THE CARDIOPROTECTIVE EFFECTS OF FIBROBLAST GROWTH FACTOR-2 AGAINST ISCHEMIA-REPERFUSION INJURY IN THE ISOLATED RAT HEART

BY

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

UNIVERSITY OF MANITOBA

WINNIPEG, MANITOBA

SEPTEMBER, 1997

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

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ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. Elissavet Kardami, whose wisdom and knowledge in science has guided me to persevere in becoming a dedicated researcher. She helped create in me the necessary cognitive processes by which a scientist approaches a problem. She modeled to me the abilities of inductive and deductive reasoning necessary for scientists to hypothesize, experiment and theorize via the scientific process. She reminded us that as researchers our responsibility lies in furthering human knowledge to benefit the world around us. Her creativity and enthusiasm in cardiovascular research is indeed inspiring and will no doubt guide me in my future endeavors in research.

I would like to thank the following collaborators in my research project whose contributions were essential in fulfilling this FGF-2 cardioprotection story: Dr. G.N. Pierce and his technician T. Maddaford for the constant flow Langendorff preparation and ATP assays; Dr. W.C. Cole and his technician C. McPherson for the right ventricular wall perfusion preparation, measurements of APD_{90} and back perfusion pressure; Dr. V. Panagia, his student, Dr. C-H. Yu and his post-doctoral fellow Dr. P.A Tappia for the preparation of cardiac sarcolemma and cytosol and PLC activity assays; Dr. N.S. Dhalla and Dr. R. Sethi for the pilot experiments in Langendorff preparations; Dr. P. Zahradka for the PKC activity assays; my fellow Ph.D. student B. Doble for successfully producing recombinant FGF-2 in our lab and being a wiz with computers; and R.R. Fandrich for his technical experience and wicked western blot gels.

I would like to further thank the members of me advisory committee for their guidance, support and interaction: Dr. G.N. Pierce, Dr. N. Khalil, Dr. A Lucas, Dr. A. Zarain-Herzberg and Dr. B.S. Tuana.

Thanks also to the members of the Kardami lab past and present: Rob, Brad, Lei, Guangping, Pierre, Yijing, Lena, Sujata, Otto, Jin, Tanya, Andy, Janice, Sarah, Neela, Roxanne, Jit, Justin, Jason and Rodel, whose long and short term friendship and camaraderie made this lab an enjoyable and exciting place to work in.

Thanks also to my fellow members of the Institute of Cardiovascular Sciences whose friendship and dedication was a tremendous asset in shaping my academic career and helping make the St. Boniface Research Center a stimulating and friendly place to do research.

I would like to especially state my appreciation to my parents and family for their love and support in those long and trying years and to my daughter Raquelle for keeping my head from the clouds and reminding me I am still her Daddy.

ABSTRACT

Ischemic heart disease is a major cause of death in industrialized societies. Myocardial ischemia occurs when there is an interruption of blood flow to the heart. Restoration of blood flow, termed reperfusion, is required to salvage the ischemic heart from further injury. Reperfusion itself may cause further damage exacerbating the injury induced by ischemia. Ischemia-reperfusion (I-R) injury is seen during heart attacks, and may can occur in patients undergoing heart transplantation, angioplasty, coronary bypass surgery and anti-thrombolytic drug therapy. Thus interventions which increase cardiac resistance to I-R injury are of clinical interest.

FGF-2 is a multifunctional protein which is present in the heart and other organs, and exerts its biological effects by binding to tyrosine kinase receptors at the cell surface. In addition to being a potent mitogen, FGF-2 appears capable of protecting differentiated neuronal cells from injury, degradation and death. In cultured cardiomyocytes, FGF-2 administration prevented cell damage induced by H_2O_2 and serum starvation. We therefore examined whether FGF-2 would act in a cardioprotective manner in cardiac I-R injury.

We used *ex-vivo* adult perfused rat heart models subjected to global ischemia followed by reperfusion. These models included isolated whole heart Langendorff preparations perfused under conditions of constant flow or constant pressure and the isolated right ventricular wall preparation perfused under conditions of constant flow. All experiments were conducted under normothermic conditions. FGF-2 was administered either prior to the induction of ischemia to address its effects on normal cardiac function, and on recovery from I-R, or during reperfusion to examine if it could be protective after

ischemia. Contractile function was measured using a pressure and force transducer. Under both constant flow and constant pressure perfusion conditions and in all model systems, FGF-2 administered prior to ischemia induced significant improvement in recovery of contractile function over respective controls as indicated by measurements of DF, DP, resting tension, EDP, ±dP/dt, and ±dF/dt. FGF-2-treated hearts released less CPK (an indicator of myocardial damage) in the perfusate compared to controls, attesting to improved myocardial integrity. FGF-2 administration to the right ventricular wall model prior to ischemia induced a significant recovery in contractile function and a complete recovery of APD_{90} compared to controls. Thus FGF-2 protects isolated hearts from the ionic imbalances and the cellular damages which occur during I-R injury. In addition, FGF-2 administration prior to ischemia induced a negative inotropic effect, indicated by measurements of DF, DP and ±dP/dt in right ventricular preparations perfused under conditions of constant flow, and whole ventricular preparations perfused under conditions of constant pressure. Finally, administration of FGF-2 after ischemia and during reperfusion promoted a significant recovery in contractile function (DF, DP, resting tension, EDP, ±dP/dt, and ±dF/dt) over controls, demonstrating that FGF-2 continues to be cardioprotective against I-R injury even when administered in the reperfusion phase only.

FGF-2 was found to induce increased levels of ATP and CP prior to ischemia and a significant preservation of ATP and CP levels after I-R compared to control hearts. In addition better preservation of ATP and especially CP levels was observed in perfused hearts treated with FGF-2 during reperfusion only, compared to controls. Increases in

ATP and CP levels upon FGF-2 administration before ischemia are likely the result of the FGF-2-induced negative inotropic effect and are probably contributing to the subsequent protection against contractile dysfunction after I-R, compared to controls. FGF-2, however, is likely to exert additional effects which result in protection of the myocardium from injury, as indicated by its effects when administered during reperfusion.

FGF-2 is reported to activate PKC in some systems, and PKC activation by several effectors and by ischemic preconditioning is believed to mediate cardioprotection. We therefore examined whether inhibition of PKC by a specific blocker, chelerythrine chloride, would affect FGF-2-induced cardioprotection. Our results demonstrated that the improvement in the recovery of contractile function and negative inotropic effect induced by FGF-2 administration prior to I-R injury, were abolished by chelerythrine. It would thus appear that PKC activation is necessary for the cardioprotective effect of FGF-2.

Measurements of the effect of FGF-2 on back perfusion pressure indicated a very minor vasodilatory effect compared to the effect of SNP, suggesting that FGF-2 induced vasodilation may play only a minor role in FGF-2 induced cardioprotection.

We then used immunoblotting and immunofluorescent staining on FGF-2 perfused hearts to obtain some information as to potential cell types affected by administered FGF-2 in the myocardium. FGF-2 administration resulted in a 4-fold increase in heart-associated FGF-2 over controls, and in strong increases in anti-FGF-2 staining in blood vessels, capillaries and around cardiomyocytes implicating all the participating cell types as targets for direct FGF-2 action.

In addition, we obtained evidence that administered FGF-2 can activate signaling cascades in cardiomyocytes: Immunofluorescence staining of cardiac tissues sections with specific antibodies revealed increased levels of phosphorylated tyrosine residues associated with cardiomyocyte intercalated disks in areas of increased anti-FGF-2 pericellular staining, consistent with a cause and effect relationship. FGF-2, administered by direct injection to the heart apex, increased overall tyrosine phosphorylation as assessed by western blotting.

In addition to the use of a PKC blocker, we sought more direct evidence for PKC activation by FGF-2 in the cardiomyocytes in situ. To this end, we obtained purified sarcolemmal and cytosolic preparations and analyzed them for PKC enzymatic activity and PKC translocation (and thus activation) at the sarcolemma. FGF-2 induced a significant increase in PKC enzymatic activity associated with sarcolemmal membranes, an increase that was abolished by chelerythrine. As assessed by western blotting, FGF-2 administration induced increased levels of PKC subtypes -α, -δ, -ε, and -ζ at the sarcolemma. Administration of chelerythrine alone resulted in decreased levels of PKC-ζ and possibly PKC-ε in association with the sarcolemma, while levels of subtypes -α and -δ did not change substantially. Administration of FGF-2 to chelerythrine-treated hearts resulted, in all cases, in reduced levels of sarcolemmal associated PKC compared to samples from FGF-2-only treated hearts. Immunolocalization studies showed a translocation and presumably activation of PKC-δ to the cardiomyocyte sarcolemma confirming data from subcellular fractionation. Since FGF-2 induced translocation of PKC

isoforms -α, -δ, -ε, and -ζ coincides with increased PKC activity, it is likely that some or all of these PKC isoforms participate in FGF-2 induced cardioprotection.

βARK1, a serine/threonine kinase, regulates contractility of the heart by phosphorylating α1-adrenergic receptors, uncoupling them from their G-proteins. Since this process can be activated by PKC and PKC is activated by FGF-2, we investigated whether administered FGF-2 can activate βARK1 in the isolated perfused heart. Results from western blotting analysis show increased levels of βARK1 at the sarcolemma of FGF-2 perfused hearts. This increased translocation to sarcolemmal membranes most likely indicates βARK1 activation by FGF-2. Indeed, this FGF-2-induced increase in sarcolemma associated βARK1 coincides with FGF-2 induced negative inotropic effect and may thus represent a mechanism by which FGF-2 affects contractile function.

The effects of FGF-2 on additional enzymatic systems *in situ* were also examined. Measurements of total inositol phosphate showed increased total PLC activity at both the sarcolemma and cytosolic fractions of FGF-treated hearts compared to control hearts. Western blotting with specific antibodies to PLC-γ1 (linked to tyrosine kinase receptors) and to δ1 and β3 (linked to G-protein signaling) showed that FGF-2 induced changes in subcellular distribution of all PLC subtypes. Our data point at a correlation between increased PLC activity at the sarcolemma and increased levels of PLC-β3 and/or PLC-δ1-like enzymes at that site, suggesting that FGF-2 may have stimulated the activity of these G-linked enzymes implicating for the first time FGF-2 in G-protein signaling in the heart.

To determine whether MAPK, a mediator of FGF-2 signaling in various cell systems, including isolated cardiomyocytes was activated by FGF-2 in perfused hearts, Western blotting was performed on purified sarcolemmal and cytosolic preparations from control, FGF-treated, FGF-treated in the presence of chelerythrine and chelerythrine alone treated hearts, using antibodies specific for the p42 and p44 MAPK and their dually phosphorylated and thus activated forms. Our data showed that FGF-2 caused an increase in MAPK levels at the sarcolemma suggesting the presence of potential MAPK-targets at this location. In addition, Western blot results show increased dually phosphorylated MAPK isoforms at both cytosolic and sarcolemmal fractions with FGF-2 treatment demonstrating that FGF-2 activates MAPK in cardiac myocytes *in situ*. This activation persisted even in the presence of chelerythrine, suggesting that activation of MAPK may not be essential for the cardioprotective effect of FGF-2. Our data also indicated that in contrast to popular belief, p42 and p44 MAPK activation does not occur downstream of PKC.

Finally, FGF-2 was shown to increase nuclear localization of the proto oncogene c-fos, in both myocytes and smooth muscle cells, and to induce changes in distribution of the proto oncogene c-jun and the non-receptor tyrosine kinase src.

Since FGF-2 administration to the heart during reperfusion was also protective against I-R injury, we looked for changes in the subcellular distribution of some signal transduction proteins to see whether they may be involved in cardioprotection under these conditions. Preliminary data have indicated that FGF-2- induced changes in the localization of PLC-β3 and c-fos in the reperfused heart after ischemia are similar to those

induced by this factor in the pre-ischemic heart. This would suggest c-fos and the Gq protein activated PLC-β3 may participate in the FGF-2 induced cardioprotective effect against reperfusion injury.

In conclusion, we have shown that FGF-2 is cardioprotective against I-R injury in a chelerythrine-inhibitable fashion, implicating PKC in the mechanism of protection. This cardioprotection induced by FGF-2 against I-R may be receptor mediated as indicated by increased levels of phosphorylated tyrosine residues upon FGF-2 administration. In addition, we have provided evidence indicating that FGF-2 administration results in translocation and presumably activation of several PKC subtypes within cardiomyocytes, in the context of the whole heart, suggesting that FGF-2 may directly increase resistance of these cells to injury *in situ*. This FGF-2-induced cardioprotection seems to be independent of MAPK activation and may involve the activation and/or translocation of the various PLC isoforms, βARK1, c- fos, c-jun and src.

Possible clinical applications for the cardioprotective effects of FGF-2 against I-R injury include: administration immediately after a heart attack; during restoration of blood flow to ischemic regions upon angioplasty or coronary bypass surgery; or during heart transplantation. More research into how FGF-2 protects against I-R injury may lead to an optimization of FGF-2 therapy and the utilization of endogenous FGF-2 to combat cardiomyocyte damage.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
AP	Action potential
APD	Action potential duration
aPKC	Atypical protein kinase C
ATP	Adenosine triphosphate
AV	Atrio-ventricular
βARK1	Beta-adrenergic receptor kinase-1
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Clonal DNA
CEC	Chelerythrine chloride
cGMP	Cyclic guanidine monophosphate
Chl	Chelerythrine chloride
CNS	Central nervous system
Cl^-	Chloride
CNS	Central nervous system
cPKC	Conventional protein kinase C
CO_2	Carbon dioxide
CP	Creatine phosphate
CPK	Creatine phosphokinase

Co.	Company
DAG	Diacyl glycerol
DF	Developed force
dF/dt	Derivative of the rate of change in developed force
DNA	2-Deoxyribonucleic acid
DOG	1,2-Dioctanoyl-sn-glycerol
DP	Developed pressure
dP/dt	Derivative of the rate of change in developed pressure
ECG	Electrocardiogram
ECM	Extra cellular matrix
EDP	End diastolic pressure
EDRF	Endothelial relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetra-acetic acid
ERK	Extracellular-signal-regulated kinases
Fig.	Figure
FGF-2	Fibroblast growth factor-2
FGFR	Fibroblast growth factor recptor
G protein	GTP-binding protein
GTP	Guanidine triphosphate
HETE	Hydroxyeicosatetraenoic acid

HSPG	Heparan sulphate proteoglycan
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IgG	Immunoglobulin G
I_{Na}	Sodium current
I_{ki}	Inward rectifying K^+ channel
IP3	Inositol 1,4,5 triphosphate
I-R	Ischemia-reperfusion
K^+	Potassium
K^+_{ATP}	ATP-sensitive potassium channels
Kv	Delayed rectifier potassium channel
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
L-NMMA	L-N-monomethylarginine
L-NAME	NG-nitro-L-arginine methyl ester
LVEDP	Left ventricular end diastolic pressure
MAPK	Mitogen activated phosphokinase
MARKS	Myistoylated alanine-rich protein kinase C substrate
MBP	Myelin basic protein
MDA	Malonyldialdehyde
min.	Minutes
mRNA	Messenger ribonucleic acid

β-MHC	Beta-myosin heavy chain
NO	Nitric oxide
nPKC	Novel protein kinase C
NOS	Nitric oxide synthase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC12	Rat adrenal pheochromocytoma cell line
PDGF	Platelet derived growth factor
pI	Isoelectric point
PI3	Phosphatidylinositol 3 kinase
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PIP2	Phosphatidylinositol-4,5-diphosphate
PI	Phosphatidylinositol
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13 -acetate
PMSF	Phenylmethyl-sulfonyl fluoride
PtdIns	Phosphatidylinositide
PtdSer	Phosphatidyl Serine
rDNA	Ribosomal deoxyribonuceic acid
RT	Resting tension

RTK	Receptor tyrosine kinase
S2	Rabbit anti-FGF-2 serum characterized in reference Kardmi et al, 1990
SA	Sino-atrial
SDS	Sodium dodecyl sulphate
SE	Standard error
α-SKA	Alpha-skelatal actin
SL	Sarcolemma
SNP	Sodium nitroprusside
SPT	8-(p-sulfophenyl)theophylline
SR	Sarcoplasmic reticulum
STMR	Seven member spanning receptor
TBST	Tris buffered saline with tween
TCF	Ternary complex factor
TEMED	N,N,N',N'-Tetra-methyl-ethylenediamine
TGF-β1	Transforming growth factor beta-1
t-PA	Tissue plasminogen activator
Tyr	Tyrosine
VEGF	Vascular endothelial cell growth factor

UNITS OF MEASUREMENT

°C	Degrees celcius
cmH_20	Centimeters of water
cpm	Counts per minute
g	Gram
Hz	Hertz
kDa	Kilodalton
l	Liters
M	Molarity
mM	Millimolarity
mg	Milligrams
mmHg	Millimeters of mercury
min.	Minutes
ml	Milliliters
ms	Milliseconds
µ	Micro-
µg	Microgram
µl	Microliter
µm (µmol)	Micromole
%	Percent
wt.	Weight

CHAPTER 1

LITERATURE REVIEW

1.1 ISCHEMIC INJURY

Clinically, ischemic heart disease is a complex disorder which is manifested in many forms and levels depending on the cause, length of time, and severity of the ischemia. In the heart, ischemia occurs when there is an imbalance between oxygen demand of the working heart and the amount of blood and oxygen supplying it. This imbalance usually occurs when coronary arteries situated along the epicardial surface experience a partial or total blockage of their lumen. As a result, myocardial infarction (cell death) can occur [1,8]. Concurrent with the decrease in blood supply to the working heart, the supply of metabolic substrates (such as glucose and oxygen) become diminished and catabolites (such as H^+ and lactate) accumulate in the injured region since no blood flow is present to wash them out [1,8,10]. Thus, as reviewed by Opie et al [1] and others [8, 10, 91] underperfused dysfunctional ischemic tissues are characterized biochemically and physiologically by: depressed arterial blood flow; the occurrence of hypoxia; disruption of the aerobic respiration processes; depletion of cardiomyocyte substrate supplies; the presence of anaerobic glycolysis; the accumulation of metabolic end products of ischemia; depressed contractile function; cardiac arrhythmia and altered action potential profile [1,8,10,91].

Contractile failure, during ischemia, is characterized by a decrease in contractile force and in rates of contraction and relaxation [1,10,53,94]. Patients suffering contractile

failure exhibit decreased cardiac output, poor circulation of blood and oxygen to tissues and eventual cardiac failure [1].

A number of mechanisms have been proposed to explain the occurrence of contractile failure during times of oxygen stress and ischemia. The reduction of cytosolic ATP and glucose during ischemia, for instance has been proposed to contribute to contractile failure [1,10,79]. With this depletion of cytosolic ATP and glucose, increased action potential duration (APD) shortening occurs and Ca^{2+} release from the cytosol is prevented. Contractile failure ensues as increased intracellular Ca^{2+} eventually leads to contracture and decreased cardiac performance [1,10,53].

Alternatively, the accumulation of ischemic products are thought to also contribute towards ischemia-induced contractile failure. Studies have shown that with the intercellular buildup of metabolic waste products (such as lactic acids, H^+ ions, inorganic phosphates and CO_2), acidosis can occur within the cell [1,10,79]. Increased cytosolic H^+ ions, in turn, are thought to replace Ca^{2+} ions from their binding sites on the thin contractile filaments, thus inhibiting the actin-myosin cross-bridging and reducing myocardial contractility.

Alterations in action potential and ECG patterns due to ischemia have also been shown to be clinically manifested by patients with ischemic disease. These alterations in action potential are thought to occur because of depressed levels of intracellular ATP which, in turn, can induce the activation of K^+_{ATP} channels and thus shorten the action potential duration [36]. As well, with increased extracellular K^+ accumulation outside the

myocyte, an increased resting membrane potential and decreased amplitude of phase 0 of the action potential is seen in these ischemic cardiomyocytes [37-40].

Increased intracellular Ca^{2+}concentration in myocytes during ischemia has been correlated with the development of ventricular fibrillation and other arrhythmias [4-7]. According to Mochwat et al. [2] this increased intracellular Ca^{2+}during ischemia has been shown to be due to the effects of long-chain acylcarnitines and lysophosphatidylcholine via three potential mechanisms: (1) inhibition of Na/K-ATPase leading to increase in $[Na^+]i$ and thereby activating Na^+/Ca^{2+} exchange; (2) inhibition of Ca^{2+}-ATPase activity in the SR, leading to a reduction in net Ca^{2+} uptake and increased intracellular concentration of Ca^{2+}; and (3) delayed inactivation of the Na^+ channel leading to an increase in intracellular Na^+, and enhanced Na^+/Ca^{2+} exchange [2].

1.1.2 Reversible and irreversible ischemia

According to Ip et al. [8], reversible ischemia is described as the initial period of ischemia where cardiomyocytes may recover fully from permanent ischemic damage if reperfused between 10 to 20 minutes after coronary artery occlusion. Metabolically, during this period of reversible ischemia, hearts show a rapid depletion of myocardial high-energy phosphate compounds along with glycogen stores. In addition, other biological changes occur, such as peripheral aggregation of nuclear chromatin, and mild tissue and myocyte swelling. However, despite these changes, reversibly ischemic cardiomyocytes still show viably intact sarcolemmal and cell membranes. Upon reperfusion these reversibly injured myocytes display a restoration of high energy phosphate compounds and a reversal of any ischemically induced structural changes [8].

Irreversible ischemia occurs during prolonged periods of coronary occlusion (>20 min.). In this case, irreversibly injured myocytes displayed a disruption of their sarcolemma, disorganization of mitochondrial cristae, and cessation of anaerobic glycolysis. Loss of the cytoskeletal protein vinculin from the periphery of cardiomyocytes is an early sign of irreversible ischeamia [95].

1.1.3 Experimental models of ischemia

As reviewed by Karmazyn [10], there have been different experimental models of ischemic injury ranging from in vitro isolated cell model to in vivo whole animals. The primary in vivo ischemic model of injury used is the regional infarction model of the in situ heart. The advantage of this model is that it has been shown to mimic clinical ischemic conditions closely. The disadvantage, however, is that one cannot control systemic effects of ischemia such as collateral blood flow, and the presence of nerve endings, endothelial cells and other tissues. These systemic effects prevent researchers from manipulating experimental conditions whereby ischemic effects on cardiomyocytes alone can be examined [10].

In vitro models, on the other hand, allowed researchers to examine the effects of I-R injury on cardiac myocytes and coronary vascular cells without the participation of blood borne factors. Examples of these in vitro models included the hypoxic or ischemic Langendorff perfused heart [11-13] and the isolated adult cardiomyocytes [14], subjected to hypoxia, metabolic inhibition, or ischemia. However, the disadvantage of using perfused heart preparations was shown to include the development of reperfusion deficits (no reflow) in the subendocardium of ischemia-reperfused hearts following the development of

ATP depletion-induced rigor contracture [54,55]. In addition, the isolated cardiomyocyte has the disadvantage of possessing possible residual damage caused by the isolation procedure itself. Also, both the perfused heart and the suspended isolated myocyte hypoxic models were shown to differ from true in vivo ischemia in that although there is a limited amount of oxygen and substrate to the in vitro isolated heart, there is still a continuous flow or a large extracellular space to wash out metabolic end products.

Despite the differences and disadvantages of the various experimental models, all of the models were shown to produce a severe depletion of ATP prior to irreversible injury [56-61, 64, 67-70, 72]. This severe depletion of ATP with ischemia implied that the irreversible ischemic injury is directly or indirectly a result of ATP depletion from within the cardiomyocyte.

1.1.4 Global ischemia in experimental animal models or during surgical bypass surgery and heart transplantation

One type of myocardial ischemic model which is seen both clinically and experimentally is the global ischemic model. In this case, the whole heart is deprived of its coronary flow and oxygen supply, for example, by cross-clamping the aorta of isolated perfused animal hearts [1,10,65] or during cold cardioplegic arrest of hearts during surgical coronary artery bypass or heart transplants [1]. This results in arrest of cardiac mechanical work and provides a standard laboratory model of severe total ischemia that can be followed readily by restoration of the coronary flow with reperfusion. Never the-less, similar to regional ischemia, global ischemia was also shown to display heterogeneity

of phenotype since microzones of total ischemia can develop side by side with other zones of ischemia in which cardiac tissue is much better perfused [9].

1.1.5 Ultrastructural changes in cardiomyocytes during ischemia

Irreversible injury and rupture of myocyte membranes is thought to occur as a result of the stresses imposed on the myocyte by the hypercontracture [70-74], osmotic swelling [64, 75] and weakened cell membrane [75,76] that occurs during ischemia at a time of depleted energy production. Decreased energy production is thought to lead to alterations in ionic regulation and increased Ca^{2+} influx into the cell, hypercontracture and formation of contraction bands, cytoskeletal disruption and thus plasma membrane detachment and bleb formation.

Irreversibly injured myocytes display detachment of gap junctional connections between cells [65]; detachment of sacomeres from intercalated discs and from one another at the Z bands [62,66]; formation of plasma membrane blebs [54,62,63]; detachment of intermediate filaments at Z and M lines at the sarcolemmal and outer nuclear membranes, and at cellular organelles (such as mitochondria) [77]. In addition, immunofluorescence studies have shown decreased cytoskeletal proteins, such as vinculin and α-actinin in irreversibly injured ischemic myocytes [77,78].

1.1.6 Alterations in metabolic energy production with ischemia

According to Opie [15], during the initial few seconds of ischemia as the remaining oxygen in the tissue is diminished, there is a shift in the physiological way energy is produced in the myocardial cell. Instead of energy being produced via fatty acid degradation in the mitochondria, the ischemic cardiomyocyte shifts its production of

energy towards anaerobic glycolysis [15]. One consequence of this metabolic shift is that the amount of ATP produced by the cell is decreased because anaerobic glycolysis yields only five percent of the regular amount of ATP. This decreased ATP production was correlated with the depletion of intracellular glycogen stores during ischemia as glucose supply in the tissue diminishes [16,17].

As ischemia becomes more severe, glycolytic metabolites such as protons, inorganic phosphates, $NADH_2$ and lactate are no longer washed out. These accumulated glycolytic metabolites lead to a decreased intracellular pH, inhibition of glycolytic enzymes, such as phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase, and further decreased ATP production [18-20]. In response, ischemic cells initially counteract this process by activating various pH regulating cellular ion transport systems such as lactate transport [21], Na^+ dependent HCO_3^- transport [22] and Na/H exchange [23,24]. However, as the ischemic damage becomes more severe, ATP production and intracellular pH becomes further decreased, intracellular sodium is increased, and there is a decrease in function of the various pH regulating transport proteins. This dysfunction was attributed to the accumulation of protons in the extracellular space [25,26], decreased availability of HCO_3^-, decreased lactate gradients across the cell and decreased availability of ATP due to decreased ischemic intracellular pH (or pH_i) [16].

Several consequences have been shown for the ischemically-induced decreases in intracellular myocardial pH and ATP. One consequence seen is a decreased myocardial contractility observed very soon after the beginning of ischemia [16,27-29]. Since ATP is required for the contractile process, a reduction of myocardial contraction can be expected

with ATP deficiency. However, the reported drop of ATP of only about 10 to 15% [16,30] in this first phase of ischemia does not correlate well with the observed fast reduction in myocardial contraction. This decreased contractility during early ischemia can still be explained by the possible existence of a critical ATP pool. Due to its close proximity to the sarcolemma, researchers [10] speculated that SLM-derived ATP may act as a critical modulator of membrane-associate ATP-dependent processes involved in contractility, such as Na^+/K^+ or Ca^{2+} ATPase activity or the maintenance of ionic channels. Therefore, contractile dysfunction seen during early ischemia may be attributed to the depletion of SLM-derived ATP pools, possibly as a consequence of intracellular phosphate accumulation [10]. In contrast to ATP, the faster decrease in intracellular pH [24,31-33] seen during the onset of ischemia, gave a much better correlation with the rapid decline in contractile dysfunction. This better correlation between rapidly diminishing intracellular pH and myocardial contractility during ischemia was in agreement with diminished binding of Ca^{2+} to the contractile elements in acidified myocardial cells [16,28,34] and reduced activity of the myosin-ATPase at low pH [16,34,35]. In addition, myocardial contractility was very sensitive to only small reductions in intracellular pH [16,33].

Another consequence of cytosolic ATP depletion is the accumulation of extracellular potassium and shortening of the action potential due mainly to the opening of the K^+ATP channels [36]. This opening of the K^+ATP channels has been shown in several animal models of ischemia including hypoxic myocytes [37], globally ischemic hearts [38] and regionally ischemic hearts [39,40] and is thought to be an endogenous

cardioprotective response by the heart to reduce arrhythmia, precondition the heart, maintain vascular tone and increase coronary flow [42].

In addition, with diminishing intracellular ATP concentrations, ATP dependent Na-K ATPase pump becomes inactivated during early ischemia. This inactivation of Na-K ATPase pumps has been shown to contribute to the accumulation of extracellular K^+ caused by activated K^+ATP channels. With inactivation of these pumps, intracellular Na^+ is no longer pumped out of the cell against its concentration gradient and extracellular K^+ ions are no longer pumped back into the cell in exchange for Na^+. Furthermore, other potassium channels during ischemia, such as those regulated by arachidonic acid and intracellular sodium, were shown to also play a partial role in the extrusion of K^+ out of the cell [41], which in turn, help promote the shortening of the action potential and contribute to the cardioprotective response by the myocardial cell.

1.1.7 Ionic changes in the cardiomyocyte during ischemia

With depletion of high energy phosphate compounds and fall of intracellular pH during ischemia, other ionic changes were shown to occur in the intracellular and extracellular milieu with respect to Ca^{2+} and Na^+. Researchers have shown that there is an intracellular rise in Na^+ and Ca^{2+} during ischemia. These researchers hypothesized that this increased cytosolic Na^+ and subsequent Ca^{2+} rise may play an important role in the development of myocardial ischemic injury [43]. As of yet though, it is unclear what is the relationship between the time course in rise of $[Na^+]_i$ and $[Ca^{2+}]_i$ with the time course of ischemic injury. However, studies have suggested that the mechanism for the rise in $[Na^+]_i$ and $[Ca^{2+}]_i$ during ischemia involves Na^+/H^+ exchange coupled to Na^+/Ca^{2+} exchange [44].

During ischemia, with the fall of intracellular pH by increased anaerobic glycolysis, there is a rise in intracellular sodium after 10 min. of ischemia, probably due to activation of the Na^+/H^+ exchanger. This increase in $[Na^+]_i$ is complemented by the decreased activity of the Na^+/K^+-ATPase [44] preventing intracellular Na^+ extrusion. Subsequently, a parallel increase in $[Ca^{2+}]_i$ is observed, presumably due to the activation of the Na^+/Ca^{2+} exchange in response to increased $[Na^+]i$ [45]. This rise in intracellular Ca^{2+} is further augmented by the decreased sarcoplasmic recticulum Ca^{2+}-ATPase activity caused by decreased intracellular ATP levels so that cytosolic Ca^{2+} is no longer accumulated by the sarcoplasmic reticulum [45].

