. (2003) Pathophysiology of congestive heart failure. *Reviews in cardiovascular medicine* **4 Suppl 2**, S14-20

3. Mendis, S., Puska, P., Norrving B. (2011) Global Atlas on Cardiovascular Disease Prevention and Control.

. Geneva, Switzerland

4. Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**, 801-809

5. Libby, P., Ridker, P. M., and Hansson, G. K. (2011) Progress and challenges in translating the biology of atherosclerosis. *Nature* **473**, 317-325

6. Tabas, I., Williams, K. J., and Boren, J. (2007) Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation* **116**, 1832-1844

7. Moore, K. J., and Tabas, I. (2011) Macrophages in the pathogenesis of atherosclerosis. *Cell* **145**, 341-355

8. Packard, R. R., and Libby, P. (2008) Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clinical chemistry* **54**, 24-38

9. Tabas, I. (2010) Macrophage death and defective inflammation resolution in atherosclerosis. *Nature reviews. Immunology* **10**, 36-46

10. Virmani, R., Burke, A. P., Kolodgie, F. D., and Farb, A. (2002) Vulnerable plaque: the pathology of unstable coronary lesions. *Journal of interventional cardiology* **15**, 439-446

11. Hadi, H. A., Carr, C. S., and Al Suwaidi, J. (2005) Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vascular health and risk management* **1**, 183-198

12. Davignon, J., and Ganz, P. (2004) Role of endothelial dysfunction in atherosclerosis. *Circulation* **109**, III27-32

13. Szmitko, P. E., Wang, C. H., Weisel, R. D., de Almeida, J. R., Anderson, T. J., and Verma, S. (2003) New markers of inflammation and endothelial cell activation: Part I. *Circulation* **108**, 1917-1923

14. Vanhoutte, P. M., and Boulanger, C. M. (1995) Endothelium-dependent responses in hypertension. *Hypertension research : official journal of the Japanese Society of Hypertension* **18**, 87-98

15. Creager, M. A., Cooke, J. P., Mendelsohn, M. E., Gallagher, S. J., Coleman, S. M., Loscalzo, J., and Dzau, V. J. (1990) Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. *J Clin Invest* **86**, 228-234

16. Munzel, T., Sinning, C., Post, F., Warnholtz, A., and Schulz, E. (2008) Pathophysiology, diagnosis and prognostic implications of endothelial dysfunction. *Annals of medicine* **40**, 180-196

17. Schachinger, V., Britten, M. B., and Zeiher, A. M. (2000) Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* **101**, 1899-1906

18. Halcox, J. P., Schenke, W. H., Zalos, G., Mincemoyer, R., Prasad, A., Waclawiw, M. A., Nour, K. R., and Quyyumi, A. A. (2002) Prognostic value of coronary vascular endothelial dysfunction. *Circulation* **106**, 653-658

19. Bugiardini, R., Manfrini, O., Pizzi, C., Fontana, F., and Morgagni, G. (2004) Endothelial function predicts future development of coronary artery disease: a study of women with chest pain and normal coronary angiograms. *Circulation* **109**, 2518-2523

20. De Caterina, R., Libby, P., Peng, H. B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone, M. A., Jr., Shin, W. S., and Liao, J. K. (1995) Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* **96**, 60-68

21. Gauthier, T. W., Scalia, R., Murohara, T., Guo, J. P., and Lefer, A. M. (1995) Nitric oxide protects against leukocyte-endothelium interactions in the early stages of hypercholesterolemia. *Arterioscler Thromb Vasc Biol* **15**, 1652-1659

22. Peng, H. B., Rajavashisth, T. B., Libby, P., and Liao, J. K. (1995) Nitric oxide inhibits macrophage-colony stimulating factor gene transcription in vascular endothelial cells. *J Biol Chem* **270**, 17050-17055

23. de Graaf, J. C., Banga, J. D., Moncada, S., Palmer, R. M., de Groot, P. G., and Sixma, J. J. (1992) Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. *Circulation* **85**, 2284-2290

24. Marks, D. S., Vita, J. A., Folts, J. D., Keaney, J. F., Jr., Welch, G. N., and Loscalzo, J. (1995) Inhibition of neointimal proliferation in rabbits after vascular injury by a single treatment with a protein adduct of nitric oxide. *J Clin Invest* **96**, 2630-2638

25. Draijer, R., Atsma, D. E., van der Laarse, A., and van Hinsbergh, V. W. (1995) cGMP and nitric oxide modulate thrombin-induced endothelial permeability. Regulation via different pathways in human aortic and umbilical vein endothelial cells. *Circ Res* **76**, 199-208

26. Tsihlis, N. D., Oustwani, C. S., Vavra, A. K., Jiang, Q., Keefer, L. K., and Kibbe, M. R. (2011) Nitric oxide inhibits vascular smooth muscle cell proliferation and neointimal hyperplasia by increasing the ubiquitination and degradation of UbcH10. *Cell biochemistry and biophysics* **60**, 89-97

27. Cornwell, T. L., Arnold, E., Boerth, N. J., and Lincoln, T. M. (1994) Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *The American journal of physiology* **267**, C1405-1413

28. Graves, J. E., Greenwood, I. A., and Large, W. A. (2000) Tonic regulation of vascular tone by nitric oxide and chloride ions in rat isolated small coronary arteries. *American journal of physiology. Heart and circulatory physiology* **279**, H2604-2611

29. Vallance, P., Collier, J., and Moncada, S. (1989) Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* **2**, 997-1000

30. Moncada, S., and Higgs, A. (1993) The L-arginine-nitric oxide pathway. *N Engl J Med* **329**, 2002-2012

31. Forstermann, U., Closs, E. I., Pollock, J. S., Nakane, M., Schwarz, P., Gath, I., and Kleinert, H. (1994) Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension* **23**, 1121-1131

32. Fleming, I., and Busse, R. (2003) Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *American journal of physiology. Regulatory, integrative and comparative physiology* **284**, R1-12

33. Chatterjee, A., Black, S. M., and Catravas, J. D. (2008) Endothelial nitric oxide (NO) and its pathophysiologic regulation. *Vascular pharmacology* **49**, 134-140

34. Shaul, P. W. (2002) Regulation of endothelial nitric oxide synthase: location, location, location. *Annual review of physiology* **64**, 749-774

35. Boo, Y. C., and Jo, H. (2003) Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases. *American journal of physiology. Cell physiology* **285**, C499-508

36. Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G., and Michel, T. (1996) Acylation targets emdothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem* **271**, 6518-6522

37. Govers, R., Bevers, L., de Bree, P., and Rabelink, T. J. (2002) Endothelial nitric oxide synthase activity is linked to its presence at cell-cell contacts. *The Biochemical journal* **361**, 193-201

38. Govers, R., van der Sluijs, P., van Donselaar, E., Slot, J. W., and Rabelink, T. J. (2002) Endothelial nitric oxide synthase and its negative regulator caveolin-1 localize to distinct perinuclear organelles. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **50**, 779-788

39. Gao, S., Chen, J., Brodsky, S. V., Huang, H., Adler, S., Lee, J. H., Dhadwal, N., Cohen-Gould, L., Gross, S. S., and Goligorsky, M. S. (2004) Docking of endothelial nitric oxide synthase (eNOS) to the mitochondrial outer membrane: a pentabasic amino acid sequence in the autoinhibitory domain of eNOS targets a proteinase K-cleavable peptide on the cytoplasmic face of mitochondria. *J Biol Chem* **279**, 15968-15974

40. Dusserre, N., L'Heureux, N., Bell, K. S., Stevens, H. Y., Yeh, J., Otte, L. A., Loufrani, L., and Frangos, J. A. (2004) PECAM-1 interacts with nitric oxide

synthase in human endothelial cells: implication for flow-induced nitric oxide synthase activation. *Arterioscler Thromb Vasc Biol* **24**, 1796-1802

41. Uematsu, M., Ohara, Y., Navas, J. P., Nishida, K., Murphy, T. J., Alexander, R. W., Nerem, R. M., and Harrison, D. G. (1995) Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. *The American journal of physiology* **269**, C1371-1378

42. Noris, M., Morigi, M., Donadelli, R., Aiello, S., Foppolo, M., Todeschini, M., Orisio, S., Remuzzi, G., and Remuzzi, A. (1995) Nitric oxide synthesis by cultured endothelial cells is modulated by flow conditions. *Circ Res* **76**, 536-543

43. Woodman, C. R., Muller, J. M., Rush, J. W., Laughlin, M. H., and Price, E. M. (1999) Flow regulation of ecNOS and Cu/Zn SOD mRNA expression in porcine coronary arterioles. *The American journal of physiology* **276**, H1058-1063

44. Garcia-Cardena, G., Comander, J., Anderson, K. R., Blackman, B. R., and Gimbrone, M. A., Jr. (2001) Biomechanical activation of vascular endothelium as a determinant of its functional phenotype. *Proc Natl Acad Sci U S A* **98**, 4478-4485

45. Boo, Y. C., Sorescu, G., Boyd, N., Shiojima, I., Walsh, K., Du, J., and Jo, H. (2002) Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A. *J Biol Chem* **277**, 3388-3396

46. Uhlmann, S., Friedrichs, U., Eichler, W., Hoffmann, S., and Wiedemann, P. (2001) Direct measurement of VEGF-induced nitric oxide production by choroidal endothelial cells. *Microvascular research* **62**, 179-189

47. Gooch, K. J., and Frangos, J. A. (1996) Flow- and bradykinin-induced nitric oxide production by endothelial cells is independent of membrane potential. *The American journal of physiology* **270**, C546-551

48. Florian, M., Lu, Y., Angle, M., and Magder, S. (2004) Estrogen induced changes in Akt-dependent activation of endothelial nitric oxide synthase and vasodilation. *Steroids* **69**, 637-645

49. Davies, P. F., Civelek, M., Fang, Y., and Fleming, I. (2013) The atherosusceptible endothelium: Endothelial phenotypes in complex hemodynamic shear stress regions in vivo. *Cardiovasc Res*

50. Liao, J. K., Shin, W. S., Lee, W. Y., and Clark, S. L. (1995) Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J Biol Chem* **270**, 319-324

51. Alonso, J., Sanchez de Miguel, L., Monton, M., Casado, S., and Lopez-Farre, A. (1997) Endothelial cytosolic proteins bind to the 3' untranslated region of endothelial nitric oxide synthase mRNA: regulation by tumor necrosis factor alpha. *Molecular and cellular biology* **17**, 5719-5726

52. Oemar, B. S., Tschudi, M. R., Godoy, N., Brovkovich, V., Malinski, T., and Luscher, T. F. (1998) Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. *Circulation* **97**, 2494-2498

53. Wilcox, J. N., Subramanian, R. R., Sundell, C. L., Tracey, W. R., Pollock, J. S., Harrison, D. G., and Marsden, P. A. (1997) Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol* **17**, 2479-2488

54. Fukuchi, M., and Giaid, A. (1999) Endothelial expression of endothelial nitric oxide synthase and endothelin-1 in human coronary artery disease. Specific reference to underlying lesion. *Laboratory investigation; a journal of technical methods and pathology* **79**, 659-670

55. Won, D., Zhu, S. N., Chen, M., Teichert, A. M., Fish, J. E., Matouk, C. C., Bonert, M., Ojha, M., Marsden, P. A., and Cybulsky, M. I. (2007) Relative reduction of endothelial nitric-oxide synthase expression and transcription in atherosclerosis-prone regions of the mouse aorta and in an in vitro model of disturbed flow. *Am J Pathol* **171**, 1691-1704

56. Kanazawa, K., Kawashima, S., Mikami, S., Miwa, Y., Hirata, K., Suematsu, M., Hayashi, Y., Itoh, H., and Yokoyama, M. (1996) Endothelial constitutive nitric oxide synthase protein and mRNA increased in rabbit atherosclerotic aorta despite impaired endothelium-dependent vascular relaxation. *Am J Pathol* **148**, 1949-1956

57. d'Uscio, L. V., Smith, L. A., and Katusic, Z. S. (2001) Hypercholesterolemia impairs endothelium-dependent relaxations in common carotid arteries of apolipoprotein e-deficient mice. *Stroke; a journal of cerebral circulation* **32**, 2658-2664

58. Cayatte, A. J., Palacino, J. J., Horten, K., and Cohen, R. A. (1994) Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. *Arterioscler Thromb* **14**, 753-759

59. Kauser, K., da Cunha, V., Fitch, R., Mallari, C., and Rubanyi, G. M. (2000) Role of endogenous nitric oxide in progression of atherosclerosis in apolipoprotein E-deficient mice. *American journal of physiology. Heart and circulatory physiology* **278**, H1679-1685

60. Knowles, J. W., Reddick, R. L., Jennette, J. C., Shesely, E. G., Smithies, O., and Maeda, N. (2000) Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. *J Clin Invest* **105**, 451-458

61. Kuhlencordt, P. J., Gyurko, R., Han, F., Scherrer-Crosbie, M., Aretz, T. H., Hajjar, R., Picard, M. H., and Huang, P. L. (2001) Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation* **104**, 448-454

62. Clempus, R. E., and Griendling, K. K. (2006) Reactive oxygen species signaling in vascular smooth muscle cells. *Cardiovasc Res* **71**, 216-225

63. Guzik, T. J., Sadowski, J., Guzik, B., Jopek, A., Kapelak, B., Przybylowski, P., Wierzbicki, K., Korbut, R., Harrison, D. G., and Channon, K. M. (2006) Coronary artery superoxide production and nox isoform expression in human coronary artery disease. *Arterioscler Thromb Vasc Biol* **26**, 333-339

64. Azumi, H., Inoue, N., Takeshita, S., Rikitake, Y., Kawashima, S., Hayashi, Y., Itoh, H., and Yokoyama, M. (1999) Expression of NADH/NADPH oxidase p22phox in human coronary arteries. *Circulation* **100**, 1494-1498

65. Yang, Z., and Ming, X. F. (2006) Recent advances in understanding endothelial dysfunction in atherosclerosis. *JCMR* **4**

, 53-65

66. Owens, G. K., Kumar, M. S., and Wamhoff, B. R. (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* **84**, 767-801

67. Davis-Dusenbery, B. N., Wu, C., and Hata, A. (2011) Micromanaging vascular smooth muscle cell differentiation and phenotypic modulation. *Arterioscler Thromb Vasc Biol* **31**, 2370-2377

68. Owens, G. K. (1995) Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* **75**, 487-517

69. Chamley-Campbell, J. H., Campbell, G. R., and Ross, R. (1981) Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. *The Journal of cell biology* **89**, 379-383

70. Boerth, N. J., Dey, N. B., Cornwell, T. L., and Lincoln, T. M. (1997) Cyclic GMP-dependent protein kinase regulates vascular smooth muscle cell phenotype. *J Vasc Res* **34**, 245-259

71. Christen, T., Bochaton-Piallat, M. L., Neuville, P., Rensen, S., Redard, M., van Eys, G., and Gabbiani, G. (1999) Cultured porcine coronary artery smooth muscle cells. A new model with advanced differentiation. *Circ Res* **85**, 99-107

72. Frid, M. G., Moiseeva, E. P., and Stenmark, K. R. (1994) Multiple phenotypically distinct smooth muscle cell populations exist in the adult and developing bovine pulmonary arterial media in vivo. *Circ Res* **75**, 669-681

73. Mosse, P. R., Campbell, G. R., and Campbell, J. H. (1986) Smooth muscle phenotypic expression in human carotid arteries. II. Atherosclerosis-free diffuse intimal thickenings compared with the media. *Arteriosclerosis* **6**, 664-669

74. Aikawa, M., Yamaguchi, H., Yazaki, Y., and Nagai, R. (1995) Smooth muscle phenotypes in developing and atherosclerotic human arteries demonstrated by myosin expression. *Journal of atherosclerosis and thrombosis* **2**, 14-23

75. Aikawa, M., Sivam, P. N., Kuro-o, M., Kimura, K., Nakahara, K., Takewaki, S., Ueda, M., Yamaguchi, H., Yazaki, Y., Periasamy, M., and et al. (1993) Human smooth muscle myosin heavy chain isoforms as molecular markers for vascular development and atherosclerosis. *Circ Res* **73**, 1000-1012

76. Sata, M., Saiura, A., Kunisato, A., Tojo, A., Okada, S., Tokuhisa, T., Hirai, H., Makuuchi, M., Hirata, Y., and Nagai, R. (2002) Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med* **8**, 403-409

77. Bentzon, J. F., Sondergaard, C. S., Kassem, M., and Falk, E. (2007) Smooth muscle cells healing atherosclerotic plaque disruptions are of local, not blood, origin in apolipoprotein E knockout mice. *Circulation* **116**, 2053-2061

78. Daniel, J. M., Bielenberg, W., Stieger, P., Weinert, S., Tillmanns, H., and Sedding, D. G. (2010) Time-course analysis on the differentiation of bone marrow-derived progenitor cells into smooth muscle cells during neointima formation. *Arterioscler Thromb Vasc Biol* **30**, 1890-1896

79. Iwata, H., Manabe, I., Fujiu, K., Yamamoto, T., Takeda, N., Eguchi, K., Furuya, A., Kuro-o, M., Sata, M., and Nagai, R. (2010) Bone marrow-derived cells

contribute to vascular inflammation but do not differentiate into smooth muscle cell lineages. *Circulation* **122**, 2048-2057

