INSULIN-LIKE GROWTH FACTOR EFFECTS ON VASCULAR SMOOTH MUSCLE CELLS ARE IN PART MODULATED VIA A G PROTEIN-COUPLED PATHWAY

by

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ABSTRACT

An important part of repair processes activated by vascular injury is the recruitment of vascular smooth muscle cells (SMC) from the existing contractile coat. Phenotypic modulation of SMCs enables these cells to proliferate and migrate into the vessel intima. Despite its importance in vessel repair, this plasticity of SMCs can also promote both the pathogenesis of atherosclerosis as well as neointimal formation following revascularization- induced injury.

Vascular growth factors are major contributors to the migratory and proliferative responses to injury. IGF-1 is one such growth factor that elicits a response via its receptor, the IGF-1R, a classical tyrosine kinase receptor. However, it has been suggested that the IGF-1R may also be coupled to a heterotrimeric G protein and can thus initiate cellular responses via this alternate pathway. The objective of this study was to investigate the structural aspects of IGR-1R coupling to a heterotrimeric G protein in SMCs, as well as the contribution of this pathway to the cellular responses.

In a porcine primary SMC culture model, IGF-1R co-precipitated with both the α- and β-subunits of a G protein, with the latter demonstrating activation dependent precipitation. The specific G_{α} class activated by IGF-1R was $G_{\alpha i}$, in a manner that was independent of the activity of the tyrosine kinase. Both $G_{\alpha i1}$ and $G_{\alpha i2}$ directly interacted with the receptor. $G_{\beta\gamma}$ mediated the activation of MAPK and its inhibition was sufficient to attenuate both the proliferation and migration of SMCs *in vitro*. In contrast, the contribution of $G_{\alpha i}$ was related to regulation of protein translation and histone modification.

The data supports the conclusion that IGF-1 regulates the phenotype of vascular SMCs at least partially via a non-classical G protein-coupled receptor. Investigation into the individual subunits of the G protein complex led to the elucidation of a model in which both components play an integral role in the IGF-1 response, independent of the receptor tyrosine kinase activity. In one case, an interplay of specific $G_{\alpha i}$-subunits leads to modulation of the VSMC translational and transcriptional responses, while in the other, release of the $G_{\beta\gamma}$-subunit activated the MAPK response in a manner that significantly contributes to both the migration and proliferation of SMCs.

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ABBREVIATIONS

- aa: Amino Acid
- AB/AM: Antibiotic/Antimycotic
- ACE: Angiotensin Converting Enzyme
- AGS: Activators of G protein signaling
- A/L Buffer: Assay/Lysis Buffer
- βARKct: β-Adrenergic Receptor Kinase C-terminal
- cAMP: cylic Adenosine Mono Phosphate
- ANOVA: Analysis of Variance
- APS: Ammonium Persulfate
- BrdU: Bromo-deoxyuridine
- BSA: Bovine Serum Albumin
- CMFDA: 5-chloromethylfluorescein diacetate
- CREB: cAMP Response Element Binding Protein
- DMEM: Dulbecco's Modified Eagles Medium
- EC: Endothelial Cells
- ECL: Enhanced Chemi-luminescence
- EDTA: Ethylene Diamine Tetraacetic Acid
- EGF: Epidermal Growth Factor
- EGFR: Epithelial Growth Factor Receptor
- EGTA: Ethylene Glycol Tetraacetic Acid
- eIF2B: Eukaryotic Translation Initiation Factor 2B
- eIF4E: Eukaryotic Translation Initiation Factor 4

- 5’-FAM: 5-Carboxyfluorescein Amidite
- bFGF: basic Fibroblast Growth Factor
- FBS: Fetal Bovine Serum
- hG protein: Heterotrimeric Guanine nucleotide-binding protein
- GF: Growth Factor
- GH: Growth Hormone
- HB-EGF: Heparin Binding Epidermal Growth Factor
- HIV: Human Immunodeficiency Virus
- HRP: Horseradish Peroroxidase
- ICAM: Inter-Cellular Adhesion Molecule
- IFN: Interferon
- IGF-1: Insulin-like Growth Factor-1
- IGF-1R: Insulin-like Growth Factor-1 Receptor
- IGFBP: Insulin-like Growth Factor Binding Protein
- IL: Interleukin
- IP: Immunoprecipitation
- IR: Insulin Receptor
- LDL: Low Density Lipoprotein
- oxLDL: Oxidized LDL
- mmLDL: Minimally modified LDL
- MAPK: Mitogen Activated Protein Kinase
- MCP-1: Monocyte Chemoattractant Protein
- MCSF: Macrophage Colony Stimulating Factor

- MI: Myocardial Infaction
- MMP: Matrix metalloproteinase
- MOPS: 3-(N-morpholino) Propanesulfonic Acid
- MWM: Molecular Weight Marker
- PDGF: Platelet-Derived Growth Factor
- PDGFR: Platelet-Derived Growth Factor Receptor
- NE: Norepinephrine
- NI: Neointimal
- PI3K: Phosphatidylinositol-3-Kinase
- PFA: Paraformaldehyde
- PMSF: Phenylmethanesulphonylfluoride
- PP1-α (catalytic): Protein Serine Phosphatase 1 - catalytic subunit - α isoform
- PTD: Protein Transduction Domain
- PTX: Pertussis Toxin
- PVDF: Polyvinylidene Fluoride
- PTSA: Pyruvate, Transferrin, Selenium, Ascorbic Acid
- ROS: Reactive Oxygen Species
- RTK: Receptor Tyrosine Kinase
- SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamine Gel Electrophoresis
- SMC: Smooth Muscle Cell
- SMαA: Smooth Muscle α-Actin
- SM-MHC: Smooth Muscle- Myosin Heavy Chain
- SRF: Serum Response Factor

- TAT: Trans-Activating Transcriptional Activator
- TAT-$G_{\alpha i}$: TAT-conjugated $G_{\alpha i}$ C-terminal peptide inhibitor
- TBS-T: Tris-Buffered Saline-Tween
- TEMED: tetramethylethylenediamine
- TGF: Transforming Growth Factor
- TNF: Tumour Necrosis Factor
- VCAM: Vascular Cell Adhesion Molecule
- VEGF: Vascular Endothelial Growth Factor
- VEGFR: Vascular Endothelial Growth Factor Receptor
- VSMC: Vascular Smooth Muscle Cell

LITERATURE REVIEW

1. Introduction

Many growth factors are carried through the body's vascular system, exerting their effects on the various cell types encountered along the way. Within the vasculature itself, and specifically within the smooth muscle tissue, it is thought that many play a role in exacerbating atherogenesis, although the specific effects and interactions of their respective signaling networks are still incompletely understood. In particular, the Insulin-like Growth Factor-1 (IGF-1) has remained a minor player in the vast amount of literature pertaining to growth factor effects on smooth muscle and the resulting phenotypic switch, despite its well recognized importance as a growth regulator in the cancer field. An important reason for this is IGF-1's close homology to insulin, which has implicated IGF-1 in the metabolic disease diabetes, often resulting in a blurring of the importance of IGF-1 as a growth agent. This research examines structural aspects governing the novel G protein coupled pathway of cellular signaling resulting from IGF-1 stimulation of smooth muscle cells, and how this signaling cascade influences the processes underlying the development of atherosclerosis.

2. Atherosclerosis

2.1. Introduction

Atherosclerosis is a common vascular disease thought to be the major underlying cause behind the clinical events seen in myocardial infarctions (MI), strokes and peripheral vascular disease, as well as in major clinical complications such as restenosis

and vein graft arterialisation (Fuster et al., 2009). Its development involves a complex interplay of genetic, metabolic, cellular and immunological factors (Siu, 2010).

Atherosclerosis is a form of arteriosclerosis, which signifies thickening (sclerosis) of the arteries (arterio). Atherosclerosis specifically refers to thickening and loss of elasticity in the walls of the large to medium-sized arteries (Hahn & Schwartz, 2009; Ross, 1999). Atherosclerosis manifests as lesions, which are most intense in the coronary arteries and abdominal aorta. Though the onset of the clinical manifestations tends to occur in middle-aged individuals, the evolution of atherosclerosis is chronic and is thought to initiate as early as childhood (Santos et al., 2008). It is now thought to be the major cause of death, not only in the Western world, but also worldwide (Hof & von Eckardstein, 2009). Indeed, the costs of atherosclerosis and cardiovascular disease are staggering and ever increasing, now reaching 400 billion dollars annually in the United States alone (Lands, 2009).

The starting point for gross evolution of atherosclerotic lesions is the "fatty streak", a region of intimal thickening due to an accumulation of inflammatory cells and lipid deposits on the arterial wall (Katsiki et al., 2009). From the initial lesions, a "fibrous plaque" may develop. These consist of raised intimal lesions made up of a lipid core surrounded by vascular smooth muscle cells (SMCs) which have migrated from the media to the subendothelial space and secrete excess extracellular matrix proteins and collagen (Stoneman & Bennett, 2004). Their lipid core consists of foam cells formed from accumulated macrophages and SMCs which have taken up oxidized low-density lipoprotein (LDL) via the scavenger receptor (Woollard & Geissmann, 2010; Yu et al., 2010). Oxidized LDL (oxLDL) originates as LDL, which enters vessel walls by passive

diffusion, a process dependent on ApoB and the LDL receptor. LDL becomes trapped in the subendothelial space, where it is oxidized by reactive oxygen species, first to minimally modified LDL (mmLDL) and further to oxLDL (Gleissner et al., 2007). While oxLDL leads to foam cell formation, mmLDL stimulates endothelial cells (ECs) to express inflammatory cytokines and adhesion molecules, leading to further monocyte recruitment (Saha et al., 2009). Thus, the presence of LDL in the subendothelial space is crucial to the maintenance of the chronic degenerative environment created within the atherosclerotic plaque. Evolution of a vascular lesion past a fibrous plaque leads to the formation of a "complicated lesion". Such a lesion is altered by hemorrhage, calcification and neovascularization. The lesion core often becomes necrotic, leading to erosion and mural thrombosis (Ross & Glomset, 1973). In fact, although angina pectoris is usually a symptom of vessel narrowing, acute cardiovascular events are generally thought to result from plaque rupture and thrombosis (Davies & Thomas, 1984; Shah, 2003).

2.2. Important cell types

The normal artery wall is made up of three tubular layers surrounding the central lumen. The tunica intima is the innermost layer, consisting of a thin sheet of endothelial cells supported on a basement membrane. The middle layer, the tunica media, consists largely of many overlapping layers of smooth muscle cells. It is separated from the other layers by the internal and external elastic laminae, which are sheets of the extracellular matrix protein elastin. The final and outermost layer is called the tunica externa (or

adventitia), and is composed primarily of collagen fibers for vessel support (Moyes & Schulte, 2006).

2.2.1. Endothelial cells

The endothelial layer which makes up the tunica intima was long thought to simply act as an inert barrier that separated the vessel wall from the circulating blood components. It is now known to be an important and dynamic organ, playing a crucial role in the regulation of vascular tone and homeostasis (Versari et al., 2009). Its homeostatic role encompasses regulation of vascular SMC proliferation and migration, as well as the delicate equilibriums maintaining the body's inflammatory, thrombogenic and coagulative responses (Mensah, 2007). A damaged or dysfunctional endothelial layer can undergo functional and structural alterations, which can lead to disruption of this balance, predisposing the vasculature to an increasingly athero-prone phenotype that exhibits more vasoconstriction, leukocyte adhesion, platelet activation, mitogenesis, oxidation, apoptosis, thrombosis and vascular inflammation (Bai et al., 2010; Verma & Anderson, 2002). Indeed, endothelial dysfunction has been closely associated with cardiovascular events (Landmesser & Drexler 2005). In addition, it has been associated with the presence of all established risk factors for coronary heart disease, and precedes the development of atherosclerosis (Mensah, 2007).

The defining feature of what is known as "endothelial dysfunction" is impaired nitric oxide (NO) bioavailability, leading to impaired endothelium-dependent vasodilation, and resulting most frequently from increased breakdown of endothelial NO synthase (eNOS) by reactive oxygen species. (Versari et al., 2009). However, the term

also encompasses general defects in all of the homeostatic mechanisms. In particular, dysfunction is accompanied by higher turnover rates (Foteinos et al., 2008), secondary to increased apoptosis (Dimmeler et al., 1998). EC dysfunction is thought to be the initial step in the continuous cycle leading to the development of atherosclerotic lesions (Chien, 2008a). Increased synthesis of adhesion molecules such as Vascular cell adhesion molecule (VCAM) and Inter-cellular adhesion molecule (ICAM), as well as inflammatory cytokines such as Monocyte chemo-attractant protein (MCP-1), is part of the local inflammatory microenvironment created by the dysfunctional endothelium (Doran et al., 2008). Together with increased formation of reactive oxygen species (ROS), transcytosis of monocytes to the subendothelial space sets the stage for differentiation into macrophages, foam cell formation and SMC phenotypic switching.

While the mechanisms behind endothelial dysfunction are not fully known, mechanical forces are thought to play an important role, since arterial branch points are highly susceptible to plaque development (Chien, 2008b). Here, shear flow, which vascular endothelial cells respond to by releasing NO (Jin et al., 2003), is not laminar, but disturbed (Hahn & Schwartz, 2009). This disturbed flow includes pulsatile (Ku et al., 1985) and turbulent flow, as well as low shear flow. These flow patterns can be further exacerbated by pathology such as hypertension, which affects encountered pressure. It has been suggested that these branch sites are susceptible to a chronic maladaptive response, being unable to adapt to disturbed flow and leading to failure of the ECs to maintain their normal quiescent phenotype and function. Disturbed flow also results in the higher cell turnover rates which accompany EC dysfunction (Davies et al., 1986). Though it is not yet clear how forces are transduced to biochemical signals, potential

mechanotransducers include the cytoskeleton (Hartog et al., 2007), adhesion receptors (Tzima et al., 2005) and other luminal membrane proteins such as G proteins (Gudi et al., 1998) and ion channels (Maroto et al., 2005)

2.2.2. Vascular smooth muscle cells (VSMCs)

The highly specialized environment that is the arterial vessel wall maintains its intrinsic ability to respond to injury, thus enabling repair and restoration of vessel integrity, primarily due to vascular SMCs (VSMCs). These have a prominent role in this repair process, as they are recruited from the existing contractile coat, and are also responsible for the production of new connective tissue (Ross & Fuster, 1996). An important repercussion of this, however, is that SMCs must retain the ability to re-enter the cell cycle and proliferate throughout life (Mallika et al., 2007; Muto et al., 2007), a characteristic that has implicated them in the development of vascular diseases and post-surgery repair.

An important study by Katsuda et al. (1992) revealed that, with respect to numbers, smooth muscle cells were by far the predominant cell type found in atherosclerotic plaques. However, the implication that SMCs are important in lesion formation was first outlined in 1973 in a landmark paper by Ross and colleagues (Ross & Glomset, 1973), who proposed a breakthrough hypothesis, namely the "SMC response to injury". They argued that focal accumulation of SMCs is central to the process of lesion development since it is involved in both the deposition of lipid and the accumulation of extracellular connective tissue. Furthermore, they postulated that:

> "local injury to the endothelium increases the concentration of plasma proteins in the vicinity of medial smooth muscle cells and in response to

> some of these proteins the cells migrate into the intima and proliferate. […] with continued injury to the endothelium, the smooth muscle cells that have already migrated into the intima are stimulated to proliferate further, and a critical balance between cell proliferation and cell destruction may determine whether the lesion enlarges…" (Ross & Glomset, 1973)

Although the current view of lesion development has deviated from this original hypothesis, the basis of vascular injury research and treatment was forever altered, with increased focus now being given to SMCs and their proliferation and migration.

It is now thought that endothelial dysfunction, as previously described, precedes and possibly activates these SMC responses. The origin of the SMCs that participate in intima formation has also recently deviated from the long-standing belief that intimal SMCs are derived from preexisting medial SMCs. Evidence now suggests that ECs (DeRuiter et al., 1997) and adventitial fibroblasts (Sartore et al., 2001) may give rise to SMC-like cells within the intima to thereby contribute to injury repair. In addition, there is accumulating evidence that circulating bone marrow-derived cells invest in the intima following injury, and express at least some SMC markers (Sata et al., 2002; Simper et al., 2002). Interestingly, Han et al. (2001) conducted a study of scratch induced vascular injuries of varying degrees. They found major investment of bone marrow-derived cells in severe injury, but none in minor injury cases where there was endothelial denudation but minimal damage to the medial SMCs. This suggested that bone marrow-derived cells may be recruited only to complement resident SMCs. However, although it now appears clear that that bone marrow (BM)-derived cells *can* invest in neointima (NI) formation, several studies have raised doubts as to whether these undergo full differentiation into mature SMCs (Hu et al., 2002; Sata et al., 2002). Their importance also continues to be

controversial as it has been shown that NI can form in cultured vessels, in the complete absence of any blood elements (Wilson et al., 1999).

It is now well understood that the role of the SMCs in atherosclerosis appears to vary with the stage of the disease. In early events, SMCs are involved in a maladaptive role in lesion development and progression via their proliferative and migratory responses. However, over 50% of acute cardiac events occur in arteries having a non-significant degree of stenosis (≤50%), underlying the relatively minor role of plaque size in determining stability (Versari et al., 2007). Thus, in late-stage disease, SMCs likely play a beneficial role within the fibrous plaque, forming a physiological, stabilizing cap. Indeed, it has been shown that rupture-prone vulnerable plaques are characterized by a necrotic core and a very thin fibrous cap containing reduced numbers of SMCs, and that plaques specifically tend to rupture at sites of reduced SMC content (Davies et al., 1993). Vascular SMC apoptosis has been shown to occur in advanced atherosclerotic lesions (Han et al., 1995), and is thought to be a prominent pathway leading to plaque destabilization (Bennett, 1999). Thus, agents that are present and involved in regulating apoptosis within the plaque remain attractive targets for acute MI research.

2.2.3. Inflammatory cells

In the past, it was suggested that the two major characteristics of atherosclerotic lesions included the intimal proliferation of SMCs, as described above, and the inflammatory reaction (Nilsson, 1993). These two characteristics are, in fact, not mutually exclusive, as both SMCs and ECs secrete and respond to cytokines involved in

the inflammatory process, which further activates the proliferative response (Nilsson, 1993).

Indeed, it is now well established that circulating inflammatory cells play an important part in both the initiation and the progression of vascular disease. There is substantial evidence that inflammatory cascades are initiated by a wide range of cellular effectors which promote atherogenesis (Weber et al., 2008). In addition, inflammation is now seen as a key regulatory process linking multiple risk factors for atherosclerosis (Libby et al., 2009). Though macrophages are thought to be the predominant inflammatory species, T cells (Munro et al., 1987), B cells (Caligiuri et al., 2002; Zhou & Hansson, 1999), neutrophils (Zernecke et al., 2008), mast cells (Sun et al., 2007) and dendritic cells (Bobryshev & Lord, 1995) have all been detected in both human and mouse aortae. Some, like the B cells and neutrophils may be at least partially atheroprotective, while the T cells and mast cells, as well as the very important monocyte-derived macrophages, are negatively involved in every step of atherogenesis.

In the middle of the 19th century, the German scientist Rudolf Virchow studied and described inflammatory changes in atherosclerotic vessel walls, and postulated their role as being critical for atherogenesis (Virchow, 1989). Subsequently however, the direction of atherosclerotic plaque research took a turn and became focused on cholesterol, metabolism and diet. Thus, it wasn't until much later that this aspect of vascular disease was further investigated (Mayerl et al., 2006).

The presence of leukocytes within atherosclerotic plaque was first characterized by Gerrity et al. (1979) who studied the aortic intima of cholesterol-fed pigs. They found monocytes to be present in the very earliest stages of developing plaque. Monocytes

under homeostasis represent 10% of circulating leukocytes (Auffray et al., 2009), originate from the bone marrow (Fogg et al., 2006), and do not proliferate (Woollard & Geissmann 2010). However, during inflammation, they can migrate from the blood to the subendothelial space in response to tissue-derived signals (Ley et al., 2007), among which is the very potent monocyte chemoattractant protein-1 (MCP-1). Once infiltrated, they can phagocytose other cells and toxic molecules and produce inflammatory cytokines (Geissmann et al., 2008). In addition, they can differentiate into inflammatory dendritic cells or macrophages, and further into foam cells, as determined by the inflammatory milieu (Tacke & Randolph, 2006).

Heterogeneity within the monocyte population has led to the study and identification of monocyte subtypes in both mice and humans, varying in chemokine receptor and adhesion molecule expression. The most proinflammatory subtype, recognized by high levels of a marker known as Ly6C in mice (Swirski et al., 2007), homes to the atherosclerotic plaque, where it expresses high levels of proinflammatory cytokines and other macrophage mediators, including matrix metalloproteinases (MMPs) (Libby et al., 2009). Although a directly corresponding human subtype has not yet been identified, the $CD14^{hi}CD16^{lo}$ monocytes which express CCR2, CD62L and CD64A have been associated with the inflammatory subset (Weber et al., 2000, Woollard & Geissmann, 2010). A study by Swirski et al. (2006) found that monocytes were continually recruited to plaque, and that this accumulation increased proportionally with lesion size. This study directly correlated monocyte recruitment with progression of atherosclerosis. A second very interesting study found that inhibition of the potent MCP-1/CCR2 axis via a gene-eluting anti-MCP-1 stenting strategy in rabbits and monkeys

(Egashira et al., 2007) significantly decreased neointimal formation. Conversely, another study found that emigration of monocyte-derived DC-like cells was found in regressing lesions, and that increased differentiation into these cells decreased lesion size (Llodra et al., 2004). Taken together, these studies together suggest that plaque progression may be the result of both increased monocyte recruitment and decreased monocyte emigration from lesions. Thus, the importance of monocytes in atherogenesis has been well documented. However, while the initial infiltration step is important, it is the subsequent differentiation to macrophages which is recognized as crucial for plaque progression.

Monocyte to macrophage differentiation is a process dependent on a combination of both the distinct subtype and the inflammatory milieu. Interactions with the extracellular matrix as well as with the cytokines Macrophage colony stimulating factor (MCSF) and members of the Tumour Necrosis Factor (TNF) family are known to drive the process (Choudhury et al., 2005). This differentiation is characterized by significant changes in the gene expression program, including increases in scavenger receptors. They, along with smooth muscle cells, are able to take up oxidized LDL which accumulates as cholesteryl esters in characteristic foamy deposits (Li & Glass, 2002). These foam cells then become a major contributor to pathology via ROS production and increased expression of cytokines, including TNFs, Interleukins (ILs) & Interferons (IFNs), of enzymes, including serine/cysteine proteinases and MMPs, and of growth factors, including Platelet-derived growth factor (PDGF), Vascular endothelial growth factor (VEGF), Epidermal growth factor (EGF) and Insulin-like growth factor (IGF) (Saha et al., 2009). Through this degenerative cycle, the macrophage has been implicated in every stage of atherogenesis, from the early inflammatory events to the late-stage

necrotic and ruptured plaque, which often contains high numbers of prothrombotic MMP- and Tissue Factor-secreting macrophages (Davies et al., 1993).