1.1.8 Altered action potential profiles with ischemia

With the changing intracellular ionic milieu and energy requirements, changes in action potential profile were shown to occur. For instance, a shortening of AP duration (APD) was observed during acute myocardial ischemia. This APD shortening was attributed to an increase in K^+ outward current [46] and a decrease in slow inward current resulting in the loss of intracellular K^+ [47] from the myocyte and buildup of extracellular K^+ [48]. These altered levels of intracellular and extracellular K^+ from the myocyte is partially mediated by: (1) decreased ATP levels, which in turn, mediate the opening of the K^+_{ATP} channels [49] thus decreasing the myocyte APD; (2) co-ionic loss of potassium with negatively charged lactate and phosphate ions from the myocardial cell, and (3) an inhibition of Na -K pump due to decreasing levels of local ATP [1]. Thus, with the increased conductance of K^+ out of the cell, there is a hyperpolarization of the resting membrane potential away from the voltage required for optimal opening of the Na^+

channel resulting in a lessened upstroke and duration of the ischemic AP [1]. This ischemic AP profile seems to be species specific since it was observed during ischemia in rabbits [50], dogs [51], and guinea pigs [52] but not in rats [53].

1.1.9 Protein and enzymatic markers for myocardial damage during ischemia

In addition to alterations of the biochemical and ionic milieu, a number of changes in serum enzyme concentrations was shown to occur upon ischemically induced myocyte damage. These altered serum enzymes can be effectively used as indices for myocardial ischemia and infarction. According to Sobel et al. [79], excreted serum enzymes were shown to originate: either directly from the ischemically injured heart; from other organs which are subjected to diminished perfusion after myocardial infarction; or from connective tissue or blood elements in the ischemic heart [79]. For instance, in the ischemic heart, intracellular mitochondrial enzymes such as creatine phosphokinase (CPK) [80-83] and lactate dehydrogenase (LDH) [82-86] become depleted from injured necrotic myocytes. According to Sobel et al. [79], the depletion of LDH and CPK is a consequence of both enzymatic degradation from ischemic cells and leakage of intracellular enzymes through damaged membranes, thereby increasing their serum concentrations [79]. Other mitochondrial enzymes which may be used as indices for ischemica-induced myocardial damage include transaminase and adenolate kinase [79]. These ischemic marker enzymes usually exist in different isoforms depending on the tissue from which they originated. For instance, in the heart, the predominant isoforms for CPK and LDH were shown to be CPK-MB [87-95] and LDH-I [94], respectively.

Besides enzymes, other factors secreted into the serum that can be used as a measure of the severity of myocardial ischemia and infarction are noradrenaline [81], glutathione [82], histamine [86], lactate [83], cardiac troponin T [90] and malonyldialdehyde (MDA) [89]. The release of these protein factors from the heart can be easily quantitated from perfusates of isolated hearts, serum from diseased patients and whole animal models; and from direct measurement of the ischemic tissue itself.

1.2.10 REFERENCES: Chapter 1.1 Ischemia

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1.2 REPERFUSION

1.2.1 Introduction

To stop the damaging effects of ischemic heart disease ischemic tissue must be reperfused rapidly to replace the depleted oxygen and energy supply and remove the accumulated metabolic wastes. A variety of reperfusion interventions have been used clinically to treat ischemic heart disease [1]. These treatments included: anti-thrombolytic agents such as streptokinase and urokinase [2]; coronary bypass surgery [3,4]; and balloon angioplasty [3,5]. These various interventions, if performed immediately after the initial infarct, have been shown to promote recovery of the cardiac function and stop further myocyte necrosis [6]. However, although reperfusion of ischemic tissues is necessary to restore cardiac function, the reperfusion process itself can also induce serious compromise to myocyte viability and function leading to further injury. The mechanism for this paradoxical event of reperfusion injury remains to be fully elucidated.

1.2.2 The role of ischemia in determining cardiac injury during reperfusion

In determining a mechanism of I-R (ischemia-reperfusion) injury, investigators have found that one cannot totally separate the events that occur during ischemia from those that occur during reperfusion. Indeed, the extent of myocardial recovery with reperfusion has been shown to be dependent on the type and severity of ischemia, the length of ischemia and the extent of collateral blood flow [1,7,8,9].

However, it is this dependency of reperfusion injury on the severity and duration of ischemia that has led to the controversy of whether or not reperfusion injury

really exists as a distinct entity or whether it represents an acceleration of cell death of already lethally injured tissue. This controversy, is compounded by the fact that many of the interventions used to reduce reperfusion injury must be implemented before ischemia prior to reflow. This phenomenon is particularly evident with agents such as calcium blockers [11] which are shown to protect the heart only when administered before ischemia. Regardless of the involvement of ischemia in the generation of reperfusion injury, reperfusion injury itself is associated with distinct events including free radical generation, neutrophil activation, enhanced eicosanoid synthesis, activation of the Na^+-H^+ exchanger, and abrupt Ca^{2+} influx, all of which could contribute to cell injury. For a recent review see reference [13]. Free radical generation in particular, was shown to be a distinct reperfusion event since rapid free radical generation is seen only during times of coronary blood flow restoration [12].

1.2.3 Experimental models of reperfusion injury

Reperfusion injury has been demonstrated in a number of experimental models. In *in vivo* studies of reperfusion injury, animals are subjected to coronary artery ligation for various periods of time followed by reperfusion. In *in vitro studies*, as outlined in a review paper by Karmazyn [6], isolated heart and cell preparations are used to study reperfusion injury [6]. In these isolated preparations, global or zero-flow ischemia is induced by a total cessation of coronary flow, or low-flow ischemia was induced by a partial interruption of coronary flow.

However, as reviewed by Karmazyn [6], a number of inherent advantages and disadvantages are seen with each type of I-R injury model. In isolated heart

preparations, the use of a saline based perfusion buffer has allowed investigators to elucidate possible mechanisms of reperfusion injury outside the involvement of blood-born factors. However, the disadvantage of excluding blood-born factors, such as neutrophils and platelets, from the experimental condition is that their contributions to the development of reperfusion injury is not examined. In comparison, *in vivo* regional ischemia-reperfusion animal models, such as the rat coronary artery ligation model, was found to be an excellent representation of reperfusion injury in the clinical setting. However, the disadvantage to *in vivo* models is that it does not allow determination of the exact mechanisms of I-R injury and protection by various agents [6]. Regardless of the experimental model employed, all I-R injured myocytes were shown to display similar characteristics such as: ultrastructural changes, electrical disturbances, defects in energy metabolism and ionic and biochemical irregularities [6].

1.2.4 Reperfusion injury in the clinical setting

Reperfusion of ischemically injured tissues has been shown to occur under different clinical conditions. These conditions have been extensively outlined in a review paper by Czubryt and Pierce [13]. In this paper, the authors propose four types of clinical conditions of reperfusion injury: coronary artery vasospasm, coronary angioplasty, cardiac by-pass surgery, and thrombolytic therapy [13]. In coronary arterial vasospasm, reperfusion of post- ischemic tissue is achieved upon spontaneous or drug-induced relaxation of vasospastic vessels [13-16]. In balloon angioplasty, the removal of an atherosclerotic block from the coronary artery using a balloon catheter has been shown to successfully restore the blood flow to oxygen deprived tissues

[3,4,19]. In anticoagulant therapy, the use of anticoagulants following ischemia is shown to restore blood flow to the ischemic tissue through the enzymatic degradation of the arterial occlusion [3,4,19]. In cardiac by-pass surgery, the heart is usually paced under conditions of low-flow ischemia for varying periods of time prior to restoration of blood flow and attachment of the new arterial graft [3,4,19].

1.2.5 Biological features of the post-ischemic heart

The biological features of reperfusion of the ischemic heart was shown to be dependent on the time point reperfusion is initiated. For instance, myocardial stunning is a reversible type of ischemia that is defined as "a transient left ventricular dysfunction that persists after reperfusion, despite the absence of irreversible damage and the restoration of normal or near-normal coronary flow" [38]. In other words, in stunned hearts, reflow is initiated during the reversible phase of ischemia and characterized by a preservation of cell viability, resumption of aerobic respiration, proper washout of accumulated metabolites, the disappearance of electrocardiographic changes, and restoration of electrolytic and water content [19]. The exact mechanism for stunning in reversibly injured hearts during reperfusion is unknown. However, depressed or stunned hearts were shown to display depressed adenine nucleotide pools [20-22] and increased intracellular calcium [18]. This increase in intracellular calcium and depression of adenine nucleotide pools may, in turn, affect the energy metabolism and the myofibrillar contractility of reperfused hearts.

In contrast, rapid and striking changes have been shown to occur upon early reperfusion of irreversibly injured myocytes [23,24]. Sarcomeres become

supercontracted and form contraction bands. Sarcolemmal plasma membrane become severely damaged leading to increased intracellular calcium accumulation and leakage of creatine kinase out of the cell. With increased intracellular calcium accumulation, increased calcium deposits are observed within the mitochondria [25]. Changes in cell volume are shown to also increase swelling of the cell [19]. This cell swelling is characterized by increased intracellular edema, and ultrastructural changes such as the appearance of intracellular vacuoles, mitochondrial swelling and sarcolemmal blebs [19,23,24].

When reflow is initiated during late periods of irreversible ischemia, further detrimental changes occur. For instance, the no-reflow phenomenon of coronary arteries was shown to occur in ischemic myocardium after 60-90 minutes of ischemia [26,27], particularly in areas of the myocardium in which significant capillary or myocyte swelling is present at the time of reperfusion [26-29]. This no- reflow phenomenon of the coronary arteries and capillaries is characterized by an endothelial vasodilator dysfunction and increased neutrophil adherence [31]. Furthermore, after 3 hours of reperfusion, the extent of myocyte injury was shown to be dependent on the location of ischemic cells within the myocardial wall [30]. As reviewed by Reimer et al. [30], ischemic myocytes in the subepicardial region of the myocardium are found to be more salvageable after a period of 30-90 minutes of ischemia followed by 3 hours of reperfusion. In contrast, in the midwall of the myocardium, although the vasculature tissue was salvageable, myocyte necrosis occurred. In the subendocardial region, both myocyte necrosis, vasculature injury and no reflow phenomenon occurred [30]. Thus in

irreversibly injured hearts, reflow can cause further deterioration in myocytes and coronary vessels.

1.2.6 Reperfusion-induced vascular dysfunction

Microvascular dysfunction has also been shown to occur during reperfusion of the ischemic myocardium [1]. With long periods of ischemia (>30 minutes) reperfusion of the coronary vasculature has been shown to lead to a sustained reduction in blood flow when compared with pre-ischemic levels. This reduction in blood flow has been commonly referred to as the "no-reflow" phenomenon [31]. As previously mentioned, the possible mechanisms for this reflow phenomenon have been postulated to involve: (1) ischemia-reperfusion induced endothelial cell dysfunction; (2) ischemic contracture of the myocardium which squeezes the coronary arteries and prevents normal flow [31,32]; and (3) increased basal tone of coronary vasculature and obstruction by neutrophils [33,35].

One mechanism for this I-R induced microvascular dysfunction can be related to the impairment of the endothelial vasodilator reserve [35]. Studies have shown, that endothelium derived mediators, such as nitric oxide and prostacyclin regulate vessel tone by opposing thrombus formation and vessel occlusion and by inhibiting adhesion and aggregation of neutrophils [36,37]. However, during ischemia-reperfusion, nitric oxide and prostacyclin release is reduced by endothelial cells within the ischemic region. This decrease in vasodilator release is correlated with an increased constrictor tone, increased adherence of platelets and neutrophils to the coronary endothelium and

eventual decrease in coronary flow. As a result, a decreased recovery in myocardial fucntion is observed upon reperfusion [36,37].

1.2.7 Mechanism for reperfusion induced injury of cardiomyocytes

In terms of cardiomyocytes themselves, studies have shown a variety of mechanisms which are responsible for mediating the myocardial dysfunction and necrosis that occur upon reperfusion of ischemic tissue. These mechanisms are thought to act in concert with one another to produce reperfusion damage. The two primary mechanisms of importance in mediating ischemia-reperfusion damage have been shown to involve increased intracellular calcium concentrations (or calcium overload) in myocytes; and the generation of free radicals upon reperfusion of the post-ischemic heart. Other mediators of reperfusion injury described in the literature all involve directly or indirectly either the generation of free radicals or further increases in intracellular calcium. Table 1 depicts some of the known mediators summarized by Karmazyn et al. [6] for the generation of reperfusion injury in the myocyte:

Table 1: Some factors potentially mediating myocardial reperfusion injury [6]

Oxygen free radicals

Calcium

Adrenergic receptors

Amphiphiles

Arachidonic acid and its metabolites

Glycolytic products

Defective aerobic metabolism

Osmotic factors

Intracellular ionic derangement

Loss of membrane integrity

1.2.8 Role of calcium in the development of reperfusion injury

Calcium entry into the post ischemic myocyte is a major factor in the generation of ischemia-reperfusion injury. Numerous studies so far have documented a rise in intracellular calcium in ischemic cardiomyocytes after reperfusion [39-42] and that damage occurs as a result of this rise. This intracellular rise was described by Zimmerman and Hulsmann [43] as the calcium paradox event. In this paradoxical event, with the increased intracellular calcium during reperfusion, tissue damage, enzyme release, contracture and reduction in high energy phosphate stores occurs. The mechanism by which increased calcium ions enter the ischemic cardiomyocyte during reperfusion and cause injury has been the focus of many studies.

One mode of calcium entry into reperfused ischemic heart was shown to involve the Na^+-H^+ exchanger [17]. The Na^+-H^+exchanger is an ion transport or antiport protein which catalyzes the transport of intracellular H^+ across the cardiac sarcolemmal membrane in a 1:1 exchange for extracellular Na^+ [44,45]. The mechanism for this exchange involves ischemia-induced acidosis within the ischemic myocyte. This intracellular acidic environment activates the Na^+-H^+ exchanger [46,47]. In addition to ischemia-induced acidosis, the Na^+-H^+ exchanger becomes activated during reperfusion via a ischemia-induced increase of phosphoinositide turnover [48]. This

increased phosphoinositide turnover induces an increased production of diacyl glycerol and IP3. This is significant since PKC is activated by diacyl glycerol and that PKC activation is correlated with increased activation of the Na^+-H^+ exchanger in numerous tissues [57-60]. With Na^+-H^+ exchange activity stimulated, there is a rise of intracellular [Na]i within the cell as H^+ is extruded extracellularly down its concentration gradient. The Na/Ca exchanger, in turn becomes activated in response to the rise in intracellular Na^+. This exchanger acts to increase intracellular Ca^{2+} as it extrudes excess Na^+ out of the cell [51]. In addition, this pathway of Ca^{2+} entry is augmented by the inhibition of Na^+/K^+ ATPase by the prior ischemia. Inhibition of Na^+/K^+ ATPase increases intracellular Na^+ by preventing the cell extrusion of Na^+ in exchange for K^+ by this ATP driven pump [40]. A number of studies have been published to support this pathway of Ca^{2+} intrusion into the myocyte cell during reperfusion [40,51,52]. In addition, when Na^+-H^+ [13] and Na/Ca [51] exchangers were inhibited, there was a better recovery of contractile parameters following ischemia-reperfusion. Thus, as reviewed by Pierce et al. [13], the Na^+-H^+, Na/Ca antiport systems as well as the Na/K-ATPase pump play an important role in the development of reperfusion injury through increased intracellular calcium.

Other modes of Ca^{2+} entry into the myocyte were shown to involve the voltage operated Ca^{2+} channel [52]; activation of ß-adrenergic receptor [53]; phosphorylation of calcium channels by PKC [54] and decrease in ATP stores and production [54]. The involvement of voltage operated Ca^{2+} channels in the intracellular rise of Ca^{2+}_i during reperfusion was first shown by Nayler et al. [52] who blocked Ca^{2+} entry into post-

ischemic hearts using nifedipine. In addition, ß-adrenergic stimulation was also shown to enhance the probability of Ca^{2+} channel opening. Furthermore, Otani et al. [53], showed that during reperfusion, phosphorylation of Ca^{2+} channels by PKC in post-ischemic myocytes enhanced Ca^{2+} channel opening. Intracellular rise of Ca^{2+} was also shown to be enhanced by the depletion of glycolytic ATP stores [55]. This ATP depletion was shown to decrease the extrusion of cytoplasmic Ca^{2+} by the ATP dependent sarcoplasmic recticulum Ca^{2+}-ATPase, thus enhancing the reperfusion induced calcium overload within post-ischemic myocytes.

However, recent research has shown that glycogen depletion during preconditioning may actually enhance recovery against ischemia reperfusion injury. For instance, in various rat heart models, improved contractile recovery, as a result of preconditioning [61] is correlated with rapid glycogen depletion. On the other hand, other researchers have shown that: rapid glycogen depletion does not participate in the protective effects of preconditioning and agents which mimick preconditioning (eg. adenososine and bradykinin) in isolated rat [62] and rabbit hearts [63]; that elevated glycogen levels in isolated rat heart actually improves contractile recovery after treatment with hydrogen peroxide [64]; and that reduced glycogenolysis is correlated with a reduction in ischemia-induced acidosis and improved contractile recovery[65].

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1.3 CARDIOPROTECTIVE TREATMENTS AGAINST ISCHEMIA-REPERFUSION INJURY

A number of treatments have been shown to be cardioprotective against ischemia-reperfusion injury. These agents included: Ca^{2+} channel blockers (such as diltiazem [1], verapamil [4,5] and nifedipine [2,3]); β-blockers (such as carvedil [6]); angiotensin converting enzyme (ACE) inhibitors (such as enalapril [7]); Na^{+}-H^{+} exchange inhibitors (such as amiloride [8] and its analogues dimethylamiloride [9] and methylisobutylamiloride [10]); free radical scavengers (such as vitamin E, vitamin C, and glutathione [11]); nitric oxide derivatives (such as L-arginine [12] and sodium nitroprusside [13]); preconditioning [14]; PKC activators [14]; adenosine [15-17]; and K^{+}_{ATP} channel openers [18]. These treatments were shown to work via a number of different mechanisms which share a commonality in the end result, that is, the reduction of calcium and, depending on the agent, a decrease in free radical damage before, during and after ischemia-reperfusion.

How each one of these cardioprotective agents works has been the subject of extensive research to protect the heart against ischemia-reperfusion damage. Many studies have used various in vitro and in vivo models of I-R injury, including cell cultures in which reperfusion injury was stimulated by adding H_2O_2 to the culture medium [19,28]; ex vivo I-R experiments on isolated animal heart preparations using a Langendorff perfusion apparatus [20]; in vivo animal models in which temporary regional coronary occlusion was induced [21].

Evidence for the protective effects of these treatments was obtained by employing various blockers and/or activators of the specific channels, receptors or signal transduction

proteins which induced or prevented cardioprotection against I-R. K_{ATP} channel openers, such as bimakalim [21], were also shown to be cardioprotective against I-R injury [18]. Bimakalim acted by reducing the cell's action potential duration (APD), which, in turn, limited the amount of time calcium channels remain open during the action potential's plateau phase. The protective effect of these K_{ATP} channel openers was inhibited by such K_{ATP} channel blockers as glibenclamide [26] and 5-hydroxydeconoate [27]. In addition, ischemic preconditioning of the heart was shown to also induce cardioprotection against I-R injury by a mechanism involving adenosine-induced A1 receptors, PKC activation and K_{ATP} channel opening [23].

Cardioprotective treatments studied so far have differed in their effectiveness against I-R injury depending on whether they are administered before ischemia or upon reperfusion. Calcium channel blockers [2,3,22] and most K^{+}_{ATP} channel openers [28] were shown to be effective only upon pre-ischemic treatment of hearts. The only K^{+}_{ATP} channel opener shown to be cardioprotective when administered not only before, but also after ischemia, was nicorandil [29]. This was attributed mainly to the ability of nicorandil to activate K^{+}_{ATP} channels before ischemia as well as, during reperfusion, due to its nitrite property which confers vasodilation and inhibition of neutrophil activity. Other cardioprotective agents which have been shown to protect the heart against I-R injury when administered before ischemia or upon reperfusion included: nitric oxide donors (such as L-arginine [25]), antioxidants [30,31], and Na/H exchange inhibitors [9,35,36].

The mechanisms used by these various agents to confer cardioprotection against reperfusion injury were shown to be different. Nitric oxide and its donors (L-arginine)

induce cardioprotection in isolated neonatal lamb [33] and rat [34] hearts when administered directly after the ischemic injury largely through the inhibition of neutrophil-mediated injury and adherence to coronary vessels, improved coronary flow and improved endothelial cell function [33,34] In addition, administration of free radical scavengers (such as catalase, superoxide dismutase, reduced glutathione, glutathione peroxidases and the xanthine oxidase inhibitors (allopurinol and oxypurinol)) to postischemic myocardial tissues, were shown to prevent reperfusion injury [32]. These agents worked by scavenging free radicals (such as 0^{2-}, OH- and H_2O_2) either produced locally in ischemic and reperfused myocardial tissues or from infiltrating free radical generating neutrophils during reperfusion [31].

Na/H exchange inhibitors are shown to be cardioprotective against I-R injury when administered before as well as after ischemia [9,35,36]. Studies employing isolated rat hearts showed that post-ischemic treatment with Na/H exchange inhibitors (such as 5-(N,N-dimethyl)amiloride) was able to induce protection by inhibiting Na entry into the ischemic or post-ischemic cell [36]. With decreased Na_i, there is a reduction in the Na/Ca exchange activity. Reduced Na/Ca exchange activity, in turn leads to reduced Ca entry into the cell and thus, calcium overload is attenuated [37].

Thus, the use of exogenous treatments which either block or activate specific channels, receptors or signal transduction proteins has led researchers to discover new and different pathways and mechanisms of I-R injury as well as different modes of cardioprotection. In this literature review, the different modes of cardioprotection against I-R that will be concentrated upon will be: the role of NO and its derivatives; K_{ATP} channel

openers; and PKC and preconditioning. Although, the other modes of cardioprotection, such as free radical scavengers and Na/H exchange blockers may represent possible modes by which FGF-2 may exert its cardioprotective effects against I-R injury, nothing in the literature has yet directly linked these with FGF-2. In contrast, FGF-2 has been shown to promote the release of NO [39,40], activate the opening of K^+_{ATP} channels [39], and activate PKC [38].

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1.4 PRECONDITIONING AND ROLE OF PROTEIN KINASE C (PKC)

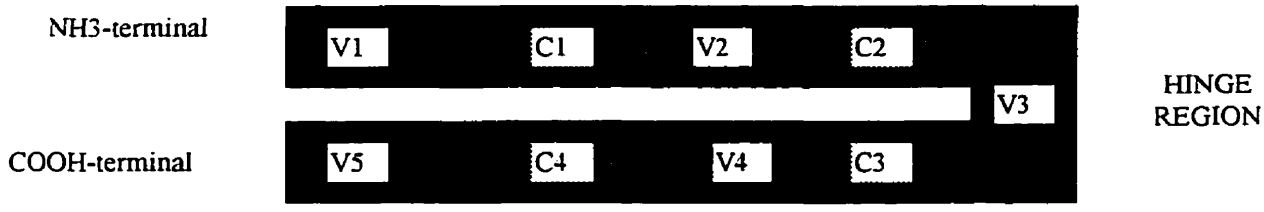
Ischemic preconditioning is defined as a protective adaptation by the heart against long periods of ischemic injury when it is pre-exposed to shorter periods of ischemic stress. Preconditioned hearts display reduced cellular damage and physiologically detrimental ventricular arrhythmias upon reperfusion [16]. Understanding the preconditioning process may lead to better treatments against I-R injury in the clinical setting (such as during bypass surgery and heart transplantation [17]). In the clinical setting, preconditioning of the heart with a short burst of ischemia-reperfusion or utilizing a pharmocological agent which simulates preconditioning may be advantageous when the heart is exposed to prolonged ischemia during myocardial infarction, or surgical procedure.

At the moment, the mechanism of preconditioning is not elucidated. Various studies have shown that ischemic preconditioning was not dependent on: altered myocardial blood flow [18]; attenuation of oxidant generation [19]; myocardial stunning [20]; altered glycolytic flux [21]; activation of an endogenous mitochondrial ATPase inhibitor [22]; increased prostacyclin [23]; nitric oxide synthesis [24].

A growing body of evidence has implicated adenosine and adenosine receptor activation in ischemic preconditioning [6-9,12,13,26-28,30]. Adenosine receptor activation was shown to: reduce leukocyte adhesion to postcapillary venules in I-R tissues [26,27]; modulate neutrophillic oxidative metabolism and inhibit platelet aggregation [28]. In ischemic preconditioning studies utilizing isolated rabbit hearts [9,13] or cardiomyoctes [8], the cardioprotective effects of preconditioning were either mimicked or augmented by

the addition of adenosine [26] or adenosine agonists [8] to the perfusate, or blocked by inhibitors to adenosine A1 receptors, such as 8-(p-sulfophenyl)theophylline (SPT) [6,8]. In these studies, the activation of adenosine release generated smaller infarct sizes [6,13] and increased functional recovery [7,12,30] over that of their respective controls.

Protein kinase C (PKC) has also been shown to be a important mediator of preconditioning. PKC is a family of phospholipid-dependent serine/threonine kinases. Structurally, PKC consists of up to five variable (V1-V5) regions and four conserved (C1-C4) regions as described in the figure below:



The V1, C1, V2, and C2 regions comprise the amino-terminal regulatory domain which is linked through the V3 "hinge" region to the carboxyl-terminal catalytic domain consisting of the C3, V4, C4, and V5 regions. C1 region has been shown to contain a pseudosubstrate site which, in the absence of activators, maintains the inactive state of the enzyme through its interaction with the catalytic ATP binding site on the C3 region. In addition, the C1 region contains one or two Zn^{2+}-finger-like Cys-rich motifs which bind the physiological activators of PKC (such as diacylglycerol or DAG) and their analogues (e.g., phorbol 12-myristate 13 -acetate or PMA). The C2 region consists of acidic amino

acids which function in binding Ca^{2+} to the enzyme. The C4 region functions in transfering the phosphate group from the ATP bound C3 region to various protein substrates along the signal transduction pathway. Phosphorylation of the PKC is also required for PKC activity and occurs either prior to or rapidly upon ligand binding [44].

PKC isoenzymes differ in their structure, cofactor requirement, and substrate specificity [29]. As of to date, twelve PKC isoenzymes have been identified. There are the conventional PKCs (cPKC) which consist of PKC-α, -β_I, -β_{II} and -γ and are Ca^{2+} - dependent enzymes. This subfamily was shown to possess the full primary sequence described above and is activated by phosphatidyl serine (PtdSer), Ca^{2+}, and DAG (or PMA) [29,44]. The next family of PKCs, called the novel PKCs (nPKC) consists of PKC-δ, -ε, -η, μ and -θ [29,44]. This subfamily of PKCs are considered to be Ca^{2+} - independent since they lack the C2 region in their primary structure and thus are not dependent on Ca^{2+} for activation. However, members of this subfamily still require PtdSer and DAG (or PMA) for activation [44]. The third family discovered was the atypical PKCs (aPKC). This family consisted of PKC-ζ, ι and -λ and its primary structure lacks the C2 region and one of the two Zn^{2+}-finger-like Cys-rich motifs in the C1 region. Thus, this, subfamily of PKCs is independent of Ca2+, DAG or phorbol esters for activation [29]. However, PtdSer and phosphatidylinositol-3,4,5-triphosphate (PIP3) have been shown to activate this sub family [44].

Activation of members of the cPKC and nPKC family is shown to involve the movement of this enzyme from the cytosol to the cell membrane [46,47] where it

phosphorylates proteins containing the consensus sequence X-Arg-X-X-Ser/Thr-X-Arg-X [45].

In the heart, nPKC-δ, nPKC-ε, cPKC-α and aPKCζ have been detected [48,49,50] and their relative amounts shown to be developmentally regulated, decreasing as the cell approaches its adult phenotype [51,52]. PKC was shown to be involed in processes such as: the development of hypertrophy and activation of the early immediate transcription factor genes [53,54]; phosphorylation of myofibrillar proteins such as myosin light chain [55], Troponin I,T and C [56]; L-type Ca^{2+} channels [57,58], Na^{+} channels [59], K^{+} channels [60] and Cl^{-} channels [61]. PKC's effect on these above mentioned proteins would suggest that its activation may play a role in the regulation of cardiac contractility. However, experiments conducted so far by different researchers have produced mixed results from negative to positive to no change in contraction upon PKC activation [62]. These variations in experiments have so far been attributed to differences in the experimental model chosen [44].

Recently, research into characterizing the function of PKC in the heart has focussed on its potential role during ischemic preconditioning. Studies utilizing PKC agonists such as 1,2-dioctanoyl-sn-glycerol (DOG) [30], diacylglycerol [12], and phorbol 12-myristate 13-acetate (PMA) [4,7,9] on isolated rat or rabbit cardiomyocytes [7,9], isolated rat hearts [12], or in vivo rabbit hearts [4] mimicked protective effects of preconditioning against I-R injury. However, these agonists are used to activate all PKC isoforms since they work by binding to the DAG binding site present on all of PKCs. This agonist binding, in turn, activates PKC by facilitating the binding of Ca^{2+} ions and

phosphotidylserine to the PKC enzyme [64]. However, studies which utilized more specific activators to various isoforms of PKC such as ingernol (an epsilon and delta-PKC isoenzyme selective activator) and thymeleatoxin (an alpha, beta, gamma-PKC isoenzyme selective activator) showed that preconditioning in isolated myocytes was enhanced with ingernol but not with thymeleatoxin [9]. Since both ingernol and thymeleatoxin have been shown to be relatively specific for their respective PKC isoforms [43] these results would suggest that preconditioning is dependent on the activation of the epsilon or delta PKC isoforms, although inhibitors for these specific isoforms were not used by these researchers to strengthen their claim [9]

Further evidence for PKC involvement in preconditioning was obtained in studies utilizing various specific PKC inhibitors. For instance, studies of ischemic preconditioning in isolated rat [42] and rabbit [6,8,9,13] hearts or cardiomyocytes [8]; in vivo rabbits [4] and rats [1,3]; and human myocardium [30] have shown that PKC inhibitors such as chelerythrine chloride [3,30,42], polymyxin B [4,6,13] and calphostin C [1,8] can block the cardioprotective effects of ischemic preconditioning by inhibiting PKC activation. These hearts, in turn displayed increased infarct size [1,3,6,13], cell damage [8] and decreased functional recovery [1,30] compared to control hearts after I-R injury.

In addition to specific activators to PKC, other mediators have also been implicated in ischemic preconditioning. These include: α-adrenergic agonists (such as norepinephrine [5], and phenylephrine [13]), via the α1 adrenergic receptor [12,13]; prostaglandin E1 and E2 [31]; bradykinin via the B2 receptor [32], and angiotensin [6], via the AT1 receptors. These mediators have all been shown to be cardioprotective

through the activation of PKC [5,6,12,13, 31,32] and the further downstream activation of K^{+}_{ATP} channels [30,31,33].

However, the involvement of PKC in preconditioning is not without its opponents. For instance, Przyklenk et al. [65] found in their in vivo dog models, that PKC inhibitors H-7 and polymyxin B failed to prevent the benificial effects of preconditioning nor was there any visual translocation of PKC isoforms to the membrane. As well, Moolman et al. [66] in their isolated rat heart model failed to block the protective effects of preconditioning using the PKC inhibitors chelerythrine and bisindolylmaleimide. Reasons for this conflicting data are uncertain. Various reasons have been proposed ranging from different animal species to dosages of agonist/inhibitors to the type of experimental manipulation [67,68].

Furthermore, there is an increased likelihood that Gi protein may be involved in the preconditioning process in light of the fact that α1 adrenergic stimulation [6,12,13], prostaglandin induced bradykinin-B2 receptor activation [33] and adenosine A1-receptor activation [6], were implicated in mediating preconditioning's cardioprotective effects. This Gi protein coupling was demonstrated in Langendorff perfused rat hearts during α1-adrenergic receptor stimulation [6] and in canine myocytes during adenosine-A1 receptor stimulation [34].