80. Lacolley, P., Regnault, V., Nicoletti, A., Li, Z., and Michel, J. B. (2012) The vascular smooth muscle cell in arterial pathology: a cell that can take on multiple roles. *Cardiovasc Res* **95**, 194-204

81. Johnson, J. L. (2007) Matrix metalloproteinases: influence on smooth muscle cells and atherosclerotic plaque stability. *Expert review of cardiovascular therapy* **5**, 265-282

82. Ketelhuth, D. F., and Back, M. (2011) The role of matrix metalloproteinases in atherothrombosis. *Current atherosclerosis reports* **13**, 162-169

83. Miano, J. M., Long, X., and Fujiwara, K. (2007) Serum response factor: master regulator of the actin cytoskeleton and contractile apparatus. *American journal of physiology. Cell physiology* **292**, C70-81

84. Wamhoff, B. R., Hoofnagle, M. H., Burns, A., Sinha, S., McDonald, O. G., and Owens, G. K. (2004) A G/C element mediates repression of the SM22alpha promoter within phenotypically modulated smooth muscle cells in experimental atherosclerosis. *Circ Res* **95**, 981-988

85. Cherepanova, O. A., Pidkovka, N. A., Sarmento, O. F., Yoshida, T., Gan, Q., Adiguzel, E., Bendeck, M. P., Berliner, J., Leitinger, N., and Owens, G. K. (2009) Oxidized phospholipids induce type VIII collagen expression and vascular smooth muscle cell migration. *Circ Res* **104**, 609-618

86. Cordes, K. R., Sheehy, N. T., White, M. P., Berry, E. C., Morton, S. U., Muth, A. N., Lee, T. H., Miano, J. M., Ivey, K. N., and Srivastava, D. (2009) miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature* **460**, 705-710

87. Boettger, T., Beetz, N., Kostin, S., Schneider, J., Kruger, M., Hein, L., and Braun, T. (2009) Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster. *J Clin Invest* **119**, 2634-2647

88. Elia, L., Quintavalle, M., Zhang, J., Contu, R., Cossu, L., Latronico, M. V., Peterson, K. L., Indolfi, C., Catalucci, D., Chen, J., Courtneidge, S. A., and Condorelli, G. (2009) The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. *Cell death and differentiation* **16**, 1590-1598

89. Davis, B. N., Hilyard, A. C., Nguyen, P. H., Lagna, G., and Hata, A. (2009) Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype. *J Biol Chem* **284**, 3728-3738

90. Blank, R. S., and Owens, G. K. (1990) Platelet-derived growth factor regulates actin isoform expression and growth state in cultured rat aortic smooth muscle cells. *Journal of cellular physiology* **142**, 635-642

91. Corjay, M. H., Thompson, M. M., Lynch, K. R., and Owens, G. K. (1989) Differential effect of platelet-derived growth factor- versus serum-induced growth on smooth muscle alpha-actin and nonmuscle beta-actin mRNA expression in cultured rat aortic smooth muscle cells. *J Biol Chem* **264**, 10501-10506

92. Ferns, G. A., Raines, E. W., Sprugel, K. H., Motani, A. S., Reidy, M. A., and Ross, R. (1991) Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* **253**, 1129-1132

93. Jawien, A., Bowen-Pope, D. F., Lindner, V., Schwartz, S. M., and Clowes, A. W. (1992) Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J Clin Invest* **89**, 507-511

94. Sano, H., Sudo, T., Yokode, M., Murayama, T., Kataoka, H., Takakura, N., Nishikawa, S., Nishikawa, S. I., and Kita, T. (2001) Functional blockade of platelet-derived growth factor receptor-beta but not of receptor-alpha prevents vascular smooth muscle cell accumulation in fibrous cap lesions in apolipoprotein E-deficient mice. *Circulation* **103**, 2955-2960

95. ten Dijke, P., and Arthur, H. M. (2007) Extracellular control of TGFbeta signalling in vascular development and disease. *Nature reviews. Molecular cell biology* **8**, 857-869

96. Lagna, G., Ku, M. M., Nguyen, P. H., Neuman, N. A., Davis, B. N., and Hata, A. (2007) Control of phenotypic plasticity of smooth muscle cells by bone morphogenetic protein signaling through the myocardin-related transcription factors. *J Biol Chem* **282**, 37244-37255

97. Mallat, Z., Gojova, A., Marchiol-Fournigault, C., Esposito, B., Kamate, C., Merval, R., Fradelizi, D., and Tedgui, A. (2001) Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. *Circ Res* **89**, 930-934

98. Stegemann, J. P., Hong, H., and Nerem, R. M. (2005) Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype. *J Appl Physiol* **98**, 2321-2327

99. Hedin, U., and Thyberg, J. (1987) Plasma fibronectin promotes modulation of arterial smooth-muscle cells from contractile to synthetic phenotype. *Differentiation; research in biological diversity* **33**, 239-246

100. Qin, H., Ishiwata, T., Wang, R., Kudo, M., Yokoyama, M., Naito, Z., and Asano, G. (2000) Effects of extracellular matrix on phenotype modulation and MAPK transduction of rat aortic smooth muscle cells in vitro. *Exp Mol Pathol* **69**, 79-90

101. Koyama, H., Raines, E. W., Bornfeldt, K. E., Roberts, J. M., and Ross, R. (1996) Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. *Cell* **87**, 1069-1078

102. Li, S., Lao, J., Chen, B. P., Li, Y. S., Zhao, Y., Chu, J., Chen, K. D., Tsou, T. C., Peck, K., and Chien, S. (2003) Genomic analysis of smooth muscle cells in 3-dimensional collagen matrix. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17**, 97-99

103. Labat-Robert, J., Szendroi, M., Godeau, G., and Robert, L. (1985) Comparative distribution patterns of type I and III collagens and fibronectin in human arteriosclerotic aorta. *Pathologie-biologie* **33**, 261-265

104. Rohwedder, I., Montanez, E., Beckmann, K., Bengtsson, E., Duner, P., Nilsson, J., Soehnlein, O., and Fassler, R. (2012) Plasma fibronectin deficiency impedes atherosclerosis progression and fibrous cap formation. *EMBO molecular medicine* **4**, 564-576

105. Wight, T. N. (1995) The extracellular matrix and atherosclerosis. *Current opinion in lipidology* **6**, 326-334

106. Newby, A. C. (2005) Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. *Physiol Rev* **85**, 1-31

107. Hergenreider, E., Heydt, S., Treguer, K., Boettger, T., Horrevoets, A. J., Zeiher, A. M., Scheffer, M. P., Frangakis, A. S., Yin, X., Mayr, M., Braun, T., Urbich, C., Boon, R. A., and Dimmeler, S. (2012) Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nature cell biology* **14**, 249-256

108. Ueba, H., Kawakami, M., and Yaginuma, T. (1997) Shear stress as an inhibitor of vascular smooth muscle cell proliferation. Role of transforming growth factor-beta 1 and tissue-type plasminogen activator. *Arterioscler Thromb Vasc Biol* **17**, 1512-1516

109. Qi, Y. X., Jiang, J., Jiang, X. H., Wang, X. D., Ji, S. Y., Han, Y., Long, D. K., Shen, B. R., Yan, Z. Q., Chien, S., and Jiang, Z. L. (2011) PDGF-BB and TGF-{beta}1 on cross-talk between endothelial and smooth muscle cells in vascular remodeling induced by low shear stress. *Proc Natl Acad Sci U S A* **108**, 1908-1913

110. Owens, G. K., and Schwartz, S. M. (1982) Alterations in vascular smooth muscle mass in the spontaneously hypertensive rat. Role of cellular hypertrophy, hyperploidy, and hyperplasia. *Circ Res* **51**, 280-289

111. Lee, R. M., Triggle, C. R., Cheung, D. W., and Coughlin, M. D. (1987) Structural and functional consequence of neonatal sympathectomy on the blood vessels of spontaneously hypertensive rats. *Hypertension* **10**, 328-338

112. Wilson, E., Mai, Q., Sudhir, K., Weiss, R. H., and Ives, H. E. (1993) Mechanical strain induces growth of vascular smooth muscle cells via autocrine action of PDGF. *The Journal of cell biology* **123**, 741-747

113. Richard, M. N., Deniset, J. F., Kneesh, A. L., Blackwood, D., and Pierce, G. N. (2007) Mechanical stretching stimulates smooth muscle cell growth, nuclear protein import, and nuclear pore expression through mitogen-activated protein kinase activation. *J Biol Chem* **282**, 23081-23088

114. Zettler, M. E., Prociuk, M. A., Austria, J. A., Massaeli, H., Zhong, G., and Pierce, G. N. (2003) OxLDL stimulates cell proliferation through a general induction of cell cycle proteins. *American journal of physiology. Heart and circulatory physiology* **284**, H644-653

115. Chahine, M. N., Blackwood, D. P., Dibrov, E., Richard, M. N., and Pierce, G. N. (2009) Oxidized LDL affects smooth muscle cell growth through MAPK-mediated actions on nuclear protein import. *J Mol Cell Cardiol* **46**, 431-441

116. Cooper, A. L., and Beasley, D. (1999) Hypoxia stimulates proliferation and interleukin-1alpha production in human vascular smooth muscle cells. *The American journal of physiology* **277**, H1326-1337

117. Rao, G. N., and Berk, B. C. (1992) Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ Res* **70**, 593-599

118. Baas, A. S., and Berk, B. C. (1995) Differential activation of mitogen-activated protein kinases by H2O2 and O2- in vascular smooth muscle cells. *Circ Res* **77**, 29-36

119. de Graaf, R., Kloppenburg, G., Kitslaar, P. J., Bruggeman, C. A., and Stassen, F. (2006) Human heat shock protein 60 stimulates vascular smooth muscle cell proliferation through Toll-like receptors 2 and 4. *Microbes Infect* **8**, 1859-1865

120. Gerrity, R. G., Naito, H. K., Richardson, M., and Schwartz, C. J. (1979) Dietary induced atherogenesis in swine. Morphology of the intima in prelesion stages. *Am J Pathol* **95**, 775-792

121. Ylitalo, R., Oksala, O., Yla-Herttuala, S., and Ylitalo, P. (1994) Effects of clodronate (dichloromethylene bisphosphonate) on the development of experimental atherosclerosis in rabbits. *The Journal of laboratory and clinical medicine* **123**, 769-776

122. Glass, C. K., and Witztum, J. L. (2001) Atherosclerosis. the road ahead. *Cell* **104**, 503-516

123. Wang, X., H.L. Collins, M. Ranaletta, I.V. Fuki, J.T. Billheimer, G.H. Rothblat, A.R. Tall, and D.J. Rader. (2007) Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. *J. Clin. Invest* **117**, 2216-2224

124. Shibata, N., and Glass, C. K. (2009) Regulation of macrophage function in inflammation and atherosclerosis. *J Lipid Res* **50 Suppl**, S277-281

125. Edfeklt, K., J. Swedenborg, G.K. Hansson, and Z.Q. Yan. (2002) Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation* **105**, 1158-1161

126. Bjorkbacka, H., Kunjathoor, V. V., Moore, K. J., Koehn, S., Ordija, C. M., Lee, M. A., Means, T., Halmen, K., Luster, A. D., Golenbock, D. T., and Freeman, M. W. (2004) Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. *Nat Med* **10**, 416-421

127. Mullick, A. E., Tobias, P. S., and Curtiss, L. K. (2005) Modulation of atherosclerosis in mice by Toll-like receptor 2. *J Clin Invest* **115**, 3149-3156

128. Lindstedt, K., Mayranpaa MI, Kovanen PT. (2007) Mast cells in vulnerable atherosclerotic plaques-a view to a kill. *J. Cell. Mol. Med.* **11**, 739-758

129. Sun, J., Sukhova, G. K., Wolters, P. J., Yang, M., Kitamoto, S., Libby, P., MacFarlane, L. A., Mallen-St Clair, J., and Shi, G. P. (2007) Mast cells promote atherosclerosis by releasing proinflammatory cytokines. *Nat Med* **13**, 719-724

130. Heikkila, H. M., Trosien, J., Metso, J., Jauhiainen, M., Pentikainen, M. O., Kovanen, P. T., and Lindstedt, K. A. (2009) Mast cells promote atherosclerosis by inducing both an atherogenic lipid profile and vascular inflammation. *J Cell Biochem*

131. Lee M, C. L., Chiesa G, Franceschini G, Kovanen PT. (2002) Mast cell chymase degrades apoE and apoA-II in apoA-I-knockout mouse plasma and reduces its ability to promote cellular cholesterol efflux. *Arterioscler Thromb Vasc Biol* **22**, 1475-1481

132. Galkina, E., and Ley, K. (2009) Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol* **27**, 165-197

133. Jongstra-Bilen, J., Haidari, M., Zhu, S. N., Chen, M., Guha, D., and Cybulsky, M. I. (2006) Low-grade chronic inflammation in regions of the normal mouse arterial intima predisposed to atherosclerosis. *J Exp Med* **203**, 2073-2083

134. Keaney, J. F., Jr. (2011) Immune modulation of atherosclerosis. *Circulation* **124**, e559-560

135. Hansson, G. K., Holm, J., and Jonasson, L. (1989) Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am J Pathol* **135**, 169-175

136. Paulsson, G., Zhou, X., Tornquist, E., and Hansson, G. K. (2000) Oligoclonal T cell expansions in atherosclerotic lesions of apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **20**, 10-17

137. Rossmann, A., Henderson, B., Heidecker, B., Seiler, R., Fraedrich, G., Singh, M., Parson, W., Keller, M., Grubeck-Loebenstein, B., and Wick, G. (2008) T-cells from advanced atherosclerotic lesions recognize hHSP60 and have a restricted T-cell receptor repertoire. *Exp Gerontol* **43**, 229-237

138. Mosorin, M., Surcel, H. M., Laurila, A., Lehtinen, M., Karttunen, R., Juvonen, J., Paavonen, J., Morrison, R. P., Saikku, P., and Juvonen, T. (2000) Detection of Chlamydia pneumoniae-reactive T lymphocytes in human atherosclerotic plaques of carotid artery. *Arterioscler Thromb Vasc Biol* **20**, 1061-1067

139. Bui, Q. T., Prempeh, M., and Wilensky, R. L. (2009) Atherosclerotic plaque development. *Int J Biochem Cell Biol* **41**, 2109-2113

140. Romagnani, S. (2000) T-cell subsets (Th1 versus Th2). *Ann Allergy Asthma Immunol* **85**, 9-18; quiz 18, 21

141. Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., Shimizu, J., Takahashi, T., and Nomura, T. (2006) Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* **212**, 8-27

142. Mallat, Z., Gojova, A., Brun, V., Esposito, B., Fournier, N., Cottrez, F., Tedgui, A., and Groux, H. (2003) Induction of a regulatory T cell type 1 response reduces the development of atherosclerosis in apolipoprotein E-knockout mice. *Circulation* **108**, 1232-1237

143. Caligiuri, G., Nicoletti, A., Poirier, B., and Hansson, G. K. (2002) Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest* **109**, 745-753

144. Major, A. S., Fazio, S., and Linton, M. F. (2002) B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. *Arterioscler Thromb Vasc Biol* **22**, 1892-1898

145. Binder, C. J., Shaw, P. X., Chang, M. K., Boullier, A., Hartvigsen, K., Horkko, S., Miller, Y. I., Woelkers, D. A., Corr, M., and Witztum, J. L. (2005) The role of natural antibodies in atherogenesis. *J Lipid Res* **46**, 1353-1363

146. Fabricant, C. G., Fabricant, J., Litrenta, M. M., and Minick, C. R. (1978) Virus-induced atherosclerosis. *J Exp Med* **148**, 335-340

147. Adam, E., Melnick, J. L., Probtsfield, J. L., Petrie, B. L., Burek, J., Bailey, K. R., McCollum, C. H., and DeBakey, M. E. (1987) High levels of cytomegalovirus

antibody in patients requiring vascular surgery for atherosclerosis. *Lancet* **2**, 291-293

148. Bruggeman, C. A. (2000) Does Cytomegalovirus Play a Role in Atherosclerosis? *Herpes* **7**, 51-54

149. Lo, J., Abbara, S., Shturman, L., Soni, A., Wei, J., Rocha-Filho, J. A., Nasir, K., and Grinspoon, S. K. (2010) Increased prevalence of subclinical coronary atherosclerosis detected by coronary computed tomography angiography in HIV-infected men. *AIDS* **24**, 243-253

150. Mendall, M. A., Goggin, P. M., Molineaux, N., Levy, J., Toosy, T., Strachan, D., Camm, A. J., and Northfield, T. C. (1994) Relation of Helicobacter pylori infection and coronary heart disease. *Br Heart J* **71**, 437-439

151. Moutsopoulos, N. M., and Madianos, P. N. (2006) Low-grade inflammation in chronic infectious diseases: paradigm of periodontal infections. *Ann N Y Acad Sci* **1088**, 251-264

152. Saikku, P., Leinonen, M., Mattila, K., Ekman, M. R., Nieminen, M. S., Makela, P. H., Huttunen, J. K., and Valtonen, V. (1988) Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* **2**, 983-986

153. Sorlie, P. D., Adam, E., Melnick, S. L., Folsom, A., Skelton, T., Chambless, L. E., Barnes, R., and Melnick, J. L. (1994) Cytomegalovirus/herpesvirus and carotid atherosclerosis: the ARIC Study. *J Med Virol* **42**, 33-37