3. Arterial smooth muscle cells

3.1. Morphology and function

The smooth muscle cell is a specialized non-striated contractile cell that lines the walls of many of the body's organs, including blood vessels. It is a truly specialized cell, expressing a unique repertoire of contractile proteins, ion channels and signaling molecules, which come together to coordinate cellular functions (Owens et al., 2004). Its principal function lies in resistance vessel contraction for regulation of blood pressure and blood flow distribution (Hilgers & Webb, 2005). Even terminally differentiated smooth muscle cells retain remarkable phenotypic plasticity, a fact that has confounded the process of deciphering the molecular events in their differentiation. Indeed, changes in local environmental cues can greatly influence cellular phenotype, inducing profound and reversible changes in gene expression and, therefore, in phenotype. This bidirectional switch in phenotype was suggested in 1967 by Wissler (Wissler, 1967), and was based on the assumption that SMCs are integral to atherosclerosis. While two extreme phenotypes are commonly described, a range of more subtly altered, intermediate phenotypes are also seen. The first extreme phenotypic state is termed the "contractile" phenotype, most commonly seen in adult vessels, which defines a cell of small size, with a spindle-like, elongated morphology. The SMCs in this state have elevated levels of contractile proteins, and maintain a very low proliferation rate as well as a low synthetic activity (Rudd & Weissberg, 2002; Rzucidlo et al., 2007). The second

state is referred to as the "synthetic" phenotype. A synthetic SMC is characterized by increased cell size and extracellular matrix production as well as decreased contractile protein expression. For this reason, a cell in this state is often termed as being "undifferentiated". A synthetic SMC is also hyperproliferative, has increased migration and is exquisitely responsive to circulating vascular growth factors (Owens, 1996). While this phenotypic plasticity is crucial for development of the vascular system and vessel remodeling after injury, it predisposes the cell to adverse phenotypic switching due to pathogenic environmental cues (Owens, 1995).

Importantly, however, it is now appreciated that proliferation and differentiation are not mutually exclusive. Thus, during late embryogenesis, SMCs have a very high rate of proliferation (Cook et al., 1994) and also have a rapid induction of SMC differentiation genes (Owens & Thompson, 1986). Conversely, in late-stage atherosclerosis, SMCs have very low proliferation rates similar to that of fully differentiated SMCs (O'Brien et al., 1993), while being highly phenotypically modulated (Wilcox, 1992). Clearly then, modulation of phenotype remains a very complex phenomenon, and the two states described above are far from capturing a complete picture.

3.2. Smooth muscle cell markers

Lineage-specific markers, that is, selective genes that contribute to the differentiated functions of smooth muscle cells to the exclusion of all others, have proven extremely challenging to discover for SMCs. This is partly because of the diverse functions of the SMC, but also because most SMC markers, with the possible exception

of smooth muscle myosin heavy chain (SM-MHC), although specific in adults, are expressed by other cell types at least transiently during development, tissue repair, or disease (Hungerford & Little, 1999). Thus, many cells have been misidentified as SMCs, or, alternatively, SMCs have not been identified due to temporary loss of expression of markers as part of the disease process (Owens et al., 2004). However, some genes have emerged as reliable differentiation markers.

The most commonly used markers are the smooth muscle isoforms of the contractile apparatus, including SM-α-actin (SMαA) and SM-MHC. SMαA makes up 40% of total cell protein and 70% of actin present in VSMCs (Hungerford & Little, 1999). However, it is transiently expressed in both cardiac and skeletal muscle during development (Woodcock-Mitchell et al., 1988), and during disease or injury, can be expressed in other cell types (Adachi et al., 1998; Cintorino et al., 1989; Cintorino et al., 1991; Sartore et al., 2001).

Other popular SMC markers follow similar patterns, either being expressed during development of other muscle types, in fibroblasts/myofibroblasts or in disease. Such is the case with SM22α, a calponin-like protein of unknown function (Li et al., 1996), with H_1 calponin, a calcium regulatory protein (Sakamoto et al., 2002; Samaha et al., 1996) and with metavinculin, an intracellular protein found at sites of myofilament insertion into cell membranes (Belkin et al., 1988). Smoothelin appears to be selectively expressed in differentiated SMCs thus far, though expression in many relevant cells types has not been examined (Owens et al., 2004), and it has been shown to be expressed in prostate stromal cells in response to basic Fibroblast growth factor (bFGF) and Transforming growth factor-β2 (TGF-β2) (Tang et al., 2002). SM-MHC appears to be

the most reliable differentiation marker to date. A study by Miano et al. (1994) employed *in situ* hybridization analysis of SM-MHC throughout development and maturation of whole mouse embryos and found that its expression restricted to SMCs. There have been some reports of SM-MHC expression in myofibroblasts, ECs or tumor cells (Borrione et al., 1990; Lazard et al., 1993), though these results are challenged by the finding that the antibody used cross reacts with a non-muscle isoform of MHC (Owens et al., 2004).

A novel concept that has been explored is the identification of SMC markers that vary during the response to injury for specific identification of phenotypically modulated SMCs. Kuro-o et al. (1991) showed that a relatively specific marker of modulated SMCs is the non-muscle MHC isoform, though it does appear to be expressed in neuronal cell lines (Itoh & Adelstein, 1995). This could prove to be a useful tool for the study of phenotypically modulated SMCs within the context of atherosclerosis and restenosis.

Little is currently known as to the order of appearance of smooth muscle differentiation markers, and their individual roles in SMC differentiation. Thus, the developmental program of the SMC lineage remains unclear. However, various mutagenesis studies have revealed that expression of almost all putative marker genes is dependent upon the presence of a specific element within either their promoter or intronic sequence, specifically a $CC(AT)_6GG$ motif, abbreviated CArG. Such is the case of SMαA (Mack & Owens, 1999), SM22α (Kim et al., 1997), desmin (Mericskay et al., 2000) and SM-MHC, though interestingly, mutations in each of the three conserved CArG elements of SM-MHC resulted in different phenotypic end points (Manabe & Owens, 2001).

CArG elements bind serum response factor (SRF), a transcription factor that undergoes dimerization and binds DNA through a MADS box domain (Shore & Sharrocks, 1994). Paradoxically, SRF is a ubiquitously expressed transcription factor which can regulate a variety of genes in addition to the SMC-specific genes, including the immediate early gene *c-fos* (Shore & Sharrocks, 1994) and various skeletal- and cardiac-muscle specific genes (Sartorelli et al., 1993). A number of solutions to this have been proposed, and the answer may lie in a combination of any number of these (Reviewed in (Owens et al., 2004; Owens, 2007)).

3.3. Smooth muscle culture models

The study of vascular smooth muscle has so far been restricted by the limited number of culture models available. Importantly, aside from some genetically modified null or deficient mice such as the ApoE (+/-) (van Ree et al., 1994) or the LDL receptor (-/-) mice (Ishibashi et al., 1993), the popular laboratory rodent models such as mice and rats are considered to be especially resistant to atherosclerotic lesions, in particular to those with proliferative vascular involvement (Andrus et al., 1956). In addition, the major cause of acute angina, namely plaque rupture and thrombosis, is rare in these models (Calara et al., 2001). Therefore, the relevance of studies done on these SMCs is confounded by the fact that, at least within the context of vascular disease development, major differences exist between the physiological responses of rodent SMCs as compared to that of human SMCs. While the reasons underlying this are not completely understood, studies have shown that they cannot be attributed exclusively to differences in species metabolism. Differences are observed at the molecular level, as demonstrated

in the studies by Yau et. al (1996, 2001) who showed completely opposing responses between rodent and porcine SMCs to Bradykinin. Other differences lie within the Renin-Angiotensin System (RAS), as various Angiotensin Converting Enzyme (ACE) inhibitor trials which were promising in the rat model (Sun & Mendelsohn, 1991) did not translate to any benefit in humans (Desmet et al., 1994). Despite this, a majority of SMC studies have examined rodent cell culture models, which have nevertheless given much insight into SMC morphology and function. The embryonic, rat-derived A7r5 and A10 cell lines (Kimes & Brandt, 1976) and the rat pulmonary artery SMC line PAC1 (Rothman et al., 1992) are examples of such culture models.

Considering this, attempts have been made to establish alternative culture models. In one interesting study, Li et al. (1999) established an anchorage-dependent, human SMC clonal line, isolated from a segment of internal thoracic artery. They studied phenotypic switching between a proliferating, migratory state to a contractile one. This line was viable beyond forty passages, while retaining its SMC characteristics.

Apart from classic cell lines or established clonal lines, primary cells remain as a powerful, physiologically relevant tool for culture studies. A number of relevant cells are commercially available, but with limited passages, studies can become extremely expensive. Thus, a few common laboratory methods have been developed to establish primary SMC cultures directly from vessels (Campbell & Campbell, 1993). One such method involves an enzymatic dispersion of vessels. However, this generalized digestion does not allow cell type separation and can be harmful to cell membranes and receptors. Alternatively, vessel wall microdissection allows cell-type specific isolation but is laborious and has been shown to yield significantly less fully differentiated SMCs (Opitz

et al., 2004). In 1997, Saward & Zahradka established a third method of preparing primary cultures using porcine coronary artery explants. They employed the distinct temporal migratory patterns of vessel cell populations to collect a ≥95% homogenous SMC population (Saward & Zahradka, 1997). This methodology presents an advantage over the existing methods by creating nearly homogenous, fully differentiated preparations, while preserving cell integrity and avoiding direct microdissection. In addition, it is a powerful technique as it allows selection of the relevant, migrating subpopulation of SMCs within the vessel wall.

4. Vascular growth factors

4.1. Their role in atherogenesis

Vascular growth factors (GFs) are important environmental factors which play a major role in atherogenesis, in particular via their actions on SMC phenotypic modulation. Some have been shown to promote the differentiated and anti-atherogenic contractile phenotype, while others tip the equilibrium towards an undifferentiated, proliferative and migratory phenotype. Others still have indirect effects through surrounding cells, including ECs. While some are cytokines that act systemically on athero-prone sites while circulating through the body, it is thought that many act in an autocrine and paracrine manner. These GFs become an integral part of the lesion microenvironement by exert local effects on both the cells which synthesize them and those in the immediate surrounding. GFs are numerous and have proven to be effective cell communication tools, each one acting through specific and complex pathways. In addition, implication of one factor in a certain stage of the disease does not necessarily

implicate it in all stages. Thus, our current understanding of their actions within lesions is incomplete with respect to phenotypic modulation as well as with respect to interactions with inflammatory cell mediators, lipids, lipid peroxidation products and ROS. However, certain GFs have been identified as potentially important and, through extensive studies, have provided some clues to their role in lesion development

4.2. Growth factor overview

4.2.1. Platelet Derived Growth Factor

PDGF is a potent chemoattractant and mitogen for cells of mesenchymal origin, such as fibroblasts and SMCs. It is produced by activated platelets and released during blood clotting or when platelets adhere at sites of vessel injury to initiate a vessel repair response (Deuel & Huang, 1984). Interestingly, it has also been shown to be secreted by lesion macrophages, and by a number of both normal and transformed cell types (Martinet et al., 1986; Ross, et al., 1986; Uutela et al., 2001). PDGF consists of either disulfide linked homo- or hetero-dimers of two chains, A and B, or of homo-dimers exclusively of two other chains, C and D (Reigstad et al., 2005). It acts through two tyrosine kinase receptors, PDGFR-α and PDGFR-β which initiate similar but non-identical cellular responses (Heldin et al., 1998). These dimerize following activation and hybrid receptor formation is also thought to occur. PDGF has been shown to induce rapid downregulation of SM-selective markers in cultured SMCs (Blank & Owens, 1990; Corjay et al., 1989), and, importantly, several post-injury models have detected PDGF chains as well as their receptors in human coronary arteries (Tanizawa et al., 1996; Ueda et al., 1996). Sano et al. (2001) suggested a critical role for PDGF in atherogenesis when

they used antibodies against PDGFR-α and –β in ApoE(-/-) mice fed a Western diet. Their results showed a 67% reduction in lesion size and 80% reduction in SMC neointimal investment with PDGFR-β blockage, as compared to control ApoE(-/-) mice. Another study by Kozaki et al. (2002) looked at these same ApoE(-/-) mice long-term, after a lethal irradiation and reconstitution of blood cells using PDGF-deficient embryonic liver cells. They found an interesting temporal importance for the PDGF axis in lesion development, with lesion differences at 35 weeks, which were indistinguishable at 45 weeks.

4.2.2. Vascular Endothelial Growth Factor

The VEGF family of growth factors is a subfamily of the PDGF family. The major member, VEGF-A is a 165 amino acid (aa) peptide, which signals through the tyrosine kinase receptors VEGF receptor (VEGFR)-1/2. It is known as an important physiological mediator of angiogenesis, with a particularly potent effect on endothelial cells, but also has an effect on inflammatory cells (Holm et al., 2009). The role of VEGF also appears to be two-fold. It plays a role in plaque development, stimulating the formation of immature neovessels (Dunmore et al., 2007), which not only provide nutrients to the plaque but also contribute, either via hemorrhage or via distribution of inflammatory cells, to plaque instability (Virmani et al., 2005). However, it is also important for reendothelialization after injury, as well as maintenance of endothelial integrity, as VEGF gene-eluting stents have been shown to decrease NI formation (Asahara et al., 1996; Walter et al., 2004), thus resulting in long-term decrease of SMC proliferation and inflammatory activity.

4.2.3. Transforming Growth Factor-β

Transforming Growth Factor-β (TGF-β) is a 390 aa peptide that signals through any one of two tyrosine kinase receptor subtypes I and II. It has been shown to promote SMC differentiation via upregulation of SMC markers (Hautmann et al., 1997). In addition, its levels are rapidly increased after balloon injury in animal models (Majesky et al., 1991). Though its role in lesion formation remains unclear, there is evidence suggesting a protective role for TGF-β. Patients with unstable angina will often present with severely low levels of plasma TGF-β (Grainger et al., 1995), and studies in ApoE(-/-) mice have presented convincing evidence that TGF-β may be critical for SMC matrix production and the development of a stable plaque (Lutgens et al., 2002; Mallat et al., 2001; Owens et al., 2004).

4.2.4. Epidermal Growth Factor

The Epidermal Growth Factor (EGF) is a 53 aa growth factor, one of six family members that originate as a transmembrane glycoprotein precursor with an extracellular growth factor sequence. Cleavage by MMPs releases the active growth factor, which acts on many cell types through the EGF receptor (EGFR), a member of the tyrosine kinase receptor family (Dreux et al., 2006). EGF, and other family members such as heparin binding-EGF (HB-EGF) and TGF-α, are expressed by cells involved in atherogenesis, including ECs (Gospodarowicz et al., 1978), SMCs (Bhargava et al., 1979), and T lymphocytes (Blotnick et al., 1994). In addition, they have been shown to contribute to phenotypic switching (Yamanaka et al., 2001), and while EGF is thought to be important

for monocyte chemotaxis and macrophage proliferation (Lamb et al., 2004), HP-EGF is thought to be an important chemoattractant and mitogen (Higashiyama et al., 1991). Furthermore, in differentiated macrophages and SMCs, it is upregulated in response to oxLDL (Ouchi et al., 1997) and upregulates other growth factors (Peifley et al., 1996). The EGFR and other family members also appear to be central in the growth responses of other important vascular factors including angiotensin II (AngII) (Eguchi et al., 1998) and endothelin-1 (ET-1) (Iwasaki et al., 1998) via guanine nucleotide-binding protein (G protein) EGFR transactivation (Kalmes et al., 2001). Thus, EGF appears to contribute to atherogenesis via many different mechanisms.

4.2.5. Basic Fibroblast Growth Factor / Fibroblast Growth Factor-2

Basic Fibroblast Growth Factor (bFGF), also known as FGF-2, is a heparin-binding factor, part of the FGF family, which binds the tyrosine kinase FGF receptors. It is thought to act on SMCs and ECs to stimulate angiogenesis (Przybylski, 2009), with a synergistic effect with VEGF (Asahara et al., 1995). As with VEGF, the role of FGF-2 in atherogenesis remains elusive. While it may have a pro-atherogenic role as it has been shown to promote SMC proliferation (Lindner et al., 1991), an experimental model has suggested a protective effect by reversing endothelial dysfunction (Meurice et al., 1996). A more recent study of diet-induced atherosclerosis further supported a protective role for FGF-2 in the early stages of plaque development, showing improved endothelium dependent relaxation and decreased plaque macrophage content with bFGF administration (Six et al., 2004).

5. The IGF-1 axis

5.1. Physiological overview

5.1.1. IGF-1

Insulin-like growth factor-1 is a single chain, 70 aa peptide cytokine that is homologous to insulin in sequence, translating to three-dimensional structure homology (Rinderknecht & Humbel, 1978). Along with IGF-2, it is part of a growth factor family termed the IGFs that has effects on most cell types (Laviola et al., 2007). The *IGF-1* gene has been mapped to chromosome 12 in humans (Tricoli et al., 1984) and gives rise to two different mRNAs, suggesting a role for mRNA processing in IGF-1 expression (Rotwein, 1986). At the cellular level, it is important for progression through the G_1/S phase of the cell cycle, and it is known to be a potent inhibitor of apoptosis. It is not surprising, therefore, that disruptions within the IGF-1 axis are observed in many cancers (Reviewed in (Werner & Bruchim, 2009)).

IGF-1 has both endocrine and paracrine/autocrine effects in the body. Its endocrine effects were discovered first, and are therefore the most extensively studied. Endocrine IGF-1 has primarily growth promoting and differentiation functions, and plays a fundamental role in both pre- and post-natal development (Delafontaine et al., 2004). Indeed, IGF-1(-/-) mice exhibited a severe growth deficiency of 60% at birth, which was also often lethal shortly after birth, depending on the genetic background of the mice (Liu et al., 1993). Circulating IGF-1 is synthesized by the liver, under the control of growth hormone (GH) originating from the anterior pituitary. The "somatomedin hypothesis", suggested by Daughaday and collegues (Daughaday & Rotwein, 1989; Daughaday et al., 1972), refers to a liver origin of IGF-1 in response to pulsatile growth hormone (GH)

secretion (Jansson et al., 1985), resulting in longitudinal bone growth in an endocrine manner. This original hypothesis has been challenged by many studies, including an important study by Lupu et al. (2001), which established an IGF-1-independent effect of GH on post-natal body growth. However, this classical endocrine loop does have some well established and critically important effects, among which are some which are non-overlapping with locally produced IGF-1. One very important role of endocrine IGF-1 is negative feedback on the pituitary. Major phenotypes of loss of this negative feedback result primarily from increased GH levels and include insulin resistance and increased liver size. In addition, humoral IGF-1 has a number of direct effects on a variety of tissues, including insulin sensitizing effects in muscle, liver and fat tissues. It is required for specific brain functions such as mediating the effect of exercise on anxiety and spatial learning, and for clearance of brain β-amyloid. It plays a role in mediating blood pressure and endothelial dysfunction, and is also associated with stimulation of kidney size (Reviewed in (Ohlsson et al., 2009)).

Although it was shown that many tissues synthesize IGF-1 mRNA (Daughaday & Rotwein, 1989), direct evidence for the importance of paracrine and autocrine actions of IGF-1 was first demonstrated by Yakar et al. (1999). They used a Cre/loxP approach to selectively delete liver IGF-1. This resulted in a 75% decrease in serum IGF-1, but growth as measured by body weight, length and femoral length did not differ significantly. In addition, many cell types have been shown to rely on autocrine IGF-1 for appropriate differentiation (Laviola et al., 2007, Tollefsen et al., 1989), another unique aspect of IGF-1 signaling. Indeed, unlike other growth factors, IGF-1 stimulates

both proliferation and differentiation of cells in culture, albeit with a temporal separation of these effects (Ewton et al., 1994).

In the vascular system, IGF-1 has a strong influence on smooth muscle cells. It is primarily known as a survival factor (Bai et al., 1999), but is also a chemoattractant and a mitogen (Bornfeldt et al., 1994; Hsieh et al., 2003). In addition, it is involved in the regulation of many crucial processes in VSMCs including contractility, protein synthesis, differentiation and glucose uptake (Boulware et al., 1992; Sowers, 1997). In particular, its role as a survival factor was first extensively studied in the cancer field for its oncogenic potential. However, this role takes on a different outlook in vascular disease where the balance between cell survival and cell death contributes to atherosclerotic plaque stability (Jia et al., 2007). Indeed, it has been shown that in advanced atherosclerotic plaques, IGF-1 and IGF-1R expression is low or absent in intimal regions with macrophage infiltration, thereby contributing to increased apoptosis (Okura et al., 2001).

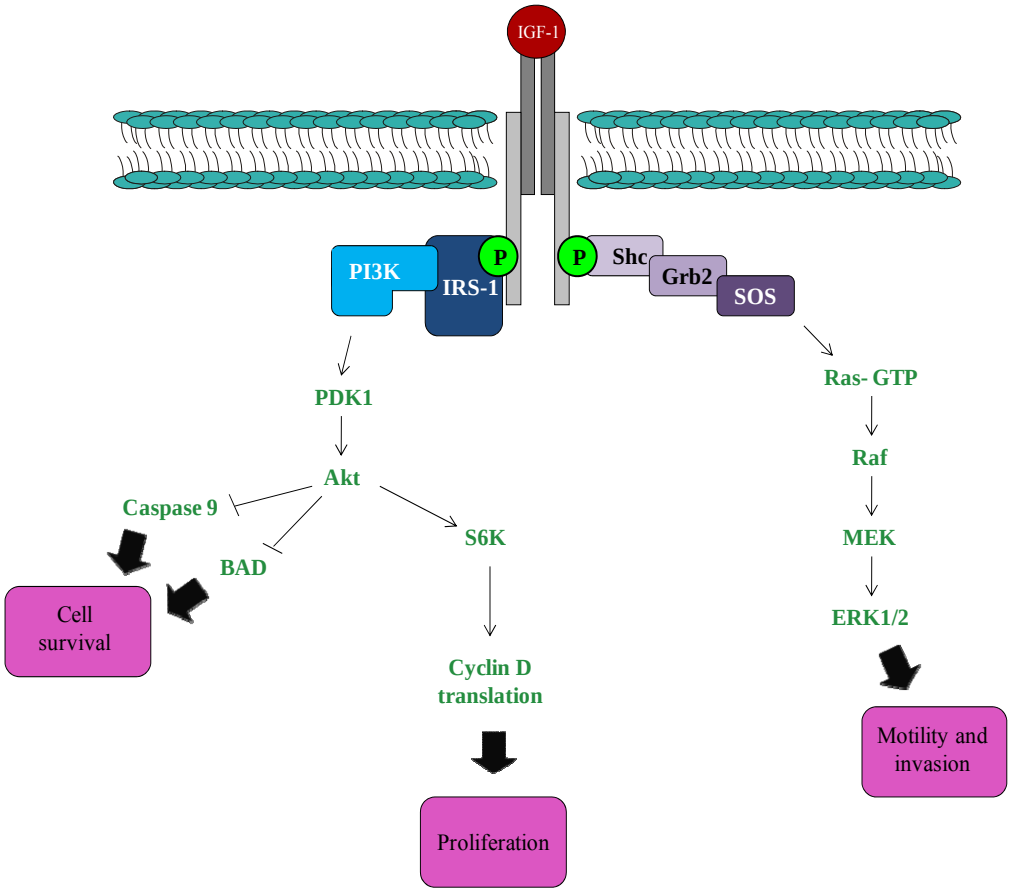
5.1.2. IGF-1 Receptor and downstream signaling pathways

Being peptide hormones, the actions of the IGFs are mediated by specific membrane receptors. The IGF receptor type 1 (IGF-1R) is homologous to the insulin receptor (IR), consisting of a heterodimer of α and β subunits, which further dimerizes and creates an $\alpha_2\beta_2$ tetramer-receptor, even in the non-activated state (LeRoith et al., 1995). It is synthesized as a pre-proreceptor containing a 30-residue signal peptide that is cleaved during translation. Before going through Golgi transport to the membrane, it is glycosylated, folded and dimerized. Within the Golgi, it undergoes furin cleavage to

yield the final α and β subunits, which form disulfide-linked dimers (Riedemann & Macaulay, 2006). The α subunits are extracellular and contain the IGF-1 binding site, while the β subunit contains the transmembrane segment as well as the cytoplasmic C-terminal. In accordance with its homology to the insulin receptor, this cytoplasmic domain also contains its intrinsic tyrosine kinase activity and a corresponding ATP binding site. The IGF-1R binds IGF-1 primarily, but also IGF-2 and insulin with 10- and 100-fold lower affinity, respectively. Similarly, while the IGFs are able to bind the insulin receptor, it is with much lower affinity. Thus, the IGF-1R is thought to be the major receptor by which IGF-1 signaling is activated.