The involvement of Gi protein in the preconditioning pathway is important in that Gi protein activation has been implicated in the activation of membrane phospholipases [35] and involved in the adenosine A1-receptor induced activation of PLC [36]. Activation of PLC is important because it has been shown to lead to the production of

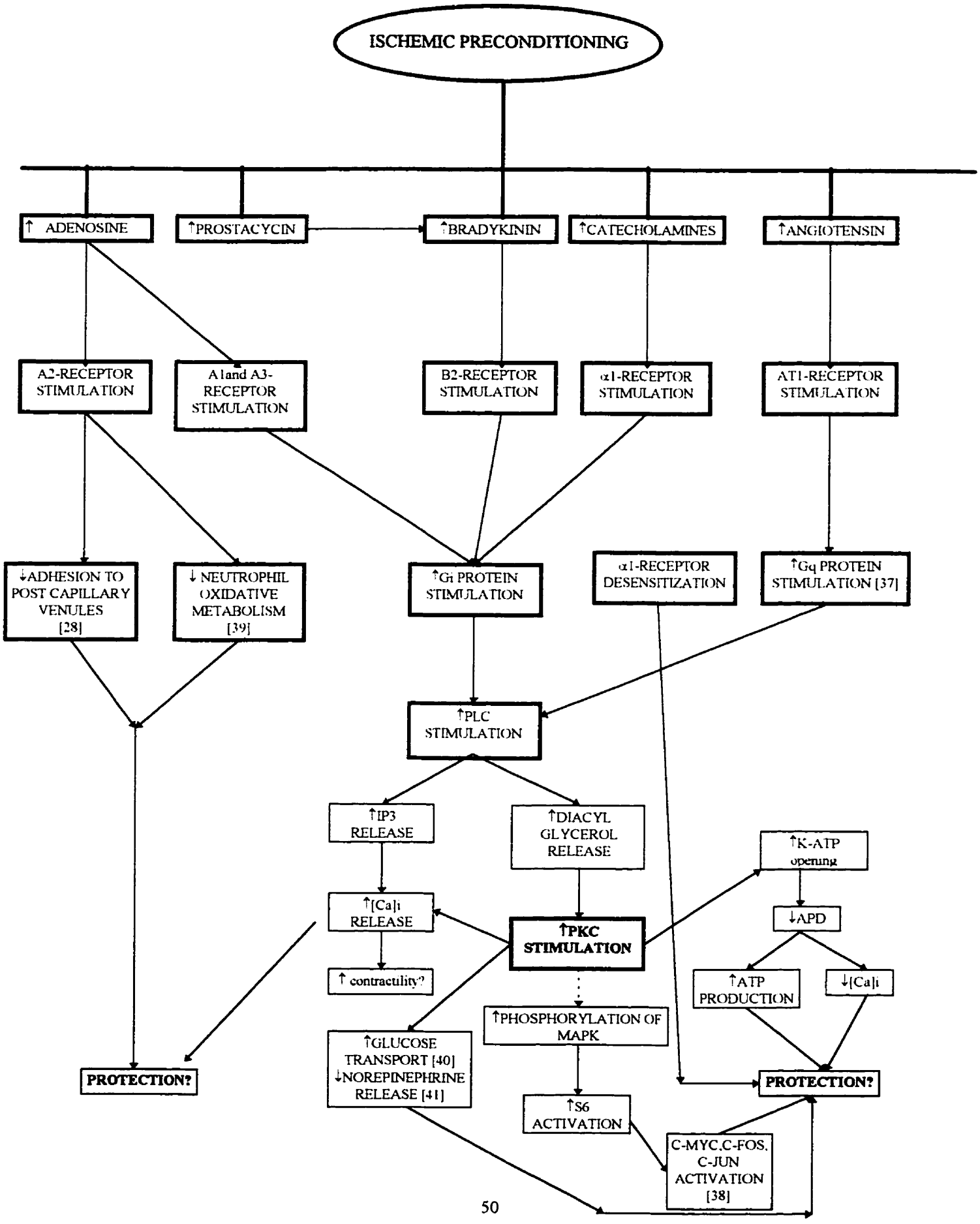
diacyl glycerol and inositol 1,4,5 triphosphate. Diacyl glycerol, in turn was shown to be a potent PKC activator and its exogenous addition to isolated rat hearts can mimic preconditioning [12].

In addition, G-protein independent activation of phospholipases by tyrosine kinase receptors may also participate in preconditioning since DAG and IP3 are also produced by these phospholipases [69,70].

Thus, a possible schema for the mechanism of ischemic preconditioning may follow the representation in Fig 1. With ischemic preconditioning, there is an increased release of adenosine, prostacyclin, angiotensin, bradykinina and norepinephrine from various vascular, cardiac and nervous tissues. This release is followed by the activation of the adenosine-A1 receptor complex; the α1-adrenergic agonist-receptor complex; and the prostacyclin-induced bradykinin-B2 receptor complex. These receptors would then activate Gi protein which in turn, activates membrane phospholipases. Increased phospholipase activity, would induce the release of diacyl glycerol, a powerful PKC activator. PKC activation can then stimulate a host of other different pathways which may mediate the cardioprotective effects of ischemic preconditioning. These PKC induced pathways include: increased glucose transport; decreased norepinephrine release; activation of the early immediate genes; K^{-}_{ATP} channel opening; increased ATP production and decreased intracellular calcium concentrations. In addition to Gi protein activation, preconditioning's protective effects could also be mediated by Gq protein activation via increased angiotensin-AT1 stimulation. Gq coupling would, in turn, also stimulate PKC activation an thus help mediate preconditioning effects.

Furthermore, PKC activation, and cardioprotection against I-R injury by ischemic preconditioning may also be precipitated through: the activation of α1-adrenergic receptor [12,13]; the adenosine-A2 receptor induced decrease of the neutrophil oxidative metabolism and adhesion to post-capillary venules [28]; and adenosine-A1 and -A3 activation in isolated rabbit cardiomyocytes [71], isolated perfused rabbit hearts [72] and conscious rabbit in vivo infarcted hearts [73].

Thus, ischemic preconditioning has been shown to induce cardioprotection in different animal and human heart models. Many different agonists are capable of mimicking the protective response of ischemic preconditioning to I-R injury. Many agonists, such as adenosine, norepinephrine, angiotensin and bradykinin, share a common signal transduction pathway through PKC as testified by the use of inhibitors or activators of PKC.


ISCHEMIC PRECONDITIONING
↑ ADENOSINE
↑PROSTACYCIN
↑BRADYKININ
↑CATECHOLAMINES
↑ANGIOTENSIN
A2-RECEPTOR STIMULATION
A1and A3-RECEPTOR STIMULATION
B2-RECEPTOR STIMULATION
α1-RECEPTOR STIMULATION
AT1-RECEPTOR STIMULATION
↓ADHESION TO POST CAPILLARY VENULES [28]
↓ NEUTROPHIL OXIDATIVE METABOLISM [39]
↑Gi PROTEIN STIMULATION
α1-RECEPTOR DESENSITIZATION
↑Gq PROTEIN STIMULATION [37]
↑PLC STIMULATION
↑IP3 RELEASE
↑DIACYL GLYCEROL RELEASE
↑K-ATP opening
↑[Ca]i RELEASE
↑PKC STIMULATION
↓APD
↑ contractility?
↑ATP PRODUCTION
↓[Ca]i
↑PHOSPHORYLATION OF MAPK
↑GLUCOSE TRANSPORT [40] ↓NOREPINEPHRINE RELEASE [41]
PROTECTION?
↑S6 ACTIVATION
C-MYC,C-FOS, C-JUN ACTIVATION [38]
PROTECTION?

1.4.1 REFERENCES: Chapter 1.4 Preconditioning and role of PKC

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1.5 FIBROBLAST GROWTH FACTOR-2 (FGF-2)

1.5.1 General Background

Fibroblast growth factor-2 (FGF-2) is a member of the larger family of fibroblast growth factors (FGF1-9) sharing 30-50% sequence homology [1,2]. This growth factor is synthesised and localised in most tissues of neurodermal, mesodermal and ectodermal origin (e.g. astrocytes, neuronal cells, muscle cells, chondrocytes, keratinocytes, platelets, macrophages, etc.) [1,2,3]. FGF-2 itself is a cationic peptide with a pI of 9.6. FGF-2 lacks an amino- terminal hydrophobic signal peptide sequence and therefore is not secreted via the conventional secretion pathways through the golgi apparatus [4,5]. FGF-2 is very well conserved between species (e.g. bovine and human FGF-2 share 97% amino acid homology to one another [6,7].

FGF-2 structure also contains 2 disulphide bonds and an inverse cell adhesion (R-G-D) sequence which facilitates in FGF-2's cell adhesive function [3]. In addition, FGF-2 has serine and threonine amino acids which become phosphorylated by either protein kinase A or C depending on the FGF-2's association with various ECM components. FGF-2's affinity for high affinity receptors, in turn, is influenced by this phosphorylation [13].

Different sizes of FGF-2 peptides have been identified ranging from the low molecular weight forms(14 to 18 kDa) to the high molecular weight forms (21.5 to 29 kDa) [4]. These different species of FGF-2 differ in their localisation in the cell and thus are thought to perform different functions. Shorter FGF-2 peptides have been found mainly in the cytoplasm and extracellular matrix but also the nucleus [14,15,16,17]. In comparison, the longer FGF-2 peptides, were found preferentially targeted to the cell

nucleus. This nuclear localisation of FGF-2 is associated with proliferative cells suggesting a possible role in cell proliferation and growth [17,19].

Multiple FGF-2 isoforms derive from initiation of translation from AUG as well as CUG-start sites. For instance, in humans, three potential "CUG" codons for FGF-2 translation, at positions -302, -329 and -344, were found to exist at the 5' terminal end of the gene resulting in the expression and synthesis of higher molecular weight FGF-2 (21.5 kDa, 22.5 kDa, and 25 kDa) [20,21]. These amino terminal extended FGF-2s contain a nuclear translocation sequence in the extended portions.

The question of the mechanism by which FGF-2 is secreted when it lacks a hydrophobic amino terminal signal sequence still remains unanswered. One theory speculated that FGF-2 is released upon cell lysis during tissue injury and cell death [30]. However, other studies have shown that cell death may not be required for FGF-2 release. These studies have shown that minor damage to the cell, without cell death, can also induce FGF-2 leakage from the cell interior [31]. More theories of FGF-2's mode of secretion ranged from evagination of the cell plasma membrane forming extracellular vesicles [5], to FGF-2 'piggy backing' on nascent heparin sulphate as a mode of transportation out of the cell [5]. Recently, FGF-2 release in the heart was shown to occur via a transient, survivable disruption (or wounding) of the cardiac myocyte plasma membrane. This cardiac myocyte wounding appears to be dependent on the normal contraction of the heart. Indeed, upon beta-adrenergic stimulation and increased heart rate, an increase in FGF-2 release is seen in these hearts [161].

1.5.2 Biological effects of FGF-2

FGF-2 is a multifunctional protein which has been studied primarily as a mitogen. In *in vitro* studies, the biological effects of FGF-2 were shown to vary according to cell type and developmental stage. FGF-2 induced transformation of NIH 3T3 [22,28] and BHK- 21 cells [23] and stimulated proliferation of numerous cell types. FGF-2 reduced the average doubling time and shortened the G1 phase of the cell cycle of vascular endothelial cells [4]. FGF-2 was also demonstrated to play a role in cell differentiation. It can act as an inducer of differentiation (e.g. capillary endothelial cells [4]) or an inhibitor of differentiation (e.g. myoblasts, chondrocytes and adipocytes [4]). In myoblasts, in particular, FGF-2 when added to cultures, decreased MyoD1 transcription and prevented fusion [32,33]. *In vitro*, FGF-2 also promoted cell adhesion [34,35]; induced mesoderm formation [36]; and promoted angiogenesis [37]. As an angiogenic agent, FGF-2 stimulates degradation of the basement membrane, promoted endothelial cell migration, tube formation and proliferation, and increased plasminogen activator and collagenase synthesis and release [4].

In vivo biological effects of FGF-2 include: early embryonic mesoderm induction [36]; regeneration (e.g. newt limbs, lens tissue and cartilage) [3]; and neurotropic and neurotrophic actions (FGF-2 promoted neurite outgrowth, differentiation and survival) [3]. FGF-2 stimulates proliferation and growth of all cell types involved in the wound healing processes *in vivo*: capillary endothelial cells [38,41], vascular smooth muscle cells [39], fibroblasts [38,39] and specialised tissue cells (chondrocytes [40] and skeletal myoblasts [42]). Thus FGF-2 also participates in the healing process of many systems such

as arterial endothelium damage [43], skin wounds [44], corneal epithelial wounds [45], CNS injury [46], and epidermal wounds [47]. More recently, increased FGF-2 mRNA synthesis and anti-FGF-2 immunoreactivity was demonstrated in models of cortical brain injury [46]; and muscle injury (specifically in myoblasts), and in degenerating and regenerating myotubes [42].

1.5.3 FGF-2 receptors

FGF-2 is a high affinity heparin binding protein [5]. Work by Steinfeld et al. [10] demonstrated that for FGF-2 to bind to its cognate receptor, distinctive FGF-2-heparan sulfate complexes must be formed [10].

Heparin- FGF-2 interactions: (i), protect FGF-2 from degradation and protease actions [8]; (ii), stabilise the tertiary structure of FGF-2[10]; (iii) act as a possible direct transducer of FGF's signalling pathway by internalization of FGF-2 into the cell [12] and; (iv) more recent studies have shown heparan sulfate proteoglycans associated with the cell surface and ECM act in concert to regulate the bioavailabililty and growth promoting activity of FGF-2. In these studies, Miao et al. [9] showed that while heparan sulfate in the subendothelial ECM functions primarily in sequestration of FGF-2 in the vicinity of responsive cells, heparan sulfate on the cell surfaces plays an active role in displacing the ECM-bound FGF-2 and its subsequent presentation to high affinity signal transducing receptors [9]. In addition, the sulfate groups on heparin are important in the binding of FGF-2 [9].

The high affinity receptors for FGF-2 are glycosylated single chain polypeptides with molecular weights ranging from 110 to 165 kDa [3,4] in numbers ranging from 0.2 to

1.0 ($x10^5$) per cell depending on cell type [3,4]. These receptors are tyrosine kinases embedded in the cellular membrane [4]. As of yet, four related gene products of the high affinity receptor have been described (FGFR1-4), each with an extensive array of splice variants [24]. This RNA splicing process has been shown to be highly regulated and tissue specific [25].

Structurally, the four FGF-2 receptor isoforms are transmembrane proteins containing either two or three Ig-like domains in the extracellular ligand binding portion. Within each isoform group, receptor variants differ from another by the presence of two-amino acid insertions. A fifth isoform has been shown to be a truncated, possibly secreted protein, containing the first IgG-like domain [25]. Another isoform, reported by Johnson et al. [26], is also a truncated form of the FGF-2 receptor, which differs from the others in that it contains the acidic region of the receptor and the Ig-like domains II and III. In the carboxy terminal, FGFR1 contains a tyrosine kinase domain which becomes autophoshorylated on the Tyr^{766} and the Tyr^{653}. Tyr^{766} in particular, once phosphoylated acts as a bind site for phospholipase C-γ (PLC-γ). Tyr^{653} on the other hand, maybe involved in regulating kinase activity [157]. In addition to autophosphorylation, the tyrosine kinase domain of the FGFR1 phosphorylates other intracellular substrates such as phospholipase Cγ [156,157,159], p89 [157], shc [157] and src [156].

1.5.4 The signal transduction mechanism of FGF-2

1.5.4.1 PC12 cells

The signal transduction mechanism for FGF-2 is complex and varied depending on the cell type or developmental stage. One cell type system that has been extensively studied is the rat adrenal pheochromocytoma cell line (PC12).

A summary of the signal transduction pathway is shown in Fig 1.2. One pathway proposed by Roivainen et al. [84] involves the activation of protein kinase C. These researchers found that addition of FGF-2 to PC12 cells induced neurite outgrowth through enhanced delta or epsilon PKC-regulated pathways with mitogen activated protein kinase (MAP) taking place downstream to the PKC.

Kremer et al. [91] and Isono et al. [86] demonstrated a PKC independent pathway of FGF-2 signaling. Upon FGF receptor activation, PLC-γ1 becomes activated, which in turn, activates a cascade of downstream signaling proteins which involve: production of diacylglycerol and inositol 3-phosphate [91]; increased activation of K252a sensitive protein kinases or phosphatases [86]; increased activation of MAPK [84,86,87,101]; decreased EGF receptor binding [87]; and finally neurite outgrowth and proliferation. Alternatively or concomitantly, a p60 src - p21 ras activated pathway was shown also to become activated in PC12 cells upon FGF-2 binding, and may also play a role in neurite outgrowth and differentiation [86,92].

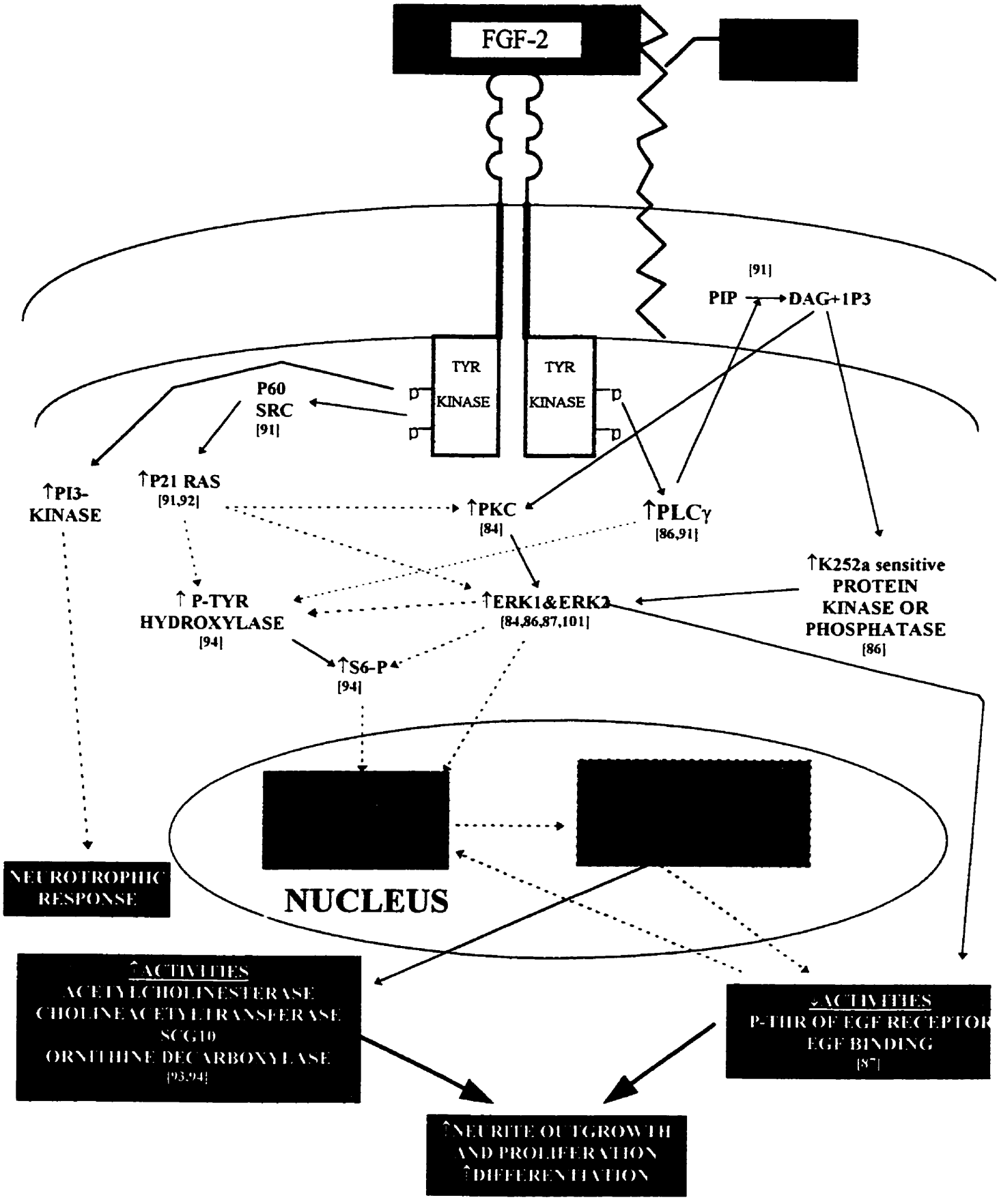
In addition, Damon et al. and others [93,94] have demonstrated further activation of other signaling proteins such as tyrosine hydroxylases and S6 [94] and the subsequent activation of primary response genes (such as: TIS1, TIS2, TIS8 [93,94]; c-fos [93,95];v-

crk [96]) and secondary response genes (such as: ornithine decarboxylase, acetyl-cholinesterase, SCG10 [93]). With gene activation by FGF-2 on PC12 cells, increased activities of acetylcholinesterase, cholineacetyltransferase, SCG10, and ornithine decarboxylase was shown to occur, followed by increased neurite outgrowth and differentiation [93,94].

Furthermore, FGF-2 has been demonstrated to protect PC12 cells from injury [17,18]. In studies conducted by Raffioni et al. [99], FGF-2 was shown to activate phosphatidylinositol 3 kinase (PI 3-kinase), which in turn induced a neurotrophic response in PC12 cells, independent of phosphatidylinositol 4-kinase activation [99].

A summary of the signal transduction of FGF-2 in PC12 cells is depicted in Figure 1.2. Solid arrows indicate substantiated signal transduction pathways. Dotted arrows indicate speculative signal transduction pathways.

Figure 1.2: PC12 cells



1.5.4.2 Endothelial cells

The signal transduction of FGF-2 in endothelial cells has also proven to be just as complex and uncertain as in neuronal cells. The biological effects of FGF-2 in these cells range from chemotaxis and cell migration [102,103,106,109] to induction of DNA synthesis and proliferation [102-105,110,111]. How FGF-2 accomplishes these various biological effects in endothelial cells has been the subject of many experiments and has been shown to differ among the various types of endothelial cells [105]. However among all the different endothelial cells types, FGF-2 must first bind and activate its tyrosine kinase receptor [102-111]. Upon stimulation of the FGF-2 receptor, a number of different signal transduction pathways are activated depending on the type of endothelial sell being studied.

In bovine aortic and adrenal cortex capillary endothelial cells [103,106,110,111] and mouse brain endothelial cells [109], motility and plasminogen activation are stimulated by FGF-2 via a Ca^{2+} dependent/protein kinase C independent pathway [104] (Fig.1.3). In this pathway, activation of phospholipase A_2 by FGF-2 leads to the release of arachidonic acid [103,106,110,111] and endothelial cell migration. How $PLCA_2$ is activated is still uncertain, however recent research points to the involvement of both pertussin toxin sensitive G proteins (Go or Gi) [106] and MAPK [102,103] upstream of the PLA_2.

Additionally, nuclear transloction of the FGF-2/FGF receptor complex was also found to be associated with the induction of endothelial cell motility [102]. However, nuclear translocation was associated with increased DNA synthesis [102], which, in turn,

may contribute to the angiogenesis of endothelial cells by increasing proliferation [102,107] and increasing the production of plasminogen activator [104,109].

As well, Ca^{2+} independent PKC pathways have also been implicated in the induction of endothelial cell motility. Sa et al. [103] speculated that activation of PKC may indeed be involved in endothelial cell motility by activating p42 MAPK isoform, which, in turn, was shown to be upstream to PLA2 activation [103] in bovine aortic endothelial cells. Similarly, in experiments with human endothelial cells, FGF-2 was shown to stimulate the Ca^{2+} independent novel PKC isoforms (δ,η,θ) [105]. Activation of these isoforms was linked to increased endothelial cell motility [105,110] possibly via the activation of MAPK [103].

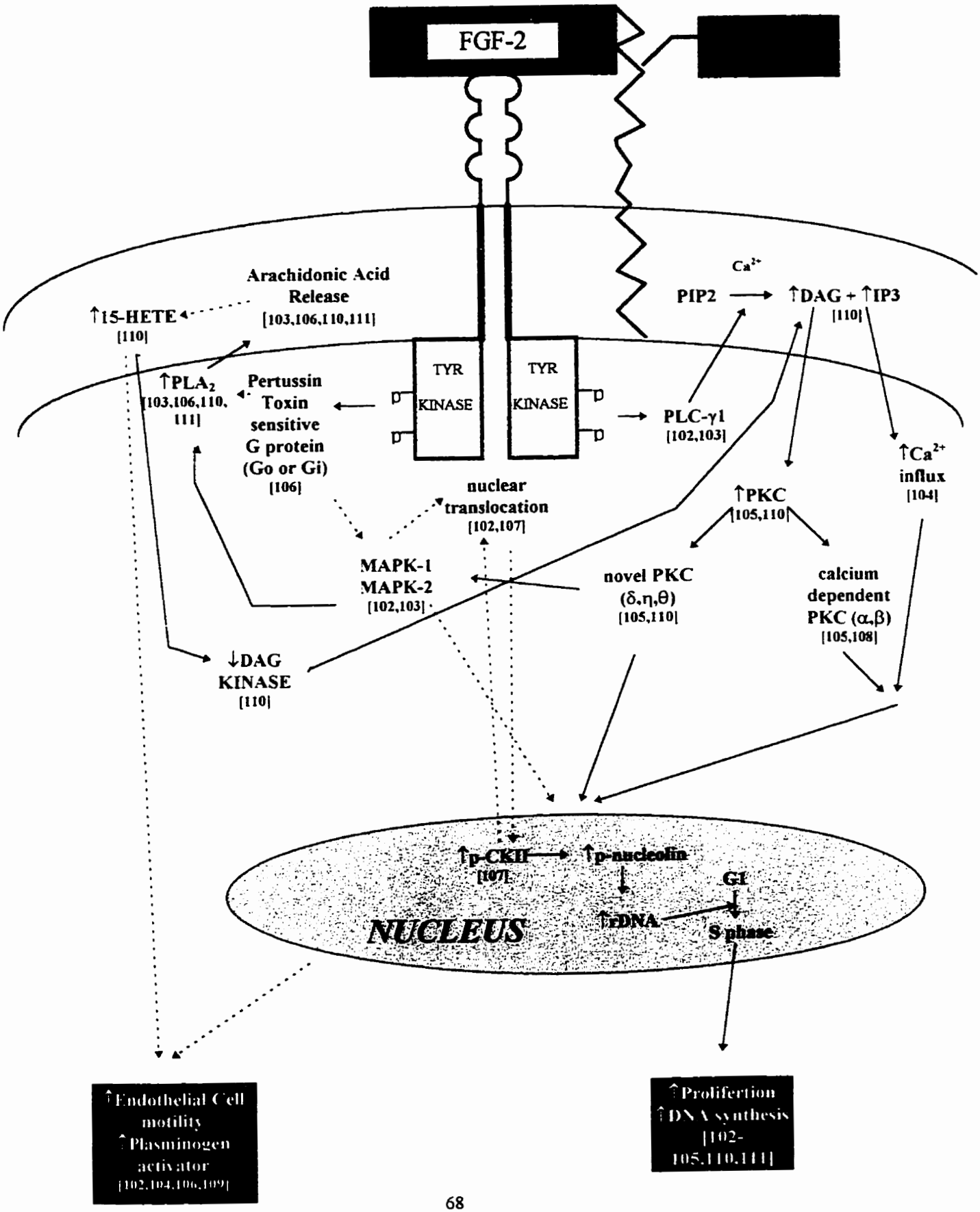
FGF-2 activation of its receptor has also been linked to increased proliferation of endothelial cells [102,103,104,105,110,111]. In bovine coronary venular [102] and bovine aortic [107] endothelial cells, FGF-2 binding to its receptor led to a nuclear translocation of the FGF-2-receptor complex. Once in the nucleus, translocation was shown to increase phosphorylation of the protein kinase CKII. CKII, in turn, phosphorylates the nucleolin protein. With phosphorylation of nucleolin, there was an increase in DNA synthesis [102] along with increased rDNA transcription and a G1 to S phase transition of the quiescent endothelial cells [107].

Other proliferative pathways activated in endothelial cells by FGF-2 were shown to involve protein kinase C. In human umbilical cord [105], bovine adrenal cortex capillary [110] and bovine aortic [108] endothelial cells, PKC activation led to an increase in cellular proliferation. This proliferation was shown to be Ca^{2+} dependent [104] suggesting

the involvement of the Ca^{2+}-dependent PKC isoforms [105,108]. However, this calcium dependency of endothelial cells is cell type specific since PKC activation of adrenal cortex capillary endothelial cells did not require Ca^{2+} to induce proliferation [110].

A summary of the signal transduction of FGF-2 in endothelial cells is depicted in Figure 1.3. solid arrows indicate substantiated signal transduction pathways. Dotted arrows indicate hypothetical signal transduction pathways.

Figure 1.3: Endothelial cells



1.5.4.3 Smooth muscle cells

In smooth muscle cells, the biological effects of FGF-2 include the induction of cell proliferation [112,110,133,135,140,142-146,148], hypertrophic growth [112], migration [129,148] and phenotypic trantransformation into a more proliferative state [116]. The result of these various biological effects *in vivo* is increased angiogenesis [122,128], the development of restenosis (after angioplasty) [124] and atherosclerosis [116].

The signal transduction pathway for FGF-2 in smooth muscle cells is not well characterized. Knowledge of the effects of FGF-2 in smooth muscle cells consist mainly of its synergistic actions and release induced by other growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF). The flow chart in Figure 1.4 depicts the signal transduction pathway for FGF-2 in smooth muscle cells from signals that induce its release to the final biological effects induced by FGF-2.

A number of factors which contribute to the development of atherosclerosis and smooth muscle cellular growth have been shown to induce the release of FGF-2 into the extracellular milieu. These agents include: Thromboxane A2 [112]; Interleukin-1β [123]; sodium nitroprusside [123]; and Angiotensin II [112,114,115]. Enzymes such as glycosyl-PI specific phospholipase C (PI-PLC) have also shown to induce the release of FGF-2 in bovine aortic smooth muscle by cleaving the gylcosyl-PI anchored cell surface heparan sulfate proteoglycan where FGF-2 is bound [153].

Mechanical strain [119], atherosclerosis [133], and smooth muscle [123,124,133] and endothelial cell [144] injury (eg. due to angioplasty [124] or nitric oxide toxicity

[123]) have also been shown to contribute to the release of FGF-2 from smooth muscle cells. Injury to endothelial cells, for example, causes the release of membrane attack complex complement C5b. Complement C5b then attaches to smooth muscle cells where it damages the cell membrane and thus facilitates the release of FGF-2 from its intracellular stores [144].

Increased release of FGF-2 into the extracellular milieu was shown to occur also during times of hypoxic stress. Hypoxia was shown to attract macrophages to the site of ischemia where they would release FGF-2 to the surrounding vascular tissues [122]. In addition, hypoxia has been shown to induce endothelial cells to release FGF-2, which inturn, acts as an autocrine factor or paracrine factor on the surrounding endothelial and smooth muscle cells [143].

Once FGF-2 is released into the extracellular milieu, it then binds to its tyrosine kinase receptor on smooth muscle cells [116,118,110,120,125-129,142]. In smooth muscle cells, FGFR1 was shown to be the predominate receptor isoform for FGF-2 [151]. Thus FGF-2 acts in both an autocrine and paracrine fashion to induce its biological effects on smooth muscle cells [119,122].