154. Thom, D. H., Grayston, J. T., Siscovick, D. S., Wang, S. P., Weiss, N. S., and Daling, J. R. (1992) Association of prior infection with Chlamydia pneumoniae and angiographically demonstrated coronary artery disease. *JAMA* **268**, 68-72

155. Grayston, J. T., Kuo, C. C., Coulson, A. S., Campbell, L. A., Lawrence, R. D., Lee, M. J., Strandness, E. D., and Wang, S. P. (1995) Chlamydia pneumoniae (TWAR) in atherosclerosis of the carotid artery. *Circulation* **92**, 3397-3400

156. Gyorkey, F., Melnick, J. L., Guinn, G. A., Gyorkey, P., and DeBakey, M. E. (1984) Herpesviridae in the endothelial and smooth muscle cells of the proximal aorta in arteriosclerotic patients. *Exp Mol Pathol* **40**, 328-339

157. Hendrix, M. G., Salimans, M. M., van Boven, C. P., and Bruggeman, C. A. (1990) High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. *Am J Pathol* **136**, 23-28

158. Ramirez, J. A. (1996) Isolation of Chlamydia pneumoniae from the coronary artery of a patient with coronary atherosclerosis. The Chlamydia pneumoniae/Atherosclerosis Study Group. *Ann Intern Med* **125**, 979-982

159. Epstein, S. E., Zhou, Y. F., and Zhu, J. (1999) Infection and atherosclerosis: emerging mechanistic paradigms. *Circulation* **100**, e20-28

160. Dechend, R., Maass, M., Gieffers, J., Dietz, R., Scheidereit, C., Leutz, A., and Gulba, D. C. (1999) Chlamydia pneumoniae infection of vascular smooth muscle and endothelial cells activates NF-kappaB and induces tissue factor and PAI-1 expression: a potential link to accelerated arteriosclerosis. *Circulation* **100**, 1369-1373

161. Hirono, S., Dibrov, E., Hurtado, C., Kostenuk, A., Ducas, R., and Pierce, G. N. (2003) Chlamydia pneumoniae stimulates proliferation of vascular smooth muscle cells through induction of endogenous heat shock protein 60. *Circ Res* **93**, 710-716

162. Kaukoranta-Tolvanen, S. S., Ronni, T., Leinonen, M., Saikku, P., and Laitinen, K. (1996) Expression of adhesion molecules on endothelial cells stimulated by Chlamydia pneumoniae. *Microb Pathog* **21**, 407-411

163. Rodel, J., Prochnau, D., Prager, K., Pentcheva, E., Hartmann, M., and Straube, E. (2003) Increased production of matrix metalloproteinases 1 and 3 by smooth muscle cells upon infection with Chlamydia pneumoniae. *FEMS Immunol Med Microbiol* **38**, 159-164

164. Span, A. H., Mullers, W., Miltenburg, A. M., and Bruggeman, C. A. (1991) Cytomegalovirus induced PMN adherence in relation to an ELAM-1 antigen present on infected endothelial cell monolayers. *Immunology* **72**, 355-360

165. Speir, E., Yu, Z. X., Ferrans, V. J., Huang, E. S., and Epstein, S. E. (1998) Aspirin attenuates cytomegalovirus infectivity and gene expression mediated by cyclooxygenase-2 in coronary artery smooth muscle cells. *Circ Res* **83**, 210-216

166. Chen, S., Sorrentino, R., Shimada, K., Bulut, Y., Doherty, T. M., Crother, T. R., and Arditi, M. (2008) Chlamydia pneumoniae-induced foam cell formation requires MyD88-dependent and -independent signaling and is reciprocally modulated by liver X receptor activation. *J Immunol* **181**, 7186-7193

167. Jiang, S. J., Kuo, C. C., Berry, M. W., Lee, A. W., and Campbell, L. A. (2008) Identification and characterization of Chlamydia pneumoniae-specific proteins

that activate tumor necrosis factor alpha production in RAW 264.7 murine macrophages. *Infect Immun* **76**, 1558-1564

168. Sasu, S., LaVerda, D., Qureshi, N., Golenbock, D. T., and Beasley, D. (2001) Chlamydia pneumoniae and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ Res* **89**, 244-250

169. Triantafilou, M., Gamper, F. G., Lepper, P. M., Mouratis, M. A., Schumann, C., Harokopakis, E., Schifferle, R. E., Hajishengallis, G., and Triantafilou, K. (2007) Lipopolysaccharides from atherosclerosis-associated bacteria antagonize TLR4, induce formation of TLR2/1/CD36 complexes in lipid rafts and trigger TLR2-induced inflammatory responses in human vascular endothelial cells. *Cell Microbiol* **9**, 2030-2039

170. Young, R. A., and Elliott, T. J. (1989) Stress proteins, infection, and immune surveillance. *Cell* **59**, 5-8

171. Zhu, J., Quyyumi, A. A., Rott, D., Csako, G., Wu, H., Halcox, J., and Epstein, S. E. (2001) Antibodies to human heat-shock protein 60 are associated with the presence and severity of coronary artery disease: evidence for an autoimmune component of atherogenesis. *Circulation* **103**, 1071-1075

172. Slade, G. D., Offenbacher, S., Beck, J. D., Heiss, G., and Pankow, J. S. (2000) Acute-phase inflammatory response to periodontal disease in the US population. *J Dent Res* **79**, 49-57

173. Grayston, J. T., Aldous, M. B., Easton, A., Wang, S. P., Kuo, C. C., Campbell, L. A., and Altman, J. (1993) Evidence that Chlamydia pneumoniae causes pneumonia and bronchitis. *J Infect Dis* **168**, 1231-1235

174. Kuo, C. C., Jackson, L. A., Campbell, L. A., and Grayston, J. T. (1995) Chlamydia pneumoniae (TWAR). *Clinical microbiology reviews* **8**, 451-461

175. Hahn, D. L., Dodge, R. W., and Golubjatnikov, R. (1991) Association of Chlamydia pneumoniae (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. *JAMA* **266**, 225-230

176. Von Hertzen, L., Alakarppa, H., Koskinen, R., Liippo, K., Surcel, H. M., Leinonen, M., and Saikku, P. (1997) Chlamydia pneumoniae infection in patients with chronic obstructive pulmonary disease. *Epidemiology and infection* **118**, 155-164

177. Saikku, P., Leinonen, M., Tenkanen, L., Linnanmaki, E., Ekman, M. R., Manninen, V., Manttari, M., Frick, M. H., and Huttunen, J. K. (1992) Chronic Chlamydia pneumoniae infection as a risk factor for coronary heart disease in the Helsinki Heart Study. *Ann Intern Med* **116**, 273-278

178. Blasi, F., Tarsia, P., and Aliberti, S. (2009) Chlamydophila pneumoniae. *Clin Microbiol Infect* **15**, 29-35

179. Burillo, A., and Bouza, E. (2010) Chlamydophila pneumoniae. *Infectious disease clinics of North America* **24**, 61-71

180. Gupta, S. K., and Sarosi, G. A. (2001) The role of atypical pathogens in community-acquired pneumonia. *The Medical clinics of North America* **85**, 1349-1365, vii

181. Dowell, S. F., Peeling, R. W., Boman, J., Carlone, G. M., Fields, B. S., Guarner, J., Hammerschlag, M. R., Jackson, L. A., Kuo, C. C., Maass, M., Messmer, T. O., Talkington, D. F., Tondella, M. L., and Zaki, S. R. (2001) Standardizing Chlamydia pneumoniae assays: recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **33**, 492-503

182. Kumar, S., and Hammerschlag, M. R. (2007) Acute respiratory infection due to Chlamydia pneumoniae: current status of diagnostic methods. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **44**, 568-576

183. Hammerschlag, M. R. (2000) Chlamydia pneumoniae and the lung. *Eur Respir J* **16**, 1001-1007

184. Wellinghausen, N., Straube, E., Freidank, H., von Baum, H., Marre, R., and Essig, A. (2006) Low prevalence of Chlamydia pneumoniae in adults with community-acquired pneumonia. *International journal of medical microbiology : IJMM* **296**, 485-491

185. Senn, L., Hammerschlag, M. R., and Greub, G. (2005) Therapeutic approaches to Chlamydia infections. *Expert opinion on pharmacotherapy* **6**, 2281-2290

186. Block, S., Hedrick, J., Hammerschlag, M. R., Cassell, G. H., and Craft, J. C. (1995) Mycoplasma pneumoniae and Chlamydia pneumoniae in pediatric community-acquired pneumonia: comparative efficacy and safety of

clarithromycin vs. erythromycin ethylsuccinate. *The Pediatric infectious disease journal* **14**, 471-477

187. Harris, J. A., Kolokathis, A., Campbell, M., Cassell, G. H., and Hammerschlag, M. R. (1998) Safety and efficacy of azithromycin in the treatment of community-acquired pneumonia in children. *The Pediatric infectious disease journal* **17**, 865-871

188. Roblin, P. M., and Hammerschlag, M. R. (1998) Microbiologic efficacy of azithromycin and susceptibilities to azithromycin of isolates of Chlamydia pneumoniae from adults and children with community-acquired pneumonia. *Antimicrob Agents Chemother* **42**, 194-196

189. Hammerschlag, M. R., and Roblin, P. M. (2000) Microbiologic efficacy of moxifloxacin for the treatment of community-acquired pneumonia due to Chlamydia pneumoniae. *International journal of antimicrobial agents* **15**, 149-152

190. Hammerschlag, M. R., and Roblin, P. M. (2000) Microbiological efficacy of levofloxacin for treatment of community-acquired pneumonia due to Chlamydia pneumoniae. *Antimicrob Agents Chemother* **44**, 1409

191. Hammerschlag, M. R., and Kohlhoff, S. A. (2012) Treatment of chlamydial infections. *Expert opinion on pharmacotherapy* **13**, 545-552

192. Mahony, J. B. (2002) Chlamydiae host cell interactions revealed using DNA microarrays. *Ann N Y Acad Sci* **975**, 192-201

193. Hammerschlag, M. R. (2002) The intracellular life of chlamydiae. *Semin Pediatr Infect Dis* **13**, 239-248

194. Coombes, B. K., and Mahony, J. B. (2002) Identification of MEK- and phosphoinositide 3-kinase-dependent signalling as essential events during Chlamydia pneumoniae invasion of HEp2 cells. *Cell Microbiol* **4**, 447-460

195. Beatty, W. L., Morrison, R. P., and Byrne, G. I. (1994) Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. *Microbiological reviews* **58**, 686-699

196. Harper, A., Pogson, C. I., Jones, M. L., and Pearce, J. H. (2000) Chlamydial development is adversely affected by minor changes in amino acid supply, blood plasma amino acid levels, and glucose deprivation. *Infect Immun* **68**, 1457-1464

197. Byrne, G. I., Ouellette, S. P., Wang, Z., Rao, J. P., Lu, L., Beatty, W. L., and Hudson, A. P. (2001) Chlamydia pneumoniae expresses genes required for DNA replication but not cytokinesis during persistent infection of HEp-2 cells. *Infect Immun* **69**, 5423-5429

198. Mathews, S., George, C., Flegg, C., Stenzel, D., and Timms, P. (2001) Differential expression of ompA, ompB, pyk, nlpD and Cpn0585 genes between normal and interferon-gamma treated cultures of Chlamydia pneumoniae. *Microb Pathog* **30**, 337-345

199. Shaw, E. I., Dooley, C. A., Fischer, E. R., Scidmore, M. A., Fields, K. A., and Hackstadt, T. (2000) Three temporal classes of gene expression during the Chlamydia trachomatis developmental cycle. *Mol Microbiol* **37**, 913-925

200. Valdivia, R. H. (2008) Chlamydia effector proteins and new insights into chlamydial cellular microbiology. *Curr Opin Microbiol* **11**, 53-59

201. Peters, J., Wilson, D. P., Myers, G., Timms, P., and Bavoil, P. M. (2007) Type III secretion a la Chlamydia. *Trends Microbiol* **15**, 241-251

202. Heuer, D., Brinkmann, V., Meyer, T. F., and Szczepek, A. J. (2003) Expression and translocation of chlamydial protease during acute and persistent infection of the epithelial HEp-2 cells with Chlamydophila (Chlamydia) pneumoniae. *Cell Microbiol* **5**, 315-322

203. Slepenkin, A., Motin, V., de la Maza, L. M., and Peterson, E. M. (2003) Temporal expression of type III secretion genes of Chlamydia pneumoniae. *Infect Immun* **71**, 2555-2562

204. Luo, J., Jia, T., Zhong, Y., Chen, D., Flores, R., and Zhong, G. (2007) Localization of the hypothetical protein Cpn0585 in the inclusion membrane of Chlamydia pneumoniae-infected cells. *Microb Pathog* **42**, 111-116

205. Luo, J., Jia, T., Flores, R., Chen, D., and Zhong, G. (2007) Hypothetical protein Cpn0308 is localized in the Chlamydia pneumoniae inclusion membrane. *Infect Immun* **75**, 497-503

206. Betts, H. J., Wolf, K., and Fields, K. A. (2009) Effector protein modulation of host cells: examples in the Chlamydia spp. arsenal. *Curr Opin Microbiol* **12**, 81-87

207. Cortes, C., Rzomp, K. A., Tvinnereim, A., Scidmore, M. A., and Wizel, B. (2007) Chlamydia pneumoniae inclusion membrane protein Cpn0585 interacts with multiple Rab GTPases. *Infect Immun* **75**, 5586-5596

208. Jewett, T. J., Fischer, E. R., Mead, D. J., and Hackstadt, T. (2006) Chlamydial TARP is a bacterial nucleator of actin. *Proc Natl Acad Sci U S A* **103**, 15599-15604

209. Savijoki, K., Alvesalo, J., Vuorela, P., Leinonen, M., and Kalkkinen, N. (2008) Proteomic analysis of Chlamydia pneumoniae-infected HL cells reveals extensive degradation of cytoskeletal proteins. *FEMS Immunol Med Microbiol* **54**, 375-384

210. Rupp, J., Gieffers, J., Klinger, M., van Zandbergen, G., Wrase, R., Maass, M., Solbach, W., Deiwick, J., and Hellwig-Burgel, T. (2007) Chlamydia pneumoniae directly interferes with HIF-1alpha stabilization in human host cells. *Cell Microbiol* **9**, 2181-2191

211. Pirbhai, M., Dong, F., Zhong, Y., Pan, K. Z., and Zhong, G. (2006) The secreted protease factor CPAF is responsible for degrading pro-apoptotic BH3-only proteins in Chlamydia trachomatis-infected cells. *J Biol Chem* **281**, 31495-31501

212. Fischer, S. F., Schwarz, C., Vier, J., and Hacker, G. (2001) Characterization of antiapoptotic activities of Chlamydia pneumoniae in human cells. *Infect Immun* **69**, 7121-7129

213. Airenne, S., Surcel, H. M., Tuukkanen, J., Leinonen, M., and Saikku, P. (2002) Chlamydia pneumoniae inhibits apoptosis in human epithelial and monocyte cell lines. *Scand J Immunol* **55**, 390-398

214. Paland, N., Rajalingam, K., Machuy, N., Szczepek, A., Wehrl, W., and Rudel, T. (2006) NF-kappaB and inhibitor of apoptosis proteins are required for apoptosis resistance of epithelial cells persistently infected with Chlamydophila pneumoniae. *Cell Microbiol* **8**, 1643-1655

215. Costa, C. P., Kirschning, C. J., Busch, D., Durr, S., Jennen, L., Heinzmann, U., Prebeck, S., Wagner, H., and Miethke, T. (2002) Role of chlamydial heat shock protein 60 in the stimulation of innate immune cells by Chlamydia pneumoniae. *Eur J Immunol* **32**, 2460-2470

216. Da Costa, C. U., Wantia, N., Kirschning, C. J., Busch, D. H., Rodriguez, N., Wagner, H., and Miethke, T. (2004) Heat shock protein 60 from Chlamydia pneumoniae elicits an unusual set of inflammatory responses via Toll-like receptor 2 and 4 in vivo. *Eur J Immunol* **34**, 2874-2884

217. Krull, M., Maass, M., Suttorp, N., and Rupp, J. (2005) Chlamydophila pneumoniae. Mechanisms of target cell infection and activation. *Thromb Haemost* **94**, 319-326

218. Watson, C., and Alp, N. J. (2008) Role of Chlamydia pneumoniae in atherosclerosis. *Clin Sci (Lond)* **114**, 509-531

219. Jackson, L. A., Campbell, L. A., Schmidt, R. A., Kuo, C. C., Cappuccio, A. L., Lee, M. J., and Grayston, J. T. (1997) Specificity of detection of Chlamydia pneumoniae in cardiovascular atheroma: evaluation of the innocent bystander hypothesis. *Am J Pathol* **150**, 1785-1790

220. Kuo, C. C., Shor, A., Campbell, L. A., Fukushi, H., Patton, D. L., and Grayston, J. T. (1993) Demonstration of Chlamydia pneumoniae in atherosclerotic lesions of coronary arteries. *J Infect Dis* **167**, 841-849

221. Maass, M., Bartels, C., Engel, P. M., Mamat, U., and Sievers, H. H. (1998) Endovascular presence of viable Chlamydia pneumoniae is a common phenomenon in coronary artery disease. *J Am Coll Cardiol* **31**, 827-832