A total of seven tyrosine residues are phosphorylated in order to initiate the cellular cascades leading to IGF-1-dependent cellular events (Krauss, 2008). Three tyrosines are first autophosphorylated within the catalytic loop domain. This results in additional phosphorylation of two tyrosines in the vicinity of the transmembrane element and two more in the region of the C-terminus, sites which then serve as binding sites for various docking proteins. Two major signaling cascades are initiated from these docking proteins. In one case, Insulin receptor substrate-1 (IRS-1) to -4 bind to the phosphorylated tyrosines, activating phosphatidylinositol-3-kinase (PI3K)-Akt signaling. This leads to activation of the cell cycle due to translation of cyclin D which promotes the transition from the G_1 to the S phase. Additionally, apoptosis is prevented via inhibition of BAD and caspase 9. Alternatively, the Src homology and collagen domain protein (Shc) may bind, leading to activation of the Ras-Raf-MAPK pathway (Figure 1) (Reviewed in (Chitnis et al., 2008)). In either case, however, the pathways are not mutually exclusive and may overlap and regulate each other at various levels.

Figure 1: *RTK-initiated IGF-1R signaling*. Current view of major cellular signaling events initiated upon IGF-1 binding to the IGF-1R. Trans-auto-phosphrylation of the receptor homodimer results in docking sites for effector protein binding. Initated cascades affect major cellular events including cell survival, proliferation and motility. Arrows represent activation and blunted ends represent inhibition. (Image adapted from Chitnis et al. (2008)).



In addition to the classical pathways downstream of the IGF-1R, it has been shown that disruption of the IGF-1R axis blocks mitogenic responses of VSMCs initiated by a number of other growth factors including EGF (Coppola et al., 1994), thrombin (Delafontaine et al., 1996) and PDGF (Clemmons, 1985). In accordance with this, AngII (Delafontaine & Lou 1993), bFGF and PDGF have been shown to upregulate IGF-1R gene transcription in SMCs (Ververis et al., 1993). Thus, it seems IGF-1/IGF-1R expression could hold a central role in the proliferative capacity of these cells.

One additional and more recently discovered effect of the IGF-1R is its role in the regulation of lifespan (Guarente & Kenyon, 2000). Interestingly, despite its role as a cellular survival factor, its activation decreases organism longevity. Indeed, IGF-1R(+/-) mice were shown to live 25% longer than wild-type mice, with no change in metabolism or fertility (Holzenberger et al., 2003). This increased longevity is thought to function through p66 Shc, one of the IGF-1R's phosphorylation targets that mediates the cellular response to oxidative stress via increases of intracellular ROS and repression of the forkhead factor FOXO3. This transcription factor, in the absence of p66 Shc, increases cellular resistance to oxidative stress (Nemoto & Finkel, 2002). To date, the p66 Shc knockout mouse is the only mutant mouse comparable to the phenotype of IGF-1R mutation, which leads to lifespan increases without any major side effects (Migliaccio et al., 1999). Interestingly, this link to oxidative stress may involve the IGF-1R in atherogenesis via a mechanism other than its growth effects. Indeed, this hypothesis has been supported by a study in p66 Shc(-/-) mice fed a high fat diet which showed a decrease in oxidative stress, in vascular cell apoptosis and in early lesion formation (Napoli et al., 2003).

Characterization of null mutants for the IGF-1R gene was done by the same group who studied the IGF-1 knockout mice. The mutants were shown to die at birth of respiratory failure and also to exhibit severe growth deficiency, demonstrated by generalized organ hypoplasia as well as developmental delays in ossification, central nervous system, and the epidermis (Liu et al., 1993)

The IGF-1R has been shown to be important in the vascular system and in vascular disease. It is present on the surface of the different cell types involved in atherosclerosis including SMCs (Pfeifle & Ditschuneit, 1983), inflammatory cells (Hochberg et al., 1992) and ECs (Bar & Boes, 1984) and, importantly, the IGF-1R has been shown to be markedly increased in human coronary atherectomy segments, along with IGF-1 and IGFBPs 1-5 (Grant et al., 1996).

5.1.3. IGF Binding Proteins

The majority of extracellular IGF-1 is bound to specific, high affinity binding proteins, as it is a hydrophilic peptide hormone. Their affinities for IGF-1 are either equal to or greater than that of the IGF-1R, such that their binding capacity establishes an additional level of regulation (Duan & Xu, 2005). Thus, they regulate IGF turnover, transport and tissue distribution. Six have thus far been isolated and characterized, IGFBP-1 to -6, in addition to a low affinity IGFBP-7, which shares only 20 to 25% identity with IGFBP1-6, but contains the highly conserved N-terminal domain (Oh et al., 1996). This N-domain contains not only the major IGF binding site, but also the C-domain variable linker, which contributes to both ligand binding and interactions with other proteins (Clemmons, 2001). One major interacting protein is the acid-labile subunit

(ALS). This subunit is often bound in a ternary complex with IGF-1 and IGFBP-3, a complex which binds up to 80-85% of serum IGF-1. The ALS has the role of retaining the complex in the vascular system, and extending their half-life (Boisclair et al., 1996). Consistent with its role as the major endocrine carrier of the IGF-1 in adults, IGFBP-3 is thought to compete with the IGF-1R for the ligands, thus acting as a growth inhibitor for extravascular tissues.

In addition to their role as carrier proteins, most IGFBPs have been shown to modulate IGF actions either by inhibiting or potentiating their effects. IGFBP-4 and -6 have consistently been found to inhibit their actions while IGF-1, -2, -3 and -5 can paradoxically do either, depending on the experimental model and method used (Duan & Xu, 2005). Interestingly, accumulating evidence also suggests ligand-independent effects of the IGFBPs. IGFBP-1 was found to bind the $\alpha_5\beta_1$ integrin, thus stimulating cell migration (Jones et al., 1993). IGFPB-3, alternatively, has been shown to inhibit cell growth in the absence of IGF-1 and in IGF-1R null cells (Butt & Williams, 2001). Studies have also shown that IGFBP-5, when added to cell cultures lacking endogenous IGF-1, can increase osteoclast formation and bone resorption activity (Kanatani et al., 2000).

5.2. Overlap with the insulin and IGF-2 axes

As is often the case *in vivo*, interactions within the IGF-1 axis are not isolated. Primary among external interactions is the insulin system. As the IGF-1 and insulin systems share both sequence and structural homology, cross-activation of one by the other, and vice-versa, is often observed. In fact, in tissues where insulin receptor

concentrations are low, such as SMCs, it has been suggested that the biological effects of insulin may be mediated through cross reaction with the IGF-1R (Bornfeldt et al., 1991; Sowers, 1997). This would impart a growth and migration-promoting component to insulin action and, conversely, in a metabolic component to IGF-1 signaling. In addition to receptor cross-activation, direct receptor contact may also be important. In 1989, two groups pursued immunological studies using the IGF-1R. They discovered the existence of a subspecies of receptors present at the cellular membrane composed of one subunit from the insulin receptor and one from the IGF-1 receptor (Moxham et al., 1989; Soos & Siddle, 1989), an interaction made possible by the high degree of homology between these individual receptors. These were termed "hybrid receptors", and have since been shown to occur widely in different mammalian tissues (Bailyes et al., 1997), forming according to the ratio of proreceptors present in cells. Importantly, functional studies revealed that these receptors behave like IGF-1R with respect to binding affinity (Seely et al., 1995). Accordingly, this results in altered insulin responsiveness of various tissues. However, little is known about the functional relevance of these hybrid receptors *in vivo* and their role in mediating insulin sensitivity of insulin-sensitive tissues. In regards to the vasculature, Engberding et al. (2009) studied vascular insulin resistance via vascular SMCs, a nonclassical insulin target tissue. They found increased IGF-1R with decreased circulating IGF-1, a situation often found in Type II diabetes mellitus (Tan & Baxter, 1986), which resulted in only a very mild insulin response via the insulin receptor. By downregulating these IGF-1Rs, they markedly increased insulin responsiveness and cellular glucose uptake. Their results also suggested the importance of a negative feedback loop for modulation of the holo/hybrid receptor ratio. Thus, at least in vascular

SMCs, the IGF-1 axis greatly influences the insulin axis, both by direct binding and by formation of hybrid receptors.

The IGF-2 system also interacts with the IGF-1 system, but in a very different way. The IGF-2R, shown to be identical to the mannose-6-phosphate receptor (MacDonald et al., 1988), is a transmembrane spanning receptor which lacks a kinase domain and possesses a high turnover rate. Interestingly, it is still unclear whether this receptor has any direct downstream cellular targets, despite binding both IGF-1 and IGF-2. Thus, it is considered to be a potential growth factor regulator, acting as a sink to the available pool of circulating IGFs (Lindsay & Evans, 2008). However important the IGF-2R may be in adults, circulating IGF-2 is thought to be most important in pre-natal development. After birth, its role has been shown to be gradually replaced by IGF-1 (Yu & Rohan, 2000).

6. G protein coupled receptors

6.1. G proteins and G protein-coupled receptors

Heterotrimeric guanine nucleotide-binding proteins (hG proteins) are assembled into a signaling complex composed of three small α, β and γ subunits. The inactive, GDP-bound α-subunit is often associated with transmembrane receptors, while the β- and γ-subunits are in contact only with the α-subunit via an α-β association. The βγ dimer is very tightly complexed, and only dissociates under denaturing conditions (Hamm, 1998). Exchange of GDP for GTP within the α-subunit by guanine nucleotide exchange factors (GEFs) leads to GDP dissociation and GTP association, as well as to the release of the $G_{\beta\gamma}$-subunits, with both components initiating unique intracellular signaling events. In such signaling networks which include receptor-bound hG proteins, the receptor will

often act as the activating GEF following ligand binding (Krauss, 2008). Interestingly, one study used millisecond time resolution measurements and established that GPCR activation occurs at least five times faster than G protein activation, suggesting a relatively inefficient GEF activity (Bunemann et al., 2003).

Twenty gene products encode the human G_α-subunits, which comprise a family of 39-52 kD proteins that share 45-80% amino acid similarity. They have been divided on the basis of sequence similarity into four classes: G_s, G_i, $G_{q/11}$ and $G_{12/13}$ (Hamm, 1998). Each subtype undergoes differential post-translational modification including palmitoylation and myristoylation for membrane association (Reviewed in (Wedegaertner et al., 1995)). A number of studies have indicated that the extreme C-terminal domain of the α-subunit is critical for both receptor specificity and contact (Conklin et al., 1993; Gilchrist et al., 1999; Sullivan et al., 1987). The six G_β-subunit peptides are small (~36 kD) and highly conserved proteins, sharing 30-83% homology. The twelve G_γ-subunits are also small proteins (6-9 kD), but are more divergent, sharing only 27-75% homology. They are also post-translationally modified by isoprenylation. This isoprenylation modification assists with effector regulation, and is also thought to be in close proximity to the G_α modification, thereby increasing its affinity for the receptor (Hamm & Gilchrist, 1996).

This stable heterotrimeric association has rendered it a common initial downstream transducer of ligand binding at the cell surface to an intracellular signaling event. It has evolutionarily given rise to a large family of G protein-coupled receptors. Indeed, nearly 80% of all known hormones signal through GPCRs, and these comprise 60% of the body's transmembrane receptors (Krauss, 2008). Since their discovery, they have come

to be known as heptahelical or serpentine receptors, owing to their characteristic structural feature of seven transmembrane helices (Krauss, 2008).

Despite the recognized importance of classical hG protein coupled signaling, the past few years have seen an increase in evidence indicating variations in the classical model for the role and functioning of hG proteins. As a primary example, a few studies have suggested that, in some cases, the heterotrimer does not dissociate for signaling, but instead undergoes a conformational rearrangement (Bunemann et at. 2003; Digby et al., 2006). G protein $\alpha_{q/11}$ may also be directly phosphorylated (Umemori et al., 1997). The discovery and characterization of a family of Activators of G protein signaling (AGS) that can stimulate hG protein activity independent of GPCRs has presented a variable in G protein signaling (Takesono et al., 1999). Evidence for a "reverse mode" for hG protein functions has also been shown, implying interaction of active G proteins with active receptors and receptor-catalyzed GTP release for G protein deactivation (Hommers et al., 2010). Another recent study also suggested a scaffolding role for a specific $G_{\alpha q}$ in the initiation of the ERK5 cascade (Garcia-Hoz et al., 2010). One more concept first suggested in the 1980's that has thus far remained underappreciated is the idea that hG proteins are coupled to, and can be activated by, non-classical and often single transmembrane receptors (reviewed in (Patel, 2004) and (Marty & Ye, 2010)).

6.2. IGF-1R as a G protein-coupled receptor

It was first suggested that there was a G protein component to IGF-1R signaling in 1987 by Nishimoto et al. (1987). They showed that IGF-1-induced calcium influx and increase in DNA synthesis could both be inhibited by pertussis toxin (PTX), a toxin that

catalyzes ADP-ribosylation of the G_{α} subunit of the $G_{\alpha i/o}$ family thereby preventing contact with GPCRs (Katada & Ui, 1982), in mouse fibroblasts. Subsequent studies in a variety of cell types including undifferentiated pre-adipocytes, myocytes and chondrocytes, also suggested a pertussis-sensitive component downstream of the IGF-1R (Poiraudeau et al., 1997; Sarbassov et al., 1997; Uehara et al., 1999). Further, Luttrel et al. (1995) showed that the participation of $G_{\beta\gamma}$ subunits derived from PTX-sensitive G proteins is required for activation of the mitogen-activated protein kinase (MAPK) signaling cascade, downstream of the IGF-1R but upstream of $p21^{ras}$ activation.

At this point, however, it was still unclear whether the IGF-1R directly activated a G protein. Rather, it was thought that the IGF-1R signal converged with a separate G protein coupled pathway through receptor transactivation early on in the cascade. Association of G proteins with the IGF-1R was first shown in 2000 by Hallak et al. (2000) and later by Dalle et al. (2001), who co-immunoprecipitated $G_{\alpha i}$ with the IGF-1R. In addition, both groups showed a decrease in $G_{\beta\gamma}$ subunit precipitation following IGF-1 activation, with either no change or, interestingly, an increase in $G_{\alpha i}$ precipitation, suggesting that this subunit may not mediate any signals downstream of the IGF-1R.

Only one more study to date has further examined the question of IGF-1R-mediated G_{α} signaling. Kuemmerle and Murthy (2001) used human intestinal smooth muscle cells to study two distinct IGF-1-mediated growth signals, namely MAPK-dependent and PI3K-dependent cascades. They showed that only the MAPK-dependent cascade had a G protein coupled component, and further supported the role of $G_{\beta\gamma}$ in this cascade. In contrast to the above studies, however, they elucidated that $G_{\alpha i2}$ was indeed activated by

the IGF-1R and may participate in growth regulation via attenuation of the growth inhibitory effects of cAMP.

An additional interesting observation made in the study by Dalle et al. (2001) was the association of β-arrestin-1 with both the IGF-1R and $G_{\alpha i}$. Although the authors were unsure of the implications of β-arrestin association in IGF-1 signaling, they speculated that it may help to bring the G protein in proximity to the receptor. Conversely, in his review, Patel (2004) hypothesized a pivotal role for β-arrestin-1 in facilitating receptor internalization (Lin et al., 1998), which is necessary for MAPK activation (Chow et al., 1998). Recently, it was shown that activation of the IGF-1R leads to ubiquitination of β-arrestin-1, activation of MAPK and cell cycle progression, even without RTK activity (Girnita et al., 2007). Although these authors did not mention G proteins, their results combined with those of Dalle et al. (2001) suggests the role of G_{α} may be related to cell cycling.

Clearly then, many questions still remain in the elucidation of G protein association with the IGF-1R. These include discovering the downstream targets of G_{α}, determining whether $G_{\alpha i2}$ is indeed specifically activated, elucidating whether the G protein is bound directly to the receptor or within a complex, and demonstrating the actual relevance of this association and subsequent MAPK activation in various relevant cells, such as SMCs.

7. Implications for Type II Diabetes Mellitus

Type II diabetes mellitus has increasingly become a prominent disease both in North America and worldwide. The Canadian Diabetes Association refers to a global epidemic,

with 285 million people worldwide affected by diabetes. In Canada, over 3 million people are affected by diabetes, and it is the contributing factor behind approximately 41 500 deaths a year (Canadian Diabetes Association 2010).

Studies have shown that cardiovascular mortality is greatly increased in patients with diabetes (Fox et al., 2007; Moss et al., 1991) and that in fact hyperglycemia is considered a risk factor for both macro- and micro-vascular disease (Stolar, 2010). One study compared lipid-rich atheromas of diabetic patients with those of non-diabetic patients. They found increased inflammation, neovascularization and reparative collagen (Type III) in the plaques from diabetic patients (Purushothaman et al., 2007). All of these processes involve synthetic, activated SMCs, either via growth factor and ECM synthesis, or via foam cell formation and their link to the inflammatory process. Evidently then, the SMC response to the physiological changes in diabetes contributes to its cardiovascular pathology.

Hyperinsulinemia is one of the major physiological changes which occur even in mild cases of Type 2 diabetes, an observation which was first made many years ago (Yalow & Berson, 1960). Though basal insulin levels may not differ significantly (Bagdade et al., 1967), a global insulin resistance, or inability of cells to respond to insulin, causes circulating insulin levels to remain elevated for a significantly extended period of time following carbohydrate consumption. Under these conditions, circulating insulin is unable to signal through its own receptor, and may instead exert effects through the IGF-1R, thereby involving this growth pathway in an otherwise metabolic disease. Since it has been suggested that the IR and IGF-1R cascades differ at the level of their different G

protein signaling components (Dalle et al., 2001), this involves G proteins in the overall process.

RATIONALE

Atherosclerosis is the major cause of stroke, critical limb ischemia and heart attacks leading to heart failure. In addition, restenosis and vein graft arterialisation are very common complications following cardiovascular interventional surgery. Since SMC accumulation is a key event in the development of all of these pathological states, understanding the body's mechanisms for communicating proliferative signals to these cells further adds to our knowledge of treatments and preventive therapies for these diseases.

The completion of this study will increase our inderstanding of the cellular signaling of IGF-1. No data are currently available on G protein signaling downstream of the IGF-1 receptor in vascular smooth muscle cells, and this study would provide information on the structural aspects of the signaling pathways as well as information on the actual physiological contribution of this pathway to the response of SMCs to IGF-1. In addition, the study will provide preliminary information as to the mechanisms by which IGF-1 signaling may contribute to the accelerated atherosclerosis observed in type II diabetes.

HYPOTHESIS

That the response of vascular smooth muscle cells to IGF-1 is in part regulated via a non-classical G protein coupled pathway, and that this novel pathway is primarily responsible for the proliferative and migratory aspects of the SMC response to IGF-1.

OBJECTIVES

The objectives of this study are to demonstrate in porcine coronary artery SMCs, that

1. Proliferative and migratory responses to IGF-1 signaling are not the sole result of the classical tyrosine kinase pathways
2. The IGF-1R interacts with a heterotrimeric G protein in vascular SMCs
3. A specific class of G_{α} is associated with the IGF-1R
4. The $G_{\beta\gamma}$ and G_{α} subunits have separate roles in the response of SMCs to IGF-1
5. Both the migratory and proliferative responses to IGF-1 are at least partially mediated by the G protein pathway.

MATERIALS AND METHODS

1- MATERIALS

Tissue culture plates from NUNC were delta surface treated (Cat. #163320 (96-well), #150628 (12-well), #172958 (100mm) & #157150 (150mm)). Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) and Antibiotic/Antimycotic (AB/AM) were all from Gibco (Invitrogen #12800-082, #12483-020 & #15240-096, respectively). Fresh porcine hearts were obtained from BJ Packers in Beausejour, Manitoba. 8 μm cell culture inserts for cell migration (Cat. #3097) and accompanying plates (Cat. #3504) were obtained from Falcon BD Biosciences.

Activators, growth factors and inhibitors were from various sources. IGF-1, AG1024 and Gallein were from Calbiochem (#407240, #121767 & #71708 respectively). LY294002 was obtained from Enzo Life Sciences (#ST-420), while PDGF-BB was from PeproTech (#100-14B), and Norepinephrine (NE) and Forskolin were from Sigma (#A7257 & #F6886 respectively).

Antibodies were obtained from various suppliers. Tables 1 and 2 depict the names, uses and sources of these antibodies.

Other molecular biology tools used include Protein G Sepharose Beads and Hoescht 33258, both from Sigma (#P3296 & #861405, respectively). 5-Carboxyfluorescein amidite (FAM)-conjugated Trans-Activating Transcriptional Activator (TAT)-G_{α} C-terminal peptides inhibitors were synthesized at the University of Calgary Peptide Services ($G_{\alpha s}$) and Biomer Technologies ($G_{\alpha i1/2}$). The respective sequences are shown in Table 3. GST-Tagged $G_{\alpha i1/2}$ and $G_{\alpha s}$ were from Abnova (#H00002770-P01/H00002771-P02 & #H00002778-P01, respectively) while the

cytoplasmic domain of the IGF-1R was obtained from Abcam (#ab71657). Oriole Fluorescent Gel Stain was from Bio-Rad (#161-0495) and 10K Centrifugal Filter Devices were from Amicon Ultra (#UFC501008).

Specific kits were also used in this study. The Bromo-deoxyuridine (BrdU) cell proliferation assay was from Chemicon International (#2750). The Cell Counting Kit-8 for cell viability was from Dojindo (CK04). $G_{\alpha s}$- & $G_{\alpha i}$-activation assays were from NewEast Biosciences (#80801 & #80301, respectively). The ProFound Pull-Down GST Protein:Protein Interaction Kit was received from Pierce (#21516).