Activation of PKC was shown to mediate the mitogenic response of smooth muscle cells to FGF-2 [112,113,117,110,120,133]. Upstream of PKC, a number of other signal transduction proteins including: pp 90 [120]; $p21^{ras}$ [141]; G-protein/cAMP activated protein kinase A [120], are activated.

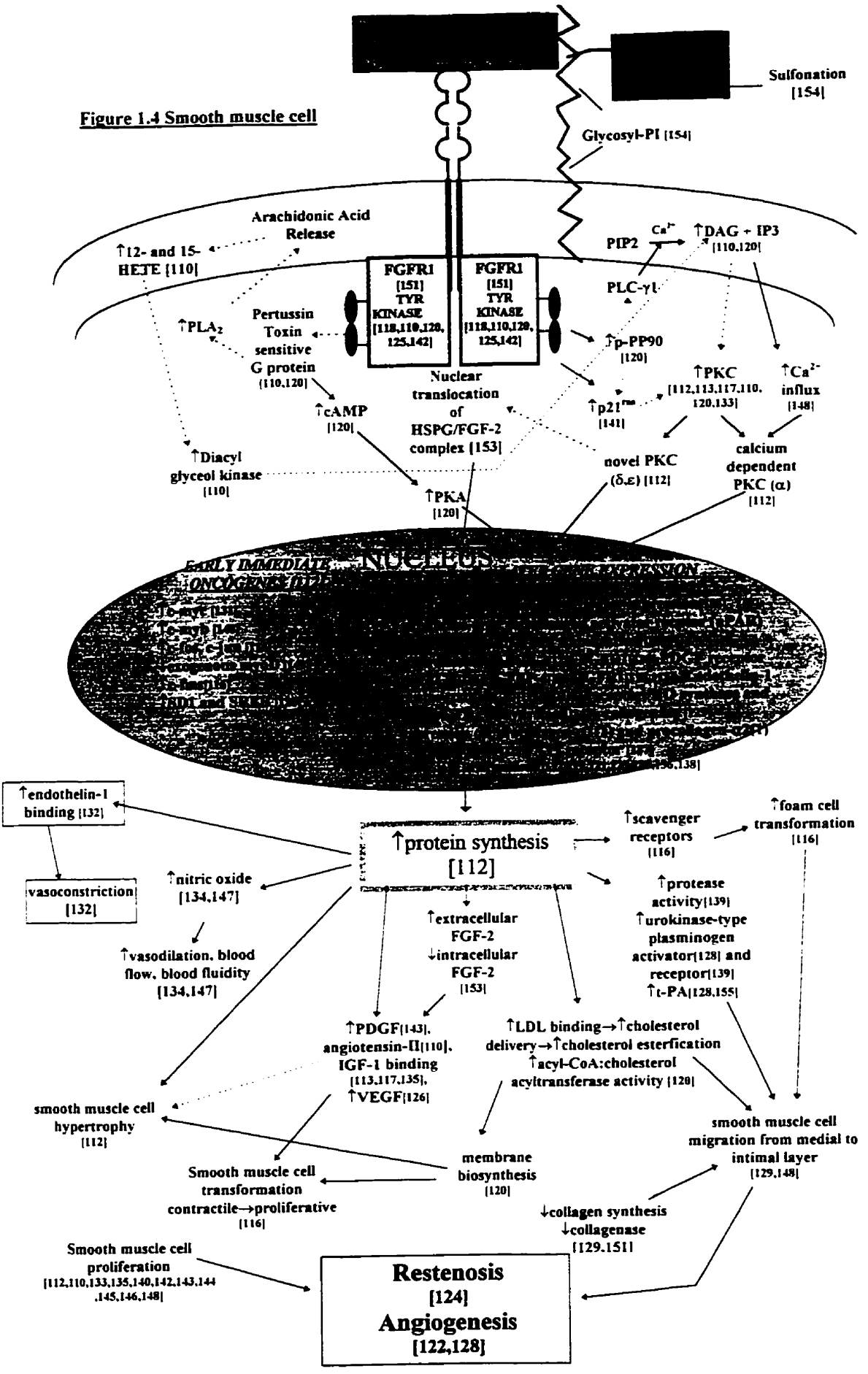
Downstream of PKC, a number of early-immediate proto-oncogenes become induced [112]. Induction of these early-immediate proto-oncogenes causes the

transcription of later genes involved in cellular proliferation and migration [112]. In rat aortic vascular smooth muscle cells, the proliferative effects of FGF-2 was correlated with increased expression of c-myc [114,131], c-fos [114], c-myb [148] and c-jun [114]. In human aortic medial smooth muscle cells, FGF-2 induced expression of c-fms [116] was correlated with increased scavenger receptor synthesis [116], formation of foam cells [116] and development of atherosclerosis [116]. In rat thoractic aortic smooth muscle cells, increased SD1 and SREB transcription factors was correlated with increased LDL receptor synthesis followed by increased cholesterol uptake and membrane synthesis [120].

FGF-2 induces the synthesis of proteins which participate in membrane biosynthesis [120]. In rat aortic vascular smooth muscle cells, FGF-2 stimulates increased LDL receptor expression. More LDL receptors, in turn, increases the delivery of cholesterol into the cell. Cholesterol is a substrate for the cholesterol esterfication substrate acyl-CoA:cholesterol acyltransferase and thus increases this enzyme's activity. As a consequence of the increased metabolism of cholesterol, there is an increase in membrane biosynthesis and thus growth of the smooth muscle cell.

Scavenger receptor biosynthesis is also increased with FGF-2 [120]. With an increase in scavenger receptors, smooth muscle cells demonstrated an increased uptake of oxidized-LDL and increased transformation into atherosclerotic foam cell phenotype [120].

Figure 1.4 Smooth muscle cell



1.5.4.4 Striated muscle

Little is known about the signal transduction pathway for FGF-2 in adult cardiomyocytes. Most of the information pertaining to FGF-2 signal transduction in myocytes has come from research using skeletal muscle cell lines [156-159,165,166,178,179] or cultured neonatal cardiomyocytes [163,164,168-173,175-177]. A schematic diagram of the known and speculated signal transduction pathway for FGF-2 in myocytes is depicted in Figure 1.5. Black solid arrows indicate substantiated signal transduction pathways in skeletal muscle cells. Black dotted arrows indicate signal transduction pathways which have been speculated in skeletal muscle cells. Red solid arrows indicate substantiated signal transduction pathways in cardiac muscle cells. Red dotted arrows indicate signal transduction pathways which have been speculated in cardiac muscle cells.

After tyrosine kinase receptor activation a variety of different possible signal transduction pathways have been shown to occur in myocytes. In rat L6 myoblasts, FGF-2 stimulation led to the phosphorylation of membrane bound p89 signal protein and the cytosolic shc [157] and the src[156] signal proteins. The phosphorylation and activation of these proteins in turn, leads to their binding to the membrane bound and cytosolic SOS-Grb2 signal complex. Activation of this complex then leads to the phosphorylation and activation of $p21^{ras}$ [156,157]. In MM14 mouse myoblasts [156], C212 mouse myoblasts [159] and rat L6 myoblasts [157], activation of $p21^{ras}$ has, in turn, been linked to the activation of c-raf [159], mitogen activated protein kinase kinase (MAPKK) [156,159], mitogen activated protein kinase (MAPK) [156,159] and the S6 signal peptide. Further

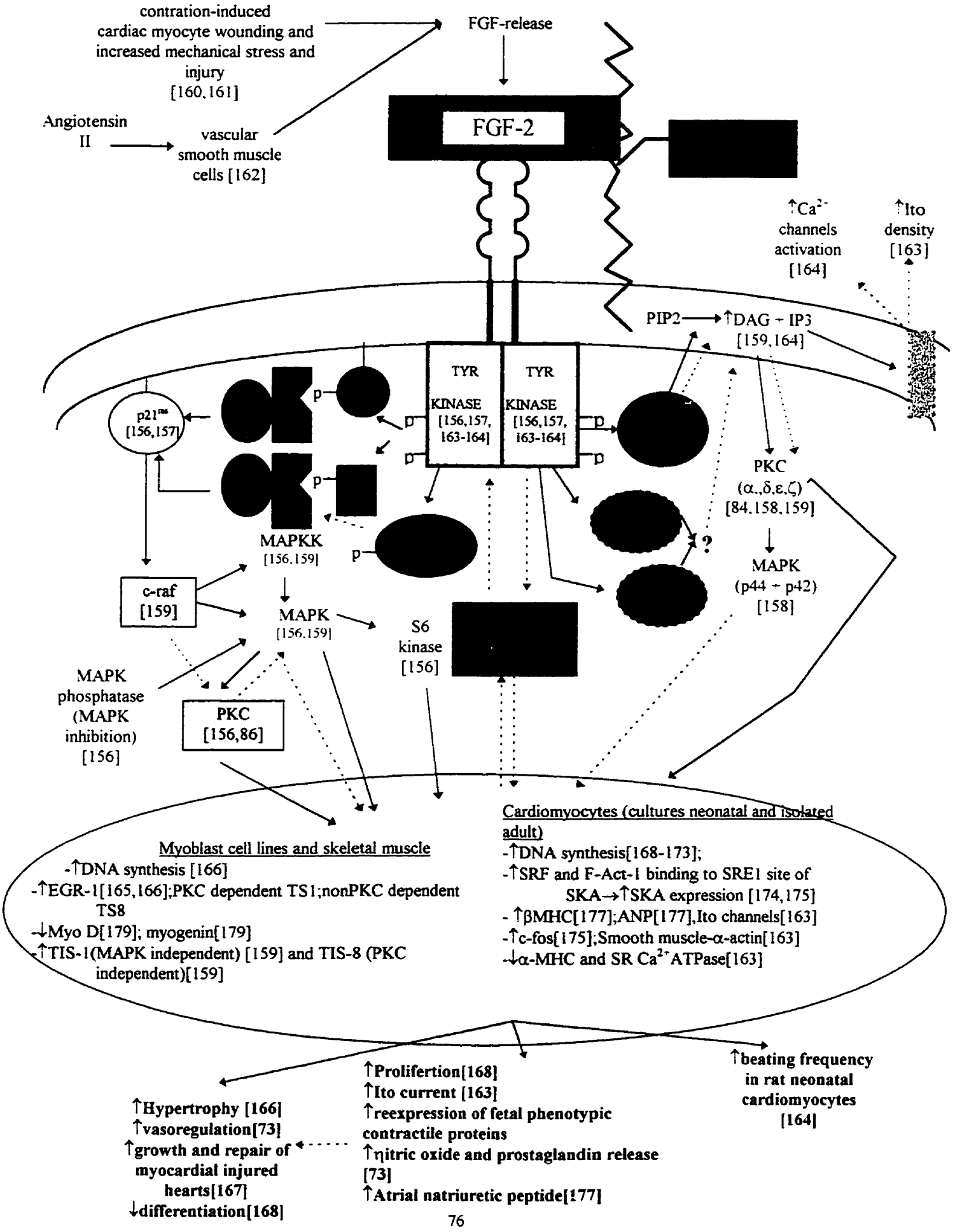
downstream signaling after MAPK activation may also include MAPK nuclear translocation [156] and the nuclear translocation of FGF-2-FGFR1 complex [156]. The activation of these MAPK mediated proteins were originally thought to be directly involved in the inhibition of differentiation and increased proliferative action of FGF-2 [157]. However, Campbell et al. [156] showed that in MM14 cells, the activation of MAPK was not required to maintain proliferation or repress muscle differentiation, but in fact, it is the activation of MAPK phosphatase which plays the role in repressing differentiation [156]. These results would suggest that although FGF-2 activates MAPKK in proliferating myoblasts, the mitogenic signal does not move on to further downstream protein kinases [156].

The involvement of protein kinase C (PKC) in mediating effects of FGF-2 in myocyte proliferation and differentiation is still speculative. In C212 mouse myoblasts, for instance, FGF-2 was shown to induce the activation of two types of TIS (Tetradecanoyl phorbol acetate inducible sequences) transcription factors, TIS1 and TIS8. However, whereas induction of TIS8 genes relies on a PKC independent pathway (Grb→c-raf→MEK→MAPK→S6 kinase→TIS8), induction of TIS1 relies on PKC activity (PLCγ1→↑diacyl glycerol→↑PKC activity→↑activation of TIS1) [4]. In addition, although TIS-1 and TIS-8 are both activated by FGF-2 in C212 myoblasts, it is still uncertain as to whether activation of these primary response genes are directly involved in mediating the differentiating and proliferating effects of FGF-2 [159].

In myoblasts, FGF-2 was shown to induce a variety of nuclear events, most likely responsible for the transduction of the dedifferentiating and proliferating effects of FGF-2. In cultured mouse muscle Sol 8 myoblasts, FGF-2 induced an increase in DNA synthesis

[166] and an increase in Egr-1 proto-oncogene expression [165]. In C2C12 mouse myoblast cells, FGF-2 induced a decrease in MyoD1 and myogenin gene expression [179] and an increase in the transcription factors TIS-1 and TIS-8 [159].

Figure 1.5 Myocytes



1.5.5 FGF-2 and receptors in the heart

In the heart, FGF-2 is synthesised and stored by various cell types including cardiomyocytes. In cardiomyocytes, FGF-2 is associated with the nucleus, cytoplasm, cell membrane, basement membrane and gap junctions [35]. This localisation of FGF-2 suggests an intimate involvement with various aspects of physiological function. In blood vessels, FGF-2 is accumulated at all cell layers [3].

Kardami et al. [14,51] have identified different forms of FGF-2 in cardiomyocytes. The 18 kDa FGF-2 species predominated in adult 5 week old rats [35,48,51]. The 22 and 24 kDa species were mainly detected in new born rats. A 25 kDa species was shown to be localised in the nucleus of proliferating cardiomyocytes [14,51]. In transfection studies by Pasumarthi et al. [17], overexpression of high and low molecular weight FGF cDNAs in chick and rat cardiomyocytes resulted in the higher molecular weight FGF-2 localising mainly in the nucleus and low molecular weight FGF-2 mainly in the cytoplasm and nucleus. Liu et al.[81] found elevated levels of 21.5- to 22 kDa forms of FGF-2 in hypothyroid compared to euthyroid cardiac ventricles suggesting that thyroid hormone may play a significant role in regulating the accumulation of high molecular weight forms of FGF-2 [81].

Upregulation of high molecular weight FGF-2 forms has been also observed in cardiac injury. Padua et al. [51] found that in the isoproternenol-induced cardiomyopathic model of injury, transient increases in the high molecular weight 21.5 and 22 kDa FGF-2 occured 24 hrs after injury in the adult ventricle. Concurrent with this observed increase, necrotic myocytes displayed increased cytoplasmic FGF-2 staining suggesting an increase

and/or release of FGF-2 from injured myocytes. After 1 week, however, 21.5 and 22 kDa FGF-2 forms were downregulated to levels similarly seen in ventricular extracts of control hearts. Localization of FGF-2 showed increased pericelular FGF-2 staining on myocytes adjacent to the scar region. Necrotic myocytes have been phagocytized and replaced by scar tissue at this time point [51].

FGFR1, the flg gene product, is the only FGF-2 receptor so far identified [52] in myocardial cells. FGFR1 was shown to exist in the heart as different RNA splice variants with either two (82-kDa, "short" form) or three (92-kDa, "long" form) extracellular immunoglobulinlike domains [52]. Recent studies on fetal and adult hearts [53,54], as well as cultured mitotic and non-mitotic neonatal cardiomyocytes [55] demonstrated a transition from the "long" to the "short" FGFR1 mRNA expression and protein isoforms as the heart progresses from fetal to adult stage [52,53,54] or from the mitotic to non-mitotic stage [52,55].

When FGF-2 binds to its high affinity tyrosine kinase receptors, increased tyrosine phosphorylation of cellular substrates is observed in both proliferating and non-proliferating cardiomyocytes in culture [24,52]. In western blot studies, proliferating myocytes showed increased tyrosine phosphorylation compared to controls in proteins migrating with an apparent molecular mass of approximately 180,110,125 and 92 kDa, while in non-mitotic myocytes, these proteins migrated with an apparent molecular mass of >200,180,140,90-100. The apparent differences in phosphorylation profiles of both cultures would indicate possible alternate FGF-2-induced signal transduction systems dependent on the proliferative state of the cell. Similarly, in ex vivo hearts treated with

FGF-2 by perfusion (see chapter 3 result section), increased tyrosine phosphorylation staining was seen in tissue sections corresponding to increased pericellular asccumulation of exogenous FGF-2 [56,57]. Low affinity heparan sulfate proteoglycan FGF-2 binding sites receptors have been identified in the heart belonging to the N-syndecan family [53,56].

Biological effects of FGF-2 in the heart are thought to be exerted via autocrine, paracrine and/or intracrine pathways [5,17,52,59]. FGF-2 may play a role in regulating cardiac cell differentiation. Kardami et al. [48], found that atrial myocytes, which are less differentiated than ventricular myocytes, are surrounded by an FGF-2- rich endomysium. As well, Schneider et al. [58] has shown that FGF-2, when added to neonatal cardiac cell cultures, induces a reversal to an earlier program of contractile and non- contractile gene expression. FGF-2, along with TGF-ß1 and FGF-1, were shown to uniformly suppress expression of the muscle- specific genes in skeletal myoblasts and biochemically differentiated myocytes. For instance, TGF-ß1 and FGF-2 are both found to selectively provoke embryonic β-MHC and α-SKA expression and inhibit adult α-MHC expression as well as adult SR Ca^{2+}-ATPase [58].

FGF-2 is able to stimulate DNA synthesis in cardiomyocytes of several species [60,61,82], an effect blocked by the addition of TGF-β1 [60]. Claycomb et al. [61] showed that FGF-2 induced increased DNA synthesis with an increase in protein synthesis and the acquisition of a more dedifferentiated phenotype in adult cardiomyocytes. Mikawa et al. [82] showed that introduction of dominant-negative FGFR1 cDNAs or cDNAs for antisense FGFR1 into early chick embryos, resulted in an inhibition of myocyte

proliferation and/or survival during the first week of chicken embryonic development but had much less effect after the second week. These results suggest that receptor-coupled FGF signaling regulates cardiac myocyte growth during tubular stages of cardiogenesis.

FGF-2 may also play a role in cell to cell recognition, adhesion and communication. It has been localised along the intercalated disc regions of cardiomyocytes [34]. Immuno electron microscopy demonstrated localization of FGF-2 to the cytoplasmic face of gap junctions [34]. More recently, Doble et al. [83] demonstrated that FGF-2 acting on connexin 43 in neonatal rat cardiomyocytes decreased cell to cell communication via the phosphorylation of a serine residue on the cytosolic face of gap junctions.

FGF-2 released by cells of the vasculature, interstitium and cardiomyocytes, may trigger capillary and vessel formation and cardiac innervation [63]. For instance, FGF-2, in conjunction with vascular endothelial growth factor, was able to stimulate endothelial cell proliferation, migration, and vascular tube formation in atrial and ventricular explants in from rat embryos [18,64]. As well, FGF-2 was able to promote smooth muscle cell migration and proliferation in coronary arteries [65,66] .

In cardiomyocytes, nuclear localisation of FGF-2 has been associated with proliferative cells suggesting a possible role in cell proliferation and growth [17,19]. This concept was supported by work done by Pasumarthi et al. [17] who transfected and overexpressed higher molecular weight FGF-2 (21.5-22-kDa) in rat neonatal cardiomyocytes. Their work showed two types of chromatin patterns in the nuclei where the expressed FGF-2 localized. In most nuclei of overexpressing cells, the pattern of chromatin clumping or fragmentation resembled that of nuclei in the prophase of mitosis.

However, a second chromatin pattern was observed in nuclei of overexpressing cells where the chromatin appeared "clumped" (similar to the pattern seen in nuclei of apoptotic cells). Thus it would seem that high molecular weight FGF-2 may exert both a proliferative response or an apoptotic one. Indeed, further evidence for the involvement of FGF-2 in nuclear and cellular division come from experiments showing the presence of endogenous FGF-2 in close association with chromatin during early prophase in the chicken [18].

In early development, FGF-2 has been localised, at first only in developing myocardial cells, appearing subsequently in the extracellular matrix [67]. Functional studies have shown that FGF-2, along with TGF-ß, FGF-1, IGF-II and other growth factors may contribute to induction of the cardiac myocyte lineage or other components of the heart. Formation of valve primordia in chick embryos (epithelial- mesenchymal induction) is elicited by TGF-ß1 [68] or cardiac extracellular matrix containing FGFs [69]. As well, in cultured precardiac endoderm cells from explanted chick embryos, FGF-2, was able to induce terminal cardiac differentiation as characterised by the exhibition of synchronous contractions and expressed cardiac alpha-actin mRNA [70].

In cardiac disease and wound healing, endogenous FGF-2 may stimulate regeneration [34,35,48,71]. An increase in cytoplasmic FGF-2 immunolabelling for necrotic myocytes with eventual increased perimyocyte staining in regions bordering the infarct area, and an overall increase in FGF-2 accumulation was observed for cardiac muscle cells in the mdx mouse model of injury and regeneration [72] and in the isoproterenol-induced cardiomyopathic rat model [51]. These observations suggest that in

response to cardiomyocyte injury there is an increase in FGF-2 expression/release in cells of the injured areas possibly playing a role in the cardiac healing process.

A number of studies have turned toward looking whether exogenous FGF-2 may affect cardiac function. FGF-2 when administered to rabbits induced peripheral vasodilation and hypotension[181]. This vasodilatory effect by FGF-2 was shown to be mediated by the initial activation of K^+ATP sensitive channels and the release from the endothelium of endothelial relaxing factor (EDRF) or nitric oxide. In addition FGF-2, injected systemically into *in vivo* rats, induced a dose dependent short-lasting decrease in blood pressure which was followed by a striking increase in blood pressure and heart rate variability [73]. These FGF-2-induced physiological effects were shown to involve an initial release of nitric oxide followed by a release of vasoactive prostanoids. In *in vivo* canine hearts, exogenous FGF-2 induced an acute vasodilation and increased coronary blood flow[74].

In *in vivo* models of canine [74,75], porcine [76,77], and rat [78] coronary occlusion, long term treatment with FGF-2 increased coronary collateral artery formation, increased recovery of contractile function, increased recovery of coronary collateral artery endothelial function and decreased infarct size. Furthermore, FGF exerted a protective effect against hydrogen peroxide or serum-starvation induced injury of cultured cardiomyocytes [57].

In the adult heart, FGF-2 receptor activation can occur upon the release of FGF-2 into the cardiac milieu either from exogenous sources (administration) or from endogenous sources. Endogenous sources of FGF-2 in the heart include mainly

cardiomyocytes [160,161], but also vascular smooth muscle cells [162], endothelial cells [143] and blood borne cells such as macrophages [122]. FGF-2 is likely released from intracellular stores through cell injury and mechanical stress [160,161] on the cell. In the heart, FGF-2 release was shown to be mediated via a contraction -induced cardiomyocyte cell wounding. In this case, pacing of the heart caused temporary reversible wounding of the myocyte membrane allowing for the passage of intracellular FGF-2 into the extracellular milieu [161]. In addition, other modes of FGF-2 release may exist in the heart. Angiotensin II, for instance, induces the release of FGF-2 from vascular smooth muscle cells via a unknown mechanism [162].

In cultured neonatal rat ventricular myocytes, FGF-2 was shown to increase the activity of voltage-dependent Ca^{2+} channels [164]. The activation of these channels, in turn, resulted in an increase in cardiomyocyte beating frequency. Inositol-3-phosphate (IP3) was thought to be one of the intermediate signals responsible for transducing this effect since addition of this compound to neonatal rat cultures also induces a similar increase in cardiomyocyte beating frequencies. Thus, presumably, activation of PLCγ1 to produce IP3 [164] and possible PKC activation by diacyl glycerol may also participate in transducing this physiological effect of FGF-2 in neonatal cardiomyocytes [164].

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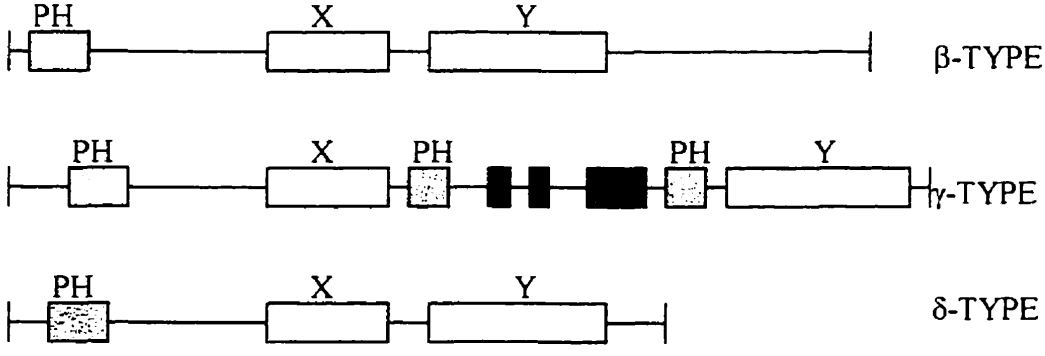
1.6 PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C (PLC)

As reviewed by Noh et al. [1] and Bristol et al. [2], phosphoinositide-specific phopholipase C (PLC) is a hydrolytic enzyme, which when activated by specific cell receptors, catalyzes the breakdown of the common inositol-containing phospholipids: phosphatidylinositol [PtdIns or PI], phosphatidylinositol 4-monophosphate [PtdIns(4)P or PIP], and PtdIns(4,5)P_2 [PIP2] [1]. The result of this hydrolytic reaction is the production of inositol 1,4,5-triphosphate (IP3) and 1,2 diacylglycerol (DAG) [1,2].

When produced, IP3 and DAG act as important biological signals. IP3, for instance, functions in the transient release of calcium upon IP3 binding to its endoplasmic reticulum receptors [2]. DAG functions as a potent protein kinase C activator [3].

There are 10 isoforms of mammalian PLC which are divided into three families: PLC-γ (1 and 2), PLC-β (1-4) and PLC-δ (1-4). These isoforms differ in their amino acid sequence, sharing anywhere from 40 to 60 percent homology within their X and Y regions [1]:



Of the three families of PLC, PLC-γ is the largest in size followed by PLC-β then PLC-δ [1]. All three families contain a pleckstrin homology domain in their amino terminal ends with only PLC-γ having an extra split PH domain between the X and Y domains. In addition, PLC-γ contains src homology domains (SH2 and SH3). These PH and SH domains function as binding domains to other signal transduction proteins which activate the PLC [1].

The various isoforms of PLC differ in terms of substrate affinity, products formed and mode of activation. For instance, in terms of selectivity for PIP2 over other phosphotidylinositols, PLC-β1 has a greater affinity than PLC-δ1, which in turn, has a greater affinity than PLC-γ1 [1]. In addition, although all three types of PLCs produce cyclic and non-cyclic inositol phosphates, PLC-β1 produces a higher ratio of cyclic versus noncyclic inositol phosphates over that of PLC-δ1, which in turn, produces a higher ratio than PLC-γ1. This ratio of cyclic versus non-cyclic inositol phosphates is important in that it is generally believed that cyclic inositol phosphates regulate cell growth [1].

Although all three families of PLC require calcium for activation, they also differ in other activation requirements. PLC-β isoforms for instance, are activated by receptors which activate the sub-family of Gq proteins [1]. These Gq proteins differ in their type of α-subunits ($G\alpha_q$, $G\alpha_{11}$, G_{14} and G_{16}), which, in turn, differ in their affinity for guinidine triphosphate (GTP) and different isoforms of PLC-βs. In vitro studies have shown that both the α-subunits of pertussis toxin-insensitive G-proteins and the βγ subunits of pertussis toxin sensitive Gq proteins are capable of activating PLC-β [10]. However these

subunits differ in their affinities for the various PLC-β isoforms with the α subunits activating PLC-β isozymes in the order of PLC-β1>PLC-β3>PLC-β2, and the βγ subunits activating PLC-β isozymes in the order of PLC-β3>PLCβ2>PLCβ1 [10]. Some agonists which activate Gq proteins include vassopressin, adrenelin and carbachol [2] and endothelin-1 [6]. Once activated, PLC-β activity can be regulated by PKC via a feedback loop mechanism [17].

PLC-β isoforms (1 and 3) have also been shown to contain PEST sequences which are common to proteins susceptible to calpain proteolysis [10,11]. Both the PLC-β1 (140 kDa) and PLC-β3 (140 and 155 kDa) were shown to be cleaved by calpain near the carboxyterminal end to produce a 100 kDa proteolytic isoform which is more susceptible to βγ-subunit activation [10,11].

In the heart, PLC-β activity has been demonstrated in cultured rat neonatal cardiomyocytes [12], adult rat cardiac muscle [5]. Here, activation of PLC-β has been correlated with an increase PKC activation and an increase in cardiac hypertrophy[3,12].

PLC-γ is activated via the phosphorylation of tyrosine residues in its SH domains by agonist activated receptor tyrosine kinases [2,7-9]. Such agonists have been shown to include: platelet derived growth factor [7], erythropoietin [16] nerve growth factor [13], epidermal growth factor [8], and fibroblast growth factor [14]. Activation of PLCγ isoforms, in particular PLCγ1, has been correlated with increased mesoderm induction [15], myocardial growth and hypertrophy [16].

Currently, the mechanism for PLC-δ activation is unclear [1]. However, recently PLC- δ1 in adult rabbit ventricles has been shown to require phosphatidic acid to bind to the membrane for activation[18].

In the heart, PLC activation has been associated with diverse functions including growth and protection on one hand and induction of ischemia-reperfusion injury on the other [1]. In addition, PLC has been shown to participate in regulating the heart's inotropic and chronotropic processes [3]. When PLC is activated in the heart by α1-adrenergic agonists, endothelin-1, angiotensin II, histamine, α-thrombin, and P2-pruinergic agonists, an increase in IP3 production occurs leading to increases in intracellular Ca2+ concentrations. Increased intracellular Ca2+ concentrations, in turn, lead to a positive inotropic effect. Increased activation of PLC also leads to the release of DAG. DAG is a potent activator of PKC. PKC, in turn, has been shown to participate in preconditioning and in reducing infarct size after a short period of ischemia in the heart [3]. PKC activation, also has been correlated with both a positive and negative inotropic effect. Why do differences in inotropism exist with PKC and PLC activation is uncertain? A number of possibilities have been proposed ranging from species differences to varying degrees of PKC isoform activation and different types of experimental manipulations [28,29]. In addition, varying degrees of PLC isoform activation and direct participation can also occur in the inotropic process which is dependent upon the type of experimental model and manipulation being performed.

As well, increased PLC activity has been shown to participate in the development of adaptive hypertrophy of the heart following chronic increased workload [3]. For instance, in cardiomyopathic hamsters [19] and stroke-prone spontaneously hypertensive

rats [20], increased PLC activity, with increased workload eventually led to increased cardiac hypertrophy.

Increased PLC activity has also been reported as potentially deleterious to the heart. For instance, in isolated rat hearts after a period of ischemia followed by reperfusion, there is an increase in PLC activity [21-23] which coincides with increased intracellular Ca^{2+} [23], arrhythmias [21,23] and decreased contractile parameters [22].