222. Jackson, L. A., Campbell, L. A., Kuo, C. C., Rodriguez, D. I., Lee, A., and Grayston, J. T. (1997) Isolation of Chlamydia pneumoniae from a carotid endarterectomy specimen. *J Infect Dis* **176**, 292-295

223. Gaydos, C. A., Summersgill, J. T., Sahney, N. N., Ramirez, J. A., and Quinn, T. C. (1996) Replication of Chlamydia pneumoniae in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect Immun* **64**, 1614-1620

224. Godzik, K. L., O'Brien, E. R., Wang, S. K., and Kuo, C. C. (1995) In vitro susceptibility of human vascular wall cells to infection with Chlamydia pneumoniae. *J Clin Microbiol* **33**, 2411-2414

225. Maass, M., Gieffers, J., and Solbach, W. (2000) Atherogenetically relevant cells support continuous growth of Chlamydia pneumoniae. *Herz* **25**, 68-72

226. Gieffers, J., van Zandbergen, G., Rupp, J., Sayk, F., Kruger, S., Ehlers, S., Solbach, W., and Maass, M. (2004) Phagocytes transmit Chlamydia pneumoniae from the lungs to the vasculature. *Eur Respir J* **23**, 506-510

227. Gueinzius, K., Magenau, A., Erath, S., Wittke, V., Urbich, C., Ferrando-May, E., Dimmeler, S., and Hermann, C. (2008) Endothelial cells are protected against phagocyte-transmitted Chlamydophila pneumoniae infections by laminar shear stress Gueinzius: Shear stress protects from C. pneumoniae infection. *Atherosclerosis* **198**, 256-263

228. Hogdahl, M., Soderlund, G., and Kihlstrom, E. (2008) Expression of chemokines and adhesion molecules in human coronary artery endothelial cells infected with Chlamydia (Chlamydophila) pneumoniae. *APMIS* **116**, 1082-1088

229. Krull, M., Klucken, A. C., Wuppermann, F. N., Fuhrmann, O., Magerl, C., Seybold, J., Hippenstiel, S., Hegemann, J. H., Jantos, C. A., and Suttorp, N. (1999) Signal transduction pathways activated in endothelial cells following infection with Chlamydia pneumoniae. *J Immunol* **162**, 4834-4841

230. Molestina, R. E., Miller, R. D., Ramirez, J. A., and Summersgill, J. T. (1999) Infection of human endothelial cells with Chlamydia pneumoniae stimulates transendothelial migration of neutrophils and monocytes. *Infect Immun* **67**, 1323-1330

231. Yang, X., Coriolan, D., Schultz, K., Golenbock, D. T., and Beasley, D. (2005) Toll-like receptor 2 mediates persistent chemokine release by Chlamydia pneumoniae-infected vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **25**, 2308-2314

232. Chen, C., Chai, H., Wang, X., Lin, P. H., and Yao, Q. (2009) Chlamydia heat shock protein 60 decreases expression of endothelial nitric oxide synthase in human and porcine coronary artery endothelial cells. *Cardiovasc Res* **83**, 768-777

233. He, P., Mei, C., Cheng, B., Liu, W., Wang, Y., and Wan, J. (2009) Chlamydia pneumoniae induces macrophage-derived foam cell formation by up-regulating acyl-coenzyme A: cholesterol acyltransferase 1. *Microbes Infect* **11**, 157-163

234. Mei, C. L., He, P., Cheng, B., Liu, W., Wang, Y. F., and Wan, J. J. (2009) Chlamydia pneumoniae induces macrophage-derived foam cell formation via PPAR alpha and PPAR gamma-dependent pathways. *Cell Biol Int* **33**, 301-308

235. Netea, M. G., Kullberg, B. J., Jacobs, L. E., Verver-Jansen, T. J., van der Ven-Jongekrijg, J., Galama, J. M., Stalenhoef, A. F., Dinarello, C. A., and Van der

Meer, J. W. (2004) Chlamydia pneumoniae stimulates IFN-gamma synthesis through MyD88-dependent, TLR2- and TLR4-independent induction of IL-18 release. *J Immunol* **173**, 1477-1482

236. Azenabor, A. A., Yang, S., Job, G., and Adedokun, O. O. (2005) Elicitation of reactive oxygen species in Chlamydia pneumoniae-stimulated macrophages: a Ca2+-dependent process involving simultaneous activation of NADPH oxidase and cytochrome oxidase genes. *Med Microbiol Immunol* **194**, 91-103

237. Carratelli, C. R., Paolillo, R., and Rizzo, A. (2007) Chlamydia pneumoniae stimulates the proliferation of HUVEC through the induction of VEGF by THP-1. *Int Immunopharmacol* **7**, 287-294

238. Dechend, R., Gieffers, J., Dietz, R., Joerres, A., Rupp, J., Luft, F. C., and Maass, M. (2003) Hydroxymethylglutaryl coenzyme A reductase inhibition reduces Chlamydia pneumoniae-induced cell interaction and activation. *Circulation* **108**, 261-265

239. Kol, A., Sukhova, G. K., Lichtman, A. H., and Libby, P. (1998) Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-alpha and matrix metalloproteinase expression. *Circulation* **98**, 300-307

240. Nazzal, D., Cantero, A. V., Therville, N., Segui, B., Negre-Salvayre, A., Thomsen, M., and Benoist, H. (2006) Chlamydia pneumoniae alters mildly oxidized low-density lipoprotein-induced cell death in human endothelial cells, leading to necrosis rather than apoptosis. *J Infect Dis* **193**, 136-145

241. Schmeck, B., Beermann, W., N'Guessan, P. D., Hocke, A. C., Opitz, B., Eitel, J., Dinh, Q. T., Witzenrath, M., Krull, M., Suttorp, N., and Hippenstiel, S. (2008) Simvastatin reduces Chlamydophila pneumoniae-mediated histone modifications and gene expression in cultured human endothelial cells. *Circ Res* **102**, 888-895

242. Coombes, B. K., and Mahony, J. B. (1999) Chlamydia pneumoniae infection of human endothelial cells induces proliferation of smooth muscle cells via an endothelial cell-derived soluble factor(s). *Infect Immun* **67**, 2909-2915

243. Rupp, J., Hellwig-Burgel, T., Wobbe, V., Seitzer, U., Brandt, E., and Maass, M. (2005) Chlamydia pneumoniae infection promotes a proliferative phenotype in the vasculature through Egr-1 activation in vitro and in vivo. *Proc Natl Acad Sci U S A* **102**, 3447-3452

244. Fryer, R. H., Schwobe, E. P., Woods, M. L., and Rodgers, G. M. (1997) Chlamydia species infect human vascular endothelial cells and induce procoagulant activity. *J Investig Med* **45**, 168-174

245. Fong, I. W., Chiu, B., Viira, E., Fong, M. W., Jang, D., and Mahony, J. (1997) Rabbit model for Chlamydia pneumoniae infection. *J Clin Microbiol* **35**, 48-52

246. Fong, I. W., Chiu, B., Viira, E., Jang, D., and Mahony, J. B. (1999) De Novo induction of atherosclerosis by Chlamydia pneumoniae in a rabbit model. *Infect Immun* **67**, 6048-6055

247. Muhlestein, J. B., Anderson, J. L., Hammond, E. H., Zhao, L., Trehan, S., Schwobe, E. P., and Carlquist, J. F. (1998) Infection with Chlamydia pneumoniae accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. *Circulation* **97**, 633-636

248. Blessing, E., Campbell, L. A., Rosenfeld, M. E., Chough, N., and Kuo, C. C. (2001) Chlamydia pneumoniae infection accelerates hyperlipidemia induced atherosclerotic lesion development in C57BL/6J mice. *Atherosclerosis* **158**, 13-17

249. Blessing, E., Lin, T. M., Campbell, L. A., Rosenfeld, M. E., Lloyd, D., and Kuo, C. (2000) Chlamydia pneumoniae induces inflammatory changes in the heart and aorta of normocholesterolemic C57BL/6J mice. *Infect Immun* **68**, 4765-4768

250. Hu, H., Pierce, G. N., and Zhong, G. (1999) The atherogenic effects of chlamydia are dependent on serum cholesterol and specific to Chlamydia pneumoniae. *J Clin Invest* **103**, 747-753

251. Liu, L., Hu, H., Ji, H., Murdin, A. D., Pierce, G. N., and Zhong, G. (2000) Chlamydia pneumoniae infection significantly exacerbates aortic atherosclerosis in an LDLR-/- mouse model within six months. *Mol Cell Biochem* **215**, 123-128

252. Deniset, J. F., and Pierce, G. N. (2010) Possibilities for therapeutic interventions in disrupting Chlamydophila pneumoniae involvement in atherosclerosis. *Fundamental & clinical pharmacology* **24**, 607-617

253. Blessing, E., Campbell, L. A., Rosenfeld, M. E., and Kuo, C. C. (2002) Chlamydia pneumoniae and hyperlipidemia are co-risk factors for atherosclerosis: infection prior to induction of hyperlipidemia does not accelerate development of atherosclerotic lesions in C57BL/6J mice. *Infect Immun* **70**, 5332-5334

254. Blessing, E., Nagano, S., Campbell, L. A., Rosenfeld, M. E., and Kuo, C. C. (2000) Effect of Chlamydia trachomatis infection on atherosclerosis in apolipoprotein E-deficient mice. *Infect Immun* **68**, 7195-7197

255. Liuba, P., Karnani, P., Pesonen, E., Paakkari, I., Forslid, A., Johansson, L., Persson, K., Wadstrom, T., and Laurini, R. (2000) Endothelial dysfunction after repeated Chlamydia pneumoniae infection in apolipoprotein E-knockout mice. *Circulation* **102**, 1039-1044

256. Liuba, P., Pesonen, E., Paakkari, I., Batra, S., Forslid, A., Kovanen, P., Pentikainen, M., Persson, K., and Sandstrom, S. (2003) Acute Chlamydia pneumoniae infection causes coronary endothelial dysfunction in pigs. *Atherosclerosis* **167**, 215-222

257. Pislaru, S. V., Van Ranst, M., Pislaru, C., Szelid, Z., Theilmeier, G., Ossewaarde, J. M., Holvoet, P., Janssens, S., Verbeken, E., and Van de Werf, F. J. (2003) Chlamydia pneumoniae induces neointima formation in coronary arteries of normal pigs. *Cardiovasc Res* **57**, 834-842

258. Chirgwin, K., Roblin, P. M., and Hammerschlag, M. R. (1989) In vitro susceptibilities of Chlamydia pneumoniae (Chlamydia sp. strain TWAR). *Antimicrob Agents Chemother* **33**, 1634-1635

259. Gieffers, J., Solbach, W., and Maass, M. (1998) In vitro susceptibilities of Chlamydia pneumoniae strains recovered from atherosclerotic coronary arteries. *Antimicrob Agents Chemother* **42**, 2762-2764

260. Schneider, C. A., Diedrichs, H., Riedel, K. D., Zimmermann, T., and Hopp, H. W. (2000) In vivo uptake of azithromycin in human coronary plaques. *Am J Cardiol* **86**, 789-791, A789

261. Fong, I. W. (1999) Value of animal models for Chlamydia pneumoniae-related atherosclerosis. *Am Heart J* **138**, S512-513

262. Jackson, L. A., Smith, N. L., Heckbert, S. R., Grayston, J. T., Siscovick, D. S., and Psaty, B. M. (1999) Lack of association between first myocardial infarction and past use of erythromycin, tetracycline, or doxycycline. *Emerg Infect Dis* **5**, 281-284

263. Meier, C. R., Derby, L. E., Jick, S. S., Vasilakis, C., and Jick, H. (1999) Antibiotics and risk of subsequent first-time acute myocardial infarction. *JAMA* **281**, 427-431

264. Liuba, P., Pesonen, E., Paakkari, I., Batra, S., Andersen, L., Forslid, A., Yla-Herttuala, S., Persson, K., Wadstrom, T., Wang, X., and Laurini, R. (2003) Co-infection with Chlamydia pneumoniae and Helicobacter pylori results in vascular endothelial dysfunction and enhanced VCAM-1 expression in apoE-knockout mice. *J Vasc Res* **40**, 115-122

265. Moazed, T. C., Campbell, L. A., Rosenfeld, M. E., Grayston, J. T., and Kuo, C. C. (1999) Chlamydia pneumoniae infection accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *J Infect Dis* **180**, 238-241

266. Burnett, M. S., Gaydos, C. A., Madico, G. E., Glad, S. M., Paigen, B., Quinn, T. C., and Epstein, S. E. (2001) Atherosclerosis in apoE knockout mice infected with multiple pathogens. *J Infect Dis* **183**, 226-231

267. Caligiuri, G., Rottenberg, M., Nicoletti, A., Wigzell, H., and Hansson, G. K. (2001) Chlamydia pneumoniae infection does not induce or modify atherosclerosis in mice. *Circulation* **103**, 2834-2838

268. Aalto-Setala, K., Laitinen, K., Erkkila, L., Leinonen, M., Jauhiainen, M., Ehnholm, C., Tamminen, M., Puolakkainen, M., Penttila, I., and Saikku, P. (2001)

Chlamydia pneumoniae does not increase atherosclerosis in the aortic root of apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **21**, 578-584

269. Ezzahiri, R., Stassen, F. R., Kurvers, H. A., van Pul, M. M., Kitslaar, P. J., and Bruggeman, C. A. (2003) Chlamydia pneumoniae infection induces an unstable atherosclerotic plaque phenotype in LDL-receptor, ApoE double knockout mice. *Eur J Vasc Endovasc Surg* **26**, 88-95

270. Burian, K., Berencsi, K., Endresz, V., Gyulai, Z., Valyi-Nagy, T., Valyi-Nagy, I., Bakay, M., Geng, Y., Virok, D., Kari, L., Hajnal-Papp, R., Trinchieri, G., and Gonczol, E. (2001) Chlamydia pneumoniae exacerbates aortic inflammatory foci caused by murine cytomegalovirus infection in normocholesterolemic mice. *Clin Diagn Lab Immunol* **8**, 1263-1266

271. Liuba, P., Pesonen, E., Forslid, A., Paakkari, I., Kornerup-Hansen, A., Kovanen, P., Pentikainen, M., Persson, K., and Ostergard, G. (2006) Protective effects of simvastatin on coronary artery function in swine with acute infection. *Atherosclerosis* **186**, 331-336

272. Gupta, S., Leatham, E. W., Carrington, D., Mendall, M. A., Kaski, J. C., and Camm, A. J. (1997) Elevated Chlamydia pneumoniae antibodies, cardiovascular events, and azithromycin in male survivors of myocardial infarction. *Circulation* **96**, 404-407

273. Muhlestein, J. B., Anderson, J. L., Carlquist, J. F., Salunkhe, K., Horne, B. D., Pearson, R. R., Bunch, T. J., Allen, A., Trehan, S., and Nielson, C. (2000) Randomized secondary prevention trial of azithromycin in patients with coronary

artery disease: primary clinical results of the ACADEMIC study. *Circulation* **102**, 1755-1760

274. O'Connor, C. M., Dunne, M. W., Pfeffer, M. A., Muhlestein, J. B., Yao, L., Gupta, S., Benner, R. J., Fisher, M. R., and Cook, T. D. (2003) Azithromycin for the secondary prevention of coronary heart disease events: the WIZARD study: a randomized controlled trial. *JAMA* **290**, 1459-1466

275. Grayston, J. T., Kronmal, R. A., Jackson, L. A., Parisi, A. F., Muhlestein, J. B., Cohen, J. D., Rogers, W. J., Crouse, J. R., Borrowdale, S. L., Schron, E., and Knirsch, C. (2005) Azithromycin for the secondary prevention of coronary events. *N Engl J Med* **352**, 1637-1645

276. Jespersen, C. M., Als-Nielsen, B., Damgaard, M., Hansen, J. F., Hansen, S., Helo, O. H., Hildebrandt, P., Hilden, J., Jensen, G. B., Kastrup, J., Kolmos, H. J., Kjoller, E., Lind, I., Nielsen, H., Petersen, L., and Gluud, C. (2006) Randomised placebo controlled multicentre trial to assess short term clarithromycin for patients with stable coronary heart disease: CLARICOR trial. *BMJ* **332**, 22-27

277. Gurfinkel, E., Bozovich, G., Beck, E., Testa, E., Livellara, B., and Mautner, B. (1999) Treatment with the antibiotic roxithromycin in patients with acute non-Q-wave coronary syndromes. The final report of the ROXIS Study. *Eur Heart J* **20**, 121-127

278. Sinisalo, J., Mattila, K., Valtonen, V., Anttonen, O., Juvonen, J., Melin, J., Vuorinen-Markkola, H., and Nieminen, M. S. (2002) Effect of 3 months of antimicrobial treatment with clarithromycin in acute non-q-wave coronary syndrome. *Circulation* **105**, 1555-1560

279. Stone, A. F., Mendall, M. A., Kaski, J. C., Edger, T. M., Risley, P., Poloniecki, J., Camm, A. J., and Northfield, T. C. (2002) Effect of treatment for Chlamydia pneumoniae and Helicobacter pylori on markers of inflammation and cardiac events in patients with acute coronary syndromes: South Thames Trial of Antibiotics in Myocardial Infarction and Unstable Angina (STAMINA). *Circulation* **106**, 1219-1223