Finally, many different instruments and computer software programs were also used. The microscope used for general tissue culture purposes was the Olympus CK-2 and the fluorescence microscopes used were the Olympus BH2 RFCA (Hoeshst) and the Olympus IX81 (TAT-$G_{\alpha i}$ inhibitor). The plate reader used for BCA absorbance readings was the $THERMO_{max}$ Microplate Reader from Molecular Devices while the plate reader used for all other absorbance readings was the Omega FLUOstar. The centrifuge used was the Eppendorf Centrifuge 5804R and the ultracentrifuge was the Optima MAX ultracentrifuge. Sonication was done using the Sonic Dismembranor model 100 from Fisher Scientific. Densitometry readings were obtained from the Bio-Rad GS-800 Calibrated Densitometer. Software used for graph drawing and statistical analysis was Origin v7.5 from Originlab Corp. Software used for image optimization was Adobe Photoshop v6.0, while software used for image representation was CorelDRAW 11.

Table 1: *List of primary antibody sources and uses*

1° antibody name	Company	Catalogue #	Dilution	Use in	Use(s)
6 ×His	Abcam	ab1187	1:200	IP buffer	IP
CREB	Cell Signaling	9197	1:1000	3% BSA-TBST	Western blot
Phospho-CREB	Cell Signaling	9191	1:500	3% BSA-TBST	Western blot
IGF-1Rβ subunit	Cell Signaling	3027	1:750 & 1:75	3% BSA-TBST & IP buffer	Western blot & IP
G protein α (pan)	Cell Signaling	3992	1:750 & 1:75	3% BSA-TBST & IP buffer	Western blot & IP
G protein α_i	NewEast Biosciences	21006	1:750	3% BSA-TBST	Western blot
G protein α_i (Active)	NewEast Bioscience	26901	1:500	1× A/L Buffer	IP
G protein α_s	Abcam	ab83735	1:750	5% Milk-PBS	Western blot
G protein α_s (Active)	NewEast Bioscience	26906	1:500	1× A/L Buffer	IP
G protein β	Abcam	ab19473	1:750	3% BSA-TBST	Western blot
p42/44 MAPK	Cell Signaling	9102	1:1000	3% BSA-TBST	Western blot
Phospho-p42/44 MAPK	Cell Signaling	9101	1:1000	3% BSA-TBST	Western blot

β-tubulin	Cell Signaling	2146	1:1000	3% BSA-TBST	Western blot

Table 2: *List of secondary antibody sources and uses*

2° antibody name	Company	Catalogue #	Dilution	Use in	Use(s)
Clean-Blot IP Detection Reagent HRP	Thermoscientific	21230	1:400 & 1:1000	1% BSA-TBST & 1% Milk-PBS	Westerns from IPs
HRP-conjugated anti-rabbit	BioRad	170-6515	1:10000 & 1:50000	1% BSA-TBST & 1% Milk-PBS	Western blot
HRP-conjugated anti-mouse	BioRad	170-6520	1:10000	1% BSA-TBST	Western blot

Table 3: *Amino acid sequences of TAT-conjugated G_α C-terminal inhibitor peptides*

Name	Sequence*
5'-FAM-TAT-$G_{\alpha s}$ C-teminal	N-RRRQRRKKKRDIIQRMHLRQYELL-C
5'-FAM-TAT-$G_{\alpha i1/2}$ C-terminal	N-RRRQRRKKRTDVIIKNNLKDCGLF-C

* N-terminal sequence corresponds to the Trans-Activating Transcriptional Activator domain of Human Immunodeficiency Virus, according to Vivès and Lebleu (2002). C-terminal sequence derived from the G protein inhibiting peptides, according to Gilchrist et al. (1999).

2- METHODS

2.1. Porcine coronary artery primary smooth muscle cell culture

Primary smooth muscle cell culture was established as previously described (Saward & Zahradka, 1997). Briefly, left anterior descending coronary arteries were excised from freshly acquired porcine hearts and incubated in SMC-PBS (consisting of 0.1 M Na_3PO_4 and 0.9% NaCl) + 10× AB/AM, (consisting of 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL of amphotericin B). Vessels were cut into thin segments, which were kept in culture at 37°C in 20% FBS DMEM + 10× AB/AM for 4 days, after which media was changed to 20% FBS DMEM + 1× AB/AM. Media was changed every 2 to 3 days from this point forward. Migration 1 (M1), consisting primarily of fibroblasts, occurred between days 5 and 9. Vessels were then transferred to a clean plate to capture the cells from migration 2 (M2) between days 10 and 14. The cells making up this migration are primarily smooth muscle cells (>95%) and were passaged for expansion, while the vessel segments were discarded. All experiments were done with passages 3, 4 and 5.

After reaching 80 to 90% confluency, cellular quiescence was induced by placing the cells into serum-free media consisting of DMEM supplemented with 1× PTSA (0.1 mM pyruvate, 5 µg/mL transferrin, 1 nM selenium and 200 µM ascorbate) and 1× AB/AM for 5 days

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Following appropriate treatments of quiescent SMCs, cells were harvested in a 2× sample buffer (consisting of 125 mM Tris-HCl pH 6.8, 2% SDS and 20% glycerol).

Protein concentrations were determined using BCA protein assay reagents (Thermoscientific #23223 & #23224, respectively) to ensure equal protein loading.

SDS-containing polyacrylamide gels consisting of both a resolving and a stacking gel of appropriate concentrations were prepared for optimal protein separation. Samples consisting of 5 μg of total protein were mixed with 10% loading dye (consisting of 1:1 β-mercaptoethanol:Bromophenol Blue), boiled for five minutes and loaded onto gels alongside a molecular weight marker (Invitrogen #10728-020). Separated protein was then transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche #03010040001), previously equilibrated by sequential treatment in methanol, dH_2O and transfer buffer (consisting of 25 mM Tris, 192 mM glycine and 20% methanol).

2.3. Immune blotting

PVDF membranes were initially incubated for one hour at room temperature with gentle shaking in blocking solution, either 3% bovine serum albumin (BSA)-TBS-T (consisting of 20 mM Tris-HCl pH 7.3, 0.15 M NaCl and 0.05% Tween-20) or 5% milk-PBS, as indicated in Table 1. Primary antibody incubations were of various times and concentrations in either 3% BSA-TBST or 5% milk-PBS, as indicated in Table 1, with gentle shaking. Overnight incubations were at 4°C while all others were at room temperature. After incubating with primary antibody, membranes were washed three times for 10 minutes using 1× TBS-T or 1× PBS (consisting of 68.4 mM NaCl, 1.34 mM KCl, 5 mM Na_2HPO_4 and 0.882 mM KH_2PO_4). Membranes were then incubated one hour with horse-radish peroxidase (HRP) conjugated secondary antibodies in either 1% BSA-TBS-T, or 1% milk-PBS, and washed three times for 5 minutes with 1× TBS-T or

1× PBS. Visualisation was done using Enhanced Chemiluminescence (ECL) Plus (GE #RPN2132) and X-Ray film (Thermoscientific #34090). All blots were then scanned and bands of interest were analyzed with the GS-800 Calibrated Densitometer. Trace OD × mm was used as the quantification measurement.

2.4. Co-immunoprecipitation

Following appropriate treatment, cells to be immunoprecipitated were lysed with immunoprecipitation (IP) buffer (consisting of 0.5 mM $MgCl_2$, 68.2 mM NaCl, 10 mM Tris-HCl pH 7.4, 5% glycerol and 0.5% Nonidet P-40) with freshly added 1 mM phenylmethanesulphonylfluoride (PMSF) and 0.4 mM sodium vanadate (Na_3VO_4). Protein concentrations were determined using the BCA protein assay reagents.

Protein G Sepharose beads were pre-washed three times using IP wash buffer (consisting of 1% BSA-TBST & 10 mM Tris-HCl pH 7.4) and resuspended at 50% in IP wash buffer. Samples of 250 μg total protein were pre-cleared using a 20% bead slurry for 30 minutes at 4°C with gentle rotation, after which samples were centrifuged at 13000 rpm at 4°C for 5 minutes, and the beads discarded. Primary antibody was then added to the supernatant at a concentration of (1:75), and incubated at 4°C for 2.5 hours with gentle rotation. 50 μL of beads were added and rotated at 4°C for 1 hr, and centrifuged down at 12000 rpm for 2 minutes. Supernatant was discarded and beads were washed four times using IP buffer. After the final wash, beads were resuspended in 2× sample buffer. Loading dye was added and the samples boiled before being centrifuged for 5 minutes at 13000 rpm. The supernatant was loaded onto an SDS-containing polyacrylamide gel, as described above.

2.5. G-alpha activation assay

The protocol was based upon the product manual for "Configuration-specific Monoclonal Antibody Based $G_{\alpha i}$ Activation Assay Kit" from NewEast Biosciences. Following treatment, cells were harvested in manufacturer's 1× Assay/Lysis (A/L) buffer (consisting of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) with freshly added 1 mM PMSF and 0.4 mM Na_3VO_4. Samples were cleared by centrifugation at 12000 rpm for 10 minutes at 4°C. Supernatants were subsequently transferred to fresh tubes.

0.5 mL of each sample was aliquoted to serve as the control treatment. 10 mM $MgCl_2$ and either 100 μM of GTPγS (negative control) or 1 mM GDP (positive control) were added to each tube. These were then incubated for 90 minutes at 30°C with gentle rotation.

0.5 mL of cell lysate from each sample was then aliquoted in two separate sets of tubes. One positive and one negative control were added to each set. Anti-active $G_{\alpha i}$ monoclonal antibody was added to one set of samples at (1:500), while anti-active $G_{\alpha s}$ was added to the other at a concentration of (1:500). A 2% protein G Sepharose bead slurry was added to each, and the samples were incubated at 4°C for one hour with gentle rotation. Beads were then pelleted by centrifugation at 4°C for 2 minutes at 12000 rpm. The pellet was washed three times with 1× Assay/Lysis buffer and resuspended in 2× sample buffer. 1% Loading dye was then added and the samples boiled for Western blotting analysis.

2.6. GST-immunoprecipitation for protein interaction

This protocol was established to study the interaction between a purified and isolated IGF-1R protein with a purified and isolated GST-tagged G_α proteins. The goal was to determine whether binding occurred directly by eliminating all possible effectors. The methods were based on the instructions manual from the "ProFound Pull-Down GST Protein:Protein Interaction Kit" from Pierce. Manufacturer's BupH TBS buffer at pH 7.2 (consisting of 25 mM Tris-HCl and 0.15 M NaCl) was first reconstituted in 500 mL of dH_20, and a (1:1) wash solution of TBS:ProFound Lysis buffer was prepared. Immobilized resin was resuspended by gentle vortexing and 50 µL were aliquoted into each Handee Spin Column. To wash the resin, 400 µL of wash solution was added to each column. Each column was first capped at both ends, inverted several times, and then uncapped, placed in collection tubes, and centrifuged one minute at 1250 rpm, 4°C. This process was repeated for a total of 5 washes. Enough columns were prepared to include a non-treated resin control, immobilized bait controls and bait treated samples.

One µg of purified GST-tagged G_α bait proteins were each diluted in 300 µL of ProFound Lysis Buffer. The column was first capped at the bottom, the GST protein was added and the top cap was then sealed. Columns were incubated 1 hour at 4°C with gentle rotation, then the caps were removed and the columns were placed in a new collection tube for centrifugation at 1250 rpm for one minute at 4°C. Columns were then washed as described above for a total of 3 washes. 1 µg of prey IGF-1R protein was diluted in 300 µL of ProFound Lysis buffer, which was then added to the appropriate bottom-capped columns. Top caps were placed and columns were again incubated for 1

hour at 4°C. Caps were removed and columns were placed in a new collection tube to be centrifuged at 1250 rpm for one minute at 4°C. Three additional washes were performed.

2.5 mL of fresh 100 mM Glutathione Elution Buffer was prepared by dissolving Glutathione into wash solution. Bottom caps were added to columns and 250 µL were added to each. Top caps were replaced and columns incubated with gentle rotation at 4°C for 30 minutes. Columns were placed into a final collection tube, caps were removed and samples were spun at 1250 rpm for 1 minute at 4°C.

For visualization of IGF-1R, equal amounts of each eluted sample were combined (1:1) with 2× sample buffer and 0.05 M NaOH and analyzed via SDS-PAGE and immune blotting. For visualization of GST-G_α proteins, the eluate was concentrated using a Centrifugal Filter Device. Samples were first added directly into the filter device, placed inside a filtrate collection tube and capped. They were then centrifuged 15 minutes at 12000 rpm, 4°C. Each filter device was then transferred upside-down to a clean, concentrate collection tube and centrifuged at 1000 rpm for 2 minutes. The concentrate was then combined (1:1) with 2× sample buffer and run on an SDS-PAGE. The gel was then stained for 90 minutes in the dark using ORIOLE fluorescent stain and photographed under UV excitation.

2.7. Competition assay

This protocol was established to study binding of G protein αi isoforms to purified and isolated His-tagged IGF-1R in a competition setting of limited receptor. The goal was to determine whether a single isoform demonstrated preferable binding to a limited amount of receptor, thereby eliminating binding of the other isoform. Thus, 0.5 µg of

purified IGF-1R was combined in 200 µL of IP buffer with 1 µg of each GST-$G_{\alpha i1}$ and GST-$G_{\alpha i2}$ and rotated at 4°C for 1 hour. Primary 6×His antibody was added at a (1:200) concentration and incubated for 2.5 hours at 4°C with rotation. 50 µL of a 50% slurry of Protein G Sepharose beads in IP wash buffer were added and rotated at 4°C for 1 hour before being centrifuged at 12000 rpm for 5 minutes. Supernatant was discarded and beads were washed four times using IP buffer. After the final wash, beads were resuspended in 20 µL of 2× sample buffer.

Loading dye was added and the samples boiled before being centrifuged for 5 minutes at 12000 rpm. The supernatant was loaded onto an SDS-containing polyacrylamide gel, as described above, alongside 1 µg of non-assayed GST-$G_{\alpha i1}$ and GST-$G_{\alpha i2}$. For visualization, gel was stained for 90 minutes in the dark using Oriole fluorescent stain and photographed under UV excitation.

2.8. BrdU cell proliferation assay

Methods were based on the datasheet for Chemicon International's "BrdU Cell Proliferation Assay" with slight modifications based on specific primary culture needs.

Cells were seeded at 1×10^5 cells/well in 100 µL of growth media consisting of 20% FBS DMEM + 1× AB/AM. They were left to attach and proliferate for 48 hours before being serum-starved using PTSA supplemented media for three days. Test reagents were then diluted at 2× the final desired concentration in the same medium, and 100 µL added directly to each well. The plate was incubated another 24 hours at 37°C before adding 20 µL/well of 1× BrdU reagent, diluted 500× in the DMEM + PTSA. A series of "background" wells did not receive any BrdU reagent, while a series of "Blank"

wells received the reagent but contained no cells. The plate was left to incubate for 24 hours at 37°C.

Media was aspirated and cells were fixed for 30 minutes with the Fixing Solution that was then removed and the plate was fully dried. The plate was then washed three times for 5 minutes with 1× wash buffer previously diluted in dH_2O, aspirating each time. Detector antibody was diluted 200× in Antibody Diluent and added to wells for one hour at room temperature before being washed, as described above. The goat anti-mouse IgG was diluted 2000× in Conjugate Diluent, and filtered using a 0.22 μm syringe filter in order to decrease background and improve precision. The conjugate was added to all wells, and the plate was then incubated for 30 minutes at room temperature and washed. Finally, the TMB peroxidase substrate was added and incubation was continued in the dark for 30 minutes at room temperature. The Acid Stop Solution was then added and cell proliferation was quantified by absorbance using the Omega FLUOstar, read at the dual emmission wavelengths of 450 and 540 nM.

2.9. Cell migration assay

Migration was measured with a modified Boyden-Chamber-like system, including cell culture housing inserts with polyethylene terephthalate (PET) membranes and companion feeder plates (See Supplemental Figure 1). Inserts were placed in the companion plates and cells were seeded inside onto the insert's PET membranes at 5×10^4 cells in growth media. Cells were left to attach and proliferate for 48 hours, before being serum-starved for four days using PTSA supplemented media. Test reagents were diluted to the final desired concentration in the DMEM + PTSA and added to the wells of

the companion feeder plates. Cells were left to incubate at 37°C for 72 hours to allow cell migration through the porous membrane.

Following completion of the incubation period, cells on the inside of the PET membranes were scratched off using a rubber policeman. The remaining cells, which represent cells that have migrated to the outside of the insert membranes, were fixed for five minutes using a 4% paraformaldehyde (PFA) solution (consisting of 1.75 mM NaOH, 0.9% NaCl, 4% PFA and 0.15 M Na_3PO_4). Membranes were then washed with SMC-PBS three times for 10 minutes. 5 µg/mL Hoechst 33258 (Sigma), diluted in SBC-PBS, was applied to the cells in the dark for two minutes. This was followed by three rounds of washing for 10 minutes with SMC-PBS.

The membranes were removed from the inserts and mounted onto microscope slides using Aqua-Mount Aqueous Mountant (Lerner Laboratories #13800), with the outside of the PET membrane facing up. The slides were left to dry in the dark and migration was quantified by reading the intensity of the fluorescence at 460 nM.

2.10. Cell viability assay (Cell counting kit-8, CCK-8)

Cells were seeded at 1×10^5 cells/well in 100 µL of growth media (20% FBS DMEM + 1× AB/AM). Time allotted for cells to attach and proliferate as well as times allotted for serum-starvation and treatments were determined by the specific assay for which cell viability was being examined.

Methods for cell counting were based on the technical manual for Dojindo's "Cell Counting Kit-8". 20 µL of the provided reagent was added to each well and incubated at

37°C for 1.5 hours. Absorbance was then read at 450 nm using the Omega FLUOstar plate reader.

2.11. Cell lysate preparation for microarray analysis

The protocol was based on the "Antibody Microarray Analysis Customer Information Package" from Kinexus. Following treatment, cells were rinsed once with ice-cold SMC-PBS and harvested with 200 µL of Kinexus buffer at pH 7.2 (consisting of 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 2 mM ethylene glycol tetraacetic acid (EGTA), 5 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100) containing freshly added 1× Halt protease inhibitor cocktail (Thermoscientific #78438), 1× Halt phosphatase inhibitor cocktail (Thermoscientific #78420) and 1 mM dithiothreitol.

Lysates were then sonicated four times for 10 seconds, with 10 second intervals on ice, followed by ultracentrifugation at 44 000 rpm for 30 minutes. The supernatant was transferred to a fresh screw cap vial and protein concentration was determined using BCA protein assay reagents.

2.12. Smooth muscle cell activation and inhibition

Whenever possible, all treatments were performed at least in triplicates. Table 4 depicts the inhibition/activation times of the chemicals used in all short term treatments. For the BrdU cell proliferation assay, the cell migration assay and the CCK-8 assay, incubation times are described in their respective methods. With the exception of the 5'-FAM-TAT-G_α C-terminal peptide inhibitors, for which determination of conditions is

described in results, the concentrations depicted in Table 4 were optimized using dose response and toxicity experiments (data not shown) and are valid for both short and long term treatments.

2.13. Statistical Analysis

Statistical analysis was performed using Origin v7.5. All means were compared using a one-way analysis of variance (ANOVA), considering a p value ≤ 0.05 as statistically significant.

Table 4: *List of chemical inhibitors and activators*

Chemical name	Incubation time	Concentration
IGF-1	12 minutes	50 nM
PDGF-BB	12 minutes	100 ng/mL
Norepinephrine (NE)	12 minutes	50 μM
Forskolin	12 minutes	10 μM
AG1024	15 minutes pre-incubation	4 μM
Gallein	15 minutes pre-incubation	50 μM
LY294002	15 minutes pre-incubation	10 μM
5'-FAM-TAT-G_α C-terminal peptide inhibitors	1 hour pre-incubation	10 μg/mL

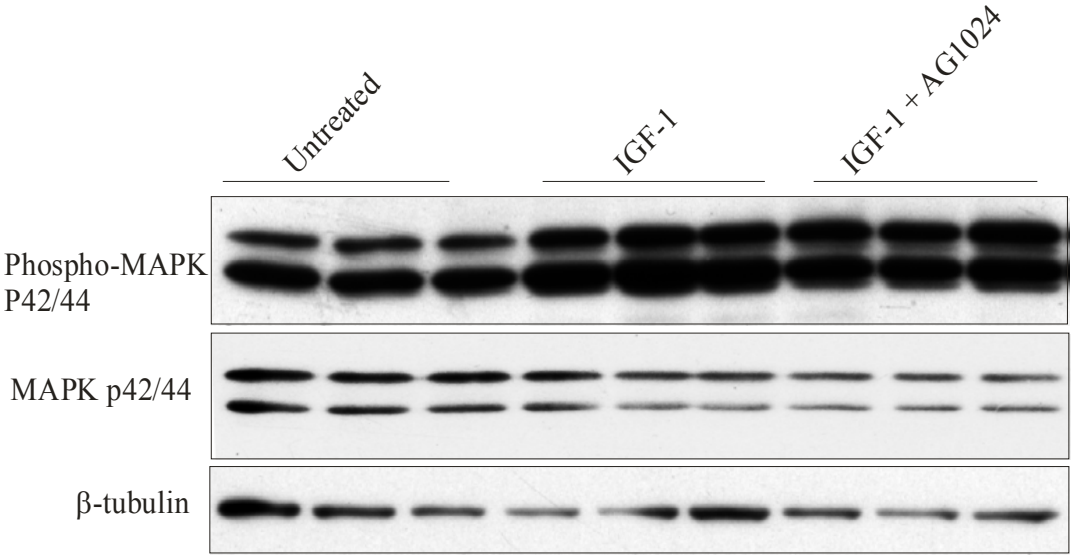
RESULTS

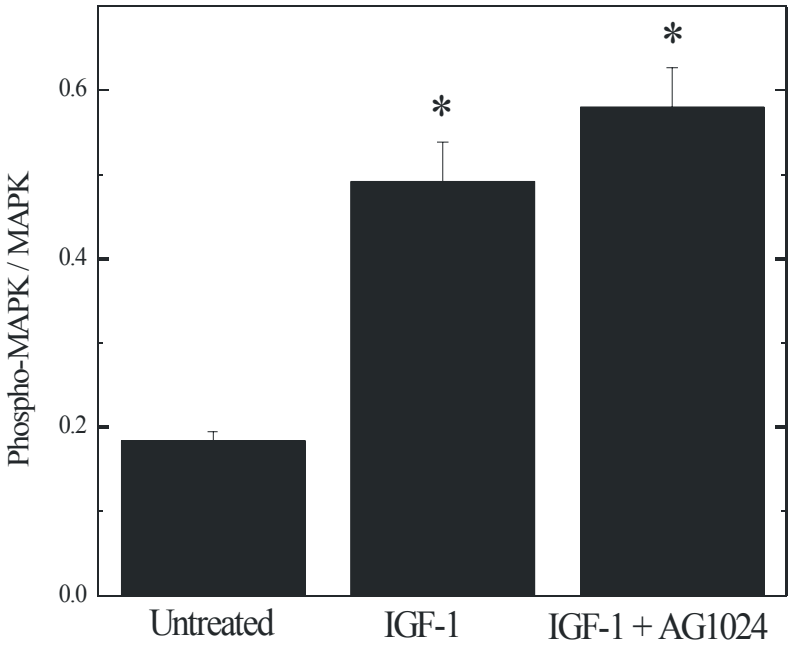
1. IGF-1-induced activation of MAPK is not attenuated by tyrosine kinase inhibition:

To investigate IGF-1-induced activation of MAPK signaling in SMCs, the phosphorylation state of MAPK in quiescent SMCs was compared to that of IGF-1 stimulated SMCs. As seen in Figure 2, IGF-1 treatment of vascular smooth muscle cells resulted in a significant increase in p42/44 MAPK phosphorylation.