Fibroblast growth factor-2 is a potent activator of PLC activity with its receptors FGFR1 having the highest affinity for PLC-γ [24]. Activation of PLC-γ1 in Xenopus embryos is correlated to mesoderm activation [25]. In PC12 cells, FGF-2 stimulation of PLC-γ1 is correlated with increase neurite outgrowth and proliferation [26]. In C212 mouse myoblasts, FGF-2 was shown to induce the activation of TIS1 transcription factors via the activation of PLC-γ1 [27].

Recent data by Mohammadi et al. [24] and Clyman et al. [14] in myoblast cultures, show that although FGF-2 does indeed activate PLC-γ1, blockage of this pathway does not block the mitogenic and chemotactic effects of FGF-2, respectively. Thus, the role of the activation of PLC by FGF-2 still remains speculative.

1.6.1 REFERENCES: Chapter 1.6 PLC

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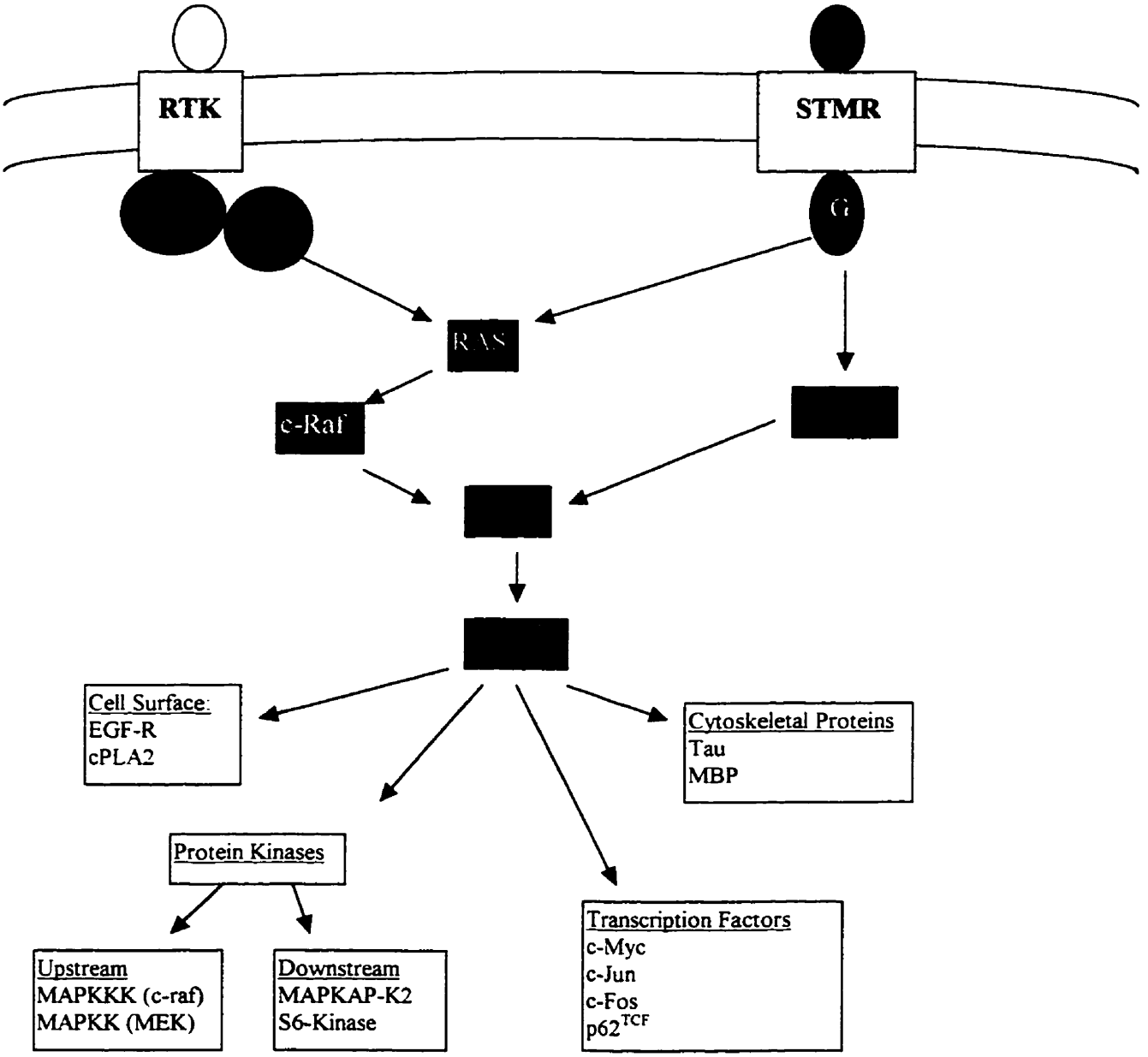
1.7 MITOGEN-ACTIVATED PROTEIN KINASE (MAPK)

MAPK is a serine/threonine kinase which is an important intermediary signal transduction protein. There are different isoforms of MAPK ranging from the extracellular-signal-regulated kinases (ERKs) and JNK/SAPK in mammals and higher eucaryotes to the HOG1 homologs in yeast [1].

As reviewed by Page et al. [2], the basic components of the classical MAPK signal pathway generally follows the following scheme:



To become active, MAPK requires dual phosphorylation of a tyrosine and threonine residues patterned in a T-x-Y motif where T represents threonine and Y represents tyrosine. X varies depending on the MAPK family [1]. In case of ERK1 and ERK2, the X residue is glutamic acid (E) [2].

As outlined in the above scheme by PAGE et al. [2], in mammalian cells, activation of the MAPK signal pathway originates with agonist binding to either a receptor tyrosine kinase (RTK) or to a seven member spanning receptor (STMR). Following RTK activation, RAS becomes activated when RTK transfers its signal through two intermediary proteins, Grb2 and mSos. Ras then interacts and activates c-raf (MAPKKK) which in turn phosphorylates MEK(MAPKK). MEK then, in turn, activates MAPK. Alternatively, STMR activation could trigger Gq protein activation which is followed downstream by either Ras or MEKK activation.

Once MAPK is activated, it can target: cell surface proteins such as epidermal growth factor receptor (EGFR) and phospholipase A2 (PLA2); cytoskeletal proteins such as ternary complex factor (TCF) and myelin basic protein (MBP); transcription factors such as c-Myc, c-Jun, c-Fos and $p62^{TCF}$; other downstream signal proteins such as MAPKAP-K2 and S6-kinase; and upstream signal proteins such as MAPKKK and MAPKK [2].

In cardiomyocytes neonatal [3] and adult [4] and non-cardiomyocyte cells of the heart, MAPK p42 and p44 are the predominant isoforms. In addition, p40 and p44/45 MAPK isoforms have also been detected in rat aortic vascular smooth muscle cells although their specific role is uncertain [2].

In cardiac myocytes, MAPK has been shown to be triggered by bradykinin [5], Endothelin [6], FGF-1 [3], stretch [7] and phenylephrine [8]. Although all these agonists trigger MAPK to induce cardiac growth and hypertrophy, modes of MAPK activation differ [2]. For instance, whereas endothelin, bradykinin and phenylephrine activate MAPK to induce hypertrophy via the translocation and activation of nPKC (-δ and ε) [5], aFGF activation of MAPK is thought be PKC independent [3].

MAPK activation has also been shown to mediate biological effects in non-myocyte cardiac cells. In fibroblasts, activation of MAPK by angiotensin II and platelet derived growth factor is correlated with increased fibroblast proliferation [9]. In rat aortic smooth muscle, MAPK activation was thought to mediate the proliferative and hypertrophic response following vascular injury [10]. In vascular endothelial cells, PKC activation was shown to mediate the activation of ERK1/ERK2 [11], which in turn, led to endothelial cell proliferation [11].

FGF-2 has also been shown to be a potent activator of MAPK. In L929 cells, FGF-2 was shown to protect cells against tumor necrosis factor alpha-mediated apoptosis via the activation of Ras and MAPK p42 [12]. In Xenopus oocytes MAPK activation was shown to mediate the mesoderm induction by FGF-2 [13]. In endothelial cells, FGF-2 induces the activation of PLA2 and release of arachidonic acid. This pathway was shown to be mediated via MAPK activation resulting in endothelial cell motility [11]. In cardiac rat neonatal myocytes, FGF-2 induction of MAPK resulted in increased hypertrophy [3].

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1.8 BETA-ADRENERGIC RECEPTOR KINASE-1 (βARK1)

βARK1 is a serine/threonine kinase which phosphorylates amino acid residues of various target receptors such as the β_1 and β_2-adrenergic receptor [1,3], α_{2A}-adrenergic receptor, muscarinic M2, and rhodopsin [2]. βARK1 (79 kDa) is one of a family of six G-protein-coupled receptor kinases responsible for receptor desensitization via a receptor-G protein uncoupling mechanism [4]. The mechanism of this receptor desensitization involves the phosphorylation of agonist-occupied ß-receptors by the specific ßARK followed by binding of the inhibitor protein ß-arrestin to the phosphorylated receptors. The result of this desensitization process is the decrease of the myocyte's contractile response to adrenergic stimulation. [4]. The Ser/Thr target sites for ßARK are most likely located in the carboxy-terminal regions of the ß-adrenergic receptors [2].

Activation of ßARK is dependent on agonist occupation of its receptor site and the $G_{\beta\gamma}$ subunit for anchoring ßARK to the receptor site on the membrane [1,7]. Some examples of agonist-receptor complexes which activate βARK include: noradrenaline [6]; Substance P [8]; acetylcholine [9]; and prostaglandin [5]. In addition, protein kinase C [1] has been shown to phosphorylate and activate βARK1 and induce its translocation from the cytosol to the membrane where β-adrenergic receptors are located.

In the heart, increased ßARK expression and activity and ß-adrenergic receptor uncoupling is associated with failing human hearts [4] and myocardial ischemia [6].

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1.9 c-FOS AND c-JUN

c-Fos and c-Jun are nuclear oncogenes which code for transcription factors which interact with one another to form the DNA binding activator protein-1 (AP-1) complex [1]. Both c-fos and c-jun contain an α-helical structure which contains repeated leucine residues. These repeated leucine residues interact with one another between the c-jun and c-fos to form as leucine zipper. This zipper allows for dimerization of the c-jun and c-fos onto an AP-1 complex [29]. It is the c-jun portion of the AP-1 complex that binds to DNA elements of late response genes containing TRE and AP-1 sites [30].

Structurally, c-fos is a member of a family of related fos proteins with a molecular weight of approximately 55 kDa and consists of 380 amino acid residues [31-33]. c-Jun also belongs to a multigene family of jun proteins which is approximately 39 kDa and consists of 334 amino acid residues [34-36].

In the heart, c-fos and c-jun have been shown to mediate the actions of various agonists and trophic factors which include: endothelin-1 [11,12]; norepinephrine [6,14]; angiotensin [2,3,11,12,15,16]; stretch receptors [2,3,14,17]; mechanical and emotional stress[6,8]; pressure overload [3,6]; hypertension and hypotension [4]; transforming growth factor-β [6,14]; hydrogen peroxide [7]; phenylephrine [9]; isoproterenol [10] and ischemia [13].

As a result of c-fos and c-jun activation a number of biological effects occur. For instance, in human patients suffering from atrial septal defect and tetrology of fallot, induction of protooncogenes such as c-fos and c-myc was correlated with increased synthesis of messengers of heat shock protein gene-HSP70 and cardiac hypertrophy [18]. In fetal and neonatal rat tissues, β-adrenergic stimulation of c-fos promoted cell

differentiation as opposed to replication [9]. In contrast, in adult rat cardiomyocytes, isoproterenol induced cardiac hypertrophy. This hypertrophy was correlated with an increase in c-fos but not c-jun expression [19]. On the other hand, α-adrenergic stimulation by phenylephrine and norepinephrine in adult rat cardiomyocytes induced cardiac hypertrophy with both upregulation of c-fos and c-jun expression [9,19]. In addition, in adult cardiomyocytes, induction of c-fos and c-jun with angiotensin [2,11,12], endothelin [11], stretch [2] and pressure [3,5,21] and volume overload [21] all led to increased hypertrophic growth. Furthermore, in adult animal hearts, induction of c-fos and c-jun by mechanical stress [6,22,23] and other trophic factors such as transforming growth factor-β and norepinephrine [6] was associated with increased re-expression of fetal contractile genes [6,22] and increased expression of atrial natriuretic factor [23].

Proto-oncogene expression in cardiomyocytes has also been associated with non-growth or differentiating effects. Das et al. [20] demonstrated that with preconditioning of isolated perfused adult rat hearts, there is an induction of proto-oncogene (c-fos and c-myc) expression along with the expression of various stress and anti-oxidant proteins. The expression of these genes was correlated with increased protection of the heart against ischemia-reperfusion injury. Similarly, Wechsler et al. [3] in their models of global myocardial stunning, demonstrated elevated expression of heat shock protein 70, c-myc and c-fos upon exposure to ischemia-reperfusion. In addition, Brand et al. [24] showed in porcine myocardium subjected to ischemia and reperfusion, slight elevations in c-jun expression and five to seven fold increases in c-fos, Egr-1 and junB expressions. The authors speculated that increased induction of these transcription factors may

represent some sort of adaptive response against ischemic stress, possibly related to a repair and/or angiogenic processes [24].

FGF-2, has also been shown to activate c-fos and c-jun. In cultured hamster lung fibroblasts, FGF-2 was found to up-regulate c-fos, c-jun and c-myc expression. The upregulation of these early-immediate genes was correlated with an increase in FGF-2-induced fibroblast mitogenicity [25]. Similarly, in bovine corneal endothelial cells, FGF-2 increased c-fos expression, which in turn was correlated to increased cellular proliferation [26]. In oligodendrocyte progenators FGF-2 induction of c-fos expression was correlated with protein kinase C activation and increased cell proliferation [27]. In cardiac muscle cells, FGF-2 was shown to upregulate c-fos expression. This expression was correlated with re-expression of fetal cardiac genes (such as skeletal α-actin, β-myosin heavy chain and atrial natriuretic factor [28].

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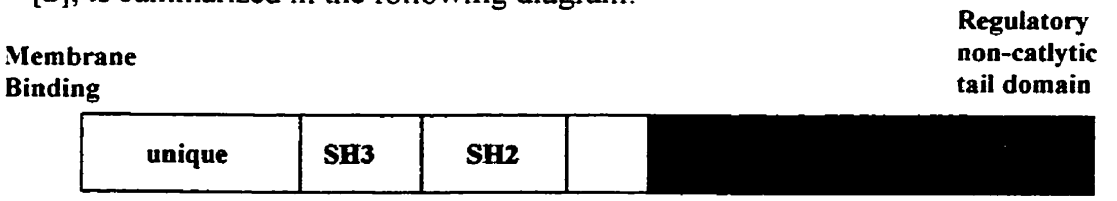
1.10 SRC

$pp60^{c\text{-}src}$ or c-src is a member of a family of nine non-receptor tyrosine kinases, whose members differ due to alternative translational initiation codons and tissue-specific splicing [1]. c-src is a peripheral membrane protein, which is involved in cellular signaling [2].

Structurally, members of this family share amino acid homology for all but 60-80 amino terminal amino acids [1]. Members of the c-src family range in size of approximately 500-530 residues. Their common structure, as outlined by Shalloway et al. [2], is summarized in the following diagram:



From the N-terminus, structural regions include: the N-terminal myristoyated signal, the SH3 and SH2 (i.e. src-like homology) domains, and a C-terminal non-catalytic tail [1]. The unique sequences represent the amino acid residues with greatest variability among the Src family members with many sites for serine/threonine protein kinases [2]. At the amino terminal end is a myristylated terminal glycine residue thought to be involved in membrane association of the c-src [3]. The two Src homology domains (SH2 and SH3) are thought to act as binding domains of Src to tyrosine phosphorylated proteins which may regulate Src activity. The catalytic kinase domain on the carboxy-terminal end contains a tyrosine 416 residue which, when phosphorylated, stimulates src activity [1,2].

Src functions as a signaling protein for a variety of agonists. Only a few examples will be mentioned here. For instance in human thrombin-stimulated platelets, Grondin et al. [4] observed an increase in pp60c-src migration from the soluble to the cytoskeletal fraction where it is thought src may activate lipid metabolizing enzymes such as phosphostidylinositol-2-kinase and phospholipase C. In NIH 3T3 fibroblast cell lines, platelet-derived growth factor (PDGF) induced the activation and cytosol to membrane translocation of c-src to the PDGF receptor [5]. In cultured aortic rat smooth muscle cells $pp60^{c\text{-}src}$ was shown to mediate angiotensin II activation of PLC-γ1. Phosphorylation and activation of PLC-γ1 by angiotensin II, in turn, induced increased smooth muscle cell production of plasminogen activator [6].

Fibroblast growth factor-2 has also been shown to activate Src in a variety of cell systems. In PC12 cells, a p60 src - p21 ras activated pathway was shown to become activated upon FGF-2 binding, and may also play a role in neurite outgrowth and differentiation [9,11,12]. In lung capillary endothelial cells and murine fibroblasts, FGF-2 stimulation of FGFR-1 led to an increase in autophosphorylation of various Src family members [10]. As well, an association of activated FGFR1 with src was reported for NIH 3T3 cells [13]. In contrast though, in porcine aortic endothelial cells and lung fibroblasts from Chinese hamster, activation of FGFR caused a reduction in the autophosphorylation of Src. In addition, these cells showed a lack of complex formation between Src and FGFR-1. This negative regulatory effect on Src activity by FGFR-1 was found to be mediated by an increase in PLC-γ and PKC activity [10]. In human umbilical vein endothelial cells, activation of the fibroblast growth factor receptor 1 (FGFR1) is correlated with increased src tyrosine kinase activity and increased proliferation [7].

In the heart, only a limited amount of research so far has concentrated on the role of Src. In cultured neonatal rat ventricular myocytes increased Src activity was shown to stimulate promoter activity of atrial natriuretic factor [8].

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HYPOTHESIS

Fibroblast Growth Factor-2 acts as a cardioprotective agent against ischemia-reperfusion injury in isolated perfused adult rat heart preparations. FGF-2 induces cardioprotection via a mechanism involving protein kinase C activation.

CHAPTER 2

EFFECT OF FGF-2 ON RECOVERY FROM ISCHEMIA AND REPERFUSION OF THE EX-VIVO PERFUSED HEART

2.1 INTRODUCTORY REMARKS

The purpose of this study was to investigate whether administration of FGF-2 to the heart by perfusion would influence recovery from I-R injury. FGF-2 is a multifunctional protein released into the extracellular environment [1,2]. Upon release, FGF-2 likely becomes associated with heparin-like components and with high affinity FGF-2 receptors, which mediate the biological effects of FGF-2 [3]. The mechanism of release of FGF-2 into the extracellular milieu is still under investigation. However, several theories point to either a release through cell death or myocyte injury [1] or through exocytotic vesicles which transport and release FGF-2 during the normal beating of cardiac muscle [4]. In adult hearts, FGF-2 is localized to extracellular, cytoplasmic and nuclear sites of cardiac myocytes, as well as non-muscle cells, suggestive of an intimate involvement with cardiac physiology [2,5-7].

As yet, there is no clear notion as to the physiological function(s) of FGF-2 in adult organs, although its ability to improve survival and prevent degeneration of cells *in vivo* and *in vitro* [8,9] points to a protective role against injury. Physiologically, FGF-2 is shown to have vasodilatory and vasorelaxing properties when administered to in vitro isolated arterial preparations and in vivo animal hearts [11-13]. FGF-2's vasodilatory and

vasorelaxing properties are thought to be exerted by activating K^+_{ATP} channels and inducing the release of nitric oxide and prostaglandin [13,14]. In addition, FGF-2 is shown to affect contractility and induce bradycardia *in vivo* in rats [15]. These vasodilatory and contractile depressant properties would be important in the myocardium subjected to ischemia-reperfusion injury in that blood flow to ischemic areas would be improved and high energy phosphate compounds would be preserved.

The Langendorff perfusion technique was used to supplement isolated whole rat hearts or right ventricles with FGF-2 either just before ischemia or upon reperfusion in the presence or absence of the PKC inhibitor chelerythrine chloride. Measurements were made of: the contractile functional parameters under constant flow and constant perfusion pressure; of creatine phosphokinase (CPK) activity in the perfusate; of the levels of ATP and CP; and of the action potential profile (APD_{90}) to determine the extent of and recovery from myocardial injury. Our results show that: (1) FGF-2 is cardioprotective when given before I-R and after ischemia, during reperfusion; (2) FGF-2 administration to the pre-ischemic heart induces a negative inotropic effect; (3) FGF-2 promotes the recovery of the APD after I-R; (4) Chelerythrine chloride blocks the cardioprotective effect against I-R induced by FGF-2; and (5) FGF-2, when adminstered prior to I-R promoted the production/preservation of ATP and CP and promoted increased recovery of ATP and CP levels after I-R compared to control hearts; and (6) FGF-2, when administered after ischemia, during reperfusion also promoted the increased recovery of ATP and CP levels compared to controls.

2.2 MATERIALS AND METHODS

2.2.1 ISCHEMIA-REPERFUSION USING WHOLE HEART LANGENDORFF PREPARATIONS

2.2.1.1 Pilot experiments using a force transducer

Male Sprague-Dawley rats (250-300g) were killed by decapitation, their hearts excised and washed in cold buffer. After trimming off the atria, extraneous fat, and connective tissue, a short cannula was introduced in the aorta and the hearts were perfused under non-recirculating constant pressure conditions [1]. The composition of the perfusion medium (Krebs-Henseleit) was (in mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 (Malinckrodt Chemical Inc., Paris, KY), KH_2PO_4 1.2 (Fisher Scientific Co., Fairlawn, N.J.), and glucose 9.0 (J.T. Baker Inc. Philipsburg, N.J.). The perfusion solution was continuously gassed with a mixture of 95% O_2 and 5% CO_2 (pH 7.4), and maintained at a temperature of $37^{o}C$. The hearts were electrically driven by an electrode placed in the AV-node with 2-ms pulses at 240 bpm or 4 Hz and a voltage of 10 % above threshold. The hearts were vented near the apex and a resting tension of 2g was applied on starting the perfusion. After an equilibration period of 25-30 min., the resting tension was increased to 5-6 g. The developed contractile force (*DF*) and derivative of the contractile force (*dF/dt*) were monitored on the Beckman Dynograph Recorder-R511A by means of a Grass FT-03 force displacement transducer. FGF-2 (5-20 μg) was dissolved in 12 ml of perfusion solution and 'injected' into the perfusion buffer at 12ml/ min. immediately before the point of entry to the heart. Hearts were then exposed to 60 min. of global ischemia under ambient conditions followed by 30 min. of reperfusion.

To minimize losses due to adherence to glassware and tubing, FGF-2 was added to a small volume of buffer which was then infused immediately to the main stream entering the aorta via retrograde perfusion. It has not been possible to quantitate the absolute amount of FGF-2 that reached the heart as a substantial proportion adheres to the cannula and tubing [11].

2.2.1.2 Creatine phosphokinase assay

Perfusate aliquots (1ml each) were collected once before global ischemia induction as well as at 1 min. intervals during 20 min. reperfusion. These samples (kept on ice) were immediately sent for CPK determination to the Biochemistry department of St. Boniface General Hospital (Winnipeg, Canada); CPK activity was assayed using a BM-Hitachi System 717 automated analyzer and BMC automated analyzer kit (Boehringer Mannheim Canada Inc., Dorval, Que).

2.2.1.3 Experiments using a balloon catheter and computerized data recording and analysis

Rat hearts were prepared and perfused essentially as described in section 2.2.1.1. Hearts were perfused under non recirculating conditions [4,5] either under a constant pressure (80 cmH_2O) [4,5,6] or constant flow rate (10 ml/min.) [6,7,8]. The hearts were vented near the apex and a resting tension of 2g (under constant flow perfusion) or a left ventricular end diastolic pressure (LVEDP) of 2 mmHg (under constant pressure perfusion) was applied on starting the perfusion. After an equilibration period of 25-30 min., the resting force was increased to 5-10g (constant flow perfusion) or the LVEDP

was increased to 5-10 mmHg depending on the maximum Frank-Starling length-tension relationship achieved [6].

Under constant flow conditions, after a 25- to 30- min. period of equilibration when the wall resting tension was gradually increased to produce optimal developed tension, global ischemia was induced by turning off the peristaltic pump. During ischemia, a plexiglass cover was placed over the entire experimental apparatus so that the humidity temperature, and O_2 content of the air could be regulated, as described [8]. Reperfusion began after 20 min. of ischemia by turning the peristaltic pump back on for approximately 60 min. The developed force (*DF*) , the derivative of the contractile force (*dF/dt*) and the resting tension (RT) were monitored on a polygraph (Linearecorder Mark VII WR3101, Graphtec, Irvine, CA). Changes in coronary arterial perfusion pressure was measured using a glass manometer situated at a point just prior to where the perfusion medium enters the heart. Under constant pressure conditions, after a 25- to 30-min. period of equilibration when LVEDP was gradually increased to produce optimal developed pressure, global ischemia was induced by clamping the flow of perfusion medium to the heart. During ischemia a glass and plexiglass cover was placed around the entire heart with a pool of heated perfusate ($37^{o}C$) just under the heart such that the humidity, temperature, and O_2 content of the air could be regulated. Reperfusion began after 30 min. of ischemia by restoring flow of perfusion medium at a constant reperfusion pressure of 80 cmH_2O for 60 min. Developed pressure (defined as systolic left ventricular pressure), EDP and its derivative (*dP/dt*) was measured with a water-filled latex balloon (Radnotti Glass Technology Inc. Monrovia, CA), which was introduced into the left

ventricle via a left atrial incision and connected to a Gould Statham pressure transducer (P23 ID) and a Biopac Acquisition System (Harvard Apparatus Canada, Saint-Laurent, Quebec). Intraballoon pressure was adjusted such that the LVEDP coincided with the ventricular peak Frank-Starling curve for contraction to obtain a stable preparation [6,9].

10 μg of FGF-2 was dissolved in 12 ml (0.825 μg/ml) of the perfusion medium. Using a peristaltic pump FGF-2-supplemented or control solution was 'injected' into the perfusion buffer at 1ml/min. immediately before the point of entry to the heart either just before ischemia or upon reperfusion. Loss during handling through adsorption to plastic tubing was accommodated by using an optimum FGF-2 concentration of 10 μg/ml which is required for maximum recovery, as previous characterized [10].

For the protein kinase C inhibitor studies, chelerythrine chloride (Research Biochemicals International; Natick,MA) was perfused into control and FGF-2-treated hearts at a concentration of 5μmol per ml, 3 min. prior to and during the 12 min. period of FGF-2 infusion.

Coronary perfusion pressure was measured using a glass manometer situated at a point just prior to where the perfusion medium enters the heart. Sodium nitroprusside was purchased from Sigma Chemical Company (St. Louis, Mo.).

2.2.2 I-R using isolated right ventricular wall and electrical recording.

2.2.2.1 Perfusion of isolated right ventricular wall

Adult Sprague-Dawley rats (300-400g) were killed by decapitation and their hearts were quickly removed and placed in a dish containing cold Krebs-Henseleit solution. The right ventricular walls were prepared and maintained *in vitro* according to a technique previously described [2]. The atria were removed, and the right ventricle was dissected from the heart, leaving the base of the aorta attached to the wall and the right coronary vasculature intact. A fine cannula was placed in the aortic opening of the right coronary artery and sutured in place, and the ventricular wall was perfused at a constant flow rate of 1.5 ml/min. with 37^{o}C Krebs-Henseleit solution. The base of the walls was pinned to the bottom of a Perspex bathing chamber with the epicardial surface up and the apex of each tissue attached to a force transducer [Gould Statham] for recording mechanical activity. The muscles were stimulated by 2 millisecond square pulses delivered from a point source at a rate of 2 Hz. Resting tension was adjusted to a level (generally 250 to 400 mg) that optimized developed tension. Action potentials recorded from arterially perfused right ventricular walls under control, ischemic and reflow conditions had a waveform similar to that reported for other preparations [2,3]. The tissues did not exhibit abnormal rhythm or decay in mechanical function for >8 hours and were stable over the period required for the present experiments. There was no evidence of ischemic dysfunction even at the extreme edges of the tissues, implying that the dissection procedure did not damage the arterial vasculature of the right wall and that the entire preparation was well perfused.

After obtaining control recordings from right ventricular walls, the tissues were either (1) subjected to 60 minutes of no-flow ischemia and 60 minutes of reperfusion in the absence of treatment (untreated preparations), or (2), treated with a bolus dose of 20μ

g of FGF-2 solubilized in 30 ml of perfusion buffer and administered to right ventricular walls 15 min. prior to 60 min. of ischemia and 60 min. of reperfusion.

2.2.2.2 Electrical recording

Transmembrane potentials were recorded from ventricular walls with conventional intracellular glass microelectrodes. Impalements were made from the epicardial surface of the right ventricular walls, and action potentials were recorded under control conditions, after 60 minutes of ischemia, and at several times during reperfusion. Mechanical movement of the right ventricular wall preparations precluded continuous electrical recording from a single cell for the entire duration of the experiments; however, it was frequently possible to maintain an impalement for more than one or two sampling intervals before the micropipette was dislodged. The micropipettes (20 to 30 MΩ) were pulled from filamented capillary tubes (outer diameter, 1.2mm; World Precision Instruments) on a P-87 pipette puller (Sutter Instruments), filled with 3 mol/L KCl, and connected to a WPI Duo 773 electrometer (World Precision Instruments). Electrical and contractile activities were recorded on videotape by using a Vetter 420 analog recorder (A.R. Vetter Co.) and subsequently digitized and stored on hard disk at a sampling frequency of 2 kHz by using a TL-1-125 Labmaster A/D board (Axon Instruments), AXOTAPE data acquisition software (Axon Instruments), and an IBM AT clone computer. The following parameters of the recorded action potentials and contractions were determined from the digitized recordings: (1) resting membrane potential, (2) action potential duration (at 30% and 90% repolarization [ADP_{30} and ADP_{90}, respectively], (3) resting tension, and (4) developed tension. Action potentials were selected at random from periods when the

preparations were not arrhythmic. Only those action potentials at which the diastolic (resting membrane) potential was stable for at least three previous cycle lengths were used.

Mean±SEM values of (1) action potential parameters, and (2) resting and developed tension were determined for n=3-4 tissues. Statistical comparisons were made by using a t-EASE statistical calculation software. Unpaired t tests were used for single comparisons. A value of $p<0.05$ was considered to be significant

2.2.3 FGF-2 sources

Recombinant human FGF-2 (preparation #1) was purchased from Pepro Tech Inc. (Rocky Hill, NJ) and used for most of the experiments described. A second human recombinant FGF-2 preparation (preparation #2) was a generous gift from Dr. Andrew Baird (The Whittier Institute for Diabetes and Endocrinology, La Jolla, CA). A third preparation (preparation #3) was produced in E.coli bacteria and purified according to standard procedures [12] with some modifications (full procedure to be submitted for publication by Doble and Kardami).

2.2.4 Measurements of ATP, CP

ATP and CP measurements were performed by enzymatic analysis as described by McPherson et al. [9]. At the end of the no-flow ischemic period, tissue samples were immediately frozen in liquid nitrogen and weighed. The samples were then frozen pulverized and digested with 0.9 M perchloric acid. The solutions were homogenized with a polytron on ice and then centrifuged for 10 min. at 5000 rpm. The suspension was retained and the pellet combined with 0.2M perchloric acid prior to homogenization and

centrifugation as above. The two suspensions were combined, the pH adjusted to 6-6.5 with 3.75 M K_2CO_3 and then frozen prior to assay of high energy phosphate levels (within 1 week). ATP and CP levels were determined according to Lamprecht et al. [11]. Hexokinase (30 mg protein/ml at 135 units/mg protein; Sigma Chemicals, St. Louis, MO.) and creatine phosphokinase (165 mg protein/ml at 135 units/mg protein; Sigma Chemicals, St. Louis, MO.) were used for determination of ATP and CP, respectively.