280. Zahn, R., Schneider, S., Frilling, B., Seidl, K., Tebbe, U., Weber, M., Gottwik, M., Altmann, E., Seidel, F., Rox, J., Hoffler, U., Neuhaus, K. L., and Senges, J. (2003) Antibiotic therapy after acute myocardial infarction: a prospective randomized study. *Circulation* **107**, 1253-1259

281. Cercek, B., Shah, P. K., Noc, M., Zahger, D., Zeymer, U., Matetzky, S., Maurer, G., and Mahrer, P. (2003) Effect of short-term treatment with azithromycin on recurrent ischaemic events in patients with acute coronary syndrome in the Azithromycin in Acute Coronary Syndrome (AZACS) trial: a randomised controlled trial. *Lancet* **361**, 809-813

282. Cannon, C. P., Braunwald, E., McCabe, C. H., Grayston, J. T., Muhlestein, B., Giugliano, R. P., Cairns, R., and Skene, A. M. (2005) Antibiotic treatment of Chlamydia pneumoniae after acute coronary syndrome. *N Engl J Med* **352**, 1646-1654

283. Coombes, B. K., Johnson, D. L., and Mahony, J. B. (2002) Strategic targeting of essential host-pathogen interactions in chlamydial disease. *Curr Drug Targets Infect Disord* **2**, 201-216

284. Gieffers, J., Fullgraf, H., Jahn, J., Klinger, M., Dalhoff, K., Katus, H. A., Solbach, W., and Maass, M. (2001) Chlamydia pneumoniae infection in circulating human monocytes is refractory to antibiotic treatment. *Circulation* **103**, 351-356

285. Kutlin, A., Roblin, P. M., and Hammerschlag, M. R. (2002) Effect of prolonged treatment with azithromycin, clarithromycin, or levofloxacin on Chlamydia pneumoniae in a continuous-infection Model. *Antimicrob Agents Chemother* **46**, 409-412

286. Kinjo, K., Sato, H., Sato, H., Ohnishi, Y., Hishida, E., Nakatani, D., Mizuno, H., Ohgitani, N., Kubo, M., Shimazu, T., Akehi, N., Takeda, H., and Hori, M. (2003) Joint effects of Chlamydia pneumoniae infection and classic coronary risk factors on risk of acute myocardial infarction. *Am Heart J* **146**, 324-330

287. Fulda, S., Gorman, A. M., Hori, O., and Samali, A. (2010) Cellular stress responses: cell survival and cell death. *International journal of cell biology* **2010**, 214074

288. Kultz, D. (2003) Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. *The Journal of experimental biology* **206**, 3119-3124

289. Ritossa, F. (1962) A new puffing pattern induced by temperature shock and DNP in Drosophila. *Experientia* **18**, 571-573

290. Tissieres, A., Mitchell, H. K., and Tracy, U. M. (1974) Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. *Journal of molecular biology* **84**, 389-398

291. Samali, A., and Orrenius, S. (1998) Heat shock proteins: regulators of stress response and apoptosis. *Cell stress & chaperones* **3**, 228-236

292. Anckar, J., and Sistonen, L. (2011) Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annual review of biochemistry* **80**, 1089-1115

293. Parcellier, A., Brunet, M., Schmitt, E., Col, E., Didelot, C., Hammann, A., Nakayama, K., Nakayama, K. I., Khochbin, S., Solary, E., and Garrido, C. (2006) HSP27 favors ubiquitination and proteasomal degradation of p27Kip1 and helps S-phase re-entry in stressed cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **20**, 1179-1181

294. Zhang, H., Rajasekaran, N. S., Orosz, A., Xiao, X., Rechsteiner, M., and Benjamin, I. J. (2010) Selective degradation of aggregate-prone CryAB mutants by HSPB1 is mediated by ubiquitin-proteasome pathways. *Journal of molecular and cellular cardiology* **49**, 918-930

295. Burrows, F., Zhang, H., and Kamal, A. (2004) Hsp90 activation and cell cycle regulation. *Cell Cycle* **3**, 1530-1536

296. Pearl, L. H., and Prodromou, C. (2000) Structure and in vivo function of Hsp90. *Current opinion in structural biology* **10**, 46-51

297. Neupert, W., and Brunner, M. (2002) The protein import motor of mitochondria. *Nature reviews. Molecular cell biology* **3**, 555-565

298. Ryan, M. T., and Pfanner, N. (2002) HSP70 proteins in protein translocation. *Adv Protein Chem* **3**, 555-565

299. Didelot, C., Schmitt, E., Brunet, M., Maingret, L., Parcellier, A., and Garrido, C. (2006) Heat shock proteins: endogenous modulators of apoptotic cell death. *Handbook of experimental pharmacology*, 171-198

300. Garrido, C., Gurbuxani, S., Ravagnan, L., and Kroemer, G. (2001) Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochemical and biophysical research communications* **286**, 433-442

301. Sreedhar, A. S., and Csermely, P. (2004) Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy: a comprehensive review. *Pharmacology & therapeutics* **101**, 227-257

302. Kampinga, H. H., Hageman, J., Vos, M. J., Kubota, H., Tanguay, R. M., Bruford, E. A., Cheetham, M. E., Chen, B., and Hightower, L. E. (2009) Guidelines for the nomenclature of the human heat shock proteins. *Cell stress & chaperones* **14**, 105-111

303. Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z., and Nardai, G. (1998) The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. *Pharmacology & therapeutics* **79**, 129-168

304. Daugaard, M., Rohde, M., and Jaattela, M. (2007) The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS letters* **581**, 3702-3710

305. Felts, S. J., Owen, B. A., Nguyen, P., Trepel, J., Donner, D. B., and Toft, D. O. (2000) The hsp90-related protein TRAP1 is a mitochondrial protein with distinct functional properties. *J Biol Chem* **275**, 3305-3312

306. Marzec, M., Eletto, D., and Argon, Y. (2012) GRP94: An HSP90-like protein specialized for protein folding and quality control in the endoplasmic reticulum. *Biochimica et biophysica acta* **1823**, 774-787

307. Hartl, F. U., and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**, 1852-1858

308. Young, J. C., Barral, J. M., and Ulrich Hartl, F. (2003) More than folding: localized functions of cytosolic chaperones. *Trends in biochemical sciences* **28**, 541-547

309. Bukau, B., Deuerling, E., Pfund, C., and Craig, E. A. (2000) Getting newly synthesized proteins into shape. *Cell* **101**, 119-122

310. Pratt, W. B., and Toft, D. O. (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp Biol Med (Maywood)* **228**, 111-133

311. Ben-Zvi, A. P., and Goloubinoff, P. (2001) Review: mechanisms of disaggregation and refolding of stable protein aggregates by molecular chaperones. *Journal of structural biology* **135**, 84-93

312. Sreedhar, A. S., Kalmar, E., Csermely, P., and Shen, Y. F. (2004) Hsp90 isoforms: functions, expression and clinical importance. *FEBS letters* **562**, 11-15

313. Echeverria, P. C., and Picard, D. (2010) Molecular chaperones, essential partners of steroid hormone receptors for activity and mobility. *Biochimica et biophysica acta* **1803**, 641-649

314. Gidalevitz, T., Prahlad, V., and Morimoto, R. I. (2011) The stress of protein misfolding: from single cells to multicellular organisms. *Cold Spring Harbor perspectives in biology* **3**

315. Taipale, M., Jarosz, D. F., and Lindquist, S. (2010) HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nature reviews. Molecular cell biology* **11**, 515-528

316. Mayer, M. P., and Bukau, B. (2005) Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and molecular life sciences : CMLS* **62**, 670-684

317. Wandinger, S. K., Richter, K., and Buchner, J. (2008) The Hsp90 chaperone machinery. *J Biol Chem* **283**, 18473-18477

318. Karzai, A. W., and McMacken, R. (1996) A bipartite signaling mechanism involved in DnaJ-mediated activation of the Escherichia coli DnaK protein. *J Biol Chem* **271**, 11236-11246

319. Laufen, T., Mayer, M. P., Beisel, C., Klostermeier, D., Mogk, A., Reinstein, J., and Bukau, B. (1999) Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. *Proc Natl Acad Sci U S A* **96**, 5452-5457

320. Andreasson, C., Fiaux, J., Rampelt, H., Mayer, M. P., and Bukau, B. (2008) Hsp110 is a nucleotide-activated exchange factor for Hsp70. *J Biol Chem* **283**, 8877-8884

321. Dragovic, Z., Broadley, S. A., Shomura, Y., Bracher, A., and Hartl, F. U. (2006) Molecular chaperones of the Hsp110 family act as nucleotide exchange factors of Hsp70s. *The EMBO journal* **25**, 2519-2528

322. Buchner, J. (1999) Hsp90 & Co. - a holding for folding. *Trends in biochemical sciences* **24**, 136-141

323. Nathan, D. F., Vos, M. H., and Lindquist, S. (1997) In vivo functions of the Saccharomyces cerevisiae Hsp90 chaperone. *Proc Natl Acad Sci U S A* **94**, 12949-12956

324. Franck, E., Madsen, O., van Rheede, T., Ricard, G., Huynen, M. A., and de Jong, W. W. (2004) Evolutionary diversity of vertebrate small heat shock proteins. *Journal of molecular evolution* **59**, 792-805

325. Taylor, R. P., and Benjamin, I. J. (2005) Small heat shock proteins: a new classification scheme in mammals. *Journal of molecular and cellular cardiology* **38**, 433-444

326. Mymrikov, E. V., Seit-Nebi, A. S., and Gusev, N. B. (2011) Large potentials of small heat shock proteins. *Physiol Rev* **91**, 1123-1159

327. Haslbeck, M., Braun, N., Stromer, T., Richter, B., Model, N., Weinkauf, S., and Buchner, J. (2004) Hsp42 is the general small heat shock protein in the cytosol of Saccharomyces cerevisiae. *The EMBO journal* **23**, 638-649

328. Basha, E., Lee, G. J., Breci, L. A., Hausrath, A. C., Buan, N. R., Giese, K. C., and Vierling, E. (2004) The identity of proteins associated with a small heat shock protein during heat stress in vivo indicates that these chaperones protect a wide range of cellular functions. *J Biol Chem* **279**, 7566-7575

329. Friedrich, K. L., Giese, K. C., Buan, N. R., and Vierling, E. (2004) Interactions between small heat shock protein subunits and substrate in small heat shock protein-substrate complexes. *J Biol Chem* **279**, 1080-1089

330. Huot, J., Houle, F., Marceau, F., and Landry, J. (1997) Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circ Res* **80**, 383-392

331. Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J. N., Huot, J., and Landry, J. (1997) Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. *Journal of cell science* **110 (Pt 3)**, 357-368

332. Sun, Y., and MacRae, T. H. (2005) Small heat shock proteins: molecular structure and chaperone function. *Cellular and molecular life sciences : CMLS* **62**, 2460-2476

333. Chernik, I. S., Panasenko, O. O., Li, Y., Marston, S. B., and Gusev, N. B. (2004) pH-induced changes of the structure of small heat shock proteins with molecular mass 24/27kDa (HspB1). *Biochemical and biophysical research communications* **324**, 1199-1203

334. Lelj-Garolla, B., and Mauk, A. G. (2006) Self-association and chaperone activity of Hsp27 are thermally activated. *J Biol Chem* **281**, 8169-8174

335. Bryantsev, A. L., Kurchashova, S. Y., Golyshev, S. A., Polyakov, V. Y., Wunderink, H. F., Kanon, B., Budagova, K. R., Kabakov, A. E., and Kampinga, H. H. (2007) Regulation of stress-induced intracellular sorting and chaperone function of Hsp27 (HspB1) in mammalian cells. *The Biochemical journal* **407**, 407-417

336. Lelj-Garolla, B., and Mauk, A. G. (2005) Self-association of a small heat shock protein. *Journal of molecular biology* **345**, 631-642

337. Bova, M. P., McHaourab, H. S., Han, Y., and Fung, B. K. (2000) Subunit exchange of small heat shock proteins. Analysis of oligomer formation of alphaA-crystallin and Hsp27 by fluorescence resonance energy transfer and site-directed truncations. *J Biol Chem* **275**, 1035-1042

338. Rogalla, T., Ehrnsperger, M., Preville, X., Kotlyarov, A., Lutsch, G., Ducasse, C., Paul, C., Wieske, M., Arrigo, A. P., Buchner, J., and Gaestel, M. (1999) Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. *J Biol Chem* **274**, 18947-18956

339. Ehrnsperger, M., Graber, S., Gaestel, M., and Buchner, J. (1997) Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *The EMBO journal* **16**, 221-229

340. Haslbeck, M. (2002) sHsps and their role in the chaperone network. *Cellular and molecular life sciences : CMLS* **59**, 1649-1657

341. Maguire, M., Coates, A. R., and Henderson, B. (2002) Chaperonin 60 unfolds its secrets of cellular communication. *Cell stress & chaperones* **7**, 317-329

342. Amberger, A., Maczek, C., Jurgens, G., Michaelis, D., Schett, G., Trieb, K., Eberl, T., Jindal, S., Xu, Q., and Wick, G. (1997) Co-expression of ICAM-1, VCAM-1, ELAM-1 and Hsp60 in human arterial and venous endothelial cells in response to cytokines and oxidized low-density lipoproteins. *Cell stress & chaperones* **2**, 94-103

343. Chahine, M. N., Dibrov, E., Blackwood, D. P., and Pierce, G. N. (2012) Oxidized LDL enhances stretch-induced smooth muscle cell proliferation through

alterations in nuclear protein import. *Canadian journal of physiology and pharmacology* **90**, 1559-1568

344. Knowlton, A. A., and Srivatsa, U. (2008) Heat-shock protein 60 and cardiovascular disease: a paradoxical role. *Future Cardiol* **4**, 151-161

345. Ranford, J. C., Coates, A. R., and Henderson, B. (2000) Chaperonins are cell-signalling proteins: the unfolding biology of molecular chaperones. *Expert reviews in molecular medicine* **2**, 1-17

346. Ranson, N. A., White, H. E., and Saibil, H. R. (1998) Chaperonins. *The Biochemical journal* **333 (Pt 2)**, 233-242

347. Langer, T., Pfeifer, G., Martin, J., Baumeister, W., and Hartl, F. U. (1992) Chaperonin-mediated protein folding: GroES binds to one end of the GroEL cylinder, which accommodates the protein substrate within its central cavity. *The EMBO journal* **11**, 4757-4765

348. Zwickl, P., Pfeifer, G., Lottspeich, F., Kopp, F., Dahlmann, B., and Baumeister, W. (1990) Electron microscopy and image analysis reveal common principles of organization in two large protein complexes: groEL-type proteins and proteasomes. *Journal of structural biology* **103**, 197-203

349. Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L., and Hartl, F. U. (1991) Chaperonin-mediated protein folding at the surface of groEL through a 'molten globule'-like intermediate. *Nature* **352**, 36-42

350. Viitanen, P. V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keefe, D. P., and Lorimer, G. H. (1990) Chaperonin-facilitated refolding of ribulosebisphosphate

carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K+ dependent. *Biochemistry* **29**, 5665-5671

351. Chandra, D., Choy, G., and Tang, D. G. (2007) Cytosolic accumulation of HSP60 during apoptosis with or without apparent mitochondrial release: evidence that its pro-apoptotic or pro-survival functions involve differential interactions with caspase-3. *J Biol Chem* **282**, 31289-31301

352. Kirchhoff, S. R., Gupta, S., and Knowlton, A. A. (2002) Cytosolic heat shock protein 60, apoptosis, and myocardial injury. *Circulation* **105**, 2899-2904

353. Gupta, S., and Knowlton, A. A. (2007) HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway. *Am J Physiol Heart Circ Physiol* **292**, H3052-3056

354. Merendino, A. M., Bucchieri, F., Campanella, C., Marciano, V., Ribbene, A., David, S., Zummo, G., Burgio, G., Corona, D. F., Conway de Macario, E., Macario, A. J., and Cappello, F. (2010) Hsp60 is actively secreted by human tumor cells. *PLoS One* **5**, e9247

355. Kim, S. C., Stice, J. P., Chen, L., Jung, J. S., Gupta, S., Wang, Y., Baumgarten, G., Trial, J., and Knowlton, A. A. (2009) Extracellular heat shock protein 60, cardiac myocytes, and apoptosis. *Circ Res* **105**, 1186-1195

356. Tian, J., Guo, X., Liu, X. M., Liu, L., Weng, Q. F., Dong, S. J., Knowlton, A. A., Yuan, W. J., and Lin, L. (2013) Extracellular HSP60 induces inflammation through activating and up-regulating TLRs in cardiomyocytes. *Cardiovasc Res*

357. Tanabe, M., Sasai, N., Nagata, K., Liu, X. D., Liu, P. C., Thiele, D. J., and Nakai, A. (1999) The mammalian HSF4 gene generates both an activator and a repressor of heat shock genes by alternative splicing. *J Biol Chem* **274**, 27845-27856

358. Fujimoto, M., Hayashida, N., Katoh, T., Oshima, K., Shinkawa, T., Prakasam, R., Tan, K., Inouye, S., Takii, R., and Nakai, A. (2010) A novel mouse HSF3 has the potential to activate nonclassical heat-shock genes during heat shock. *Molecular biology of the cell* **21**, 106-116