To determine the role of the receptor tyrosine kinase (RTK) in this signal transduction cascade, quiescent SMCs were pre-incubated with the selective IGF-1/IR tyrosine kinase inhibitor Tyrphostin AG1024 (Parrizas et al., 1997) prior to IGF-1 stimulation. Figure 2 shows that AG1024 did not return MAPK phosphorylation to basal levels. This suggests that an alternate pathway may be involved in IGF-1-induced MAPK activation.

Figure 2: *Effects of IGF-1 treatment and of IGF-1R kinase inhibition on p42//44 MAPK phosphorylation*. Quiescent VSMCs cultured in 12-well dishes were pre-treated for 15 minutes with 4 μM of the IGF-1R tyrosine kinase inhibitor AG1024 and subsequently treated for 10 minutes with 50 nM IGF-1. MAPK phosphorylation, as assessed by Western blotting of 5 μg of protein from cell lysates, was quantified by scanning densitometry. Results represent means ± SE (n=3), plotted relative to total MAPK. *: significantly different from null control by one-way ANOVA, $p < 0.05$.


Untreated
IGF-1
IGF-1 + AG1024
Phospho-MAPK P42/44
MAPK p42/44
β-tubulin


Phospho-MAPK / MAPK
0.0
0.2
0.4
0.6
*
*
Untreated
IGF-1
IGF-1 + AG1024

2. The IGF-1R and different subunits of a hG protein co-precipitate:

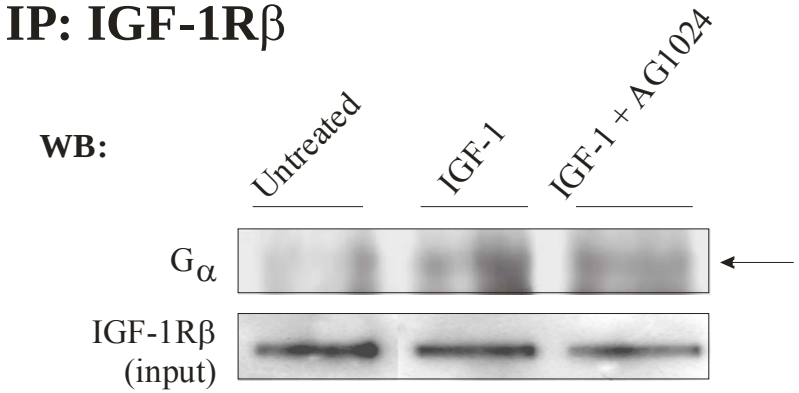
To determine whether previously made observations that implicated a hG protein in IGF-1 signaling also applied to VSMCs, interactions between the β-subunit of the IGF-1R and the different subunits of a hG protein were investigated.

Figure 3 first demonstrates interactions of the IGF-1Rβ with G protein α. Panel A shows that immunoprecipitation of IGF-1Rβ also precipitated G_{α}. This interaction appears to be present whether the VSMCs are quiescent, stimulated or stimulated in the presence of a kinase inhibitor. When the immunoprecipitation was reversed, as shown in panel B, the results were consistent since the G_{α} precipitation also precipitated the β-subunit of the IGF-1R.

Figure 4 demonstrates an interaction between the IGF-1Rβ and G protein β subunit, since immunoprecipitation of IGF-1Rβ also precipitated G_{β}. Densitometry indicated that the amount of G_{β} which precipitates with the IGF-1Rβ changes with respect to activation state of the receptor.

Taken together, these results show that the IGF-1R does indeed interact with a hG protein in VSMCs, although they do not determine whether the interaction implicates a complex or scaffold. In addition, the observed receptor activation-dependent decrease in G_{β} precipitation suggests a role for at least the β-subunit in mediating IGF-1 signaling.

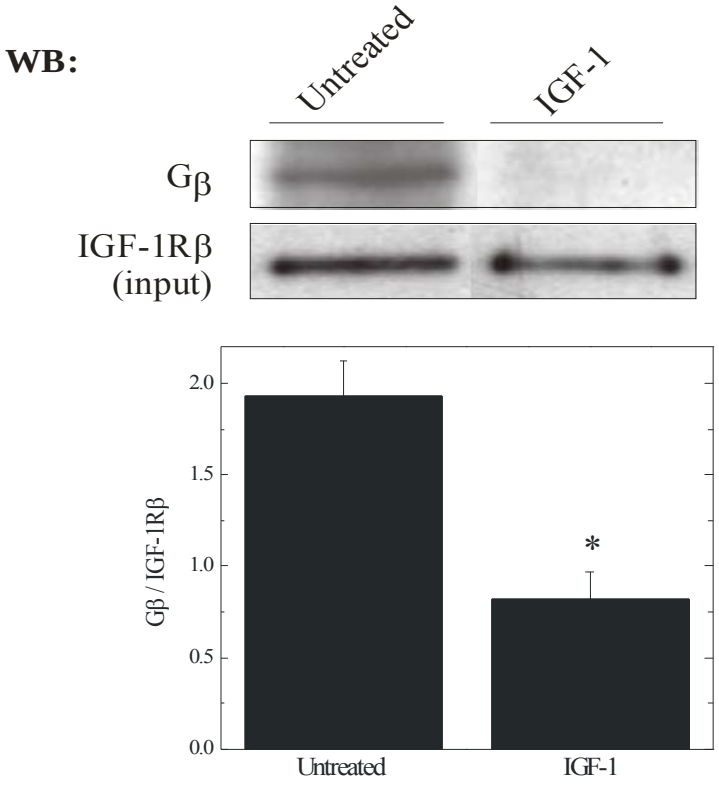
Figure 3: *Interaction of the IGF-1R β-subunit with G protein α.* Quiescent VSMCs cultured in 100 mm dishes were pre-treated with 4 μM AG1024 for 15 minutes and subsequently stimulated with 50 nM IGF-1 for 12 minutes. Cells were lysed and collected in a non-denaturing buffer, and 250 μg of total protein was immunoprecipitated with antibodies to either α-IGF-1Rβ or α-G_α (pan). Precipitates were then analyzed by immune blotting for both the immunoprecipitated protein and accompanying protein.


IP: IGF-1Rβ
WB:
Untreated
IGF-1
IGF-1 + AG1024
Gα
IGF-1Rβ
(input)


IP: Gα
WB:
Untreated
IGF-1
IGF-1 + AG1024
IGF-1Rβ
Gα (input)

Figure 4: *Interaction of the IGF-1Rβ-subunit with G protein β.* Quiescent VSMCs cultured in 100 mm dishes were treated with 50 nM IGF-1 for 12 minutes. Cells were lysed and collected in a non-denaturing buffer, and 250 μg of total protein was immunoprecipitated with an α-IGF-1Rβ antibody. Precipitates were then analyzed by Western blotting against both the immunoprecipitated protein and accompanying protein. Band intensity of G protein β relative to immunoprecipitated IGF-1Rβ was quantified by scanning densitometry. Results represent means ± SE (n=3). *: significantly different from null control by one-way ANOVA, $p < 0.05$.

IP: IGF-1Rβ


WB:
Untreated
IGF-1
Gβ
IGF-1Rβ
(input)
Gβ / IGF-1Rβ
2.0
1.5
1.0
0.5
0.0
*
Untreated
IGF-1

3. G protein α_i is activated in response to IGF-1, and is unaffected by IGF-1R kinase inhibition:

To determine which class of G protein is activated in response to IGF-1, active GTP-bound G proteins were immunoprecipitated from VSMCs that were either quiescent or IGF-1-stimulated. The antibodies used allow assessment of the extent of G protein GTP exchange, and thus activation, caused by the particular treatment. Based on the available literature, activation of $G_{\alpha i}$ was examined first.

Figure 5A shows immunoblotting of $G_{\alpha i}$ in GTP-$G_{\alpha i}$ immunoprecipitated samples. These results show that a significant increase in activation of $G_{\alpha i}$ is obtained after IGF-1 stimulation. The experimental control (panel B) utilized GTP-$G_{\alpha s}$ immunoprecipitated samples, subsequently immunoblotted against $G_{\alpha s}$. This panel shows that $G_{\alpha s}$ activation was not significantly induced by IGF-1 activation.

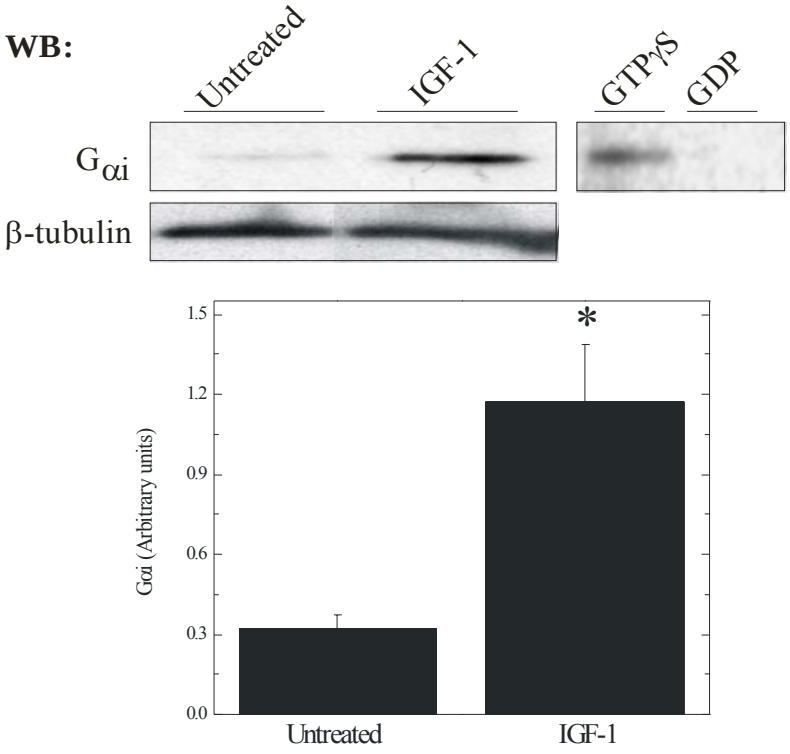
In order to establish whether the RTK was involved in this activating process, quiescent VSMCs were incubated with AG1024 to inhibit the IGF-1R tyrosine kinase prior to IGF-1 stimulation. As seen in Figure 6, $G_{\alpha i}$ was again activated, independent of the activity of the tyrosine kinase.

These results establish that $G_{\alpha i}$ was selectively activated by the IGF-1R. In addition, they suggest the presence of a new pathway for signal transduction via the IGF-1R that does not involve its RTK activity.

Figure 5: *Effects of IGF-1 activation on GTP exchange and activation of G protein α classes.* Quiescent smooth muscle cells cultured in 150 mm dishes were first treated with 50 nM IGF-1 for 12 minutes and then collected in a non-denaturing buffer. Active GTP-$G_{\alpha i}$ (panel A) or GTP-$G_{\alpha s}$ (panel B) antibodies were used for immunoprecipitation from equal volumes of samples, and quantity was measured by Western blotting against the respective G_{α}. β-tubulin loading control is representative of a blot containing equal volumes of samples. GTPγS and GDP controls represent null lysate samples incubated with either 1× GTPγS (0.1 mM) or 1× GDP (1 mM). Band intensities were quantified by scanning densitometry. Results represent means ± SE (n=3). *: significantly different from null control by one-way ANOVA, $p < 0.05$.

A

IP: Activated GTP-$G_{\alpha i}$



B

IP: Activated GTP-$G_{\alpha s}$

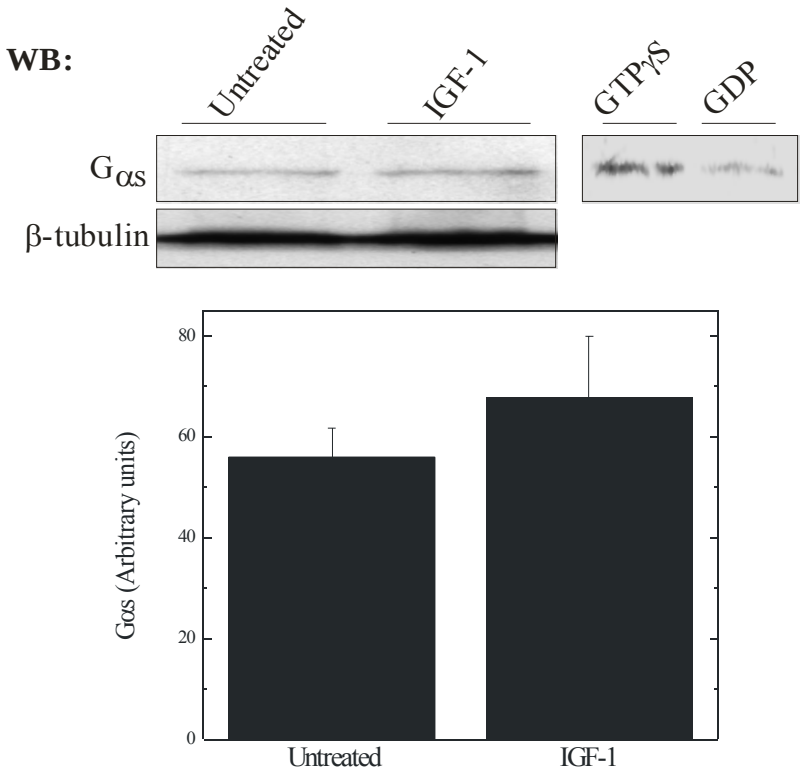
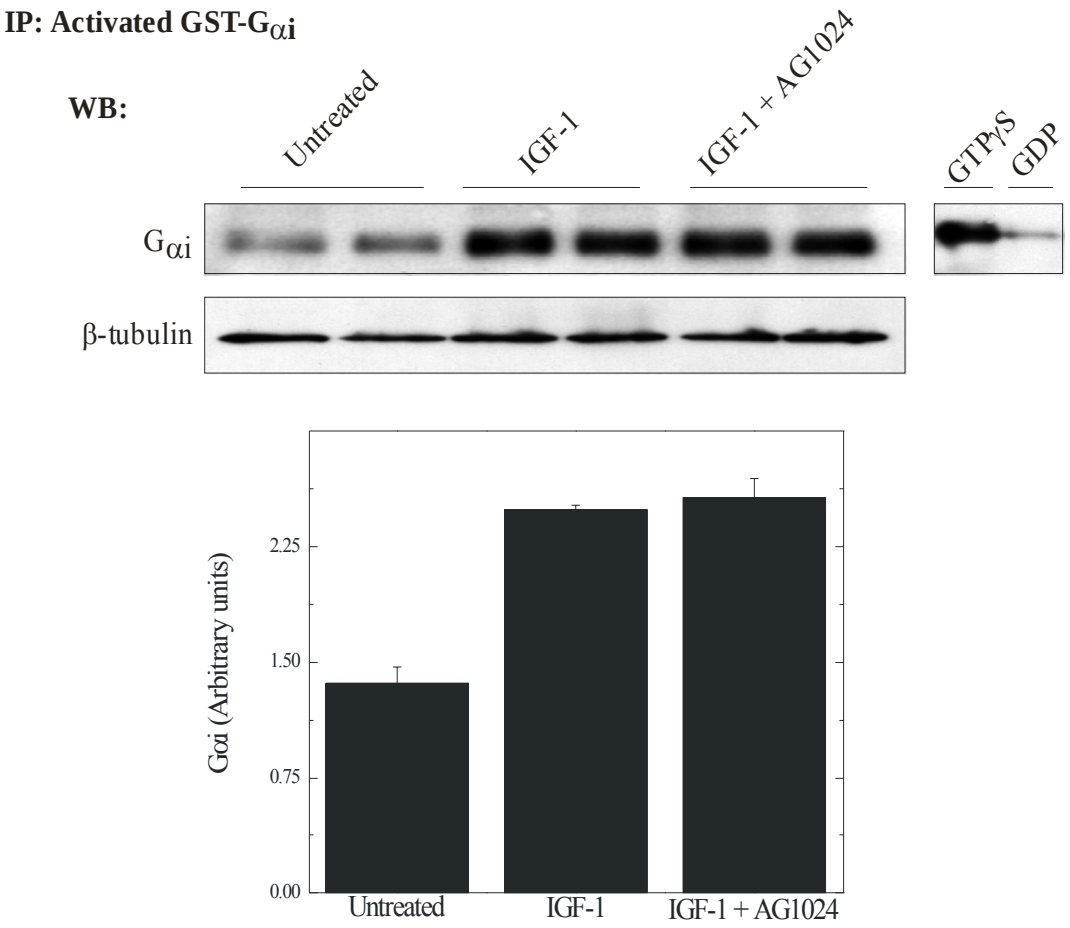


Figure 6: *Effects of IGF-1R kinase inhibition on IGF-1-induced G protein α_i activation*. Quiescent smooth muscle cells cultured in 150 mm plates were pre-treated 15 minutes with 4 μM AG1024 followed by a 12 minutes incubation with 50 nM IGF-1. Cells were then lysed and collected in a non-denaturing buffer. An active GTP-$G_{\alpha i}$ antibody was used for immunoprecipitation from equal volumes of samples, and quantity was measured by Western blotting against $G\alpha_i$. β-tubulin loading control is representative of a blot containing even volumes of samples. GTPγS and GDP controls represent null lysate samples incubated with either 1× GTPγS (0.1 mM) or 1× GDP (1 mM). Band intensities were quantified by scanning densitometry. Results represent means ± SE (n=2) and were consistent through three repetitions.


IP: Activated GST-Gαi
WB:
Untreated
IGF-1
IGF-1 + AG1024
GTPγS
GDP
Gαi
β-tubulin
Gαi (Arbitrary units)
2.25
1.50
0.75
0.00
Untreated
IGF-1
IGF-1 + AG1024

4. IGF-1R interacts directly with G protein α:

To investigate whether complexing or scaffolding proteins are necessary for interactions between the IGF-1R and its $G_{\alpha i}$, an experiment in which the association between two proteins *in vitro* can be assessed in the absence of other potential cellular effectors was performed.

In this experiment, recombinant GST-tagged G_{α} was incubated with the cytoplasmic domain of the IGF-1R β-subunit, the protein segment most likely to interact with the G protein. Since it was shown above that $G_{\alpha i}$ is specifically activated by IGF-1 stimulation of VSMCs, the interaction of the IGF-1R with different isoforms of $G_{\alpha i}$ was examined, with $G_{\alpha s}$ included as a negative control. As seen in Figure 7, both $G_{\alpha i1}$ and $G_{\alpha i2}$ were able to directly bind to the IGF-1R. The degree of background binding is shown with $G_{\alpha s}$. The results also suggest that $G_{\alpha i2}$ may bind the IGF-1R with slightly higher affinity than $G_{\alpha i1}$, as $G_{\alpha i2}$ appears to precipitate a slightly larger amount of IGF-1R

In order to determine whether the affinity of $G_{\alpha i2}$ is indeed higher than that of $G_{\alpha i1}$ for the IGF-1R, as well as to determine whether one preferably binds in a non-isolated setting, a competition assay was performed. As seen in Figure 8, when the IGF-1R is saturated with the GST-$G_{\alpha i}$ peptides, binding of $G_{\alpha i2}$ is almost completely blocked out by $G_{\alpha i1}$.

These results establish that the IGF-1R interacts directly with its G_{α} component, and that it may be either of the tested isoforms of $G_{\alpha i}$. However, they also suggest that although $G_{\alpha i2}$ may appear to have higher affinity for the IGF-1R when isolated, $G_{\alpha i1}$ preferably binds to the IGF-1R in a competitive setting.

Figure 7: *In vitro interactions of the IGF-1R with G protein α.* 1 μg of purified, recombinant GST-G_α protein, fixed on a Glutathione resin, was incubated with 1 μg of a purified, isolated peptide corresponding to the cytoplasmic domain of the β subunit IGF-1R for 1 hour. Following elution, the IGF-1R precipitation was analyzed via Western blotting, while the remaining eluate was concentrated, and the GST-G_α peptides were visualized using Oriole fluorescent gel stain under UV excitation. Results were consistent between two replicates.