2.2.5 Calculation and Statistical Analysis

All calculations and statistical analysis were performed using the Microsoft Excel Analysis Tool Pac version 5.0 (Microsoft Corporation, Cambridge, MA) and the T-ease statistical calculation software. Utilizing a paired student t-test for statistical significance (as determined by the degree of standard error), alterations in ATP and CP values (n=4-5) from FGF-2-treated and control hearts were compared at different time points (i.e. i. pre-ischemia; ii. after 30 min. ischemia; and iii. after 30 min. ischemia and 60 min. reperfusion); and versus their pre-ischemic levels.

Statistical significance for changes in coronary perfusion pressure in control and FGF-2-treated perfused hearts (n=4) were also determined using a unpaired two-tailed student t-test for statistical significance. In this case, standard error in the alterations in coronary perfusion pressure was obtained by comparing the amount of pressure change (mmHg), at one minute intervals for 12 min.) upon addition of either FGF-2 or sodium nitroprusside, with the coronary perfusion pressure values prior to perfusion with the exogenous agent. In all cases mentioned above, significant difference occurs when $p<0.05$.

2.3 RESULTS

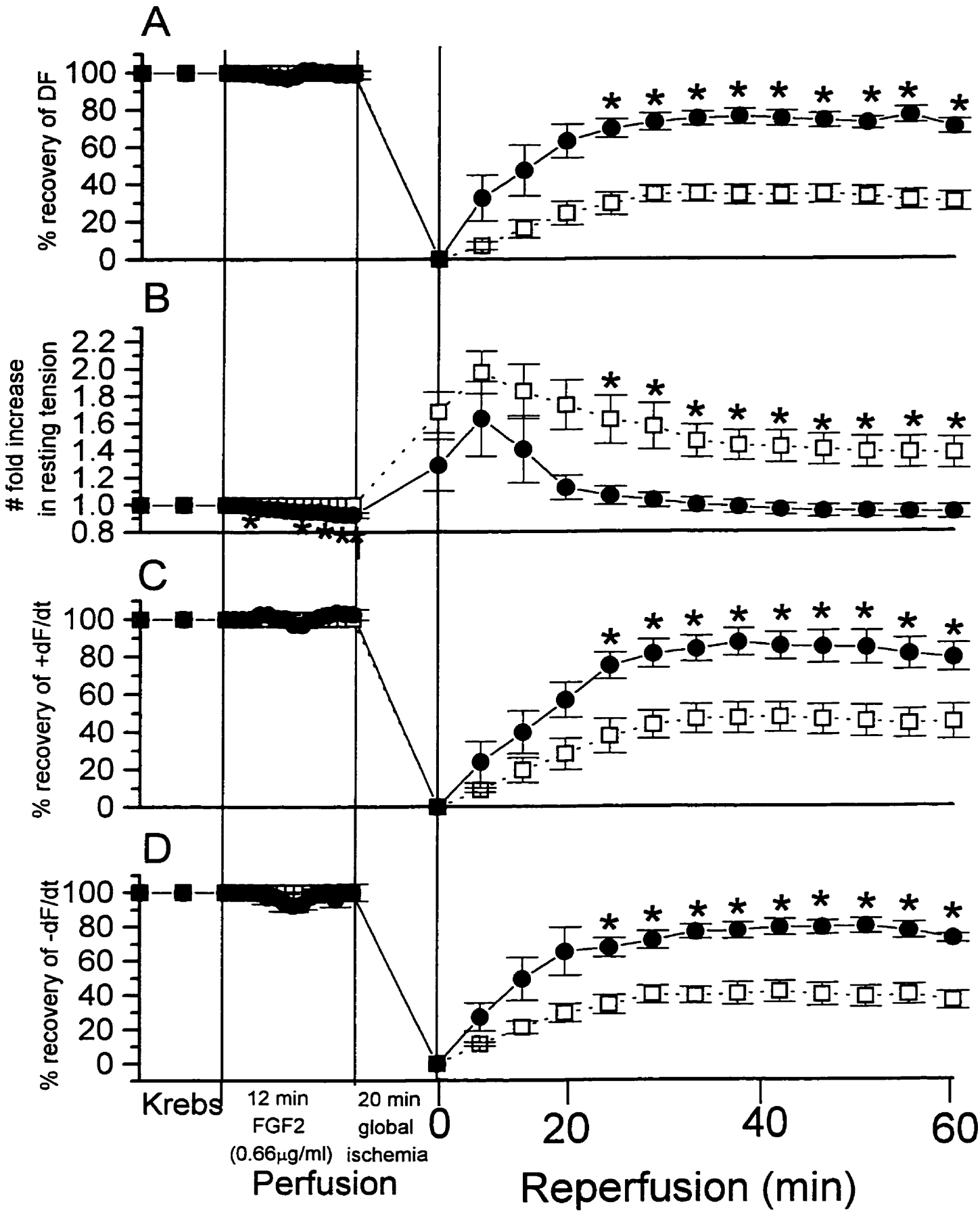
2.3.1 Langendorff model of ischemia-reperfusion (I-R) under normothermic conditions

2.3.1.1 Effect of FGF-2 on I-R under conditions of constant flow

The effect of preischemic treatment with FGF-2 on the recovery of hemodynamic function of the heart after 20 min. of global ischemia and 60 min. of reperfusion is shown in Fig. 2.1. Control group displayed a 34.1±5.1% recovery in *DF* whereas the FGF-2 treated group displayed a significantly improved recovery in *DF* of 76.4±4.1% (Fig. 2.1A) after I-R. A clear rise in resting tension (1.4±0.1 fold increase over pre-ischemic value) is seen in the control group after I-R whereas the FGF-2-treated group displayed a complete recovery of resting tension (Fig. 2.1B). Finally, the control group displayed a 47.6±7.7% recovery in +dF/dt and a 41.74±6.3% recovery in -dF/dt whereas the FGF-2 treated group displayed significantly improved recoveries of 85.5±7.3% and 79.4±4.7%, respectively after I-R (Fig. 2.1C,D).

FGF-2 administration did not induce any significant change in DF (Fig. 2.1A). FGF-2, however, did cause a statistically significant decrease by 7.4±2.1% (Fig. 2.1B) in resting tension during the same time interval, within 12 min. from treatment.

Figure 2.1: Effect of FGF-2 administration on DF (A), resting tension (B), rate of contraction, +dF/dt (C) and rate of relaxation, -dF/dt (D) of isolated rat ventricles during reperfusion after 20 min. of global ischemia, under constant flow conditions. Values are expressed as means ± SE (n=5). FGF-2 was included in perfusion medium 12 min. prior to global ischemia. FGF-2-treated and control hearts are represented by the closed circles (--•--) and open squares (—□—), respectively. Absolute values for DF and resting tension in control group (g/g wet wt) before ischemia were 17.88±2.10 and 6.08±0.39, respectively. Absolute values for +dF/dt and -dF/dt in control group (g/g wet wt/sec) before ischemia were 11.21±0.96 and 9.11±1.03, respectively. Absolute values for DF and resting tension in FGF-2-treated group (g/g wet wt) before FGF-2 perfusion were 17.85±1.49 and 5.00±0.45, respectively. Absolute values for +dF/dt and -dF/dt in FGF-2-treated group (g/g wet wt/sec) before FGF-2 perfusion were 11.59±0.70 and 8.73±0.54, respectively. Absolute values for DF and resting tension in FGF-2-treated group (g/g wet wt) before ischemia were 17.70±1.58 and 4.62±0.44, respectively. Absolute values for +dF/dt and -dF/dt in FGF-2-treated group (g/g wet wt/sec) before ischemia were 11.87±0.71 and 8.73±0.62, respectively. *P< 0.05 vs. FGF-2-untreated group.


A
% recovery of DF
100
80
60
40
20
0
B
fold increase in resting tension
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
C
% recovery of +dF/dt
D
% recovery of -dF/dt
Krebs
12 min FGF2 (0.66µg/ml)
20 min global ischemia
Perfusion
0
20
40
60
Reperfusion (min)

2.3.1.2 Effect of FGF-2 on perfusion pressure

Back perfusion pressure was measured as a function of FGF-2 administration under constant flow conditions. Results are shown in Fig. 2.2. The FGF-2 treated group displayed only a slightly drop in back pressure of approximately 1.82±0.29 mmHg compared to the back pressure before FGF-2 andministration. On the other hand, administration of the vasodilator SNP at a dosage known to cause cardioprotection (10 μMol) caused a large decrease in back pressure of approximately 12.96±2.48 mmHg compared to the back pressure before SNP administration.

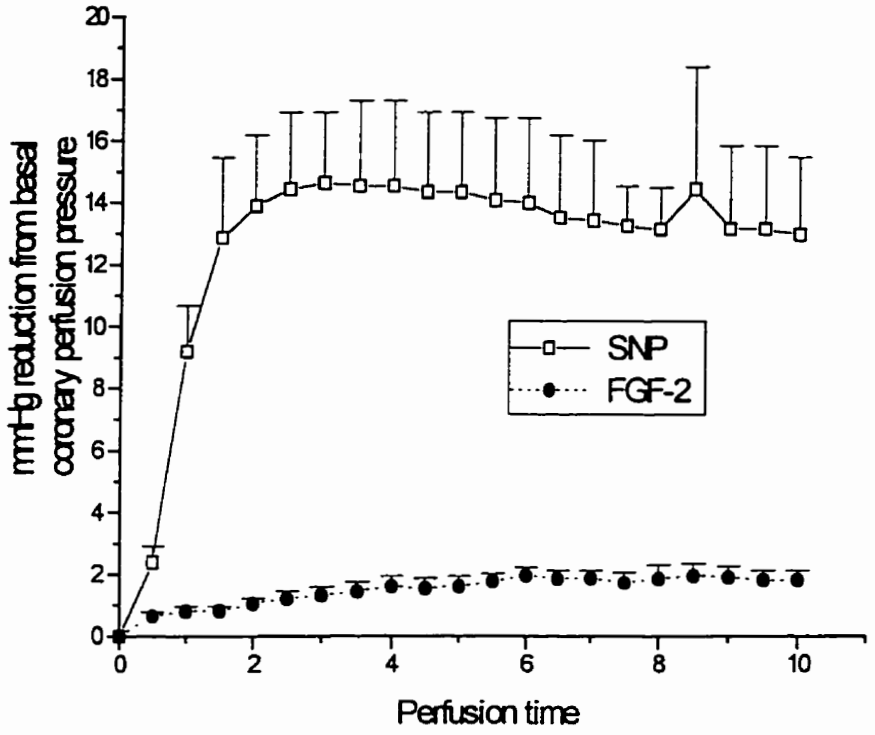


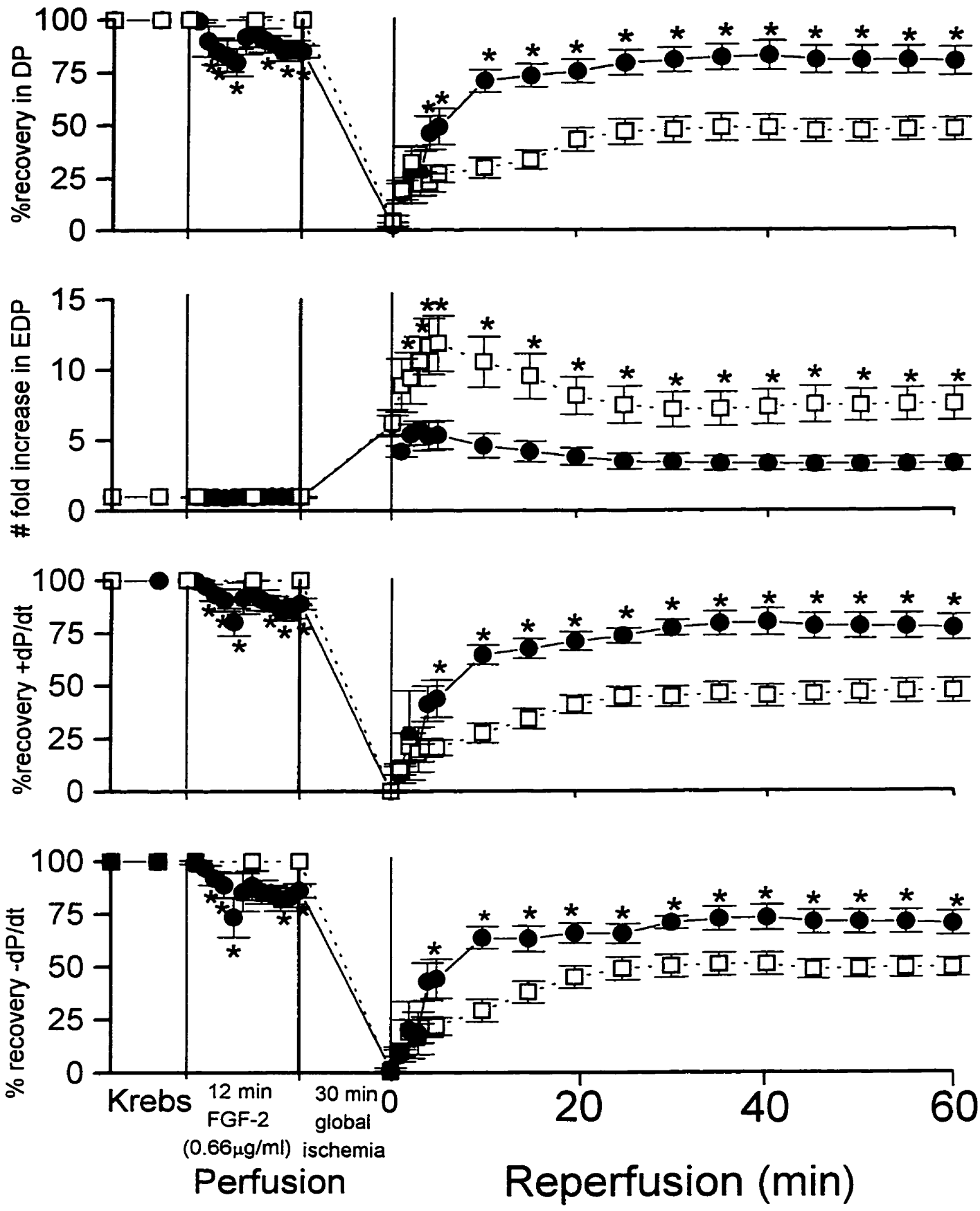
Figure 2.2: Effects of FGF-2 administration on coronary perfusion pressure of isolated rat ventricles, under constant flow conditions. Values are expressed as means ± SE (n=4). 10 μg of FGF-2 (closed circle) or 10 μ mol of SNP was administered into the perfusion medium, 12 min. prior to global ischemia under constant flow conditions. Perfused hearts treated with FGF-2 or SNP displayed a significant mean decrease in coronary perfusion pressure (mmHg±SEM) of 1.5±0.8 and 13.0±2.5, respectively. *P< 0.05.

2.3.1.3 Effect of FGF-2 on recovery from I-R under conditions of constant pressure

The effects of preischemic treatment with FGF-2 on the recovery of hemodynamic function of the heart after 30 min. of global ischemia and 60 min. of reperfusion are shown in Fig. 2.3a. After reperfusion, the control group displayed a 48.68±6.16% recovery in developed pressure (DP) compared to a significantly improved recovery of 83.11±7.03% in the treated group. A large, 7.34±1.23 fold increase in end diastolic pressure (EDP) over the pre-ischemic value, is seen in the control group after reperfusion compared to only a 3.34±0.50 fold increase in treated hearts. FGF-2 did therefore significantly depress the rise in resting tension during reperfusion in comparison to untreated hearts. A similar increase in % recovery of ±dP/dt was observed in FGF-2-treated hearts compared to controls after I-R (Fig 2.3c and d, respectively). Control hearts displayed only a 45.06±5.25% and 51.31±5.45% recovery in positive and negative dP/dt, respectively, FGF-2 perfused hearts, displayed 80.07±6.33% and 73.27±6.02% recovery, respectively.

FGF-2 administration induced a moderate negative inotropic effect, reflected in all contractile parameters measured (Fig. 2.4a, c, and d, respectively). DP decreased by 14.86±2.40% in treated hearts compared to controls, prior to induction of global ischemia (Fig. 2.4a). Similarly, positive and negative dP/dt decreased by 12.03±2.37% and a 15.40±3.15%, respectively after FGF-2 treatment (Fig. 2.4c and d, respectively). No significant change in EDP was observed during the FGF-2 perfusion. (Fig. 2.4b).

Figure 2.3: Effect of FGF-2 administration on DP (A), EDP (B), positive dP/dt (C) and negative dP/dt (D), of isolated rat ventricles after I-R under constant perfusion pressure conditions. Values are expressed as means ± SE (n=5). FGF-2 (closed circle), was included in perfusion medium for 12 min. just prior to global ischemia. Control perfused hearts (open square) contained no FGF-2 in the perfusion medium. Absolute values for DP and EDP in control group (mmHg±SE) before ischemia were 95.14±3.23 and 7.29±0.59, respectively. Absolute values for +dP/dt and -dP/dt in control group (mmHg/sec±SE) before ischemia were 3265.92±59.69 and -3266.53±83.12, respectively. Absolute values for DP and EDP in FGF-2-treated group (mmHg±SE) before ischemia were 85.65±8.98 and 6.68±0.50, respectively. Absolute values for +dP/dt and -dP/dt in the FGF-2 treated group (mmHg/sec±SE) before ischemia were 3460.48±281.80 and -3315.87±371.43, respectively. *P< 0.05 vs. FGF-2-untreated group.


%recovery in DP
100
75
50
25
0
fold increase in EDP
15
10
5
0
%recovery +dP/dt
100
75
50
25
0
% recovery -dP/dt
100
75
50
25
0
Krebs
12 min
FGF-2
(0.66µg/ml)
30 min
global
ischemia
0
20
40
60
Perfusion
Reperfusion (min)

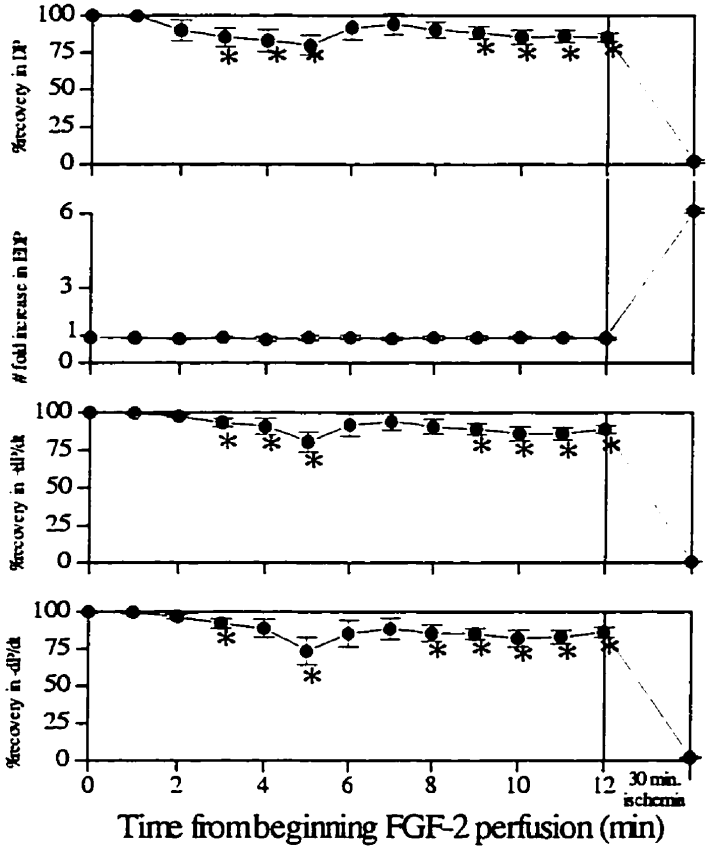
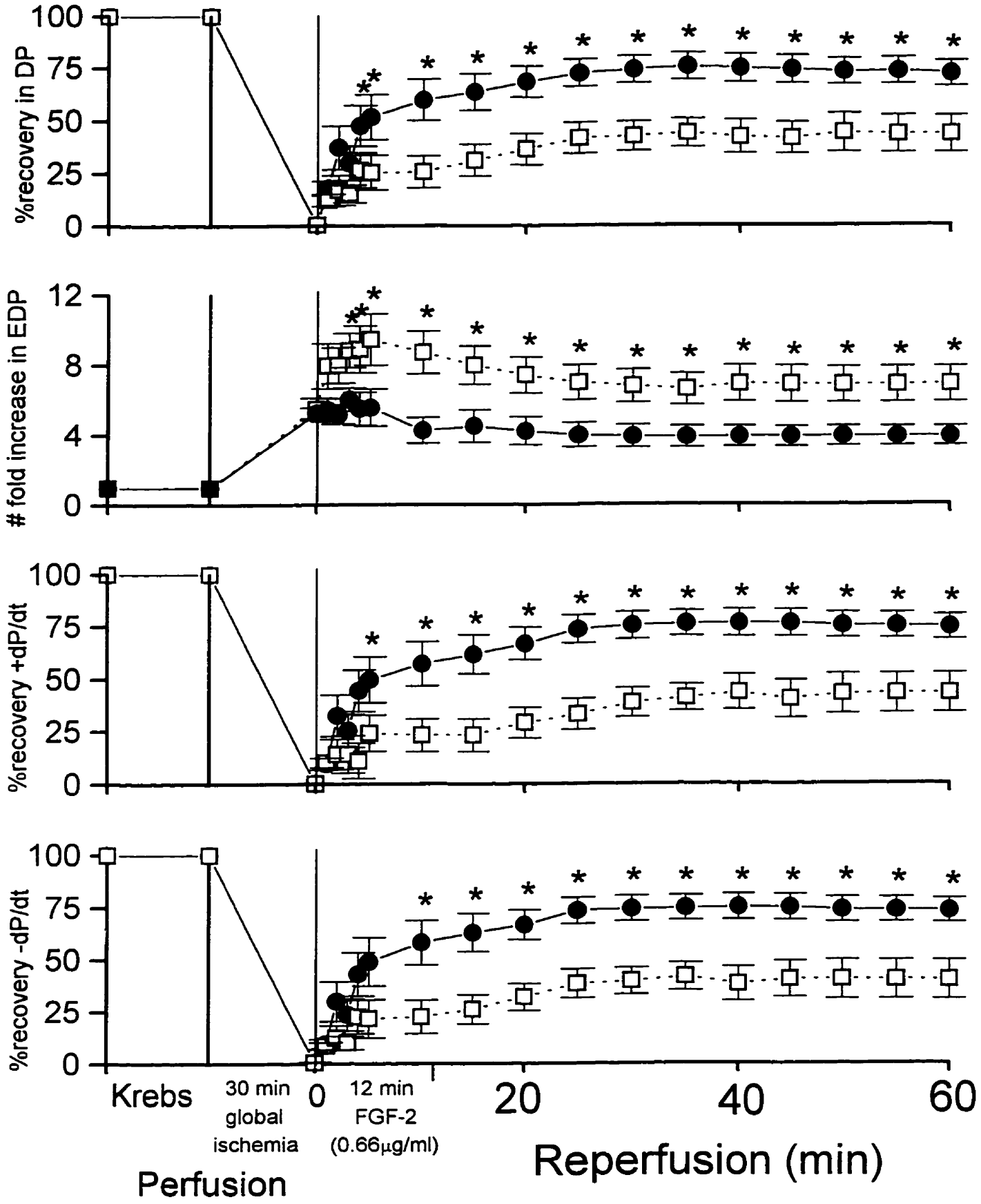


Figure 2.4: Negative inotropic effect of FGF-2 on (A), DP, (B) EDP, (C) positive dP/dt, (D) negative dP/dt under constant perfusion pressure. Values are expressed as means ± SE (n=5). FGF-2 factor was only included in perfusion medium for 12 min. just prior to global ischemia. FGF-2-treated hearts are represented by the closed circles (—●—). Just before the onset of ischemia, a drop in DP of 12.9±2.6% was observed upon FGF-2 administration. *P< 0.05. This figure represents an expansion of the initial preischemic section of Figure 2.3.

2.3.1.4 Effect of FGF-2, administered during reperfusion, on recovery from I-R

The effects of post-ischemic treatment with FGF-2 on the recovery of hemodynamic function of the heart after 30 min. of global ischemia and 60 min. of reperfusion is shown in Fig. 2.5. After reperfusion, control group displayed a 42.14±7.94% recovery in the contractile force whereas the FGF-2 treated group displayed a significantly improved recovery in DF of 75.07±5.07% (Fig. 2.5a). Similarly, control hearts displayed a 40.34±8.39% and 38.28±8.16% recovery in positive and negative dP/dt, respectively, after I-R, compared to a significantly improved recovery of 76.61±6.39% and 75.24±6.12% in FGF-2 treated hearts (Fig. 2.5c and d, respectively). In addition, a large 6.94±1.03 fold increase over preischemic values in end diastolic pressure (EDP) was seen in the control after I-R compared to a 3.93±.57 fold increase in EDP in the FGF-2 treated hearts (Fig. 2.5b).

Figure 2.5: Effect of FGF-2 on DP (A), EDP (B), positive dP/dt (C) and negative dP/dt (D) of isolated rat ventricles when administered during reperfusion after 30 min. of global ischemia, under constant pressure conditions. Values are expressed as means ± SE (n=7-8). FGF-2, was included in the reperfusion medium for 12 min. immediately after global ischemia. FGF-2-treated and control hearts are represented by the closed circles (—•—) and open squares (--□--), respectively. Absolute values for DP and EDP in control group (mmHg±SE) before ischemia were 97.20±8.21, 7.66±0.57, respectively. Absolute values for +dP/dt and -dP/dt in control group (mmHg/sec±SE) before ischemia were 3561.45±276.961 and -3454.92±232.24, respectively. For FGF-2-treated group absolute values (mmHg±SE) for DP and EDP before ischemia were 103.31±5.94 and 7.59±.46, respectively. Absolute values for +dP/dt and -dP/dt in FGF-2-treated hearts (mmHg/sec±SE) before ischemia were 3696.80±202.11 and -3520.82±192.58, respectively. *P< 0.05 vs. FGF-2-untreated group.


%recovery in DP
100
75
50
25
0
fold increase in EDP
12
8
4
0
%recovery +dP/dt
100
75
50
25
0
%recovery -dP/dt
100
75
50
25
0
Krebs
30 min
global
ischemia
0
12 min
FGF-2
(0.66μg/ml)
20
40
60
Perfusion
Reperfusion (min)

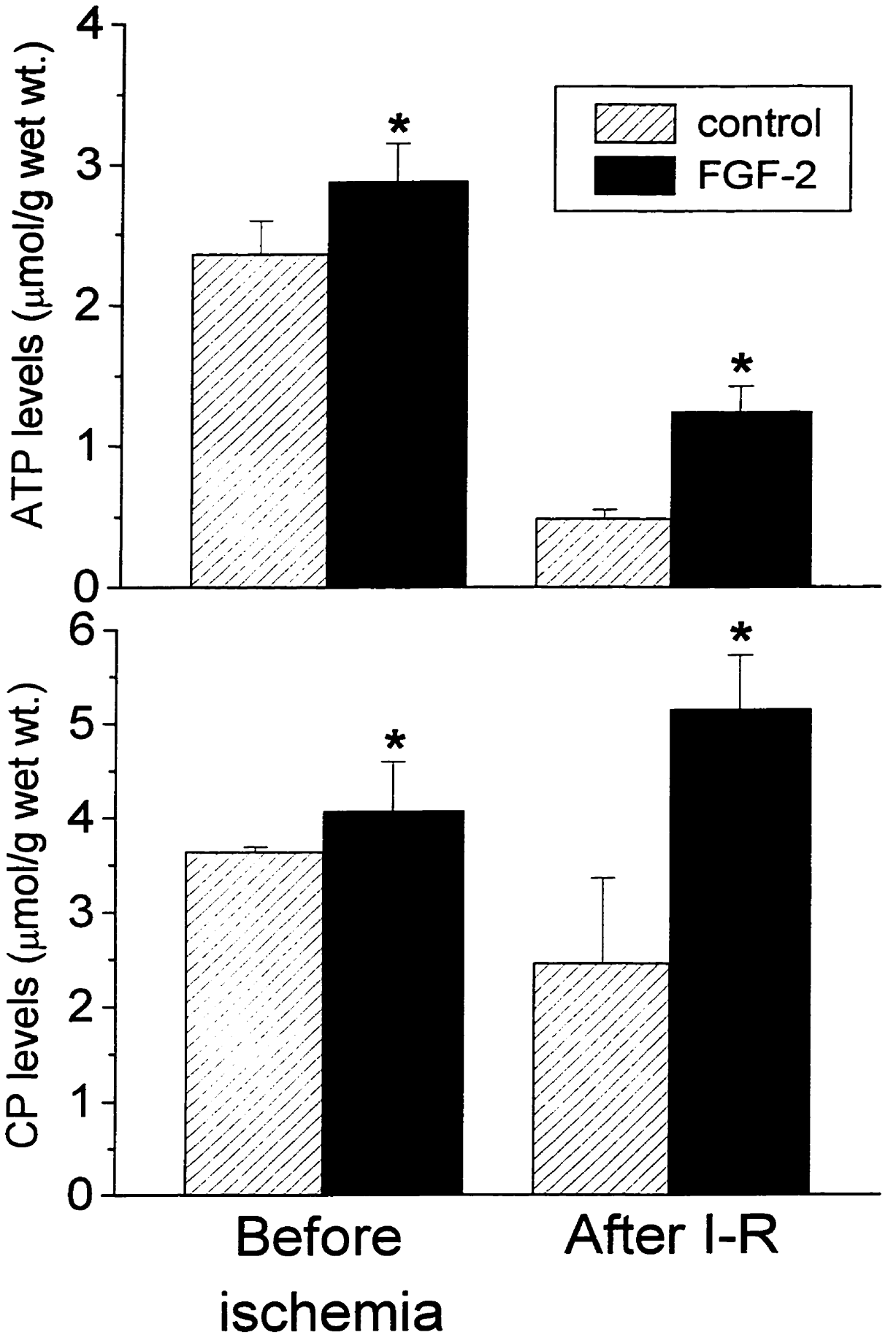
2.3.1.5 Measurements of CP and ATP levels

Figures 2.6a and b summarize the results of ATP and CP measurements, from whole ventricles subjected to: (1) perfusion with or without FGF-2 immediately before ischemia and (2) perfusion with or without FGF-2 followed by 30 min. ischemia and 60 min. reperfusion. After the equilibration period and FGF-2 administration a small but statistically significant ($p<0.05$) increase in ATP and CP levels was observed in FGF-2 perfused tissues compared to control tissues. FGF-2 perfused tissues displayed ATP and CP levels of 2.9±0.3 and 4.1±0.5 μmol/g wet wt., respectively, whereas corresponding levels in control tissue were 2.4±0.2 and 3.60±0.05 μmol/g wet wt., respectively (Fig. 2.6). After I-R, ATP and CP levels were significantly elevated in FGF-2-treated hearts compared to those of untreated hearts: ATP and CP levels in FGF-2 treated hearts were 1.2±0.2 and 5.1±0.6 μmol/g wet wt., respectively, and in control tissues 0.5±0.1 and 2.5±0.9 μmol/g wet wt., respectively. In comparison to their corresponding preischemic levels, FGF-2-treated and untreated hearts displayed a decrease in ATP levels after I-R with a mean difference of 1.7 and 2.0 μmol/g wet wt., respectively. FGF-2-treated hearts displayed a slight but statistically insignificant increase in CP levels after I-R compared to pre-ischemic levels. Untreated hearts also displayed a slight but not statistically significant decrease in CP levels after I-R compared to pre-ischemic levels with a mean difference of 1.18 μmol/g wet wt.