359. Sarge, K. D., Murphy, S. P., and Morimoto, R. I. (1993) Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Molecular and cellular biology* **13**, 1392-1407

360. Xiao, X., Zuo, X., Davis, A. A., McMillan, D. R., Curry, B. B., Richardson, J. A., and Benjamin, I. J. (1999) HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *The EMBO journal* **18**, 5943-5952

361. Page, T. J., Sikder, D., Yang, L., Pluta, L., Wolfinger, R. D., Kodadek, T., and Thomas, R. S. (2006) Genome-wide analysis of human HSF1 signaling reveals a transcriptional program linked to cellular adaptation and survival. *Molecular bioSystems* **2**, 627-639

362. Trinklein, N. D., Murray, J. I., Hartman, S. J., Botstein, D., and Myers, R. M. (2004) The role of heat shock transcription factor 1 in the genome-wide regulation of the mammalian heat shock response. *Molecular biology of the cell* **15**, 1254-1261

363. Akerfelt, M., Morimoto, R. I., and Sistonen, L. (2010) Heat shock factors: integrators of cell stress, development and lifespan. *Nature reviews. Molecular cell biology* **11**, 545-555

364. Sistonen, L., Sarge, K. D., Phillips, B., Abravaya, K., and Morimoto, R. I. (1992) Activation of heat shock factor 2 during hemin-induced differentiation of human erythroleukemia cells. *Molecular and cellular biology* **12**, 4104-4111

365. Wilkerson, D. C., Skaggs, H. S., and Sarge, K. D. (2007) HSF2 binds to the Hsp90, Hsp27, and c-Fos promoters constitutively and modulates their expression. *Cell stress & chaperones* **12**, 283-290

366. Xing, H., Wilkerson, D. C., Mayhew, C. N., Lubert, E. J., Skaggs, H. S., Goodson, M. L., Hong, Y., Park-Sarge, O. K., and Sarge, K. D. (2005) Mechanism of hsp70i gene bookmarking. *Science* **307**, 421-423

367. Ostling, P., Bjork, J. K., Roos-Mattjus, P., Mezger, V., and Sistonen, L. (2007) Heat shock factor 2 (HSF2) contributes to inducible expression of hsp genes through interplay with HSF1. *J Biol Chem* **282**, 7077-7086

368. Anckar, J., and Sistonen, L. (2007) Heat shock factor 1 as a coordinator of stress and developmental pathways. *Advances in experimental medicine and biology* **594**, 78-88

369. Zou, J., Guo, Y., Guettouche, T., Smith, D. F., and Voellmy, R. (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* **94**, 471-480

370. Guo, Y., Guettouche, T., Fenna, M., Boellmann, F., Pratt, W. B., Toft, D. O., Smith, D. F., and Voellmy, R. (2001) Evidence for a mechanism of repression of

heat shock factor 1 transcriptional activity by a multichaperone complex. *J Biol Chem* **276**, 45791-45799

371. Boyault, C., Zhang, Y., Fritah, S., Caron, C., Gilquin, B., Kwon, S. H., Garrido, C., Yao, T. P., Vourc'h, C., Matthias, P., and Khochbin, S. (2007) HDAC6 controls major cell response pathways to cytotoxic accumulation of protein aggregates. *Genes & development* **21**, 2172-2181
372. Ali, A., Bharadwaj, S., O'Carroll, R., and Ovsenek, N. (1998) HSP90 interacts with and regulates the activity of heat shock factor 1 in Xenopus oocytes. *Molecular and cellular biology* **18**, 4949-4960
373. Bharadwaj, S., Ali, A., and Ovsenek, N. (1999) Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 In vivo. *Molecular and cellular biology* **19**, 8033-8041
374. Mosser, D. D., Duchaine, J., and Massie, B. (1993) The DNA-binding activity of the human heat shock transcription factor is regulated in vivo by hsp70. *Molecular and cellular biology* **13**, 5427-5438
375. Abravaya, K., Myers, M. P., Murphy, S. P., and Morimoto, R. I. (1992) The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes & development* **6**, 1153-1164
376. Baler, R., Welch, W. J., and Voellmy, R. (1992) Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. *The Journal of cell biology* **117**, 1151-1159
377. Shi, Y., Mosser, D. D., and Morimoto, R. I. (1998) Molecular chaperones as HSF1-specific transcriptional repressors. *Genes & development* **12**, 654-666

378. Gomez, A. V., Galleguillos, D., Maass, J. C., Battaglioli, E., Kukuljan, M., and Andres, M. E. (2008) CoREST represses the heat shock response mediated by HSF1. *Molecular cell* **31**, 222-231

379. Lee, Y. J., Kim, E. H., Lee, J. S., Jeoung, D., Bae, S., Kwon, S. H., and Lee, Y. S. (2008) HSF1 as a mitotic regulator: phosphorylation of HSF1 by Plk1 is essential for mitotic progression. *Cancer research* **68**, 7550-7560

380. Boellmann, F., Guettouche, T., Guo, Y., Fenna, M., Mnayer, L., and Voellmy, R. (2004) DAXX interacts with heat shock factor 1 during stress activation and enhances its transcriptional activity. *Proc Natl Acad Sci U S A* **101**, 4100-4105

381. Holmberg, C. I., Hietakangas, V., Mikhailov, A., Rantanen, J. O., Kallio, M., Meinander, A., Hellman, J., Morrice, N., MacKintosh, C., Morimoto, R. I., Eriksson, J. E., and Sistonen, L. (2001) Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *The EMBO journal* **20**, 3800-3810

382. Kim, S. A., Yoon, J. H., Lee, S. H., and Ahn, S. G. (2005) Polo-like kinase 1 phosphorylates heat shock transcription factor 1 and mediates its nuclear translocation during heat stress. *J Biol Chem* **280**, 12653-12657

383. Kline, M. P., and Morimoto, R. I. (1997) Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. *Molecular and cellular biology* **17**, 2107-2115

384. Guettouche, T., Boellmann, F., Lane, W. S., and Voellmy, R. (2005) Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC biochemistry* **6**, 4

385. Hietakangas, V., Ahlskog, J. K., Jakobsson, A. M., Hellesuo, M., Sahlberg, N. M., Holmberg, C. I., Mikhailov, A., Palvimo, J. J., Pirkkala, L., and Sistonen, L. (2003) Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. *Molecular and cellular biology* **23**, 2953-2968

386. Hietakangas, V., Anckar, J., Blomster, H. A., Fujimoto, M., Palvimo, J. J., Nakai, A., and Sistonen, L. (2006) PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc Natl Acad Sci U S A* **103**, 45-50

387. Westerheide, S. D., Anckar, J., Stevens, S. M., Jr., Sistonen, L., and Morimoto, R. I. (2009) Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. *Science* **323**, 1063-1066

388. Wang, X., Khaleque, M. A., Zhao, M. J., Zhong, R., Gaestel, M., and Calderwood, S. K. (2006) Phosphorylation of HSF1 by MAPK-activated protein kinase 2 on serine 121, inhibits transcriptional activity and promotes HSP90 binding. *J Biol Chem* **281**, 782-791

389. Hong, Y., Rogers, R., Matunis, M. J., Mayhew, C. N., Goodson, M. L., Park-Sarge, O. K., and Sarge, K. D. (2001) Regulation of heat shock transcription factor 1 by stress-induced SUMO-1 modification. *J Biol Chem* **276**, 40263-40267

390. Chu, B., Soncin, F., Price, B. D., Stevenson, M. A., and Calderwood, S. K. (1996) Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *J Biol Chem* **271**, 30847-30857

391. Chu, B., Zhong, R., Soncin, F., Stevenson, M. A., and Calderwood, S. K. (1998) Transcriptional activity of heat shock factor 1 at 37 degrees C is repressed through phosphorylation on two distinct serine residues by glycogen synthase kinase 3 and protein kinases Calpha and Czeta. *J Biol Chem* **273**, 18640-18646

392. Dai, R., Frejtag, W., He, B., Zhang, Y., and Mivechi, N. F. (2000) c-Jun NH2-terminal kinase targeting and phosphorylation of heat shock factor-1 suppress its transcriptional activity. *J Biol Chem* **275**, 18210-18218

393. Knauf, U., Newton, E. M., Kyriakis, J., and Kingston, R. E. (1996) Repression of human heat shock factor 1 activity at control temperature by phosphorylation. *Genes & development* **10**, 2782-2793

394. Xavier, I. J., Mercier, P. A., McLoughlin, C. M., Ali, A., Woodgett, J. R., and Ovsenek, N. (2000) Glycogen synthase kinase 3beta negatively regulates both DNA-binding and transcriptional activities of heat shock factor 1. *J Biol Chem* **275**, 29147-29152

395. Park, J., and Liu, A. Y. (2001) JNK phosphorylates the HSF1 transcriptional activation domain: role of JNK in the regulation of the heat shock response. *J Cell Biochem* **82**, 326-338

396. Soncin, F., Zhang, X., Chu, B., Wang, X., Asea, A., Ann Stevenson, M., Sacks, D. B., and Calderwood, S. K. (2003) Transcriptional activity and DNA binding of heat shock factor-1 involve phosphorylation on threonine 142 by CK2. *Biochemical and biophysical research communications* **303**, 700-706

397. Stephanou, A., Isenberg, D. A., Nakajima, K., and Latchman, D. S. (1999) Signal transducer and activator of transcription-1 and heat shock factor-1 interact and

activate the transcription of the Hsp-70 and Hsp-90beta gene promoters. *J Biol Chem* **274**, 1723-1728

398. Stephanou, A., Amin, V., Isenberg, D. A., Akira, S., Kishimoto, T., and Latchman, D. S. (1997) Interleukin 6 activates heat-shock protein 90 beta gene expression. *The Biochemical journal* **321 (Pt 1)**, 103-106

399. Stephanou, A., Isenberg, D. A., Akira, S., Kishimoto, T., and Latchman, D. S. (1998) The nuclear factor interleukin-6 (NF-IL6) and signal transducer and activator of transcription-3 (STAT-3) signalling pathways co-operate to mediate the activation of the hsp90beta gene by interleukin-6 but have opposite effects on its inducibility by heat shock. *The Biochemical journal* **330 (Pt 1)**, 189-195

400. Wang, Y., Chen, L., Hagiwara, N., and Knowlton, A. A. (2010) Regulation of heat shock protein 60 and 72 expression in the failing heart. *Journal of molecular and cellular cardiology* **48**, 360-366

401. Xu, Q., Luef, G., Weimann, S., Gupta, R. S., Wolf, H., and Wick, G. (1993) Staining of endothelial cells and macrophages in atherosclerotic lesions with human heat-shock protein-reactive antisera. *Arterioscler Thromb* **13**, 1763-1769

402. Berberian, P. A., Myers, W., Tytell, M., Challa, V., and Bond, M. G. (1990) Immunohistochemical localization of heat shock protein-70 in normal-appearing and atherosclerotic specimens of human arteries. *Am J Pathol* **136**, 71-80

403. Madrigal-Matute, J., Lopez-Franco, O., Blanco-Colio, L. M., Munoz-Garcia, B., Ramos-Mozo, P., Ortega, L., Egido, J., and Martin-Ventura, J. L. (2010) Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis. *Cardiovasc Res* **86**, 330-337

404. Johnson, A. D., Berberian, P. A., Tytell, M., and Bond, M. G. (1995) Differential distribution of 70-kD heat shock protein in atherosclerosis. Its potential role in arterial SMC survival. *Arterioscler Thromb Vasc Biol* **15**, 27-36

405. Businaro, R., Profumo, E., Tagliani, A., Buttari, B., Leone, S., D'Amati, G., Ippoliti, F., Leopizzi, M., D'Arcangelo, D., Capoano, R., Fumagalli, L., Salvati, B., and Rigano, R. (2009) Heat-shock protein 90: a novel autoantigen in human carotid atherosclerosis. *Atherosclerosis* **207**, 74-83

406. Park, H. K., Park, E. C., Bae, S. W., Park, M. Y., Kim, S. W., Yoo, H. S., Tudev, M., Ko, Y. H., Choi, Y. H., Kim, S., Kim, D. I., Kim, Y. W., Lee, B. B., Yoon, J. B., and Park, J. E. (2006) Expression of heat shock protein 27 in human atherosclerotic plaques and increased plasma level of heat shock protein 27 in patients with acute coronary syndrome. *Circulation* **114**, 886-893

407. Miller, H., Poon, S., Hibbert, B., Rayner, K., Chen, Y. X., and O'Brien, E. R. (2005) Modulation of estrogen signaling by the novel interaction of heat shock protein 27, a biomarker for atherosclerosis, and estrogen receptor beta: mechanistic insight into the vascular effects of estrogens. *Arterioscler Thromb Vasc Biol* **25**, e10-14

408. Kanwar, R. K., Kanwar, J. R., Wang, D., Ormrod, D. J., and Krissansen, G. W. (2001) Temporal expression of heat shock proteins 60 and 70 at lesion-prone sites during atherogenesis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* **21**, 1991-1997

409. Garcia-Arguinzonis, M., Padro, T., Lugano, R., Llorente-Cortes, V., and Badimon, L. (2010) Low-density lipoproteins induce heat shock protein 27

dephosphorylation, oligomerization, and subcellular relocalization in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **30**, 1212-1219

410. Rayner, K., Chen, Y. X., McNulty, M., Simard, T., Zhao, X., Wells, D. J., de Belleroche, J., and O'Brien, E. R. (2008) Extracellular release of the atheroprotective heat shock protein 27 is mediated by estrogen and competitively inhibits acLDL binding to scavenger receptor-A. *Circ Res* **103**, 133-141

411. Rayner, K., Sun, J., Chen, Y. X., McNulty, M., Simard, T., Zhao, X., Wells, D. J., de Belleroche, J., and O'Brien, E. R. (2009) Heat shock protein 27 protects against atherogenesis via an estrogen-dependent mechanism: role of selective estrogen receptor beta modulation. *Arterioscler Thromb Vasc Biol* **29**, 1751-1756

412. Cuerrier, C. M., Chen, Y. X., Tremblay, D., Rayner, K., McNulty, M., Zhao, X., Kennedy, C. R., de BelleRoche, J., Pelling, A. E., and O'Brien, E. R. (2013) Chronic over-expression of heat shock protein 27 attenuates atherogenesis and enhances plaque remodeling: a combined histological and mechanical assessment of aortic lesions. *PloS one* **8**, e55867

413. Salinthone, S., Ba, M., Hanson, L., Martin, J. L., Halayko, A. J., and Gerthoffer, W. T. (2007) Overexpression of human Hsp27 inhibits serum-induced proliferation in airway smooth muscle myocytes and confers resistance to hydrogen peroxide cytotoxicity. *American journal of physiology. Lung cellular and molecular physiology* **293**, L1194-1207

414. Chen, H. F., Xie, L. D., and Xu, C. S. (2009) Role of heat shock protein 27 phosphorylation in migration of vascular smooth muscle cells. *Mol Cell Biochem* **327**, 1-6

415. Rai, S., and Zulli, A. (2013) Lack of cell stress markers in fibrous cap cells in the left main coronary artery. *Histology and histopathology* **28**, 505-511

416. Okada, M., Hasebe, N., Aizawa, Y., Izawa, K., Kawabe, J., and Kikuchi, K. (2004) Thermal treatment attenuates neointimal thickening with enhanced expression of heat-shock protein 72 and suppression of oxidative stress. *Circulation* **109**, 1763-1768

417. Xu, Q., Hu, Y., Kleindienst, R., and Wick, G. (1997) Nitric oxide induces heat-shock protein 70 expression in vascular smooth muscle cells via activation of heat shock factor 1. *J Clin Invest* **100**, 1089-1097

418. Mandrekar, P., Catalano, D., Jeliazkova, V., and Kodys, K. (2008) Alcohol exposure regulates heat shock transcription factor binding and heat shock proteins 70 and 90 in monocytes and macrophages: implication for TNF-alpha regulation. *Journal of leukocyte biology* **84**, 1335-1345

419. Hsu, H. Y., Wu, H. L., Tan, S. K., Li, V. P., Wang, W. T., Hsu, J., and Cheng, C. H. (2007) Geldanamycin interferes with the 90-kDa heat shock protein, affecting lipopolysaccharide-mediated interleukin-1 expression and apoptosis within macrophages. *Molecular pharmacology* **71**, 344-356

420. Wax, S., Piecyk, M., Maritim, B., and Anderson, P. (2003) Geldanamycin inhibits the production of inflammatory cytokines in activated macrophages by reducing the stability and translation of cytokine transcripts. *Arthritis and rheumatism* **48**, 541-550

421. Kleindienst, R., Xu, Q., Willeit, J., Waldenberger, F. R., Weimann, S., and Wick, G. (1993) Immunology of atherosclerosis. Demonstration of heat shock protein 60

expression and T lymphocytes bearing alpha/beta or gamma/delta receptor in human atherosclerotic lesions. *Am J Pathol* **142**, 1927-1937

422. Hochleitner, B. W., Hochleitner, E. O., Obrist, P., Eberl, T., Amberger, A., Xu, Q., Margreiter, R., and Wick, G. (2000) Fluid shear stress induces heat shock protein 60 expression in endothelial cells in vitro and in vivo. *Arterioscler Thromb Vasc Biol* **20**, 617-623