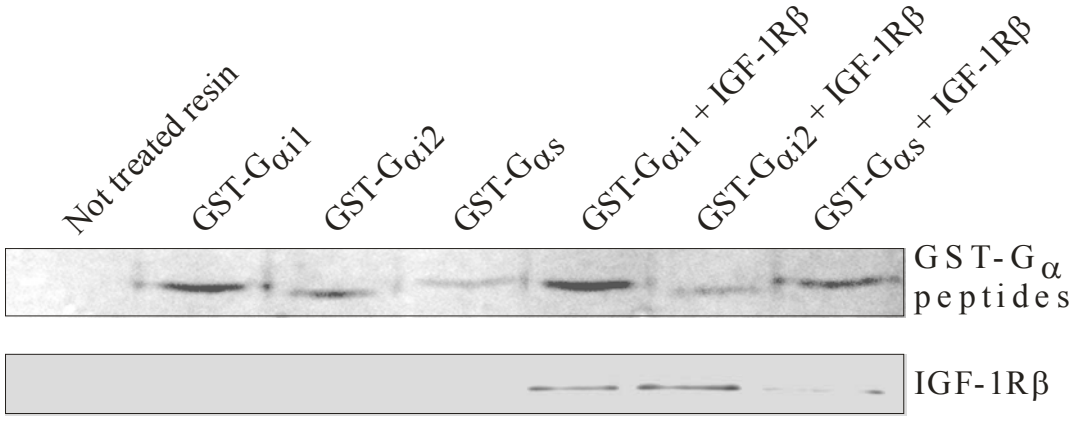
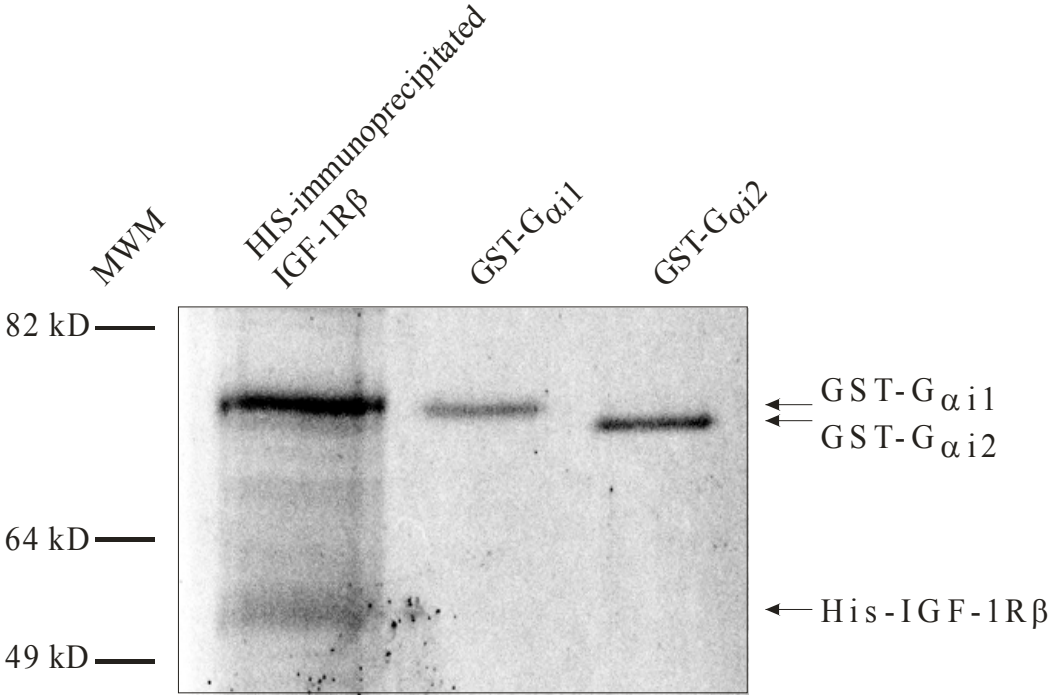

Not treated resin
GST-Gαi1
GST-Gαi2
GST-Gαs
GST-Gαi1 + IGF-1Rβ
GST-Gαi2 + IGF-1Rβ
GST-Gαs + IGF-1Rβ
GST-Gα peptides
IGF-1Rβ

Figure 8: *Effect of competition on G protein α_{i1} and G protein α_{i2} on IGF-1R binding*. 1 μg of the cytoplasmic domain of the β-subunit of the IGF-1R (55 kD) was incubated with 2 μg each of GST-$G_{\alpha i1}$ (69 kD) and GST-$G_{\alpha i2}$ (65 kD) simultaneously for 1 hour. Sample resulting from the His-tag IGF-1R immunoprecipitation was run alongside 1 μg of purified GST-$G_{\alpha i}$ proteins for molecular weight and intensity comparison. Proteins were visualized with Oriole fluorescent gel stain. Results were consistent between two replicates.

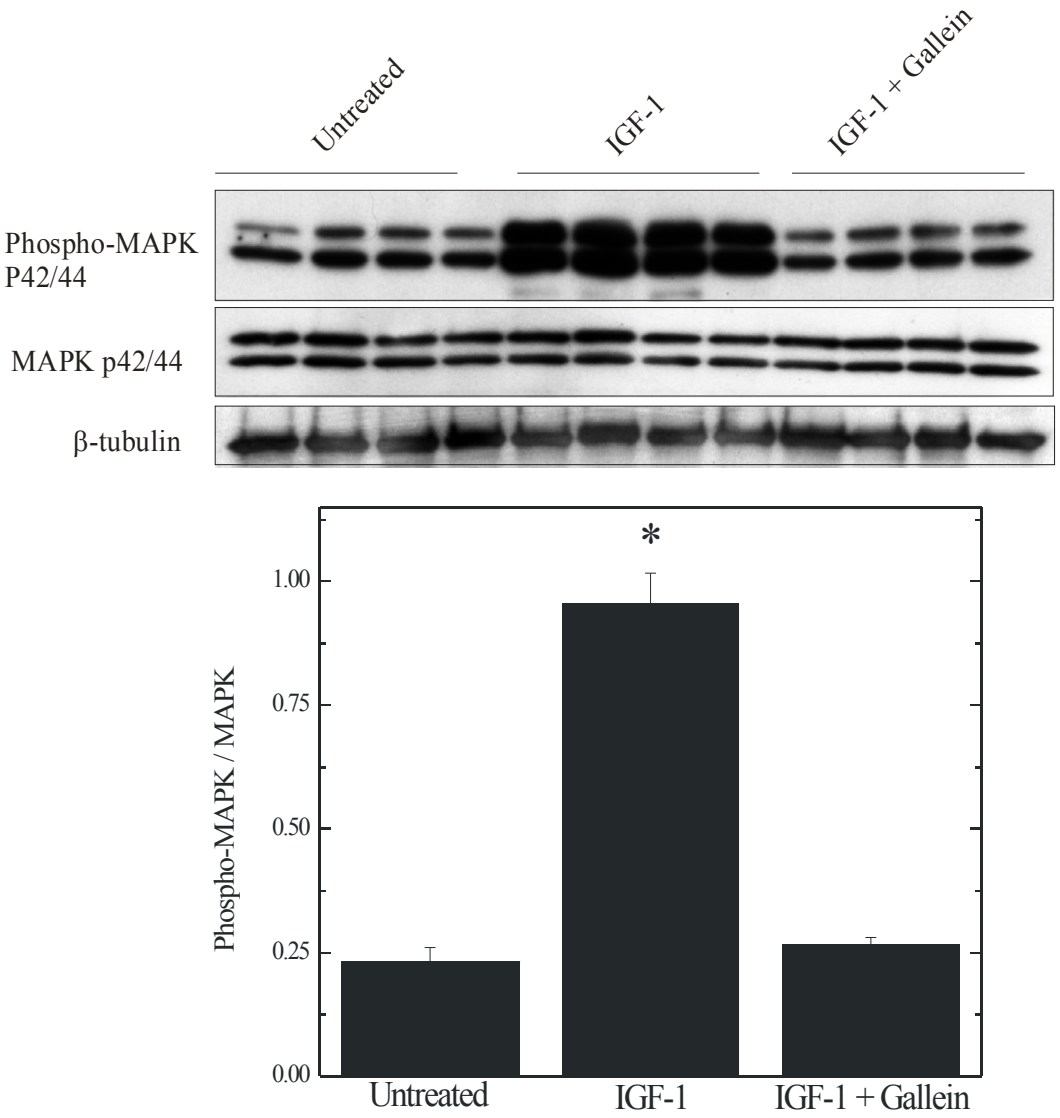

MWM
HIS-immunoprecipitated IGF-1Rβ
GST-Gαi1
GST-Gαi2
82 kD
64 kD
49 kD
GST-Gαi1
GST-Gαi2
His-IGF-1Rβ

5. The $G_{\beta\gamma}$ subunit of the hG protein is required for p42/44 MAPK activation downstream of the IGF-1R:

To determine whether $G_{\beta\gamma}$ is involved in the pathway leading to p42/44 MAPK activation by IGF-1, a small molecule inhibitor of $G_{\beta\gamma}$ signaling, Gallein (3',4',5',6'-tetrahydroxyspiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one), was used (Lehmann et al., 2008). Consistent with Figure 2, treatment of quiescent VSMCs leads to a significant increase in MAPK phosphorylation. In contrast however, pre-treatment with Gallein completely attenuated this activation, returning phosphorylation levels to that of the null control (Figure 9).

This result supports the hypothesis that $G_{\beta\gamma}$ demonstrates activation-dependent coupling to the IGF-1R and, further, identifies a component of the IGF-1 signal which is dependent upon this G protein.

Figure 9: *Effects of a $G_{\beta\gamma}$ modulator on p42/44 MAPK phosphorylation*. Quiescent VSMCs cultured in 12-well dishes were pre-treated for 15 minutes with 50 μM Gallein and subsequently treated for 12 minutes with 50 nM IGF-1. Lysates were quantified and 5 μg of protein was analyzed by Western blotting for p42/44 MAPK phosphorylation. Results, quantified by scanning densitometry, are shown as means ± SE (n=4), plotted relative to total MAPK. *: significantly different from null control by one-way ANOVA, $p < 0.05$.


Untreated
IGF-1
IGF-1 + Gallein
Phospho-MAPK P42/44
MAPK p42/44
β-tubulin
*
1.00
0.75
0.50
0.25
0.00
Phospho-MAPK / MAPK
Untreated
IGF-1
IGF-1 + Gallein

6. Modulation of $G_{\beta\gamma}$-mediated signal transduction translates into significant attenuation of pro-atherogenic VSMC activity.

To investigate whether the specific IGF-1-induced pathway leading to MAPK activation, which was found to be modulated by $G_{\beta\gamma,}$ significantly affected cellular processes relevant in atherosclerosis, both IGF-1-induced SMC proliferation and migration were studied following inhibition with Gallein.

An assay that measures the incorporation of the thymidine analog BrdU into DNA as it is replicated was used to study cell proliferation. As shown in Figure 10, cells in the null control group exhibited low, background proliferative activity. This activity was significantly increased by the growth factors IGF-1 and PDGF-BB, with the latter serving as the positive control. A potent reversible inhibitor of the PI3Ks, abbreviated LY294002 (3-kinase,2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) (Vlahos et al., 1994), was used to specifically inhibit the growth pathway initiated by the tyrosine kinase of the IGF-1R without directly inhibiting this kinase. Both LY294002 and the kinase inhibitor AG1024 completely attenuated cell proliferation, including the background activity observed in the null control. With respect to the $G_{\beta\gamma}$-modulated growth pathway, treatment with Gallein also attenuated cell proliferation, returning it to levels not significantly different from those of the null control.

A Boyden Chamber-like assay allowing cell movement across a PET membrane was used to study cell migration. In Figure 11 A, representative photographs of the PET membranes show the effect of the treatments on migration. Primary among the observations is that IGF-1 is a much less potent chemoattractant than is PDGF-BB. Also of note is a trend toward a smaller and rounder morphology in the IGF-1 treated cells.

Quantification of the numbers of cells visible in three microscope fields per treatment is shown in panel B. It can be seen that both AG1024 and Gallein were able to fully abolish SMC migration, returning it to the level observed in the null control.

In order to determine whether the significant differences in cell migration and proliferation were indeed due to the inhibitors used and not to a difference in final cell content, CCK-8 assays were performed with parallel times and treatments as those described for Figures 10 and 11. This assay measures the cell content at a given end point. Panel A of Figure 12 shows that under the conditions used for the BrdU cell proliferation assay, none of the treatments caused a significant decrease in cell content. Of note, there is a non-significant trend toward decreased cell number caused by PDGF-BB treatment. In panel B, toxicity of the cell migration assay is assessed. In all cases, IGF-1 treatment (with or without inhibitors) resulted in a slight but significant decrease in cell content. However, there were no differences between the groups.

The combination of these results leads to the conclusion that the $G_{\beta\gamma}$-modulated growth pathway which results in p42/44 MAPK activation does indeed affect critical VSMC processes, as inhibition of this pathway significantly blocks both cell migration and proliferation.

Figure 10: *Effects of $G_{\beta\gamma}$ modulation on SMC proliferation*. Quiescent VSMCs cultured in 96-well plates were incubated with respective treatments at concentrations indicated in Table 4 for 24 hours, and further incubated with 1× BrdU for 24 hours. Following assay procedure, cell proliferation was quantified using absorbance at 450 nM. Results represent mean ± SE (n=3). *: significantly different from null control; #, significantly different from IGF-1 control by one-way ANOVA, $p < 0.05$.

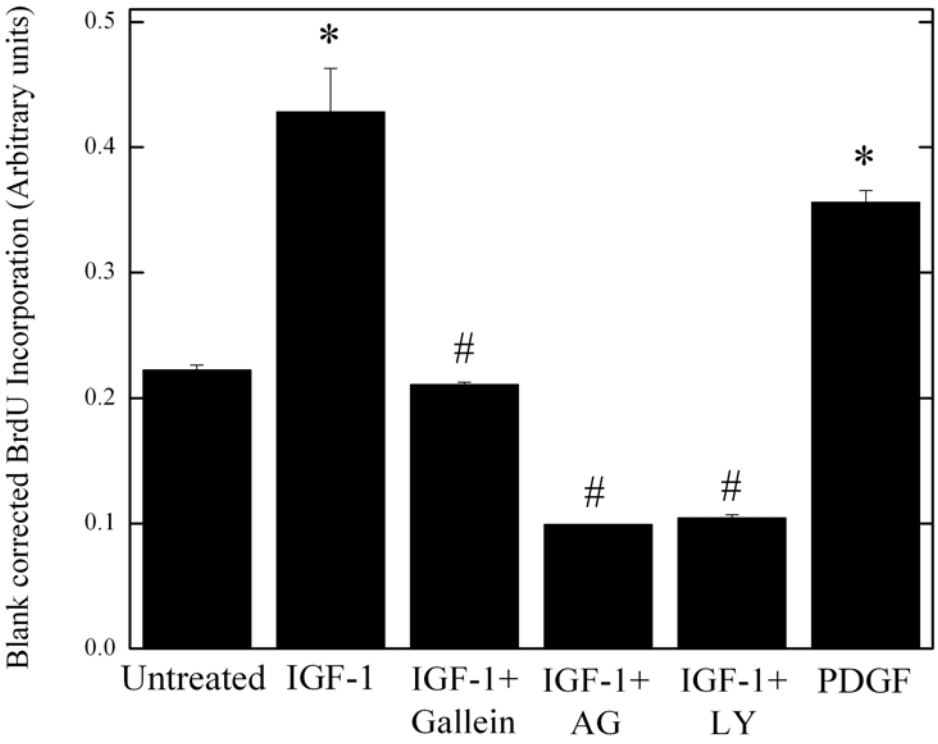
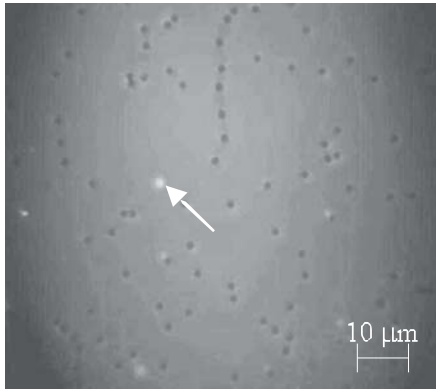
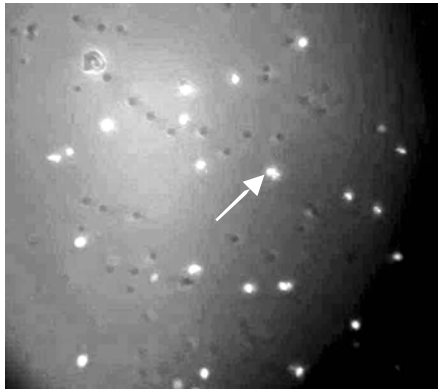

Blank corrected BrdU Incorporation (Arbitrary units)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
#
#
#
Untreated
IGF-1
IGF-1+ Gallein
IGF-1+ AG
IGF-1+ LY
PDGF

Figure 11: *Effects of $G_{\beta\gamma}$ modulation on SMC migration*. Quiescent VSMCs cultured in insert housing units for 24-well plates were incubated with respective treatments at concentrations indicated in Table 4 for 72 hours, before being fixed with 4% PFA and stained with Hoechst 33258. Migration was visualized at 460 nm and and quantified by counting three representative microscope fields at 100X (panel A). Panel B represents means number of cells in 3 fields per well ± SE (n=3 wells). *: significantly different from null control by one-way ANOVA, $p < 0.05$.

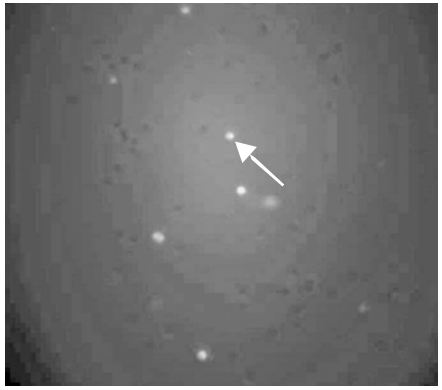
A



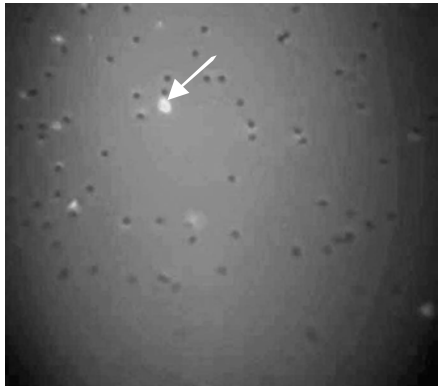
Untreated



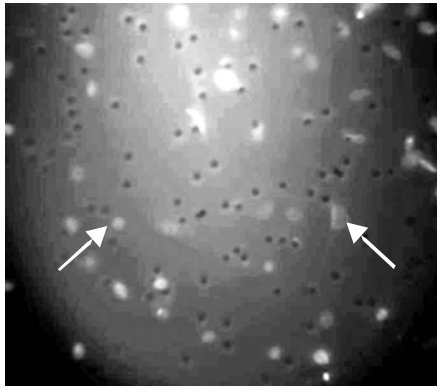
IGF-1



IGF-1 + AG1024



IGF-1 + Gallein



PDGF-BB

B

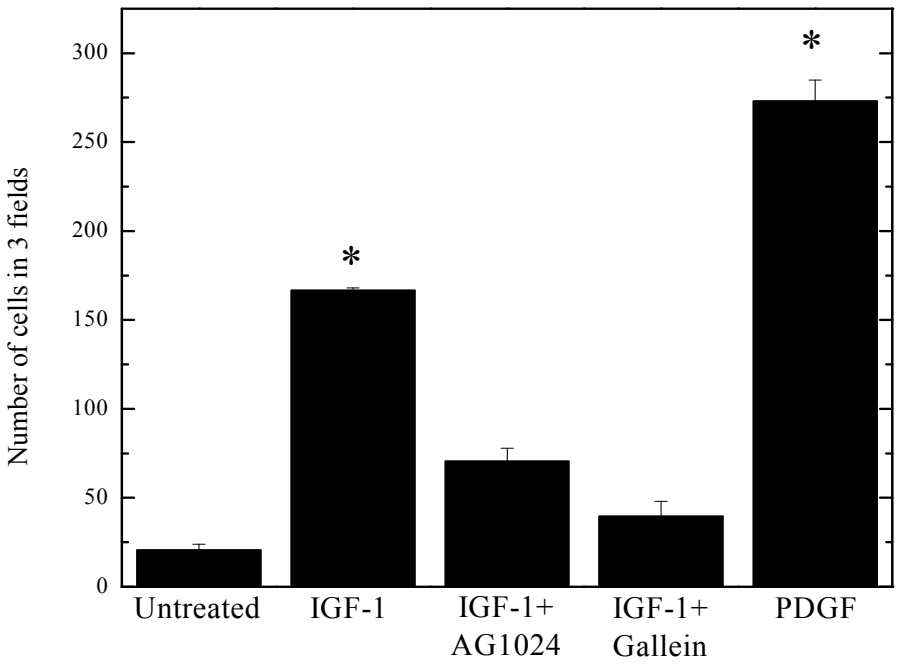
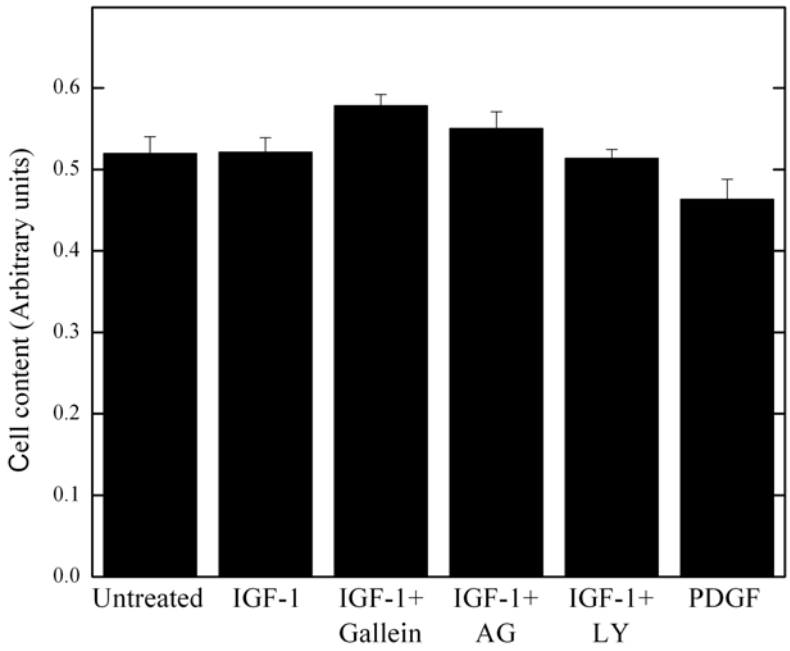
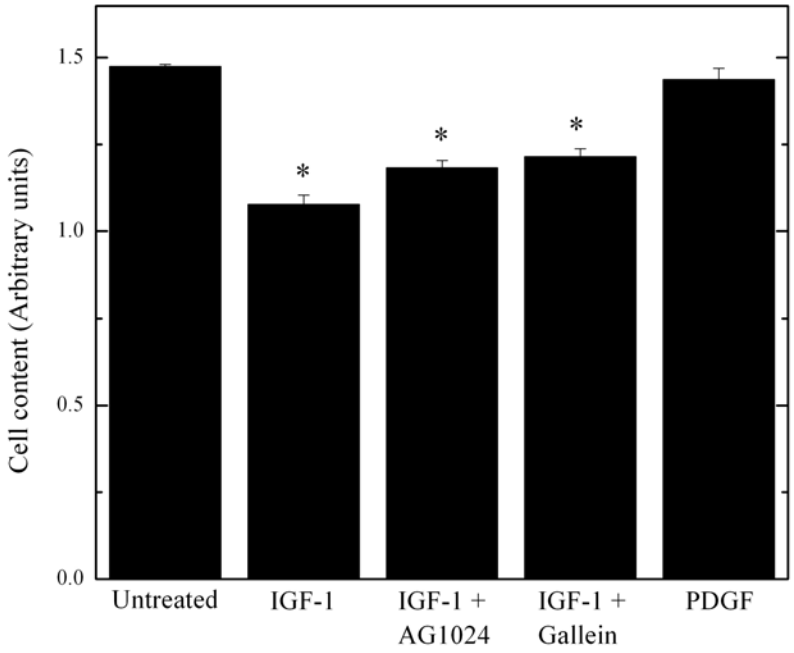

Number of cells in 3 fields
300
250
200
150
100
50
0
*
*
Untreated
IGF-1
IGF-1+
AG1024
IGF-1+
Gallein
PDGF

Figure 12: *Effects of BrdU cell proliferation and cell migration assays on final SMC content.* Quiescent VSMCs cultured in 96-well plates were treated according to the protocols outlined for the BrdU cell proliferation assay (panel A) or the cell migration assay (panel B). Cells were then incubated with the CCK-8 reagent for one hour at 37°C. Viability was assessed using intensity of signal at 450 nm. Results represent mean ± SE (n=4). *: significantly different from null control by one-way ANOVA, $p < 0.05$.