In hearts treated with FGF-2 during reperfusion (after ischemia), increased preservation in ATP and CP levels over control hearts is also seen after I-R. This is illustrated in Fig. 2.7 which shows ATP and CP content in: (1) untreated ventricles before I-R; (2) untreated ventricles after I-R; and (3) treated ventricles (FGF-2 administered

during reperfusion) after I-R. A significant decrease in ATP and CP levels was observed in untreated hearts, from a preischemic level of 2.4±0.2 and 3.60±0.05 μmol/g, respectively, to 1.1±0.2 and 2.1±0.4 μmol/g, respectively, after I-R. ATP levels in FGF-2 treated hearts, were still, significantly decreased to 1.7±0.2μmol/g wet wt. compared to preischemic levels but were significantly higher than those of untreated hearts after I-R. Conversely, CP levels in FGF-2 treated hearts after I-R (4.1± 0.7μmol/g wet wt) were significantly greater than CP levels in untreated hearts (2.1±0.4) after I-R and about equivalent to CP levels in untreated hearts before ischemia (4.1±0.5μmol/g wet wt).

Figure 2.6: Effects of FGF-2 administration on ATP (A) and CP (B) levels of perfused rat hearts. Values are expressed as means ± SE (n=5) μmol/gm wet wt. from control (cross-hatched bars) and FGF-2 (solid bars) perfused hearts, subjected to: (1) 30 min. perfusion only; and (2) 30 min. perfusion followed by 30 min. no-flow ischemia and 60 min. reperfusion. FGF-2 was introduced for 12 min. prior to ischemia. Absolute values for ATP levels for FGF-2-treated and control hearts prior to ischemia were 2.87±0.28 and 2.36±0.24, respectively. Absolute values for FGF-2-treated and control hearts after ischemia-reperfusion period were 1.24±0.18 and 0.48±0.06, respectively. Absolute values for CP levels for control and FGF-2 treated hearts prior to ischemia were 4.06±0.53 and 3.64±0.0.01, respectively. Absolute values for control and FGF-2 treated hearts after ischemia-reperfusion period were 5.14±0.59 and 2.46±0..90, respectively. *P< 0.05 vs. FGF-2-untreated group.


ATP levels (μmol/g wet wt.)
4
3
2
1
0
control
FGF-2
*
*
CP levels (μmol/g wet wt.)
6
5
4
3
2
1
0
*
*
Before ischemia
After I-R

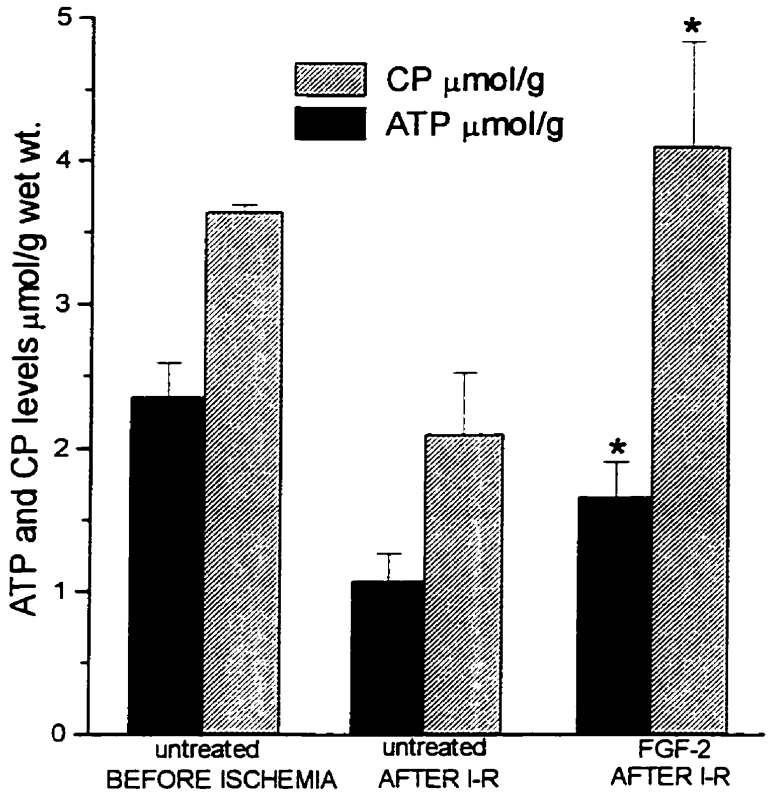


Figure 2.7: Effects of post-ischemic FGF-2 administration on ATP (Fig. 7a) and CP (Fig. 7b) levels in perfused rat hearts, under constant perfusion pressure conditions. Values are expressed as means ± SE (n=5) for CP (cross hatched bars) and ATP (solid bars) in μmol/g wet wt. from control and FGF-2 reperfused hearts subjected to (1) perfusion for 30 min.; and (2) 30 min. perfusion followed by 30 min. no-flow ischemia and 60 min. reperfusion with or without FGF-2 treatment. FGF-2 was perfused for 12 min. immediately after ischemia. Absolute values for CP and ATP levels for untreated hearts prior to ischemia were 3.64±0.05 and 2.48±0.24, respectively. Absolute values of CP for untreated and treated hearts after I-R period were 2.10±0.42 and 4.10±0.74, respectively. Absolute values of ATP for untreated and FGF-2-treated hearts after ischemia-reperfusion period were 1.08±0.19 and 1.67.10±0.24, respectively. *P< 0.05 vs. FGF-2-untreated group.

2.3.1.6 Effects of (I) chelerythrine chloride (CEC) and (ii) FGF-2/CEC on recovery from I-R. Comparison with (iii) untreated and (iv) FGF-2-only treated hearts

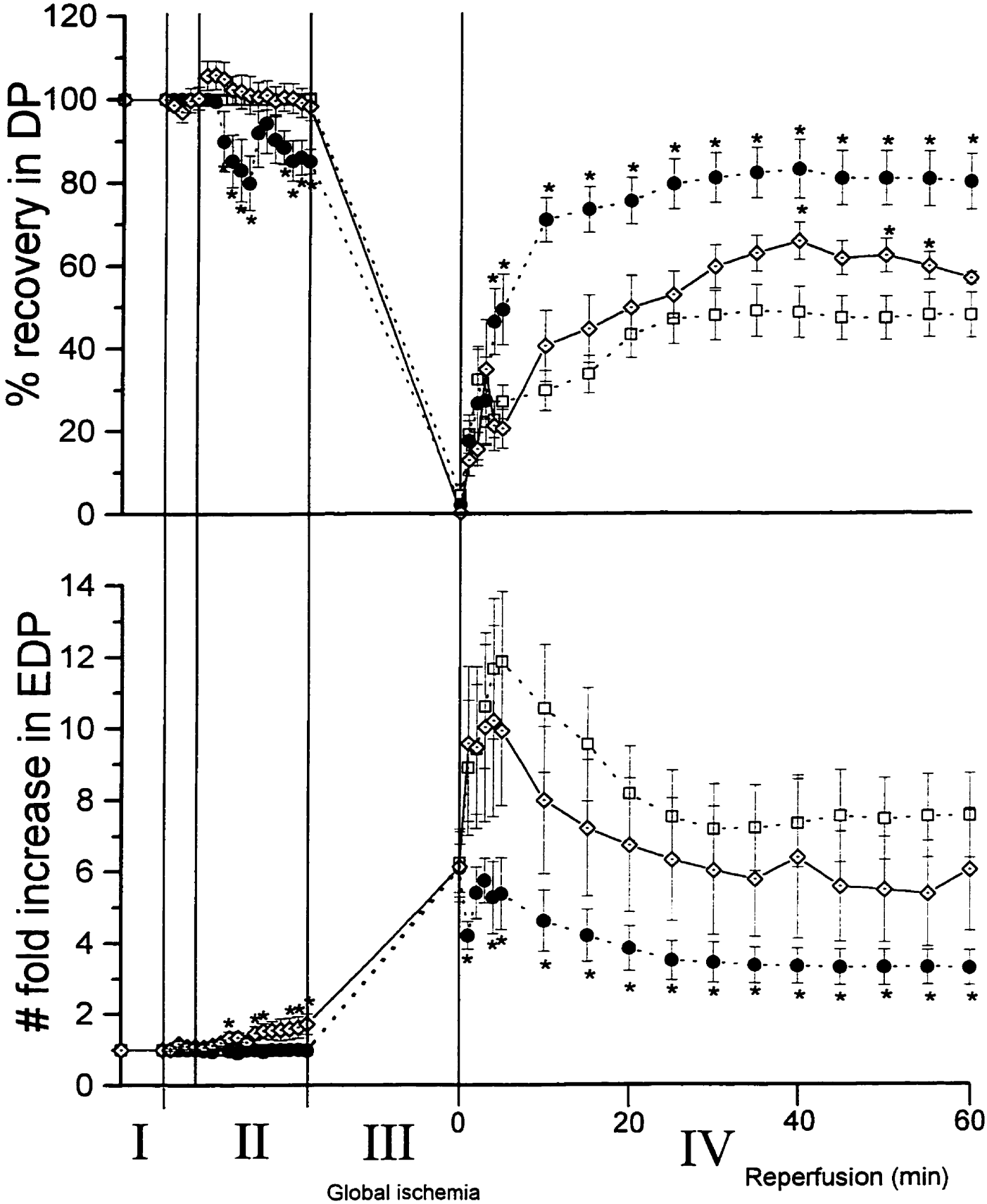
Pilot studies indicated that chelerythrine administered at 1 or 2 μmol did not have any effect on FGF-2 induced cardioprotection (data not shown). CEC was then administered to the hearts at a concentration of 5 μmol/L, for up to 15 minutes before I-R, under constant perfusion pressure conditions. Administration of CEC did not cause any significant changes in cardiac function compared to baseline control values (Fig. 2.8A). Data for control hearts and for FGF-2-treated hearts included in Fig. 2.8 and 2.9 are the same as those shown in Fig. 2.3. They are shown again to facilitate comparison with CEC-treated and CEC/FGF-2 treated hearts, and to illustrate statistical significance. After 60 minutes of reperfusion, CEC-treated or control hearts (Fig. 2.8A) displayed approximately 57% or 48% recovery of DP, respectively. This trend towards slightly increased DP recovery values in CEC-treated hearts compared to controls was observed at most time points during reperfusion (t=10-60 min.), although differences were not statistically significant for the majority of time points. Increases observed in recovery values of CEC-treated compared to control hearts although relatively small were statistically significant at three time points, at 40, 50 and 55 minutes of reperfusion.

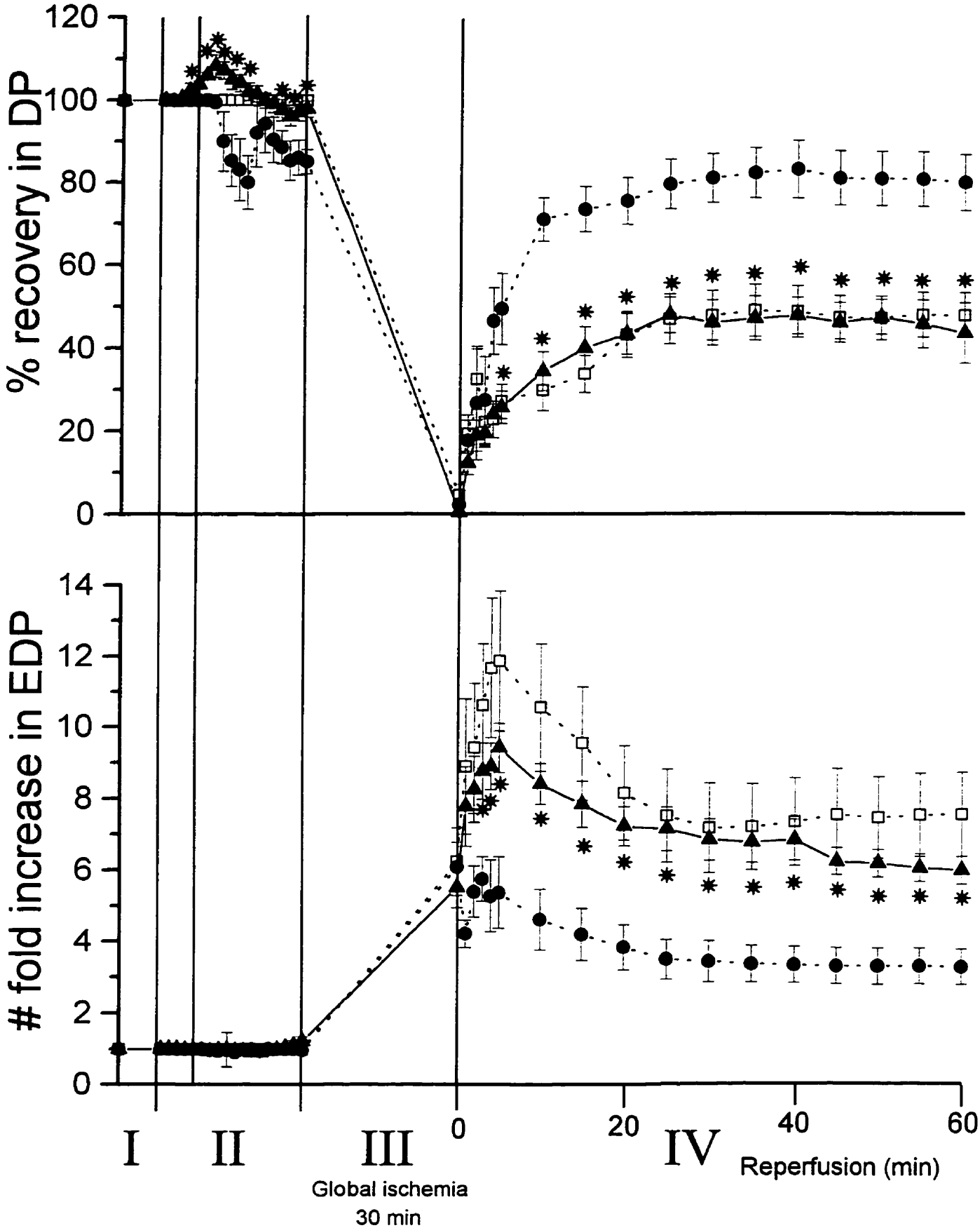
The moderate drop in DP prior to ischemia induced by FGF-2 administration alone was completely absent when FGF-2 was administered in the presence of CEC (Fig. 2.9A). Recovery of DP after I-R of hearts treated with FGF-2 in the presence of CEC was at

approximately 48% of pre-ischemic values, virtually identical to that of untreated, control hearts, and substantially lower than the 80% recovery seen in the presence of FGF-2 alone (Fig. 2.9A). Comparison of values of % recovery after I-R indicated that differences seen between FGF/CEC-treated and FGF-treated hearts were statistically significant, while those between control hearts and FGF/CEC treated hearts were not (Fig. 2.9A).

EDP after 25-60 minutes of reperfusion was increased by approximately 6-fold in CEC-treated hearts (Fig. 2.8B). This value represents a trend towards some improvement over the 7.5-fold increase seen in control hearts, although differences were not statistically significant at any point. Hearts treated with CEC-FGF-2 displayed a 6 to 7-fold increase in EDP after I-R; these values were significantly higher than those obtained by FGF-2 treatment alone, but not different from corresponding values of control or CEC-treated hearts (Fig 2.9B). A similar trend was seen in measurements of recovery in ±dP/dt (data not shown). In all contractile parameters measured, the improvement in recovery of contractile function after I-R induced by FGF-2 was abolished by CEC.

Figures 2.8 & 2.9: Effect of FGF-2, chelerythrine, and chelerythrine/FGF-2, on the relative developed pressure (DP), and left ventricular end diastolic pressure (EDP), as indicated, before and after ischemia and reperfusion of the isolated rat heart under constant pressure conditions, compared to controls. Data from control (squares, --□--) or FGF-2-treated (circles, --O--) hearts are reproduced in both Figures 2.8 & 2.9 to facilitates comparisons with data form chelerythrine treatment (Figure 2.8, diamonds, —◇—) and chelerythrine/FGF-2 treatment (Figure 2.9, triangles, —▲—) and to indicate statistical significance. The horizontal axis indicates sequence/duration of heart treatment and is divided into 4 steps: Step I represents a 30 min. period of equilibration with Krebs buffer. In step II which represents a total of 15 min., hearts are either continued to be perfused with Krebs (controls), or with FGF-2 in Krebs, or with chelerythrine in Krebs, or, finally, with chelerythrine in Krebs for 3 min. followed by FGF-2 and chelerythrine in Krebs for an additional 12 minutes. In step III, hearts are subjected to 30 min. of global ischemia, followed by (step IV), 60 min. of reperfusion. Figure 2.8: Effects of FGF-2 or chelerythrine on relative DP and EDP before and after I-R. Figure 2.9: Effect of FGF-2 or chelerythrine/FGF-2 on relative DP and EDP before and after I-R. Values are expressed as mean±SE (n=5-6). Absolute values for DP and EDP in control hearts after stabilization for 30 minutes, and prior to ischemia, were 95.1±3.2 mmHg and 7.3±0.6 mm Hg, respectively. Corresponding values in FGF-2 treated hearts were 85.7±8.9 mmHg and 6.7 ±0.5mmHg (after stabilization, immediately prior to FGF-2 administration). Corresponding values in chelerythrine-treated hearts were at 94.1±10.5 mmHg and 5.6±0.8mmHg, respectively, (after stabilization, immediately before chelerythrine administration), and at 105.8±5.7 mmHg and 6.1±0.5 mmHg in chelerythrine/FGF-2 hearts (after stabilization and prior to FGF-2 administration). Statistical analysis was done using unpaired, two-tailed student t-test, where $P<0.5$ was considered significant. In Figure 2.8, comparisons between the FGF-treated or chelerythrine-treated, and control groups (*=$P<0.5$) are indicated for each time point. In Figure 2.9, comparisons between the FGF- chelerythrine-FGF treated groups (✱=$P<0.0.5$) are indicated for each time point.


% recovery in DP
120
100
80
60
40
20
0
fold increase in EDP
14
12
10
8
6
4
2
0
I
II
III
IV
0
20
40
60
Reperfusion (min)
Global ischemia
30 min


% recovery in DP
120
100
80
60
40
20
0
fold increase in EDP
14
12
10
8
6
4
2
0
I
II
III
0
20
IV
40
60
Reperfusion (min)
Global ischemia
30 min

2.3.1.7 Measurements of creatine phosphokinase in the perfusates.

CPK activity in the coronary effluent collected in 20 min. of reperfusion after I-R under constant pressure was determined to estimate damage to the myocardium in response to the I-R challenge (Fig. 2.10). CPK values in the effluent of control hearts were over 2-fold higher than the values after FGF-2 treatment. FGF-2 treatment therefore resulted in significantly ($p<0.05$) reduced myocardial damage.

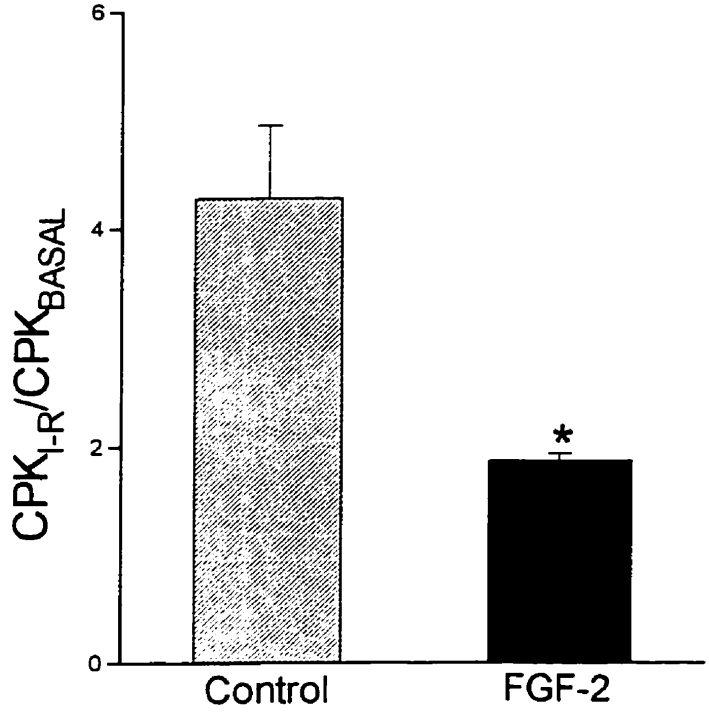


Figure 2.10: Relative levels of creatine phosphokinase (CPK) in effluents from isolated whole ventricles after I-R (CPK_{I-R}), over those before ischemia (CPK_0).

Relative CPK activities taken from coronary effluents collected at the 20 min. reperfusion time point after I-R under constant flow conditions. $P<0.05$ indicates significant difference.

2.3.2 Antigrade perfusion of the isolated right ventricular wall

2.3.2.1 Effect of FGF-2 on contractile function and recovery from I-R

The effect of FGF-2 administration (0.7 μg/ml) on DF was examined in isolated perfused adult right ventricular wall. The concentration of FGF-2 used represented the minimal concentration necessary to induce an effect, as determined in pilot studies, and was comparable to the concentrations used in perfusions of the whole heart. FGF-2 administration induced a reduction in DF of approximately 12 % compared to values from ventricles perfused with buffer only (Fig. 2.11). This reduction was sustained throughout the perfusion of FGF-2 to the tissues. After 60 min. of global ischemia, DF of both control and FGF-2 perfused tissues decreased to near zero. After 30 min. of reperfusion, a recovery of approximately 84.7±5.2% of DF was observed in FGF-2 treated tissues compared to approximately 42.5±9.0% in control tissues. Resting tension measurements during reperfusion showed no significant differences between FGF-2 treated and control tissues (Fig. 2.12) with both tissues showing a gradual recovery in resting tension during reperfusion. However, a trend toward lower resting tension values was observed in FGF-2 treated samples compared to those of control values. Our previous results using whole heart ventricles perfused with or without FGF-2 (Fig. 2.3) showing a recovery in developed pressure of 83.11±7.03% and 48.68±6.16%, respectively after I-R are consistent with the right ventricular wall perfusion data presented here.

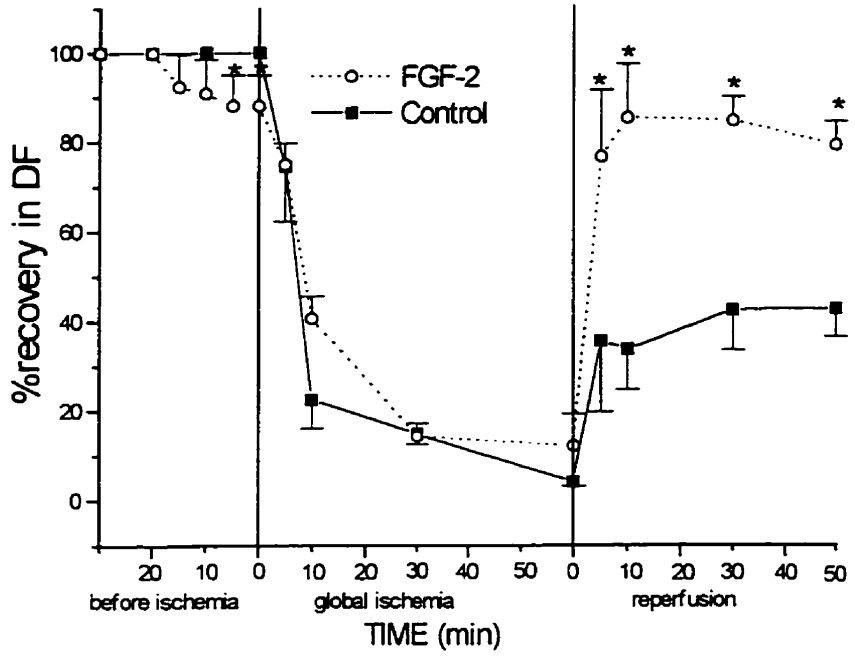


Figure 2.11: Effects of FGF-2 administration on developed tension of isolated perfused adult rat right ventricular wall before and after I-R. Values are expressed as means ± SE (n=3 for control and n=5 for FGF-2-treated tissues). FGF-2 was included in perfusion medium for 15 min. just prior to ischemia. Absolute values for developed tension in control and FGF-2-treated groups (g/g wet weight) before ischemia were 353.62±23.53mg and 500.45±87.29mg, respectively. *P< 0.05 vs. FGF-2-untreated group.

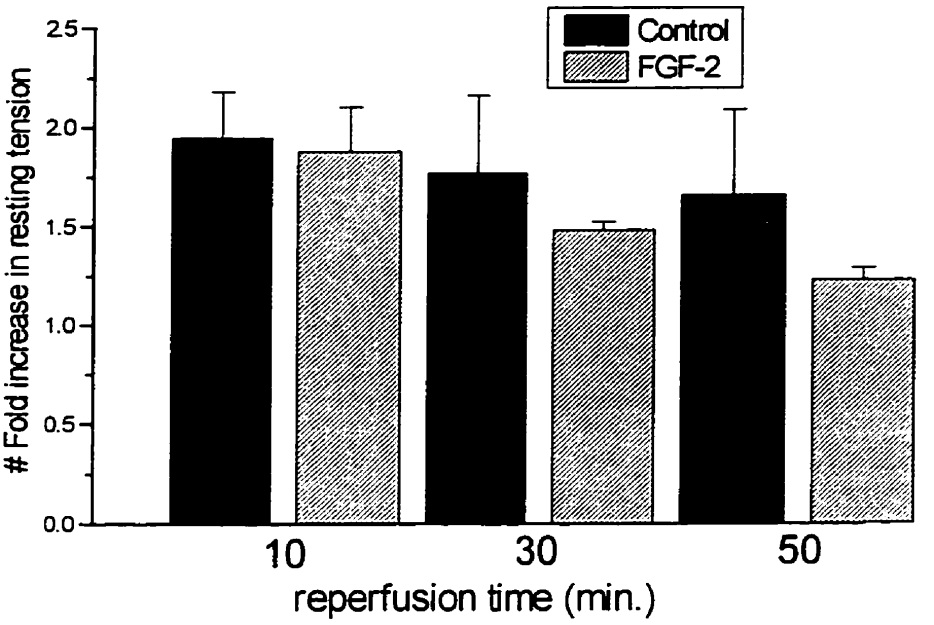


Figure 2.12: Effects of FGF-2 administration on resting tension of isolated adult rat right ventricular wall before and after I-R. Values are expressed as means ± SE (n=3 for control and n=5 for FGF-2-treated tissues). FGF-2 was only included in perfusion medium for 15 min. just prior to no-flow ischemia. Absolute values for resting tension in control and FGF-2-treated groups (g/g wet weight) before ischemia were 357.40±24.29mg and 277.86±62.46mg, respectively. *P< 0.05 vs. FGF-2-untreated group.

2.3.2.2 Effect of FGF-2 on Action Potential Duration (APD)

APD of the perfused rat right ventricular wall was examined: (1), before FGF-2 administration; (2), during FGF-2 administration; (3), during 60 min. ischemia; (4), and during 50 min. reperfusion. In comparing the APD_{90} values before FGF-2 perfusion with the APD_{90} values during FGF-2 perfusion, we see a significant difference only at the 5 min. time point of FGF-2 perfusion (Fig. 2.13), when a shortening of approximately 17% in APD_{90} was observed in FGF-2 treated hearts. By the end of the 12 min. FGF-2 perfusion period, APD_{90} values for FGF-2 treated hearts returned to pre-FGF-2 perfusion APD_{90} levels. In the 60 min. global ischemic period, we observed a slower decline in APD_{90} shortening in FGF-2 treated hearts compared to control hearts. However, by the end of the 60 min. ischemic period, APD_{90} values from both FGF-2-treated and control hearts shortened to approximately 20 % of their pre-FGF-2 APD_{90} levels. After 60 min. ischemia, and 30 min. of reperfusion, a 120±12.3% recovery was observed in FGF-2-treated hearts compared to 70.6±7.7% in control hearts.

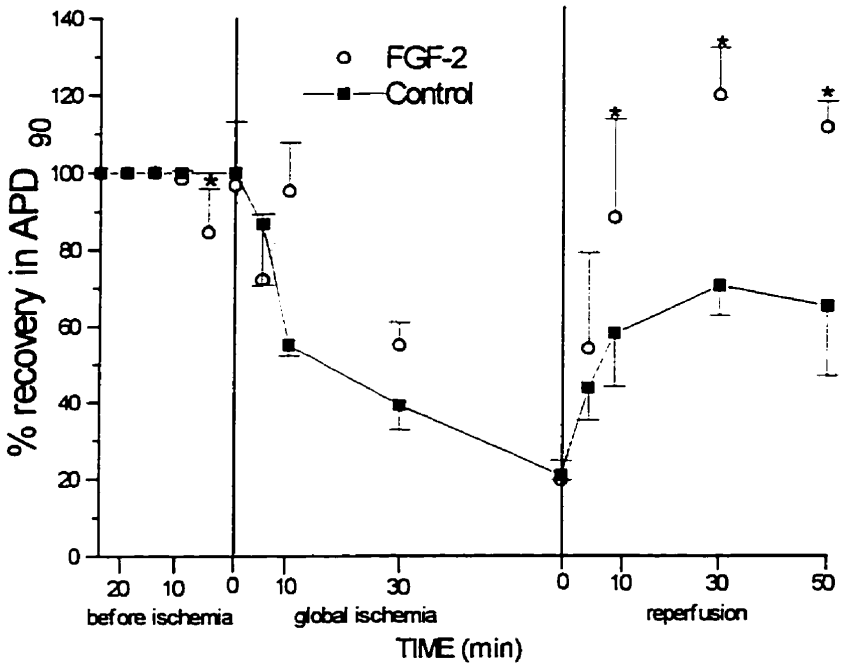


Figure 2.13: Effects of FGF-2 administration on APD of isolated adult rat right ventricular wall before and after I-R. Values are expressed as means ± SE (n=3 for control and n=5 for FGF-2-treated tissues). FGF-2 if present, was only included in perfusion medium for 15 min. just prior to no-flow ischemia. Absolute values for APD in control and FGF-2-treated groups before ischemia were 54.61±9.56ms and 43.89±3.12ms, respectively. *P< 0.05 vs. FGF-2-untreated group.

2.3.3 Effect of FGF on recovery from I-R injury. Initial experiments with heart at ambient temperature during global ischemia

2.3.3.1 Initial experiments on the effect of FGF on I-R injury

Initial experiments conducted to examine the cardioprotective effect of FGF, used a less sophisticated version of Langendorff perfusion. In this initial version, constant flow perfusion was performed at 37°C at a rate of 10 ml/min. However, due to the nature of the setup and positioning of the force transducer, global ischemia was done under ambient room temperature conditions. Measurements of contractile force were determined using a Grass FT-03 force displacement transducer connected to two hooks which were attached superficially along the midpoint on opposite sides of the heart. A dynograph Beckman Dynograph Recorder-R511A was used to record the contractile parameter measurements. Different preparations of FGF-2 were also tested in dose-response experiments in this initial setup.