423. Chahine, M. N., Deniset, J., Dibrov, E., Hirono, S., Blackwood, D. P., Austria, J. A., and Pierce, G. N. (2011) Oxidized LDL promotes the mitogenic actions of Chlamydia pneumoniae in vascular smooth muscle cells. *Cardiovasc Res* **92**, 476-483

424. Martin-Ventura, J. L., Duran, M. C., Blanco-Colio, L. M., Meilhac, O., Leclercq, A., Michel, J. B., Jensen, O. N., Hernandez-Merida, S., Tunon, J., Vivanco, F., and Egido, J. (2004) Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis. *Circulation* **110**, 2216-2219

425. Zhu, J., Quyyumi, A. A., Wu, H., Csako, G., Rott, D., Zalles-Ganley, A., Ogunmakinwa, J., Halcox, J., and Epstein, S. E. (2003) Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease. *Arterioscler Thromb Vasc Biol* **23**, 1055-1059

426. Dulin, E., Garcia-Barreno, P., and Guisasola, M. C. (2010) Extracellular heat shock protein 70 (HSPA1A) and classical vascular risk factors in a general population. *Cell stress & chaperones* **15**, 929-937

427. Martin-Ventura, J. L., Leclercq, A., Blanco-Colio, L. M., Egido, J., Rossignol, P., Meilhac, O., and Michel, J. B. (2007) Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation. *Atherosclerosis* **194**, 334-341

428. Martin-Ventura, J. L., Nicolas, V., Houard, X., Blanco-Colio, L. M., Leclercq, A., Egido, J., Vranckx, R., Michel, J. B., and Meilhac, O. (2006) Biological significance of decreased HSP27 in human atherosclerosis. *Arterioscler Thromb Vasc Biol* **26**, 1337-1343

429. Pockley, A. G., Wu, R., Lemne, C., Kiessling, R., de Faire, U., and Frostegard, J. (2000) Circulating heat shock protein 60 is associated with early cardiovascular disease. *Hypertension* **36**, 303-307

430. Xiao, Q., Mandal, K., Schett, G., Mayr, M., Wick, G., Oberhollenzer, F., Willeit, J., Kiechl, S., and Xu, Q. (2005) Association of serum-soluble heat shock protein 60 with carotid atherosclerosis: clinical significance determined in a follow-up study. *Stroke* **36**, 2571-2576

431. Zhang, X., He, M., Cheng, L., Chen, Y., Zhou, L., Zeng, H., Pockley, A. G., Hu, F. B., and Wu, T. (2008) Elevated heat shock protein 60 levels are associated with higher risk of coronary heart disease in Chinese. *Circulation* **118**, 2687-2693

432. Matsumoto, M., Dimayuga, P. C., Wang, C., Kirzner, J., Cercek, M., Yano, J., Chyu, K. Y., Shah, P. K., and Cercek, B. (2008) Exogenous heat shock protein-70 inhibits cigarette smoke-induced intimal thickening. *American journal of physiology. Regulatory, integrative and comparative physiology* **295**, R1320-1327

433. Gonzalez-Ramos, M., Calleros, L., Lopez-Ongil, S., Raoch, V., Griera, M., Rodriguez-Puyol, M., de Frutos, S., and Rodriguez-Puyol, D. (2013) HSP70 increases extracellular matrix production by human vascular smooth muscle through TGF-beta1 up-regulation. *Int J Biochem Cell Biol* **45**, 232-242

434. Yao, Y., Watson, A. D., Ji, S., and Bostrom, K. I. (2009) Heat shock protein 70 enhances vascular bone morphogenetic protein-4 signaling by binding matrix Gla protein. *Circ Res* **105**, 575-584

435. Krepuska, M., Szeberin, Z., Sotonyi, P., Sarkadi, H., Fehervari, M., Apor, A., Rimely, E., Prohaszka, Z., and Acsady, G. (2011) Serum level of soluble Hsp70 is associated with vascular calcification. *Cell stress & chaperones* **16**, 257-265

436. Kol, A., Bourcier, T., Lichtman, A. H., and Libby, P. (1999) Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. *J Clin Invest* **103**, 571-577

437. Andrie, R. P., Bauriedel, G., Braun, P., Hopp, H. W., Nickenig, G., and Skowasch, D. (2011) Prevalence of intimal heat shock protein 60 homologues in unstable angina and correlation with anti-heat shock protein antibody titers. *Basic research in cardiology* **106**, 657-665

438. Almanzar, G., Ollinger, R., Leuenberger, J., Onestingel, E., Rantner, B., Zehm, S., Cardini, B., van der Zee, R., Grundtman, C., and Wick, G. (2012) Autoreactive HSP60 epitope-specific T-cells in early human atherosclerotic lesions. *Journal of autoimmunity* **39**, 441-450

439. Koesis, J., Veres, A., Vatay, A., Duba, J., Karadi, I., Fust, G., and Prohaszka, Z. (2002) Antibodies against the human heat shock protein hsp70 in patients with severe coronary artery disease. *Immunological Investigations* **31**, 219-231

440. Xu, Q., Schett, G., Seitz, C. S., Hu, Y., Gupta, R. S., and Wick, G. (1994) Surface staining and cytotoxic activity of heat-shock protein 60 antibody in stressed aortic endothelial cells. *Circ Res* **75**, 1078-1085

441. Kreutmayer, S. B., Messner, B., Knoflach, M., Henderson, B., Niederegger, H., Bock, G., Van der Zee, R., Wick, G., and Bernhard, D. (2011) Dynamics of heat shock protein 60 in endothelial cells exposed to cigarette smoke extract. *Journal of molecular and cellular cardiology* **51**, 777-780

442. Schett, G., Xu, Q., Amberger, A., Van der Zee, R., Recheis, H., Willeit, J., and Wick, G. (1995) Autoantibodies against heat shock protein 60 mediate endothelial cytotoxicity. *J Clin Invest* **96**, 2569-2577

443. Xu, Q., Kleindienst, R., Waitz, W., Dietrich, H., and Wick, G. (1993) Increased expression of heat shock protein 65 coincides with a population of infiltrating T lymphocytes in atherosclerotic lesions of rabbits specifically responding to heat shock protein 65. *J Clin Invest* **91**, 2693-2702

444. Li, J., Zhao, X., Zhang, S., Wang, S., Du, P., and Qi, G. (2011) ApoB-100 and HSP60 peptides exert a synergetic role in inhibiting early atherosclerosis in immunized ApoE-null mice. *Protein and peptide letters* **18**, 733-740

445. van Puijvelde, G. H., van Es, T., van Wanrooij, E. J., Habets, K. L., de Vos, P., van der Zee, R., van Eden, W., van Berkel, T. J., and Kuiper, J. (2007) Induction

of oral tolerance to HSP60 or an HSP60-peptide activates T cell regulation and reduces atherosclerosis. *Arterioscler Thromb Vasc Biol* **27**, 2677-2683

446. Gorlich, D., and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm. *Annual review of cell and developmental biology* **15**, 607-660

447. Dworetzky, S. I., and Feldherr, C. M. (1988) Translocation of RNA-coated gold particles through the nuclear pores of oocytes. *The Journal of cell biology* **106**, 575-584

448. Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B. T. (2000) The yeast nuclear pore complex: composition, architecture, and transport mechanism. *The Journal of cell biology* **148**, 635-651

449. Cronshaw, J. M., Krutchinsky, A. N., Zhang, W., Chait, B. T., and Matunis, M. J. (2002) Proteomic analysis of the mammalian nuclear pore complex. *The Journal of cell biology* **158**, 915-927

450. Alber, F., Dokudovskaya, S., Veenhoff, L. M., Zhang, W., Kipper, J., Devos, D., Suprapto, A., Karni-Schmidt, O., Williams, R., Chait, B. T., Sali, A., and Rout, M. P. (2007) The molecular architecture of the nuclear pore complex. *Nature* **450**, 695-701

451. Terry, L. J., Shows, E. B., and Wente, S. R. (2007) Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport. *Science* **318**, 1412-1416

452. Fahrenkrog, B., and Aebi, U. (2003) The nuclear pore complex: nucleocytoplasmic transport and beyond. *Nature reviews. Molecular cell biology* **4**, 757-766

453. Bernad, R., van der Velde, H., Fornerod, M., and Pickersgill, H. (2004) Nup358/RanBP2 attaches to the nuclear pore complex via association with Nup88 and Nup214/CAN and plays a supporting role in CRM1-mediated nuclear protein export. *Molecular and cellular biology* **24**, 2373-2384

454. Engelsma, D., Bernad, R., Calafat, J., and Fornerod, M. (2004) Supraphysiological nuclear export signals bind CRM1 independently of RanGTP and arrest at Nup358. *The EMBO journal* **23**, 3643-3652

455. Hamada, M., Haeger, A., Jeganathan, K. B., van Ree, J. H., Malureanu, L., Walde, S., Joseph, J., Kehlenbach, R. H., and van Deursen, J. M. (2011) Ran-dependent docking of importin-beta to RanBP2/Nup358 filaments is essential for protein import and cell viability. *The Journal of cell biology* **194**, 597-612

456. Hutten, S., Flotho, A., Melchior, F., and Kehlenbach, R. H. (2008) The Nup358-RanGAP complex is required for efficient importin alpha/beta-dependent nuclear import. *Molecular biology of the cell* **19**, 2300-2310

457. Mackay, D. R., Makise, M., and Ullman, K. S. (2010) Defects in nuclear pore assembly lead to activation of an Aurora B-mediated abscission checkpoint. *The Journal of cell biology* **191**, 923-931

458. Hase, M. E., and Cordes, V. C. (2003) Direct interaction with nup153 mediates binding of Tpr to the periphery of the nuclear pore complex. *Molecular biology of the cell* **14**, 1923-1940

459. Green, D. M., Johnson, C. P., Hagan, H., and Corbett, A. H. (2003) The C-terminal domain of myosin-like protein 1 (Mlp1p) is a docking site for

heterogeneous nuclear ribonucleoproteins that are required for mRNA export. *Proc Natl Acad Sci U S A* **100**, 1010-1015

460. Shibata, S., Matsuoka, Y., and Yoneda, Y. (2002) Nucleocytoplasmic transport of proteins and poly(A)+ RNA in reconstituted Tpr-less nuclei in living mammalian cells. *Genes to cells : devoted to molecular & cellular mechanisms* **7**, 421-434

461. Strambio-De-Castillia, C., Niepel, M., and Rout, M. P. (2010) The nuclear pore complex: bridging nuclear transport and gene regulation. *Nature reviews. Molecular cell biology* **11**, 490-501

462. Pante, N., and Kann, M. (2002) Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm. *Molecular biology of the cell* **13**, 425-434

463. Fried, H., and Kutay, U. (2003) Nucleocytoplasmic transport: taking an inventory. *Cellular and molecular life sciences : CMLS* **60**, 1659-1688

464. Mosammaparast, N., and Pemberton, L. F. (2004) Karyopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends in cell biology* **14**, 547-556

465. Lange, A., Mills, R. E., Lange, C. J., Stewart, M., Devine, S. E., and Corbett, A. H. (2007) Classical nuclear localization signals: definition, function, and interaction with importin alpha. *J Biol Chem* **282**, 5101-5105

466. Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**, 615-623

467. Dingwall, C., Laskey, R. A., and . (1991) Nuclear targeting sequences-a consensus? *Trends in biochemical sciences* **16**, 478-481

468. Kalderon, D., Richardson, W. D., Markham, A. F., and Smith, A. E. (1984) Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* **311**, 33-38

469. Lanford, R. E., and Butel, J. S. (1984) Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell* **37**, 801-813

470. Lee, B. J., Cansizoglu, A. E., Suel, K. E., Louis, T. H., Zhang, Z., and Chook, Y. M. (2006) Rules for nuclear localization sequence recognition by karyopherin beta 2. *Cell* **126**, 543-558

471. Stewart, M. (2007) Molecular mechanism of the nuclear protein import cycle. *Nature reviews. Molecular cell biology* **8**, 195-208

472. Carmody, S. R., and Wente, S. R. (2009) mRNA nuclear export at a glance. *Journal of cell science* **122**, 1933-1937

473. Fagotto, F., Gluck, U., and Gumbiner, B. M. (1998) Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin. *Current biology : CB* **8**, 181-190

474. Yokoya, F., Imamoto, N., Tachibana, T., and Yoneda, Y. (1999) beta-catenin can be transported into the nucleus in a Ran-unassisted manner. *Molecular biology of the cell* **10**, 1119-1131

475. Matsuura, Y., and Stewart, M. (2004) Structural basis for the assembly of a nuclear export complex. *Nature* **432**, 872-877

476. Bischoff, F. R., and Ponstingl, H. (1991) Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature* **354**, 80-82

477. Bischoff, F. R., Klebe, C., Kretschmer, J., Wittinghofer, A., and Ponstingl, H. (1994) RanGAP1 induces GTPase activity of nuclear Ras-related Ran. *Proc Natl Acad Sci U S A* **91**, 2587-2591

478. Bischoff, F. R., Krebber, H., Smirnova, E., Dong, W., and Ponstingl, H. (1995) Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. *The EMBO journal* **14**, 705-715

479. Ribbeck, K., Lipowsky, G., Kent, H. M., Stewart, M., and Gorlich, D. (1998) NTF2 mediates nuclear import of Ran. *The EMBO journal* **17**, 6587-6598

480. Kalab, P., Pralle, A., Isacoff, E. Y., Heald, R., and Weis, K. (2006) Analysis of a RanGTP-regulated gradient in mitotic somatic cells. *Nature* **440**, 697-701

481. Fontes, M. R., Teh, T., and Kobe, B. (2000) Structural basis of recognition of monopartite and bipartite nuclear localization sequences by mammalian importin-alpha. *Journal of molecular biology* **297**, 1183-1194

482. Gorlich, D., Henklein, P., Laskey, R. A., and Hartmann, E. (1996) A 41 amino acid motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus. *The EMBO journal* **15**, 1810-1817

483. Kobe, B. (1999) Autoinhibition by an internal nuclear localization signal revealed by the crystal structure of mammalian importin alpha. *Nature structural biology* **6**, 388-397

484. Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T., and Adam, S. A. (2004) Importin alpha: a multipurpose nuclear-transport receptor. *Trends in cell biology* **14**, 505-514

485. Lee, S. J., Matsuura, Y., Liu, S. M., and Stewart, M. (2005) Structural basis for nuclear import complex dissociation by RanGTP. *Nature* **435**, 693-696

486. Matsuura, Y., and Stewart, M. (2005) Nup50/Npap60 function in nuclear protein import complex disassembly and importin recycling. *The EMBO journal* **24**, 3681-3689

487. Hood, J. K., and Silver, P. A. (1998) Cse1p is required for export of Srp1p/importin-alpha from the nucleus in Saccharomyces cerevisiae. *J Biol Chem* **273**, 35142-35146

488. Kutay, U., Bischoff, F. R., Kostka, S., Kraft, R., and Gorlich, D. (1997) Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. *Cell* **90**, 1061-1071

489. Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskill, S., Rosen, C. A., and Baldwin, A. S., Jr. (1992) I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention. *Genes & development* **6**, 1899-1913

490. Traenckner, E. B., Wilk, S., and Baeuerle, P. A. (1994) A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated form of I kappa B-alpha that is still bound to NF-kappa B. *The EMBO journal* **13**, 5433-5441

491. Xiao, C. Y., Hubner, S., Elliot, R. M., Caon, A., and Jans, D. A. (1996) A consensus cAMP-dependent protein kinase (PK-A) site in place of the CcN motif casein kinase II site simian virus 40 large T-antigen confers PK-A-mediated regulation of nuclear import. *J Biol Chem* **271**, 6451-6457

492. Hubner, S., Xiao, C. Y., and Jans, D. A. (1997) The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin. *J Biol Chem* **272**, 17191-17195

493. Kitamura, R., Sekimoto, T., Ito, S., Harada, S., Yamagata, H., Masai, H., Yoneda, Y., and Yanagi, K. (2006) Nuclear import of Epstein-Barr virus nuclear antigen 1 mediated by NPI-1 (Importin alpha5) is up- and down-regulated by phosphorylation of the nuclear localization signal for which Lys379 and Arg380 are essential. *Journal of virology* **80**, 1979-1991

494. Yang, W., and Musser, S. M. (2006) Nuclear import time and transport efficiency depend on importin beta concentration. *The Journal of cell biology* **174**, 951-961

495. Timney, B. L., Tetenbaum-Novatt, J., Agate, D. S., Williams, R., Zhang, W., Chait, B. T., and Rout, M. P. (2006) Simple kinetic relationships and nonspecific competition govern nuclear import rates in vivo. *The Journal of cell biology* **175**, 579-593

496. Riddick, G., and Macara, I. G. (2005) A systems analysis of importin-{alpha}-{beta} mediated nuclear protein import. *The Journal of cell biology* **168**, 1027-1038

497. Riddick, G., and Macara, I. G. (2007) The adapter importin-alpha provides flexible control of nuclear import at the expense of efficiency. *Molecular systems biology* **3**, 118

498. Steggerda, S. M., Black, B. E., and Paschal, B. M. (2000) Monoclonal antibodies to NTF2 inhibit nuclear protein import by preventing nuclear translocation of the GTPase Ran. *Molecular biology of the cell* **11**, 703-719