A


Cell content (Arbitrary units)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Untreated
IGF-1
IGF-1+ Gallein
IGF-1+ AG
IGF-1+ LY
PDGF

B


Cell content (Arbitrary units)
0.0
0.5
1.0
1.5
*
*
*
Untreated
IGF-1
IGF-1 + AG1024
IGF-1 + Gallein
PDGF

7. TAT-tagged G_α C-terminal peptides can enter quiescent VSMCs and inhibit GPCR signaling in a non-toxic manner:

In order to identify a potential role for the $G_{\alpha i}$ signaling component, peptides corresponding to the C-terminal 11 amino acids of $G_{\alpha i1/2}$, and $G_{\alpha s}$ as a control, were synthesized. These peptides are thought to function as competitive inhibitors of G_α binding as the residues are involved in respective their receptor binding (Martin et al., 1996; Rasenick et al., 1994; Sullivan et al., 1987). The peptides are fused at their N-terminal to the cell penetrating sequence of the Human Immuno Deficiency (HIV) virus, the TAT-sequence, in addition to 5'-carboxyfluorescein (5'-FAM) for tracking within the cells.

To first determine whether the control peptide was able to enter the cells and inhibit signaling, quiescent VSMCs were incubated with the inhibitor peptides prior to norepinephrine (NE) stimulation. Concentrations of TAT-conjugated peptide were based on various studies showing efficient, non-toxic cell penetration at concentrations varying between 5 and 20 μg/mL (Fawell et al., 1994; Han et al., 2009). As seen in Figure 13, there was a significant increase in the NE-induced activation of cyclic adenosine monophosphate (cAMP) response element-binding (CREB). Forskolin was used as a positive control of CREB activation. Results also show that pre-incubation with the TAT-$G_{\alpha s}$ peptide completely attenuated CREB phosphorylation at all tested concentrations.

Next, in order to determine whether the observed inhibition of CREB activation was indeed due to signaling inhibition and not to a decrease in final cell number, a CCK-8 assay was performed on quiescent VSMCs having incubated with the TAT-$G_{\alpha s}$

inhibitor peptide. Figure 14 shows that the peptide did not cause a significant decrease in cell content at any tested concentrations.

Having thus established the working conditions using the control peptide, the effect of the TAT-$G_{\alpha i}$ C-terminal inhibitor peptide on IGF-1 signaling was examined. The ability of this peptide to enter the cells was assessed using fluorescent imaging of the 5'-FAM tag. Figure 15 shows the fluorescence resulting from the addition of the inhibitor peptide to VSMCs. When compared to untreated null cells, the fluorescence does accumulate within the cells.

All of these results together show that the synthesized inhibitor peptides are not only able to enter the cells, bind and inhibit the intended targets, but also that they are able to do so in a manner which is not toxic to the cells.

Figure 13: *Effects of the TAT-$G_{\alpha s}$ C-terminal peptide inhibitor on norepinephrine (NE) signaling.* Quiescent VSMCs cultured in 12-well dishes were pre-treated with varying concentrations of the TAT-$G_{\alpha s}$ C-terminal peptide inhibitor for 1 hour. They were then treated with either 50 μM NE or 10 μM forskolin as a positive control and incubated for 12 minutes. Lysates were prepared and 5 μg of protein was used for analysis of CREB phosphorylation by Western blotting. These data were quantified by scanning densitometry. Results represent means ± SE (n=2), plotted relative to total CREB. Results were consistent between two replicates.

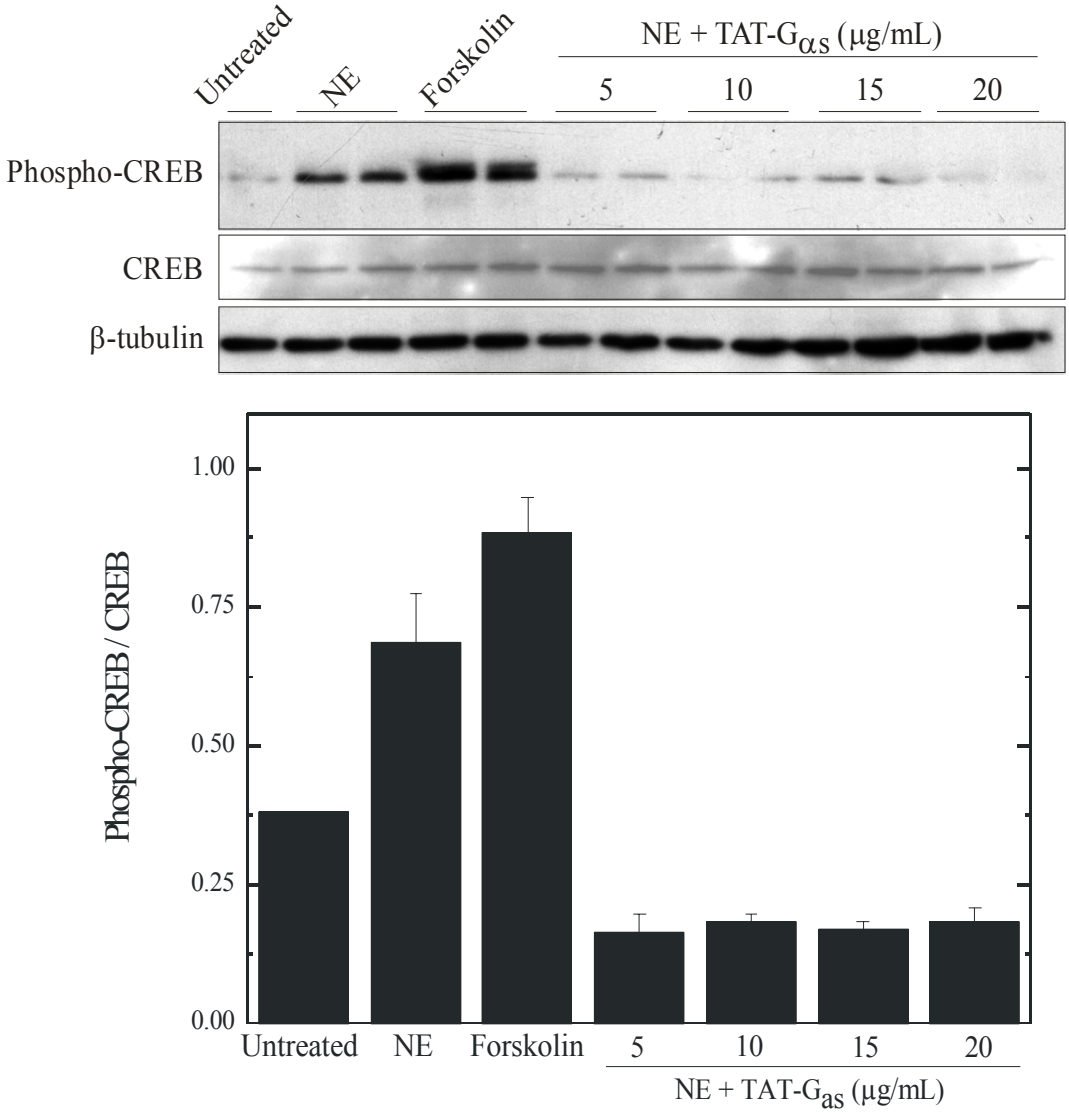

Untreated
NE
Forskolin
NE + TAT-Gαs (μg/mL)
5
10
15
20
Phospho-CREB
CREB
β-tubulin
Phospho-CREB / CREB
1.00
0.75
0.50
0.25
0.00
Untreated
NE
Forskolin
5
10
15
20
NE + TAT-Gas (μg/mL)

Figure 14: *Effects of the TAT-$G_{\alpha s}$ C-terminal peptide inhibitor on final SMC content.* Quiescent VSMCs cultured in 96-well plates were treated with the TAT-$G_{\alpha s}$ C-terminal peptide inhibitor for 1 hour. They were subsequently incubated with the CCK-8 reagent for 1 hour at 37°C. Cell viability was determined using intensity of signal at 450 nm and the results are represented as mean ± SE (n=5).

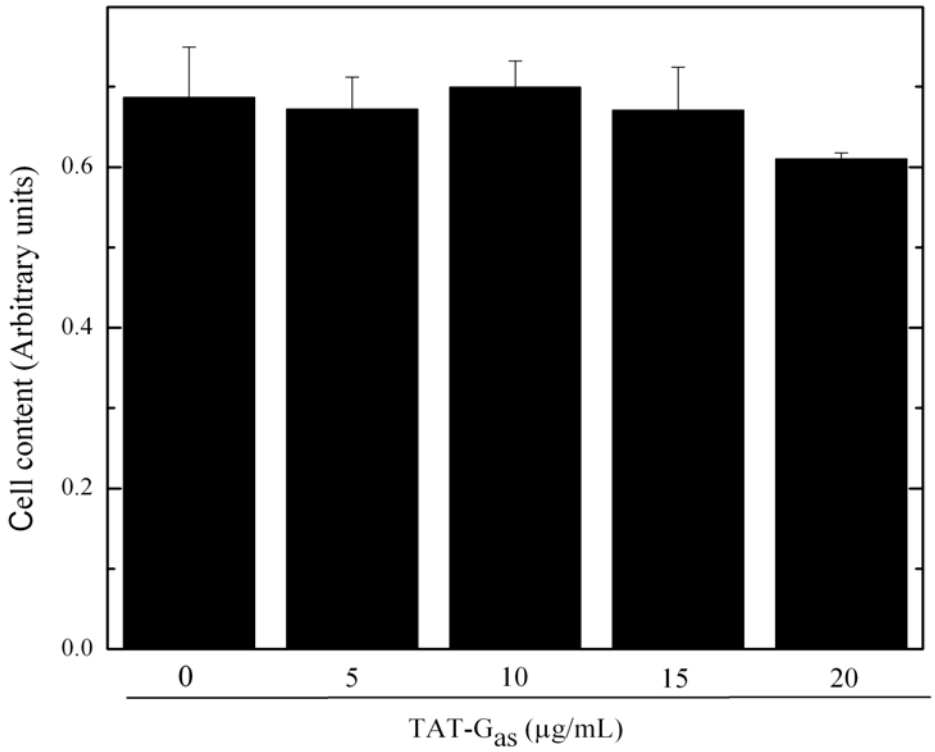
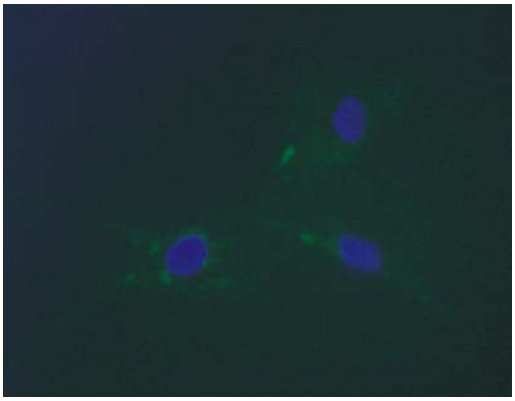
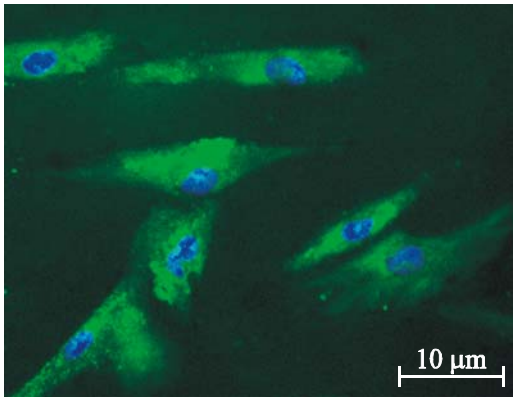

Cell content (Arbitrary units)
0.0
0.2
0.4
0.6
0
5
10
15
20
TAT-Gas (μg/mL)

Figure 15: *In-cell fluorescence of the 5'-FAM conjugated TAT-$G_{\alpha i1/2}$ C-terminal peptide inhibitor in VSMCs.* Quiescent VSMCs cultured on cover slips in 6-well plates were incubated with 3% BSA-DMEM for 30 minutes and subsequently incubated with 10 μg/mL of the peptide inhibitor for 1 hour. Fixed cover slips were then photographed at 400X using excitation filters at 488 nm (5'-FAM) and 350 nm (Hoechst).



Untreated



5'-FAM-TAT-$G_{\alpha i}$
10 µg/mL

8. The G_α subunit of the hG protein is involved in both short and long-term effects of IGF-1R signaling

To determine whether the α-subunit plays a role in signaling downstream of the IGF-1R in SMCs and to identify potential downstream mediators, a phospho-antibody microarray was performed using cell lysates from VSMCs treated with either IGF-1 or with TAT-$G_{\alpha i}$ prior to IGF-1 stimulation. The phospho-screen by Kinexus Bioinformatics allowed screening of approximately 280 potential phosphorylation sites on over 150 distinct proteins.

The significant changes detected with the phospho-screen are depicted in Table 5. The change in phosphorylation resulting from $G_{\alpha i}$ signaling inhibition prior to IGF-1 stimulation is expressed as a percentage (%) of the IGF-1 stimulated control. The two most drastic changes were seen with Eukaryotic translation initiation factor 4 (eIF4E) (83%) and Protein-serine phosphatase 1 - catalytic subunit - α isoform (PP1-α (catalytic)) (-84%). Histone phosphorylation is also affected by $G_{\alpha i}$ inhibition, suggesting that it is involved in regulating transcription. Finally, $G_{\alpha i}$ may also be involved in cross-talk between the IGF-1R and the EGFR, as EGFR phosphorylation is increased by $G_{\alpha i}$ inhibition.

These results indicate that $G_{\alpha i}$ does indeed mediate some aspects of IGF-1R signaling, both in short-term phosphorylation events, as well as in long-term protein expression levels.

Table 6 depicts the phosphorylation changes observed in the potential $G_{\alpha i}$ targets suggested by the studies of Kuemmerle and Murthy (2003) (AMPKα1/2) and Dalle et al.

(2001) (β-arrestin). At the two tested phosphorylation sites, no significant changes were induced by $G_{\alpha i}$ signaling inhibition.

Table 5: *Signal transduction effects of the TAT-$G_{\alpha i}$ C-terminal peptide inhibitor examined via phospho-microarray.*

Target protein name	Phospho Site	Z-ratio*	% Change from IGF-1 control
PP1-α (catalytic)	T320	1.73	83
EGFR	Y1197	1.44	54
Histone H3	T11	-1.53	-65
Histone H2B	S14	-1.63	-63
eIF4E	S209	-2.57	-84

* A Z-ratio of ±1.4 is considered significant, as outlined in Kinexus Bioinformatics' Kinex Antibody Microarray Report (Protein Profiling Services, 2010)

Table 6: *Effects of the TAT-$G_{\alpha i}$ C-terminal peptide inhibitor on targets suggested in the literature, examined via phospho-microarray*

Target protein name	Phospho Site	Z-ratio	% Change from IGF-1 control
AMPKα1/2	T183	0.22	-11
β-Arrestin	S412	-0.41	-17

DISCUSSION

The IGF-1 axis in the vasculature is of particular interest as it combines the importance of vascular cell growth within the context of atherosclerosis and the importance of the insulin axis within the context of diabetes. Although the signaling pathways initiated by the IGF-1R kinase have been extensively studied in various cell types, the novel idea that the receptor may also initiate signaling cascades via a hG protein has not been addressed in any of the relevant vascular cell types. The smooth muscle cells used in this study are primary cells, derived from the left anterior descending coronary artery of the pig. The pig has been established as a relevant and promising model for atherosclerosis as both their arterial structure and the atherosclerotic lesions, which develop spontaneously in wild-type animals, closely resemble that of the human (Bragdon, et al., 1957; French et al., 1965 & Reviewed in Granada et al., 2009). Thus, both their primary nature and their species of origin make their use relevant to the disease process in humans.

1. The IGF-1R is coupled to a heterotrimeric G protein in VSMCs

Very important signaling pathways in SMCs are those that lead to SMC proliferation and migration, as their accumulation is a prominent cause of atherosclerosis. Primary among important end-points is the activation of MAPK, a protein which integrates extracellular signals required for the initiation of various cascades involved in proliferation and migration. As a growth factor, IGF-1 is one of these extracellular signals which activate MAPK (Figure 2). However, inhibition of the receptor kinase using AG1024, classically the site of inititation of IGF-1 signaling (Krauss, 2008), does

not inhibit MAPK activation in VSMCs (Figure 2). This was not due to low AG1024 activity, as verified by inhibition of IGF-1R phosphorylation (data not shown). While this observation does correspond to that of at least one other group which suggested that inhibiting the IGF-1R kinase preferentially reduced Akt and not ERK phosphorylation (Vasilcanu et al., 2004), there are a variety of opposing studies reporting a decrease in activation with RTK inhibition (Camirand et al., 2005; Stromberg et al., 2006). As all of the above studies, including the one by Vasilcanu et al. (2004), were done in cancer cell lines, the role of the RTK in MAPK activation cannot yet be classified as disease-specific and would need to be further investigated.

Despite this debate, an alternate pathway such as a pathway modulated by a G protein that functions independently of the kinase is plausible, at least in SMCs. Interestingly, the observation that kinase inhibition does not affect signal initiation indicates that, should a G protein be involved, a second mechanism not involving phosphorylation must also be initiated upon IGF-1 stimulation of the IGF-1R. This could include GEF activity, or a simple conformational change which would activate the G protein. Questions also still remain as to whether this G protein pathway transactivates another receptor for MAPK activation, or whether the action is directly downstream.

While the idea that single transmembrane spanning RTKs may directly activate hG proteins is slowly being accepted in the literature, it remains that very few studies simultaneously consider both potential initiation mechanisms and few reviews simultaneously acknowledge the existence of both. With respect to the IGF-1R, other groups have shown evidence that the IGF-1R does indeed directly co-precipitate with the subunits of a hG protein (Dalle et al., 2001; Hallak et al., 2000). The study presented in

this thesis has investigated IGF-1R signaling as a mediator of cell growth in the vascular system, and demonstrates that G protein-mediated signaling is also critical within this context, as the receptor co-precipitates with both the α- and β-subunits of a hG protein (Figures 3 & 4). In addition, the results of this study support those of Dalle et al. (2001) and Hallak et al. (2000), which suggest that G_{α} uncoupling may not occur upon IGF-1 stimulation (Figure 3) while, paradoxically, a significant decrease in co-precipitation of G_{β}, postulated to represent $G_{\beta\gamma}$, is observed (Figure 4). This raises interesting questions, as the $G_{\beta\gamma}$-subunits are thought to interact only with their corresponding G_{α}-subunits and not with the receptor. Thus, for $G_{\beta\gamma}$ to be released and signal independently of G_{α}, a mechanism for transducing the signal from the receptor directly to $G_{\beta\gamma}$ must be in place. The recent study by Garcia-Hoz et al. (2010) proposed a potential solution to this issue, suggesting a scaffolding role for $G_{\alpha q}$. However, this observation which would need to be investigated in greater detail before being extended to other G_{α} classes.

2. IGF-1 stimulation of the IGF-1R activates G protein α_i, independently of kinase activation

Although a number of studies have reported that Pertussis Toxin (PTX) can inhibit a variety of IGF-1-induced cellular effects (Poiraudeau et al., 1997; Kanzaki et al., 1997), none have yet shown direct activation of $G_{\alpha i}$ following IGF-1R stimulation. In addition, with respect to MAPK, one study showed that PTX treatment alone caused increased phosphorylation in one cell type, while causing dephosphorylation below the control levels in another (Hallak et al., 2000). This highlights the fact that, not only are PTX actions cell-type dependent, but also that many of its actions are still unknown. To

avoid this issue, this study directly investigated G_α activation by measuring G_α-GTP levels, showing that $G_{\alpha i}$ is indeed activated upon IGF-1 stimulation (Figure 5A), while the control $G_{\alpha s}$ (Figure 5B) is not. Furthermore, this activation occurred regardless of RTK inhibition with AG1024 (Figure 6). This is the first report combining kinase inhibition with IGF-1-induced $G_{\alpha i}$ activation. The present observations differ from those made for the EGFR, another single transmembrane-spanning non-classical GPCR (Nair & Patel, 1993), for which G protein coupled cellular effects do require RTK activity. However, $G_{\alpha s}$ has been shown to be phosphorylated in the case of the EGFR (Poppleton et al., 1996), while GTP exchange is responsible for activating $G_{\alpha i}$ in the case of the IGF-1R (Figure 5). This further supports the idea that IGF-1R-induced initiation of $G_{\alpha i}$ signaling involves a mechanism separate from direct phosphorylation.

At this time, it has not been determined what residues within the cytoplasmic domain of the IGF-1R are potentially responsible for interaction with, and activation of, $G_{\alpha i}$. However, it has been shown that truncation of the C-terminal domain of the IGF-1R abolishes MAPK phosphorylation with minimal effects on autophosphorylation (Sehat et al., 2007). It appears therefore that the C-terminal of the receptor may be important. Studies examining segments of the C-terminal of the receptor will be necessary to uncover both the binding properties and activation mechanism of $G_{\alpha i}$.

3. A direct interaction between the IGF-1R and both G protein α_{i1} and α_{i2} is possible, with differential binding preferences.

While it now appears clear that IGF-1R signaling does overlap with that of G proteins, past experiments have shown that even co-precipitation does not clearly indicate

interaction (Sieg et al., 2000). In an interesting case of indirect G protein activation by the PDGF receptor, the group of Alderton et al. (2001) showed that $G_{\alpha i}$ was activated upon PDGF-BB stimulation. However, it involved PDGFR "tethering" to GRK2, a GPCR interacting protein, and therefore resulted in indirect activation of $G_{\alpha i}$. Thus, whether the IGF-1R is capable of accomplishing the task of directly activating the G protein will be dependent on the nature of the interaction between the two.

The interaction between the receptor and its G protein has not been studied beyond co-precipitation. In this study, direct interaction was detected *in vitro* in the absence of any potential complexing effectors (Figure 7). Binding, however, was not specific to one $G_{\alpha i}$ isoform, as both GST-$G_{\alpha i1}$ and GST-$G_{\alpha i2}$ precipitated the IGF-1R, with seemingly similar affinity, although $G_{\alpha i2}$ did appear to bind more. Further investigation placing the isoforms in a competitive setting with a limited amount of receptor presented a contradicting result, with $G_{\alpha i1}$ binding being more prevalent (Figure 8). The only other study examining $G_{\alpha i}$ isoforms in connection with the IGF-1R, that of Kuemmerle and Murthy (2001), indirectly showed activation of $G_{\alpha i2}$ by IGF-1. While their results do not perfectly coincide with those of the present study, they also do not contradict them. A more complex picture may be necessary to represent the reality of the situation. Receptor binding to more than one G_{α} is in fact not uncommon (Reviewed in (Gudermann et al., 1996)), even within the different isoforms of a certain class. Of interest, coupling to more that one G_{α}, specifically both $G_{\alpha q/11}$ (Imamura et al., 1999) and $G_{\alpha i}$ (Moxham & Malbon, 1996), has even been demonstrated in the case of the structurally similar IR. The results of the present study are consistent with a model in which the IGF-1R would preferentially bind to $G_{\alpha i1}$ at rest but could also bind $G_{\alpha i2}$ following IGF-1 stimulation and

$G_{\alpha i1}$ release. In addition, this may provide an explanation for the constitutive presence of G_{α} even following IGF-1 stimulation (Figure 3), since the antibody used was neither class nor isoform specific.