499. Furuta, M., Kose, S., Koike, M., Shimi, T., Hiraoka, Y., Yoneda, Y., Haraguchi, T., and Imamoto, N. (2004) Heat-shock induced nuclear retention and recycling inhibition of importin alpha. *Genes to cells : devoted to molecular & cellular mechanisms* **9**, 429-441

500. Kodiha, M., Banski, P., Ho-Wo-Cheong, D., and Stochaj, U. (2008) Dissection of the molecular mechanisms that control the nuclear accumulation of transport factors importin-alpha and CAS in stressed cells. *Cellular and molecular life sciences : CMLS* **65**, 1756-1767

501. Kodiha, M., Tran, D., Qian, C., Morogan, A., Presley, J. F., Brown, C. M., and Stochaj, U. (2008) Oxidative stress mislocalizes and retains transport factor importin-alpha and nucleoporins Nup153 and Nup88 in nuclei where they generate high molecular mass complexes. *Biochimica et biophysica acta* **1783**, 405-418

502. Maul, G. G., Deaven, L. L., Freed, J. J., Campbell, G. L., and Becak, W. (1980) Investigation of the determinants of nuclear pore number. *Cytogenetics and cell genetics* **26**, 175-190

503. Stoffler, D., Fahrenkrog, B., and Aebi, U. (1999) The nuclear pore complex: from molecular architecture to functional dynamics. *Current opinion in cell biology* **11**, 391-401

504. Perez-Terzic, C., Pyle, J., Jaconi, M., Stehno-Bittel, L., and Clapham, D. E. (1996) Conformational states of the nuclear pore complex induced by depletion of nuclear Ca2+ stores. *Science* **273**, 1875-1877

505. Rakowska, A., Danker, T., Schneider, S. W., and Oberleithner, H. (1998) ATP-Induced shape change of nuclear pores visualized with the atomic force microscope. *The Journal of membrane biology* **163**, 129-136

506. Perez-Terzic, C., Gacy, A. M., Bortolon, R., Dzeja, P. P., Puceat, M., Jaconi, M., Prendergast, F. G., and Terzic, A. (1999) Structural plasticity of the cardiac nuclear pore complex in response to regulators of nuclear import. *Circ Res* **84**, 1292-1301

507. D'Angelo, M. A., Anderson, D. J., Richard, E., and Hetzer, M. W. (2006) Nuclear pores form de novo from both sides of the nuclear envelope. *Science* **312**, 440-443

508. Hetzer, M. W., Walther, T. C., and Mattaj, I. W. (2005) Pushing the envelope: structure, function, and dynamics of the nuclear periphery. *Annual review of cell and developmental biology* **21**, 347-380

509. Fernandez-Martinez, J., and Rout, M. P. (2009) Nuclear pore complex biogenesis. *Current opinion in cell biology* **21**, 603-612

510. Crampton, N., Kodiha, M., Shrivastava, S., Umar, R., and Stochaj, U. (2009) Oxidative stress inhibits nuclear protein export by multiple mechanisms that target FG nucleoporins and Crm1. *Molecular biology of the cell* **20**, 5106-5116

511. Kodiha, M., Tran, D., Morogan, A., Qian, C., and Stochaj, U. (2009) Dissecting the signaling events that impact classical nuclear import and target nuclear transport factors. *PloS one* **4**, e8420

512. Kosako, H., Yamaguchi, N., Aranami, C., Ushiyama, M., Kose, S., Imamoto, N., Taniguchi, H., Nishida, E., and Hattori, S. (2009) Phosphoproteomics reveals new ERK MAP kinase targets and links ERK to nucleoporin-mediated nuclear transport. *Nature structural & molecular biology* **16**, 1026-1035

513. Faustino, R. S., Rousseau, D. C., Landry, M. N., Kostenuk, A. L., and Pierce, G. N. (2006) Effects of mitogen-activated protein kinases on nuclear protein import. *Canadian journal of physiology and pharmacology* **84**, 469-475

514. Czubryt, M. P., Austria, J. A., and Pierce, G. N. (2000) Hydrogen peroxide inhibition of nuclear protein import is mediated by the mitogen-activated protein kinase, ERK2. *The Journal of cell biology* **148**, 7-16

515. Faustino, R. S., Maddaford, T. G., and Pierce, G. N. (2011) Mitogen activated protein kinase at the nuclear pore complex. *Journal of cellular and molecular medicine* **15**, 928-937

516. Faustino, R. S., Cheung, P., Richard, M. N., Dibrov, E., Kneesch, A. L., Deniset, J. F., Chahine, M. N., Lee, K., Blackwood, D., and Pierce, G. N. (2008) Ceramide regulation of nuclear protein import. *J Lipid Res* **49**, 654-662

517. Zhang, X., Chen, S., Yoo, S., Chakrabarti, S., Zhang, T., Ke, T., Oberti, C., Yong, S. L., Fang, F., Li, L., de la Fuente, R., Wang, L., Chen, Q., and Wang, Q. K. (2008) Mutation in nuclear pore component NUP155 leads to atrial fibrillation and early sudden cardiac death. *Cell* **135**, 1017-1027

518. Perez-Terzic, C., Gacy, A. M., Bortolon, R., Dzeja, P. P., Puceat, M., Jaconi, M., Prendergast, F. G., and Terzic, A. (2001) Directed inhibition of nuclear import in cellular hypertrophy. *J Biol Chem* **276**, 20566-20571

519. Cortes, R., Rosello-Lleti, E., Rivera, M., Martinez-Dolz, L., Salvador, A., Azorin, I., and Portoles, M. (2010) Influence of heart failure on nucleocytoplasmic transport in human cardiomyocytes. *Cardiovasc Res* **85**, 464-472

520. Tarazon, E., Rivera, M., Rosello-Lleti, E., Molina-Navarro, M. M., Sanchez-Lazaro, I. J., Espana, F., Montero, J. A., Lago, F., Gonzalez-Juanatey, J. R., and Portoles, M. (2012) Heart failure induces significant changes in nuclear pore complex of human cardiomyocytes. *PloS one* **7**, e48957

521. Rosello-Lleti, E., Rivera, M., Cortes, R., Azorin, I., Sirera, R., Martinez-Dolz, L., Hove, L., Cinca, J., Lago, F., Gonzalez-Juanatey, J. R., Salvador, A., and Portoles, M. (2012) Influence of heart failure on nucleolar organization and protein expression in human hearts. *Biochemical and biophysical research communications* **418**, 222-228

522. Kuo, C., and Grayston, J. T. (1990) A sensitive cell line, HL cells, for isolation and propagation of Chlamydia pneumoniae strain TWAR. *J Infect Dis* **162**, 755-758

523. Furness, G., Graham, D. H., and Reeve, P. (1960) The titration of trachoma and inclusion blennorrhoea viruses in cell cultures. *J Gen Microbiol* **23**

524. Saward, L., and Zahradka, P. (1997) Coronary artery smooth muscle in culture: migration of heterogeneous cell populations from vessel wall. *Mol Cell Biochem* **176**

525. Deniset, J. F., Cheung, P. K., Dibrov, E., Lee, K., Steigerwald, S., and Pierce, G. N. (2010) Chlamydophila pneumoniae infection leads to smooth muscle cell proliferation and thickening in the coronary artery without contributions from a host immune response. *Am J Pathol* **176**, 1028-1037

526. Zanetti, M., d'Uscio, L. V., Peterson, T. E., Katusic, Z. S., and O'Brien, T. (2005) Analysis of superoxide anion production in tissue. *Methods Mol Med* **108**, 65-72

527. Faustino, R. S., Czubryt, M. P., and Pierce, G. N. (2002) Determining influence of oxidants on nuclear transport using digitonin-permeabilized cell assay. *Methods Enzymol* **352**, 123-134

528. Dupasquier, C. M., Weber, A. M., Ander, B. P., Rampersad, P. P., Steigerwald, S., Wigle, J. T., Mitchell, R. W., Kroeger, E. A., Gilchrist, J. S., Moghadasian, M. M., Lukas, A., and Pierce, G. N. (2006) Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. *American journal of physiology. Heart and circulatory physiology* **291**, H2987-2996

529. Deniset, J. F., Hedley, T. E., Dibrov, E., and Pierce, G. N. (2012) Chlamydophila pneumoniae infection induces alterations in vascular contractile responses. *Am J Pathol* **180**, 1264-1272

530. Francis, A. A., Deniset, J. F., Austria, J. A., Lavallee, R. K., Maddaford, G. G., Hedley, T. E., Dibrov, E., and Pierce, G. N. (2013) Effects of dietary flaxseed on atherosclerotic plaque regression. *American journal of physiology. Heart and circulatory physiology* **304**, H1743-1751

531. Byrne, G. I., and Kalayoglu, M. V. (1999) Chlamydia pneumoniae and atherosclerosis: links to the disease process. *Am Heart J* **138**, S488-490

532. Campbell, L. A., Kuo, C. C., and Grayston, J. T. (1998) Chlamydia pneumoniae and cardiovascular disease. *Emerg Infect Dis* **4**, 571-579

533. Gupta, S. (1999) Chlamydia pneumoniae, monocyte activation, and azithromycin in coronary heart disease. *Am Heart J* **138**, S539-541

534. Ouchi, K. (1999) Chlamydia pneumoniae and atherosclerosis. *Japanese journal of infectious diseases* **52**, 223-227

535. Moulder, J. W. (1991) Interaction of chlamydiae and host cells in vitro. *Microbiological reviews* **55**, 143-190

536. Hirono, S., and Pierce, G. N. (2003) Dissemination of Chlamydia pneumoniae to the vessel wall in atherosclerosis. *Mol Cell Biochem* **246**, 91-95

537. Shor, A., Kuo, C. C., and Patton, D. L. (1992) Detection of Chlamydia pneumoniae in coronary arterial fatty streaks and atheromatous plaques. *South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde* **82**, 158-161

538. Marino, J., Stoeckli, I., Walch, M., Latinovic-Golic, S., Sundstroem, H., Groscurth, P., Ziegler, U., and Dumrese, C. (2008) Chlamydophila pneumoniae derived from inclusions late in the infectious cycle induce aponecrosis in human aortic endothelial cells. *BMC microbiology* **8**, 32

539. Heltai, K., Kis, Z., Burian, K., Endresz, V., Veres, A., Ludwig, E., Gonczol, E., and Valyi-Nagy, I. (2004) Elevated antibody levels against Chlamydia pneumoniae, human HSP60 and mycobacterial HSP65 are independent risk

factors in myocardial infarction and ischaemic heart disease. *Atherosclerosis* **173**, 339-346

540. Andrie, R., Braun, P., Welsch, U., Straube, E., Hopp, H. W., Erdmann, E., Luderitz, B., and Bauriedel, G. (2003) [Chlamydial and human heat shock protein 60 homologues in acute coronary syndromes. (Auto-)immune reactions as a link between infection and atherosclerosis]. *Zeitschrift fur Kardiologie* **92**, 455-465

541. Schmidt, C., Hulthe, J., Wikstrand, J., Gnarpe, H., Gnarpe, J., Agewall, S., and Fagerberg, B. (2000) Chlamydia pneumoniae seropositivity is associated with carotid artery intima-media thickness. *Stroke; a journal of cerebral circulation* **31**, 1526-1531

542. Rodel, J., Woytas, M., Groh, A., Schmidt, K. H., Hartmann, M., Lehmann, M., and Straube, E. (2000) Production of basic fibroblast growth factor and interleukin 6 by human smooth muscle cells following infection with Chlamydia pneumoniae. *Infect Immun* **68**, 3635-3641

543. Viedt, C., Vogel, J., Athanasiou, T., Shen, W., Orth, S. R., Kubler, W., and Kreuzer, J. (2002) Monocyte chemoattractant protein-1 induces proliferation and interleukin-6 production in human smooth muscle cells by differential activation of nuclear factor-kappaB and activator protein-1. *Arterioscler Thromb Vasc Biol* **22**, 914-920

544. Selzman, C. H., Miller, S. A., Zimmerman, M. A., Gamboni-Robertson, F., Harken, A. H., and Banerjee, A. (2002) Monocyte chemotactic protein-1 directly induces human vascular smooth muscle proliferation. *American journal of physiology. Heart and circulatory physiology* **283**, H1455-1461

545. Ikeda, U., Ikeda, M., Oohara, T., Oguchi, A., Kamitani, T., Tsuruya, Y., and Kano, S. (1991) Interleukin 6 stimulates growth of vascular smooth muscle cells in a PDGF-dependent manner. *The American journal of physiology* **260**, H1713-1717

546. Puolakkainen, M., Campbell, L. A., Lin, T. M., Richards, T., Patton, D. L., and Kuo, C. C. (2003) Cell-to-cell contact of human monocytes with infected arterial smooth-muscle cells enhances growth of Chlamydia pneumoniae. *J Infect Dis* **187**, 435-440

547. Chen, L., Frister, A., Wang, S., Ludwig, A., Behr, H., Pippig, S., Li, B., Simm, A., Hofmann, B., Pilowski, C., Koch, S., Buerke, M., Rose-John, S., Werdan, K., and Loppnow, H. (2009) Interaction of vascular smooth muscle cells and monocytes by soluble factors synergistically enhances IL-6 and MCP-1 production. *American journal of physiology. Heart and circulatory physiology* **296**, H987-996

548. Orallo, F. (1996) Regulation of cytosolic calcium levels in vascular smooth muscle. *Pharmacol Ther* **69**, 153-171

549. Somlyo, A. P., and Somlyo, A. V. (1994) Signal transduction and regulation in smooth muscle. *Nature* **372**, 231-236

550. Nobe, K., and Paul, R. J. (2001) Distinct pathways of Ca(2+) sensitization in porcine coronary artery: effects of Rho-related kinase and protein kinase C inhibition on force and intracellular Ca(2+). *Circ Res* **88**, 1283-1290

551. Rzucidlo, E. M., Martin, K. A., and Powell, R. J. (2007) Regulation of vascular smooth muscle cell differentiation. *J Vasc Surg* **45 Suppl A**, A25-32

552. Hornig, B., and Drexler, H. (1997) Endothelial function and bradykinin in humans. *Drugs* **54 Suppl 5**, 42-47

553. Kawashima, S., and Yokoyama, M. (2004) Dysfunction of endothelial nitric oxide synthase and atherosclerosis. *Arterioscler Thromb Vasc Biol* **24**, 998-1005

554. Danser, A. H., Tom, B., de Vries, R., and Saxena, P. R. (2000) L-NAME-resistant bradykinin-induced relaxation in porcine coronary arteries is NO-dependent: effect of ACE inhibition. *Br J Pharmacol* **131**, 195-202

555. Bouwman, J. J., Visseren, F. L., Bevers, L. M., van der Vlist, W. E., Bouter, K. P., and Diepersloot, R. J. (2005) Azithromycin reduces Chlamydia pneumoniae-induced attenuation of eNOS and cGMP production by endothelial cells. *Eur J Clin Invest* **35**, 573-582

556. Griendling, K. K., Sorescu, D., and Ushio-Fukai, M. (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* **86**, 494-501

557. Wick, M. C., Mayerl, C., Backovic, A., van der Zee, R., Jaschke, W., Dietrich, H., and Wick, G. (2008) In vivo imaging of the effect of LPS on arterial endothelial cells: molecular imaging of heat shock protein 60 expression. *Cell Stress Chaperones* **13**, 275-285

558. Gupta, S., and Knowlton, A. A. (2005) HSP60, Bax, apoptosis and the heart. *J Cell Mol Med* **9**, 51-58

559. Chang, A. Y., Chan, J. Y., Chou, J. L., Li, F. C., Dai, K. Y., and Chan, S. H. (2006) Heat shock protein 60 in rostral ventrolateral medulla reduces cardiovascular fatality during endotoxaemia in the rat. *J Physiol* **574**, 547-564

560. Stephanou, A., and Latchman, D. S. (2011) Transcriptional modulation of heat-shock protein gene expression. *Biochem Res Int* **2011**, 238601

561. Kose, S., Furuta, M., Koike, M., Yoneda, Y., and Imamoto, N. (2005) The 70-kD heat shock cognate protein (hsc70) facilitates the nuclear export of the import receptors. *J Cell Biol* **171**, 19-25

562. Galigniana, M. D., Echeverria, P. C., Erlejman, A. G., and Piwien-Pilipuk, G. (2010) Role of molecular chaperones and TPR-domain proteins in the cytoplasmic transport of steroid receptors and their passage through the nuclear pore. *Nucleus* **1**, 299-308

563. Kaur, G., Lieu, K. G., and Jans, D. A. (2013) 70-kDa Heat Shock Cognate Protein hsc70 Mediates Calmodulin-dependent Nuclear Import of the Sex-determining Factor SRY. *J Biol Chem* **288**, 4148-4157

564. Lacolley, P., Regnault, V., Nicoletti, A., Li, Z., and Michel, J. (2012) The vascular smooth muscle cell in arterial pathology: a cell that can take on multiple roles. *Cardiovasc Res* **95**, 135-137

565. Cappello, F., Conway de Macario, E., Marasa, L., Zummo, G., and Macario, A. J. (2008) Hsp60 expression, new locations, functions and perspectives for cancer diagnosis and therapy. *Cancer Biol Ther* **7**, 801-809

566. Chahine, M. N., and Pierce, G. N. (2009) Therapeutic targeting of nuclear protein import in pathological cell conditions. *Pharmacological reviews* **61**, 358-372