4. The $G_{\beta\gamma}$-subunit is involved in receptor signaling leading to p42/44 MAPK activation, a pathway involved in regulation of both migration and proliferation of VSMCs

When Luttrel et al. (1995) first suggested that IGF-1 MAPK activation was mediated by $G_{\beta\gamma}$, he did not comment on the importance of this specific pathway in cellular activity. Work by Dalle et al. (2001) and Kuemmerle and Murthy (2001) used the C-terminal of the β-adrenergic receptor kinase (βARKct) (a peptide capable of sequestering $G_{\beta\gamma}$ (Koch et al., 1994)) and an anti-G_{β} antibody, respectively, to show significant attenuation of IGF-1-induced MAPK activation and support the conclusions attained by Luttrel et al. (1995). The results of the present study correspond with this, despite the use of an alternate strategy for $G_{\beta\gamma}$ inhibition (Figure 9).

The two major contributions of SMCs with respect to atherogenesis and restenosis lie in their proliferative and migratory responses. In their study, Kuemmerle and Murthy (2001) suggested that two distinct and additive growth pathways are initiated upon IGF-1R activation: One would be MAPK-dependent, initiated by $G_{\alpha i}$, while the other would be PI3K-dependent, initiated by the RTK. They arrived at this conclusion by targeting $G_{\alpha i}$ with PTX. In the present study, BrdU incorporation was measured in SMCs in various conditions including inhibition of the RTK, of PI3K and of $G_{\beta\gamma}$ directly. We show that $G_{\beta\gamma}$ inhibition is able to attenuate proliferation to a similar degree as RTK and PI3K inhibition (Figure 10). While this does not contradict the idea of two distinct

pathways, it suggests that, at least in vascular SMCs, they do not function in an additive manner to determine the extent of cell proliferation. Rather, they appear to be mutually exclusive pathways, each being as potent as the other. This also agrees with a model in which both kinase activation and G protein signaling activation are mutually exclusive (Figure 6). The study by Dalle et al. (2001) who measured BrdU incorporation in IGF-1 stimulated fibroblasts inhibited by βARKct reached conclusions similar to those of this study with respect to their $G_{\beta\gamma}$ target. While they did find significant inhibition of IGF-1-induced MAPK activation, they did not compare their results with RTK inhibited cells.

Cell migration in connection with this novel G protein coupled pathway has not yet been studied. The importance of migration in the development of vessel occlusion led us to test the role of IGF-1-$G_{\beta\gamma}$ signaling in migration. The results of this further support the presence of two equally potent pathways activated by IGF-1 (Figure 11).

The CCK-8 results for the cell proliferation assay differed from those for the cell migration assay. (Figure 12A & B, respectively). The cell content was similar in all treatments in the proliferation assay, despite a significant amount of proliferation in the IGF-1 and PDGF treatments. In contrast, the conditions in the protocol for the migration assay did cause a decrease in cell number. While this is interesting in itself, suggesting that the anti-apoptotic effects of IGF-1 are in fact attenuated with long term treatment, it reached the same extent in all of the groups and therefore does not change the interpretation among the groups. However, TUNEL and LIVE/DEAD assays may be helpful in evaluating the toxicity of the treatments and the respective protocols.

A notable study by the group of Iaccarino et al. (1999) investivated the role of $G_{\beta\gamma}$ signaling in revascularization. They used the βARKct peptide in a rat carotid artery

injury model *in vivo* to inhibit general $G_{\beta\gamma}$ signaling. Using an adenoviral delivery method, they demonstrated a significant attenuation of the VSMC proliferative response. This was further evidence that $G_{\beta\gamma}$ targeting holds significant potential in the attenuation of restenosis. Admittedly, IGF-1 is not the only vascular agent signaling via G proteins and $G_{\beta\gamma}$ specifically, although it is one of few vascular GFs with a demonstrated $G_{\beta\gamma}$-MAPK link. As both proliferation and migration are shown here to be significantly attenuated by $G_{\beta\gamma}$ inhibition (Figures 10 & 12), it is reasonable to speculate that inhibition of locally produced IGF-1-induced $G_{\beta\gamma}$ signaling at least contributed to the decreased neointima observed in the above study.

5. The $G_{\alpha i}$-subunit mediates IGF-1R signaling leading to phosphatase activation, to translation initiation and to histone modification.

Whether G_{α} has a role in signaling downstream of the IGF-1R is a question which has thus far remained unresolved, as some studies have suggested no change in G_{α} coupling (Dalle et al., 2001; Hallak et al., 2000), while one suggested a role in inhibiting adenylyl cyclase and thus lowering cAMP levels, which lead to cell growth (Kuemmerle & Murthy, 2001). Of note, however, is that the latter study, which was the only one suggesting a role for G_{α}, was also the only one performed in non-rodent primary cells. This, combined with the observation that more than one G_{α} could bind to the receptor (Figure 7), suggests that G_{α} signaling may indeed be active downstream of the IGF-1R in porcine VSMCs, despite no observed change in coupling (Figure 3).

In order to study IGF-1R-$G_{\alpha i}$ signaling in greater detail, there was a need to develop a specific inhibitor for $G_{\alpha i}$. The importance of the extreme C-terminal of G

proteins for receptor specificity has been known for many years, (Conklin et al., 1993; Sullivan et al., 1987), and, indeed, a peptide expressed from a bacterial vector has been shown to inhibit signaling (Gilchrist et al., 1999). Primary SMCs, however, demonstrate very low transfection efficiency, leaving a limited number of viable options available for the transfer of exogenous material into cells. Viral (adeno- or lenti-) vector delivery or conjugation of cell penetrating protein transduction domain (PTB) sequences are two strategies developed for use on hard-to-transfect cells. The TAT-sequence is one such PTB which was discovered within the HIV, and it is used by the virus for both cell penetration and replication. The cell penetrating activity of this sequence was discovered by accident in 1988 (Frankel & Pabo, 1988) and a variety of experiments have allowed shortening of the original 86 aa sequence to a 9-mer basic aa-rich sequence (described in (Vivès & Lebleu, 2002)). While the entry mechanism remains controversial, it is thought to involve absorptive endocytosis (Mann & Frankel, 1991). Since this sequence has been shown in a variety of cell types to be both versatile and non-toxic (Sugita et al., 2008) and importantly, since it has been shown to enter SMCs (Renigunta et al., 2006), it was chosen for this study and conjugated upstream of the C-terminal sequences of $G_{\alpha i}$ and the control $G_{\alpha s}$ during peptide synthesis (Table 3).

Thus, using the TAT-conjugated $G_{\alpha i}$ C-terminal peptide inhibitor (TAT-$G_{\alpha i}$), it was of interest to determine whether $G_{\alpha i}$ was active downstream of the IGF-1R. The results demonstrate that the TAT sequence is functional in the cell model used for this study (Figures 13 & 15) and that the inhibition potential of the G_{α} C-terminal is not lost within SMCs (Figure 13). Of note, no dose response was observed with the tested concentrations of the peptide. It is therefore likely that an even lower dose can be used

without compromising inhibitory potential. However, even with these potentially high concentrations, the non-toxic nature of the TAT-conjugated peptides was confirmed (Figure 14). Having established these parameters, the inhibitor was used in a phospho-antibody microarray analysis, performed with the help of Kinexus Bioinformatics. This analysis allowed screening of approximately 270 potential phospho-sites within cells which have either been IGF-1-stimulated, or IGF-1-stimulated with $G_{\alpha i}$ inhibition (Table 5).

Among the proteins whose phosphorylation in response to IGF-1 is shown to be significantly affected by $G_{\alpha i}$ inhibition is the eIF4E, a protein involved in directing ribosomes to the cap structure of mRNAs (Gross et al., 2003). The involvement of IGF-1 in stimulating protein synthesis/translation is in fact a documented concept (Rommel et al., 2001), as is the involvement of eIF4E (Burgos & Cant, 2010; Liu et al., 2006). However, this phenomenon has thus far been attributed to the involvement of the mammalian Target of Rapamycin (mTOR), a general growth signal within most cell types (reviewed in (Hay & Sonenberg, 2004). The discovery that $G_{\alpha i}$ is involved in the activation of eIF4E is a new and exciting concept. Whether $G_{\alpha i}$ is involved via mTOR activation, or alternatively, if it is involved in a separate but complementary pathway, is a question which cannot yet be answered. However, this finding does integrate IGF-1-induced G protein coupled signaling in another very important cellular growth process.

A second protein shown to be mediated by $G_{\alpha i}$ is the PP1-α catalytic subunit. This serine phosphatase is one of only four eukaryotic cytosolic serine phosphatases, and has a variety of targets depending on the particular regulatory subunit to which it is bound (Virshup & Shenolikar, 2009). As opposed to its effects of eIF4E, $G_{\alpha i}$ appears to

decrease the phosphorylation levels of the phosphatase (Table 5). However, in the case of PP1, phosphorylation at T320 results in inhibition. Therefore, IGF-1 activates the phosphatase via $G_{\alpha i}$. Because the effectors of PP1 are dependent on a regulatory subunit, the direct pathway targeted by IGF-1-$G_{\alpha i}$ cannot be determined with certainty. One group has shown that IGF-1-induced PP1 activation results in activation of eukaryotic translation initiation factor 2B (eIF2B), acting in opposition to GSK3 (Quevedo et al., 2003). This suggests a potential target for $G_{\alpha i}$-activated PP1-α, downstream of the IGF-1R and would need to be further investigated. However, it is interesting in that it further involves $G_{\alpha i}$ signaling in IGF-1-induced protein synthesis. In addition, the idea of PP1-α opposing GSK3 creates an interesting link between IGF-1 signaling and glucose metabolism. An alternative target of PP1-α is the anti-apoptotic protein BAD (Ayllon et al., 2000). Dephosphorylation of BAD leads to apoptosis, and may be a pathway by which IGF-1-stimulated cells counter the anti-apoptotic signal provided by PI3K/Akt signaling.

Histone modification is a common mechanism in the regulation of gene transcription. As reported in Table 5, IGF-1-induced $G_{\alpha i}$ signaling also targets gene expression via histone phosphorylation. Potential targets cannot be discerned with the current information as IGF-1 stimulation was acute. Investigation using a chronic IGF-1 stimulation would provide information as to which genes were regulated by IGF-1-induced $G_{\alpha i}$ signaling. A role for histone phosphorylation, and particularly H3 at T11, has also been recently identified in mitosis, specifically in early prophase to early anaphase (Preuss, et al., 2003). Although its role is unclear, it may correlate with the level of chromosome condensation or their "readiness" for separation (Hans & Dimitrov,

2001). In this context, the inhibition of phosphorylation observed with $G_{\alpha i}$ inhibition may involve this G protein-dependent pathway in the activation of a very distinct signaling cascade.

Interestingly, the results provided by the microarray analysis do not correspond with the observation of Kuemmerle and Murthy (2003), who showed inhibition of adenylyl cyclase activity by IGF-1-induced $G_{\alpha i}$ signaling. In our study, there was no observed change in AMPKα1/2 phosphorylation levels, based on the microarray analysis (Table 6). Since the C-terminal peptide inhibitor inhibits both $G_{\alpha i1}$ and $G_{\alpha i2}$, the use of different isoforms in the two studies cannot explain the different results. It is plausible, however, that this discrepancy is the result of an incomplete phospho-analysis, considering the fact that only 270 phospho sites were scanned with this array. With respect to the study by Dalle et al. (2001), it was of interest to consider the phosphorylation changes to β-arrestin. As seen in Table 6, there was no significant change in β-arrestin phosphorylation with $G_{\alpha i}$ inhibition. However, Dalle et al. (2001) studied β-arrestin association with the IGF-1R via immunoprecipitation and did not investigate its phosphorylation state. In addition, as indicated by the study of Girnita et al. (2007), the actions of β-arrestin may be modulated via ubiquitination, a post-translational modification that is not detected by this microarray analysis.

While the microarray analysis is indeed a powerful tool as it is thought to be 10-fold more sensitive than standard Western blotting (Kinexus Bioinformatics, 2010), the use of non-denatured proteins increases the risk of both false positives and false negatives. Thus, follow-up studies which include repetition and Western blotting will be necessary to fully validate these results. In addition, many holes remain within the

different putative signaling pathways as thousands of phosphorylation sites are not examined using the microarray. Nevertheless, the results provide valuable preliminary data into the signaling implications of the G_{α} component.

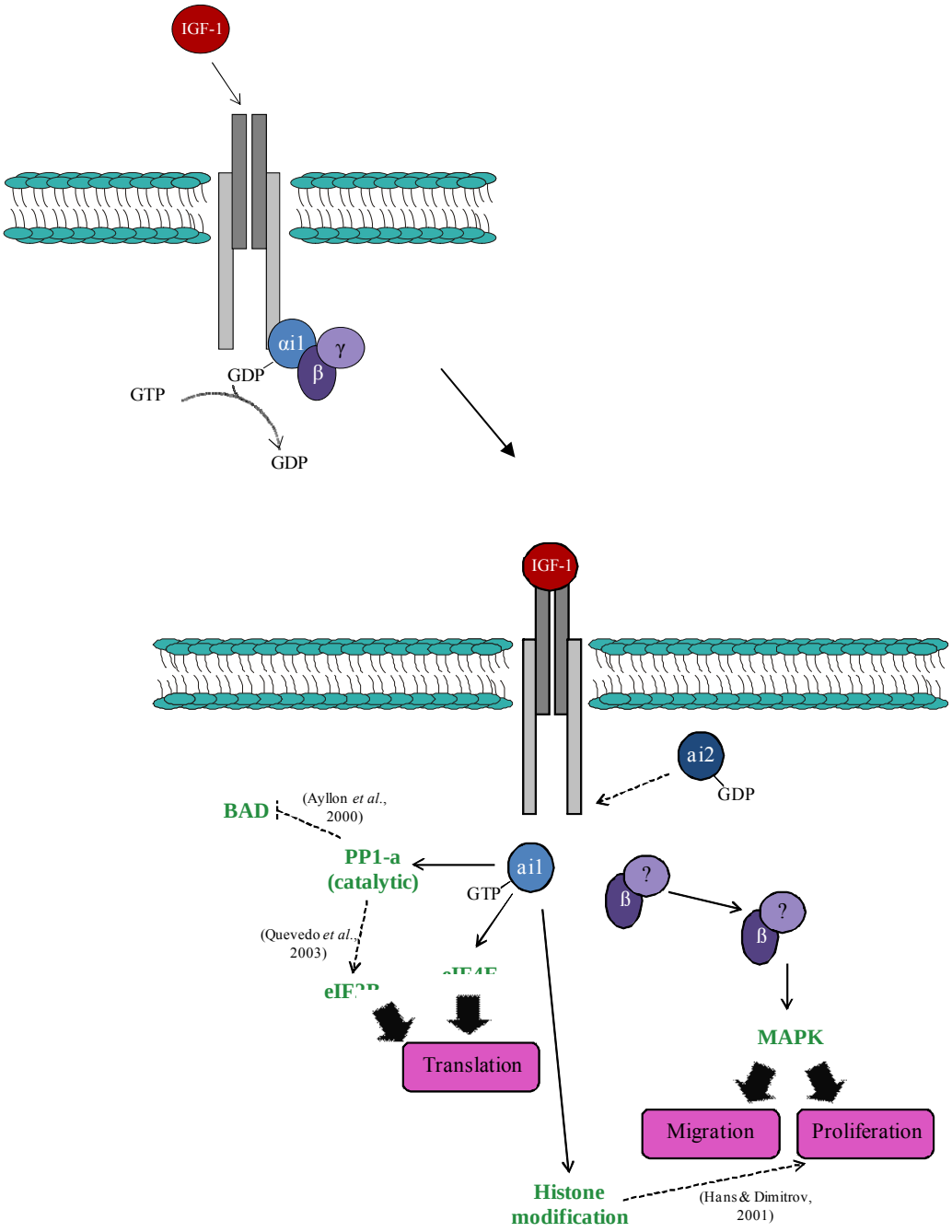
CONCLUSIONS

IGF-1R signaling in vascular SMCs is important within the general context of atherosclerosis through both its growth and anti-apoptotic effects, in addition to its unique implications in insulin signaling. The conclusion of this study has provided an overview of the structural aspects of G protein coupling to the IGF-1R in VSMCs and established a starting point for all further studies of IGF-1R signaling in vascular cells. All of our objectives were met and these allowed us to reach the following conclusions. First, it was concluded that the IGF-1R does cellular initiate responses that are modulated through a G protein in SMCs. Next, the IGF-1R is indeed coupled to a heterotrimeric G protein, and $G_{\alpha i}$ is the specific class that is activated via GTP exchange upon IGF-1R stimulation. It was determined that interaction between the receptor and its G_{α} component is direct and non-specific with respect to the two $G_{\alpha i}$ isoforms. Finally, it was shown that both G_{α} and $G_{\beta\gamma}$ components are involved in signal transduction, with $G_{\beta\gamma}$ involved in the MAPK proliferative and migratory responses of VSMCs to IGF-1, and $G_{\alpha i1}$ involved in both translational and transcriptional regulation, in addition to cytosolic phosphatase activation.

Figure 16 combines the above conclusions into a comprehensive model of G protein coupled signaling by the IGF-1R in vascular SMCs. The relevance of such basic molecular research within the context of future treatments and preventative therapies often remains elusive until the culmination of years of work is combined. This study, in conjunction with existing data regarding vascular growth factors and atherosclerosis, introduces novel signaling intermediates that may serve as targets for pharmacological intervention to block SMC proliferation and migration, and thus their pathophysiological

sequelae. The dual role of this growth factor presents it as an ideal target for protection against both diabetic atherosclerosis and post-surgery restenosis. While the relevance of this pathway *in vivo* remains unknown, the model used suggests that it is likely involved in the vascular responses in humans. Specifically, the non-metabolic nature of the pathway presents advantages for targeting diabetic hyperinsulinemia without disrupting important baseline metabolism. In addition, since the use of the TAT-conjugated peptides appears to be non-toxic, animal studies utilizing gene eluting technology remains a viable option for locally targeting this pathway. However, it is clear that much research remains before any ideas can be applied to the human disease.

Figure 16: *hG protein coupled signaling downstream of the IGF-1R in vascular smooth muscle cells.* Proposed model for hG protein coupled signaling downstream of the IGF-1R combining the conclusions reached in this study. Arrows represent activation and blunted ends represent inhibition. Solid lines indicate verified signaling pathways and the dotted lines are extrapolation suggested by non-conclusive results of this study and the existing literature. IGF-1 binding to its receptor results in $G_{\alpha i1}$ GTP exchange by an unknown mechanism, thus causing G protein dissociation and initiation of signaling by the separate subunits.



FUTURE DIRECTIONS

1. Scan members of the remaining G protein α classes for other potential binding partners of the IGF-1R

2. Elucidate the pathway by which $G_{\beta\gamma}$ leads to MAPK phosphorylation using a phospho-antibody microarray

3. Perform Western blot validation studies to confirm the results of the $G_{\alpha i}$-inhibited and $G_{\beta\gamma}$-inhibited phospho-antibody microarrays

4. Study the mechanism by which the IGF-1R initiates G protein signaling and determine important residues in the C-terminus of the receptor

5. Verify the specificity and degree of inhibition of the TAT-$G_{\alpha i1/2}$ C-terminal inhibitor peptides by use of a $G_{\alpha i}$ shRNA

6. Perform functional assays to determine whether inhibition of the $G_{\alpha i}$ pathway translates into significant changes in SMC proliferative, migratory or phenotypic responses such as cell size.

7. Expand the hypothesis to study other RTKs such as PDGF to determine whether the pathway is unique to the IGF-1R

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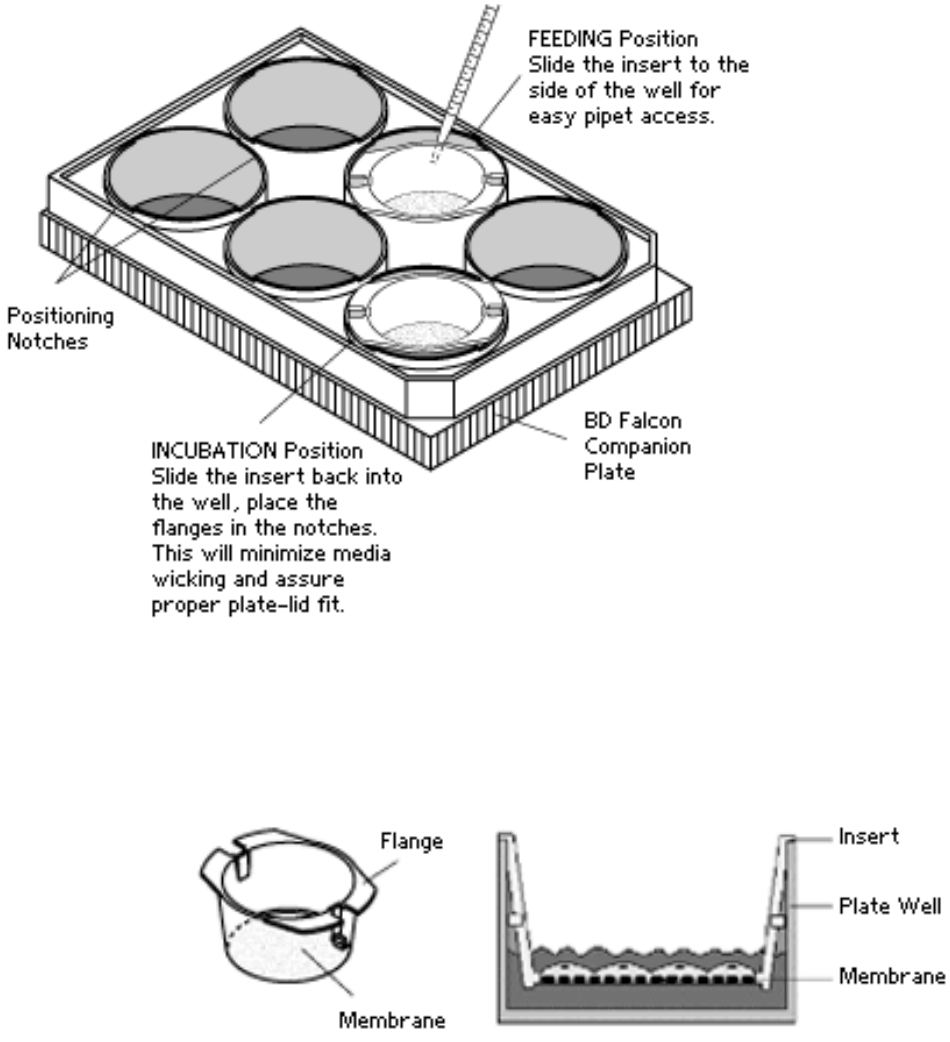
Supplemental Figure 1: *Cell migration culture insert system*

(View text for description)



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