Fig. 2.14 shows the effects of pre-ischemic treatment with FGF-2 on the recovery of hemodynamic function of the heart after I-R, as conducted in our initial Langendorff perfusion experiments. Control group displayed a 63.8±1.5% recovery in *DF* as well as 54.4 ±2.8% and 53.8±4.9% recovery in *+dF/dt* and *-dF/dt*, respectively, upon restoration of flow (Fig. 2.13 a,b and c). When hearts were treated with FGF-2 (preparation #1), recovery of *DF* and of *+dF/dt*, *-dF/dt*, reached, at 10 μg of FGF-2/heart, values of 90.0 ± 5.1% and 93.1 ± 6.0, 96.4 ± 3.7%, respectively. The increases in recovery of FGF-2-treated hearts over controls were overall dose-dependent and statistically significant. A

second human recombinant FGF-2 preparation (preparation #2) was even more potent, resulting, at 10 μg of FGF-2 /heart, in 96.5 ±3.5% recovery of *DF* and 99.8±0.2, 96.6±3.5% recovery of *+dF/dt*, *-dF/dt*, respectively (Fig. 2.14). Bovine FGF-2, purified from pituitaries was similarly protective (data not shown). The slightly different potencies with respect to cardioprotective effect displayed by the two different recombinant FGF-2 preparations may be due to age of the preparation as well as storage conditions. Prolonged storage of recombinant FGF-2 (3 months, at 0.5 mg/ml, at -70^{0}C) resulted in loss of mitogenicity, ability to bind heparin as well as cardioprotective ability (n=2, unpublished observations). These studies served to identify an optimal dosage for cardioprotection, at 10 μg /heart.

2.3.3.2 Recovery of contractile parameters upon FGF-2 administration during reperfusion only

FGF-2 (preparation #1) was administered to hearts after they had been subjected to 50 min. of ischemia, at the onset of reperfusion. This resulted in a 78.0±1.7% recovery of *DF* in FGF-2-treated hearts, compared to 64.8±5.3% recovery in controls (fig. 2.14a, broken line). The difference in recovery between control and FGF-2-treated hearts obtained when FGF-2 was administered during reperfusion was smaller (approximately half) than the one obtained when FGF-2 was administered before ischemia, but was statistically significant ($p<0.05$). Recoveries of *+dF/dt* and *-dF/dt* displayed a statistically insignificant improvement as a result of FGF-2 treatment (Fig. 2.14b and c, broken line).

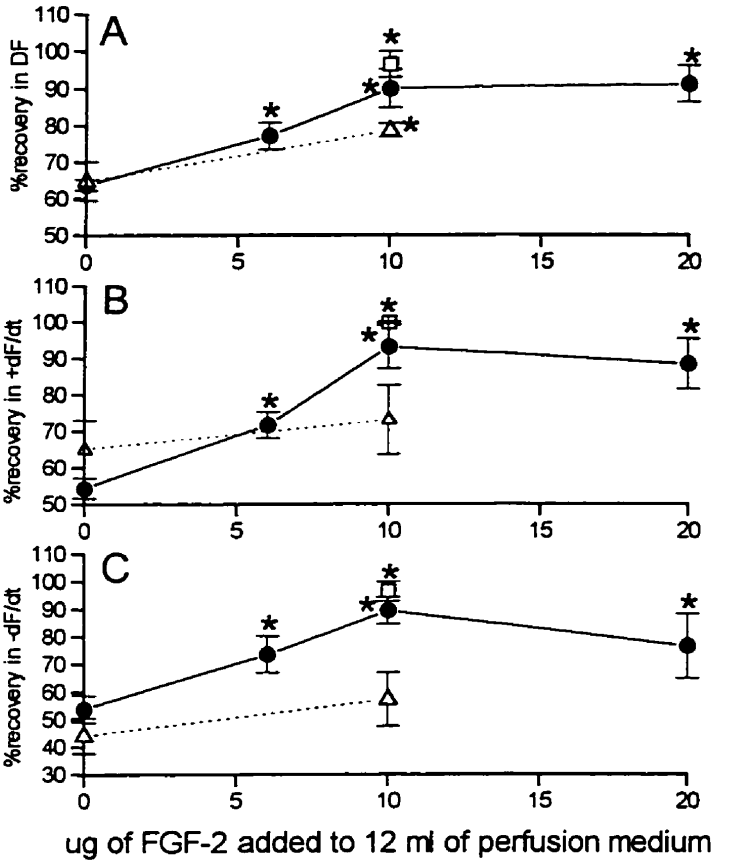


Figure 2.14: Effect of FGF-2 on recovery of cardiac mechanical function after I-R (initial studies). Percentage recovery of, (A), *DF*, (B), *+dF/dt*, and (C), *-dF/dt* is plotted as a function of the amount of FGF-2 (μg) added to the 12 ml buffer. Bars and asterisks denote standard error and statistical significance ($p < 0.05$, compared to control values at 0 μg of FGF-2), respectively. Full circles and clear triangles, data from FGF-2 preparation #1, clear squares, data from preparation #2. Continuous line, preischemic administration of FGF-2. Broken line, administration of FGF-2 during reperfusion only.

2.4 DISCUSSION

2.4.1 Introduction

Involvement of growth factors and cytokines in healthy as well as diseased myocardium is becoming increasingly evident. For example, transforming growth factor beta (TGFβ), which is synthesized and secreted by myocytes, helps to maintain their beating rate in culture [1] and inhibits circulating neutrophils from adhering to the endothelium, minimizing neutrophil-induced cell injury [2]. Interleukin-1β was shown to depress cardiac contractility by inducing production of nitric oxide [3]. FGF-2, which shares structural similarities with the interleukins [4], can also be considered to exert effects on cardiac function. In the present report we have examined the hypothesis that FGF-2 may influence recovery from myocardial injury.

We used an isolated rat heart model in which global ischemia was simulated by interruption of flow followed by reperfusion. This approach offers the advantage of examining the effects of various factors on cardiac responses to injury directly without the interference of the immune system of other pathological parameters. The disadvantages are that blood borne factors which influence and contribute to the degree of I-R injury directly or indirectly on cardiomyocytes and the vasculature are ignored thus removing the context of the study away from actual physiological conditions. In addition, unlike *in vitro* cell cultures, this model does not allow indepth study of the effects of various factors on specific cardiac cell populations such as cardiomyocytes, endothelial cells, smooth muscle cells and fibroblasts. In addition, hearts in Langendorff preparations, are to some extent exposed to short durations of ischemia upon excision of the heart from the animal and

during cannulation. Never the less, this model does allow for a controlled study whereby specific cardiac reponses to various factors can be deduced and characterized in a methodical manner.

Our initial studies provided a strong indication for a cardioprotective effect of FGF-2 when administered to the heart before I-R, but also after ischemia during reperfusion. These studies also established that various preparations of FGF-2 were all effective in inducing cardioprotection and established an appropriate dose, at 10 μg/heart, for optimal results. This dosage was used in all subsequent experiments. Because the initial experiments did not allow adequate regulation of temperature and humidity of the heart during the ischemic period, and were subject to large variability, we continued our experiments on cardioprotection by FGF-2 in a more upgraded system (balloon catheter, computerized data collection and processing) which allowed us to investigate the effects of FGF-2 on cardiac function and recovery from I-R under normothermic conditions of constant pressure or constant flow. Global ischemia was induced by interruption of flow for 20 minutes (constant flow conditions) or 30 minutes (constant pressure conditions); these time points produced a 35-45% functional recovery after reperfusion for 40-60 minutes and were selected in pilot experiments as providing baseline recovery values over which cardioprotection could be detected.

2.4.2 Induction of cardioprotection from I-R by FGF-2 administered before ischemia, under various conditions

Most I-R experiments were done under constant perfusion pressure conditions which provided the main bulk of our evidence establishing the cardioprotective effect of FGF-2. However, in order to examine if FGF-2 had a vasodilatory effect, which would manifest itself as a drop in perfusion pressure when perfusion was done under constant flow conditions, we needed to do one series of cardioprotection experiments under constant flow conditions. This demonstrated that FGF-2 continued being cardioprotective under constant flow conditions, even though it did not induce substantial vasodilation. Finally, we conducted one series of experiments in an additional perfusion system, that of the isolated right ventricular wall, to again examine if FGF-2 is protective under these conditions and to measure additional functional parameters such as APD; these experiments which confirmed the cardioprotective effect of FGF-2 were done in collaboration with Dr. W. Cole, now at the University of Calgary.

Overall and in the context of the whole heart FGF-2 consistently improved recoveries of DP, EDP, ±dP/dt, resting tension, ±dF/dt after I-R compared to controls. In addition, FGF-2 induced improved recovery in APD_{90} after I-R of the isolated right ventricular wall model. Complete recovery in APD_{90} was observed in FGF-2 treated tissues after 10 minutes reperfusion and remained elevated at approximately 120% of preischemic levels. This slight elevation could be explained by a rebound effect in the APD_{90} possible due to increased alkaline environment or possibly increase Ca^{2+} ion permeability. FGF-2 is reported to induce increased intracellular pH and intracellular

$Ca2^{+}$[5]. Regardless, this increased recovery in APD_{90} during reperfusion in FGF-2 treated tissues correlates well with the increased recovery in developed tension during reperfusion seen in FGF-2 treated tissues. This would indicate that FGF-2 may have improved contractile function by protecting the cardiomyocyte from cellular injury since APD_{90} levels were near normal suggesting preservation of the ionic homeostasis via intact membrane and ionic channel function.

Increased functional recovery after I-R could reflect some augmentation of function of the viable myocardium by FGF-2 or could reflect reduced myocyte damage from ischemia and/or reperfusion. We presumed the latter to be true since we detected decreased levels of CPK in the perfusate of FGF-2 treated hearts.

Mechanism/ATP preservation: FGF-2 administration before ischemia induced a negative inotropic effect under all experimental settings. This should promote preservation of energy stores, which is exactly what we found: ATP and CP levels were higher in FGF-2 treated hearts, compared to controls, prior to ischemia. Elevated levels of energy stores, in turn, would be expected to be beneficial towards subsequent injury.

Agents which either preserve or increase high energy phosphate levels during ischemia protect against the detrimental effects of I-R injury. K^{+}_{ATP} channel openers, such as pinacidil, enhance the shortening of the action potential duration (APD), thus indirectly preserving high-energy phosphates and reducing injury during ischemia [6]. Ca^{2+} channel antagonists, such as verapamil [7] and diltiazem [8] and other bradycardiac agents [9] have also been shown to exert a similar effect on preserving ATP and other high-energy stores during ischemia. Adenosine administration has also been shown to protect against I-

R injury with an observed increase in tissue ATP and stimulation of anaerobic glycolysis during ischemia [10]. In all cases, with an increased ATP production, an improved recovery of contractile parameters upon reperfusion was observed [7-10].

It is therefore more than likely that the FGF-2 induced cardioprotection is a consequence, at least in part, of the negative inotropic effect exerted on myocytes. Similarly, phorbol-esters, which are activators of PKC and which exert a negative inotropic effect are cardioprotective in I-R [11,12].

Mechanism/vasodilation: In addition to preservation of high energy phosphates, FGF-2 may be inducing its cardioprotective effects on the ischemic heart through its actions on the coronary vasculature. FGF-2 has been shown to be a potent hypotensive agent when administered intravenously to rabbits [13] and to cause hypotension and a slight decrease in heart rate in rats [14]. Additionally, when recombinant FGF-2 was administered to canine [15-17] and porcine [18,19] hearts, an acute vasodilation and vasorelaxation effect was observed with a concomitant increase in coronary blood flow. The vasodilatory action of FGF-2 was also shown to be mediated by the activation of K^+_{ATP} channels [13] and the release of nitric oxide into the coronary vascular milieu [13,20]. Indeed, vasodilatory agents, such as SNP [21], have been shown to be cardioprotective against I-R injury. Its mechanism of action has been shown to be through the release of nitric oxide (NO) which induces coronary vasodilation and thus improved perfusion of the myocardium [22]. Thus improved coronary perfusion prior to the ischemia may serve to attenuate the detrimental effects of ischemia by providing the heart with a store of oxygen and necessary metabolites for energy production.

In our I-R model, to examine whether the protective action of FGF-2 correlated to vasodilation, FGF-2 was administered to isolated perfused rat hearts under conditions of constant flow. Coronary perfusion pressure (or back pressure) was monitored as an indicator of increased vasodilation, and sodium nitroprusside (SNP), which is an established vasodilator, was used at concentrations reported to induce cardioprotection [21]. While SNP induced a clear drop in back perfusion pressure, the effect of FGF-2 was by comparison very small, consistent with only minor vasodilatory action. These data suggested that vasodilation may not be a major mechanism of cardioprotection by FGF-2. However, more detailed studies must be done to address the relationship between vasodilation and cardioprotection, or to determine exactly the contribution of FGF-2's minor vasodilatory activity to cardioprotection.

FGF-2 has been shown to activate K^+_{ATP} channels and induce the release of NO in vascular tissue [13]. FGF-2 induction of NO release and activation of K^+ATP channels in vascular tissue may, in turn, be responsible for the apparent physiological effects such as vasodilation [13,14,18] and the observed negative inotropic effect with FGF-2 treatment. SNP does induce a negative inotropic effect along with vasodilation. This effect would cause a decrease in work load and at the same time increase supply of oxygen and nutrients to the tissues [23].

Mechanism/PKC: FGF-2 exerts its biological effects by binding to its tyrosine kinase receptors which are considered linked to the phosphoinositde pathway via PLC-γ activation in some systems, leading thus to PKC activation [73,74]. The above pathway is by no means established for all cell types or for all possible biological roles of FGF-2. It

was important however to examine whether FGF-2 caused PKC activation in the context of our experimental system since PKC is believed to mediate another major cardioprotective process, namely that of ischemic preconditioning.

PKC stimulators such as 1,2-dioctanoyl-sn-glycerol (DOG) [24], diacylglycerol [25], and phorbol 12-myristate 13-acetate (PMA) [26-28] used on isolated rat or rabbit cardiomyocytes [27,28], isolated rat hearts [25], or *in vivo* rabbit hearts [26] have mimicked the protective effects of ischemic preconditioning. Other inducers of preconditioning such as: α-adrenergic agonists (such as norepinephrine [29], and phenylephrine [30]), via the α1 adrenergic receptor [25,30]; prostaglandin E1 and E2 [31]; Bradykinin via the B2 receptor [32], and angiotensin [33], via the AT1 receptors, all have been shown to be cardioprotective through the activation of PKC [25,29-33] and the further downstream activation of K-ATP channels [24,31].

PKC inhibitors such as chelerythrine chloride [24,34,35], polymyxin B [26,30,33] and calphostin C [36,37] block the cardioprotective effects of ischemic preconditioning. However, while the various PKC inhibitors have problems in terms of solubility and specificity, it is the water soluble chelerythrine which has emerged as the preferred choice. The advantage of using chelerythrine is that it acts on the catalytic site of, presumably, all PKC subgroups, it is very specific and it is not as toxic to the cells as other PKC inhibitors [38]. We therefore examined if FGF-2 exerted its effects on cardiac function and/or cardioprotection via the PKC pathway, by using chelerythrine as a PKC inhibitor.

Chelerythrine administered to the *ex-vivo* rat heart in our system blocked cardioprotection induced by FGF-2; in its presence, FGF-2, administration resulted in a

degree of recovery of all contractile parameters measured that was indistinguishable to that of controls, at about 50% of pre-ischemic control values. Chelerythrine also blocked the negative inotropic effect of FGF-2, indicating that in both instances, cardioprotection after I-R and decrease in developed pressure before ischemia, activation of PKC is an essential component of the mechanism involved.

It should be noted that when chelerythrine was used by itself during the pre-ischemic period it induced a slight improvement in recovery after I-R compared to controls. The reason for this is unclear. It is possible that chelerythrine exerts as yet undiscovered, non-PKC-dependent, effects on the myocardium. On the other hand, there is a preliminary report indicating cardioprotection can be induced by PKC inhibitors other than chelerythrine [39], thus supporting our findings. Since the PKC family includes at least 12 members at this point [40], it is plausible that not all of them are affected to the same extent by chelerythrine or other inhibitors and that not all of the PKC subtypes are acting in the same direction, i.e. towards cardioprotection. For example, while PKC activation is on the whole considered to protect from apoptotic death [41,42], activation of PKC-β1 or PKC-δ have been linked to the signaling mechanism actually leading to apoptosis [43,44]. Chelerythrine acting alone and blocking/decreasing activity of all PKCs may shift the balance of the overall response of the heart towards a slight cardioprotection, compared to controls.

An important issue with administration of inhibitors such as chelerythrine by retrograde perfusion is whether inhibition has actually been achieved in situ and whether all cells have been affected to the same degree. Since chelerythrine was administered to the

hearts via the same route as FGF-2, it is presumed that at the very least it came in contact with and affected the same areas, including cardiomyocytes [45]. The decreased or absent FGF-2 induced PKC activation (chapter 3) in the sarcolemmal preparations obtained after chelerythrine administration would argue in favor of the notion that chelerythrine successfully prevented or decreased FGF-2 activation, in situ.

In addition, with increased high energy phosphate compounds present in the heart upon FGF-2 treatment, increased PKC mediated phosphorylation of other signal transduction proteins can occur. All PKC isoforms were shown to contain a C4 region. This C4 region functions in transferring the phosphate group from the ATP bound C3 region to various protein substrates along the signal transduction pathway. Phosphorylation of the PKC is also required for PKC activity and occurs either prior to or rapidly upon ligand binding [46]. Thus with increased production and/or preservation of ATP with FGF-2 treatment discussed above, there is a greater likelihood of an increase in PKC activity and cardioprotection against I-R injury.

PKC was shown to be involved in processes such as: the development of hypertrophy and activation of the early immediate transcription factor genes [47,48]; phosphorylation of myofibril proteins such as myosin light chain [49], troponin I,T and C [50]; L-type Ca^{2+} channels [51,52], Na^{+} channels [53], K^{+} channels [54] and Cl^{-} channels [55]. Phosphorylation of contractile and calcium handling proteins suggests potential regulation of contractility by PKC. However, experiments conducted so far by different researchers have produced mixed results from negative to positive to no change in contraction upon PKC activation [56]. These variations in experiments have so far been

attributed to differences in the experimental model chosen [46]. They may also reflect differences in the isoform of PKC being stimulated.

PKC activation can stimulate a host of other different pathways which may mediate the cardioprotective effects of ischemic preconditioning. These PKC induced pathways include: K^{+}_{ATP} channel opening [24]; increased expression of heat shock proteins [57] and increased intracellular Ca2+-induced cardioadaptation to I-R injury [58].

2.4.3 Induction of cardioprotection from I-R by FGF-2 administered during reperfusion only

Administration of FGF-2 to the isolated perfused heart after ischemia and during reperfusion induced substantial improvement in the recovery of all contractile parameters measured, as well as in the preservation of energy stores, indicating that FGF-2 is capable of protecting cardiomyocytes froinjury incurred under these conditions. In our preliminary set of experiments, when ischemia duration was 60 minutes and it was not possible to adequately regulate temperature of the heart during ischemia, the degree of protection afforded by FGF-2 administered during reperfusion, although significant, was not as high as when FGF-2 was administered before ischemia. In the second set of experiments, however, when ischemia duration was 20 minutes and the heart temperature and humidity well controlled, FGF-2 administered during reperfusion (75% recovery in DP compared to 45% in controls) was almost as effective as when administered before ischemia (83% recovery in DP compared to 50% in controls). These results offer strong support to the notion that FGF-2 can improve the recovery of ischemic myocytes from I-R injury.

The mechanism for this FGF-2-induced recovery is at this point speculative. However, since Cuevas et al. have shown that FGF-2 induces the release of NO from the coronary milieu [13], it is could be possible that the improved contractile recovery we see with FGF-2 reperfusion after ischemia may be attributed to NO induction and improved perfusion. Indeed, other studies have shown that reperfusion with NO donors [62] or precursors to NO, such as L-arginine [63], improves the recovery of isolated perfused animal hearts against I-R injury. However, other alternative mechanisms by which FGF-2 may also protect against reperfusion-induced damage remain to be investigated. For instance, it is yet not known whether FGF-2 has any effect on the Na^+/H^+ exchanger; inhibitors of the Na^+/H^+ exchanger, such as dimethyl amiloride [64], have been shown to be effective in protecting the myocardium against I-R injury when introduced into the reperfusion medium.

It is also possible that FGF-2 may act as an antioxidant. Antioxidants, such as superoxide dismutase and catalase [65], have been shown to protect the heart against I-R injury, when introduced to isolated perfused hearts in the reperfusion medium. It is plausible that an alternative mechanism to FGF-2-induced cardioprotection may lie in the very structure of this growth factor. FGF-2 contains 4 cysteine residues whose thiol groups exist in the reduced state and may act as free radical scavengers [66]. Since other thiol containing antioxidants, such as glutathione [10] and captopril [67] have been shown to protect against I-R damage, it is plausible that FGF-2 may be acting in a similar fashion during the reperfusion, by preventing free radical oxidative reactions from destabilizing membrane integrity [65], ionic homeostasis [65] and inactivation of NO [68].

As mentioned in the previous section, the negative inotropic effect and energy store preservation induced by FGF-2 administered before ischemia are likely to cause, at least in part, the observed cardioprotection. Data from this section, however, point strongly to a component of the FGF-2 induced cardioprotection which is independent of the preischemic effects and which can occur during reperfusion. As will be shown in chapter 3, FGF-2 administered to the hearts before ischemia remains associated with the myocardium and the cardiomyocytes during I-R and can be considered to exert effects on the myocytes throughout this process.

Since FGF-2 induced negative inotropic effects before ischemia, one would expect contractility to be decreased in reperfusion rather than increased, over controls. However if FGF-2 preserves myocardial function during reperfusion by protecting the myocyte cell integrity and its contractile apparatus against reperfusion induced injury, the contractile parameters of FGF-2 treated hearts would indeed be significantly greater than that of the non-treated heart. When we compared the force of contraction, DP was slightly higher in hearts treated with FGF-2 prior to I-R (~83%) vs. during reperfusion only (~75%). This slightly lower recovery in DP could be attributed to a negative inotropic effect of FGF-2. On the other hand, FGF-2 may not exert a negative inotropic effect on ischemic cardiomyocytes. Detailed studies will be needed to address these issues. These studies may involve using isolated perfused animal and exposing them to a period of ischemia. This procedure would then be followed by a period of reperfusion, however, FGF-2 would be introduced at various time points after the initial reprefusion to see if contractility is affected.

2.4.4 Conclusion

We have provided evidence that FGF-2 administered to the isolated heart before ischemia protects the myocardium from I-R injury, that this protection is preceded and likely caused by a negative inotropic effect caused by FGF-2 and leading to preservation of energy stores, and that the effects of FGF-2 on contractility before ischemia and on recovery after I-R are blocked by the specific PKC inhibitor chelerythrine, implicating PKC in the mechanism of protection.

Our observations may lead to clinical applications targeted at better cardiac preservation. Surgical procedures, such as cardiac bypass surgery and heart transplantation would benefit from FGF-2 treatment protecting the heart against global ischemia. Indeed, understanding the signal transduction mechanism by which FGF-2 conveys cardioprotection against I-R injury may give insight to other clinical methods of protection such as preconditioning. Furthermore, elucidation of the mechanism of regulation of expression of endogenous FGF-2 and its receptor(s) in the heart may provide novel approaches for enhancement of cardiac resistance to injury. It is of interest to point out that the increases in endogenous FGF-2 reported for several models of cardiac injury [14,15,27] may, as our data suggest, also represent an endogenous self-defense mechanism.

We have shown that FGF-2 continues to be cardioprotective even when administered to ischemic myocytes, during reperfusion. This may have important clinical applications such as: administration immediately after a heart attack; upon the restoration of blood flow to ischemic regions during angioplasty or coronary bypass surgery; or

during heart transplantation. One question raised by the negative inotropic effect of FGF-2 is whether such an agent would be an appropriate therapy after a heart attack. Although we have shown that FGF-2 does induce a slight decrease in contractile function during reperfusion, its beneficial and long lasting protection against reperfusion injury may prove to be more advantageous by far. More research into how FGF-2 protects against reperfusion injury would, no doubt, lead to an optimization of FGF-2 therapy after a heart attack.

Further research is required to characterize the beneficial effects of FGF-2 during reperfusion in terms of: reperfusion arrhythmia; the types of cells protected by FGF-2; the regions and extent cardiac damage; changes in ionic homeostasis (such as intracellular Ca^{2+} and H^+); and how high energy phosphate compounds are preserved with FGF-2 treatment. In addition, the mechanisms of FGF-2-induced protection against reperfusion injury must be addressed: measurements of free radical generation with FGF-2 reperfusion; the involvement of PKC and its various isoforms; the role of other signal transduction compounds such as the PLCs, MAPK, src, c-fos, c-jun and βARK1; the role of cell membrane channels such as the K^+_{ATP} channel and the Na^+/H^+ exchanger; whether FGF-2 protects and affects vascular function; and whether FGF-2 affects the production or release of endogenous compounds known to be involved in cardioprotection such as adenosine [69,70] and NO [71,72]. Characterization and manipulation of the biological effects of FGF-2 may, in turn, lead to more effective therapies in treating against reperfusion injury.

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CHAPTER 3

STUDIES ON POTENTIAL MECHANISM OF FGF-2 INDUCED CARDIOPROTECTION

3.1 INTRODUCTORY REMARKS

As previously mentioned, the biological effects of FGF-2 are mediated by binding to high and low affinity receptor sites at the plasma membrane [1]. The low affinity FGF-2 receptors consist of heparin sulfate proteoglycans thought to function in storage and presentation of FGF-2 to its high affinity receptors [1], as well aid in the transduction of FGF-2's biological effects [1]. The high affinity receptors are responsible for mediating the biological effects of FGF-2. These receptors consist of a family of isoforms (FGFR1-4) [1] which appear to be subject to developmental and tissue specific regulation, and are tyrosine kinases [1]. Heart expresses FGFR1 as either short or long isoforms which are developmentally regulated with the short isoform being predominant in the adult heart [3,4].

The signal transduction system for FGF-2 remains to be completely elucidated. Complications in studying FGF-2 signal transduction system stem from the fact that there are different isoforms of FGF-2 as well as FGFRs depending on the cell type and stage of development. A number of signal transduction proteins and pathways are implicated in the transduction of the biological effects of FGF-2. These include the activation of PLCγ1 [62,63], raf1 [64], ras [63,64], Mapkk, mapk [65], s6 [67,17], src [66], PKC [18], and also the oncogenes (myc, c-fos c-jun, shc, Grb) [5].

When administered to animals systemically FGF-2 is shown to accumulate in various tissues including liver, heart and vasculature [5]. As shown in the previous section, FGF-2 administered to the heart by perfusion induces increased recovery from I-R injury [6,7]. In this section, we address the localization of administered FGF-2 in hearts subjected to I-R injury, to identify potential sites of direct action. We have subsequently examined potential signaling mechanisms activated in cardiomyocytes by FGF-2 starting with the activation of the tyrosine kinase receptors, and continuing downstream with the phosphoinositide PLC-PKC pathway, the MAPK kinase and the src-kinase pathways.

Our data indicate that FGF-2 administered to the hearts by retrograde perfusion has a direct tyrosine kinase receptor mediated effect on cardiac myocytes *in situ*. All three pathways mentioned become activated or affected by FGF-2 in the heart. Activation of PKC is found to be essential for cardioprotection and for the negative inotropic effect observed upon FGF administration prior to ischemia. Some evidence is provided that this may not be so for MAPK which also becomes activated by FGF-2. Effects on several PLC isozymes suggest cross-talk between tyrosine receptor and G-protein-mediated pathways. Finally, translocation, and presumably activation of ß-ARK1 puts forward the notion that FGF-2 may regulate the activity of adrenergic receptors.

3.2 MATERIAL AND METHODS

3.2.1 Detection of FGF-2, phosphotyrosine, PLCß3, PLCδ1, PKC isoforms, src, and fos in hearts treated with FGF-2 or vehicle by perfusion, ex-vivo.

Heart perfusion and FGF-2 administration has been described in detail in Chapter 2 section 2.2.2.3.1 and by Padua et. al.[6]. The various proteins were detected by immunofluoresence staining and/or by western blotting of sarcolemmal membrane fractions. Tissue sections were obtained within 10 min. of FGF (or vehicle) administration by cryosectioning and processed for immunofluorescence staining to detect FGF-2, phosphotyrosine-containing proteins, PLC-δ1, PLC-ß3, PKC-α, PKC-ε, PKC-ζ, src-kinase, c-fos.

Sarcolemmal fractions were obtained from snap-frozen hearts within 10 min. of FGF or vehicle administration and examined by western blotting for above mentioned proteins as well as ß-ARK1, MAPK, phosphorylated MAPK and c-jun. ATP and CP levels were determined at various time points, i.e. 10 min. after FGF administration, prior to I-R, after FGF-administration followed by 30 min. ischemia and after FGF perfusion, 30 min. ischemia and 60 min. reperfusion.

3.2.2 Determination of phosphotyrosine pattern in hearts injected with FGF-2 directly, in vivo

Sprague-Dawley rats, approximately 200- 250g, were placed in a anaesthetic induction chamber and exposed to 5.0% Isoflurane/95% oxygen gas to induce anaesthesia [3]. The animals were then intubated through the mouth using a 4.5 inch 16 gauge catheter as an endotracheal tube. A anaesthetic plane of approximately 2.5%

isoflurane/97.5% oxygen was maintained during the surgery with a ventilation pressure of approximately 25 cm of H_2O. After the animals were anaesthetised, the skin was incised left of the sternum, and retractors were inserted. The pericardial sac was exteriorized through the intercostal space and 2μg of recombinant FGF-2 (Prepro Tech Inc., Rocky Hill, NJ) in 50 μl of 1mM sodium orthovanadate/PBS was injected using a Hamilton syringe in 5 random spots along the apical portion of the heart. The hearts were then repositioned within the thoracic cavity and the chest wall closed without suturing. After 10 minutes, the heart is excised and dissected and placed immediately in ice-cold 1 mM $NaHCO_3$ containing 5 mM EDTA, 1mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO), 0.2 trypsin inhibitor units aprotonin/ml (Sigma Chemical Co.), 10 μM leupeptin (Sigma Chemical Co.) and 1 mM sodium orthovanadate (Sigma Chemical Co.). Control hearts were treated similarly except no FGF-2 was present in the 1mM sodium orthovanadate/PBS solution. After thorough rinsing, tissues were homogenised briefly in 10 volumes of 1mM $NaHCO_3$ solution with a homogenizer (Polytron; Brinkmann Instruments Co., Rexdale, Ont.) at low setting at 4°C to a obtain a uniform dispersion. Aliquots were removed for protein determination [3,4] and the remainder was stored in small aliquots at -70°C.

3.2.3 Immunofluorescence microscopy

3.2.3.1 Anti-FGF-2 antibodies

Antiserum S2 was raised in rabbits against a synthetic peptide containing residues [1-24] of the truncated 146 amino acid bovine brain FGF-2 conjugated to keyhole limpet hemocyanin, as previously described [11,12]. Specificity of S2 for FGF-2 has been

demonstrated repeatedly by absorption with immobilized FGF-2 [12], and was used at dilutions of 1:2000 for immunofluorescence and at 1: 10,000 for western blots.

Anti-[1-24] FGF-2 antibodies were also obtained in our laboratory in guinea pigs. These antibodies have qualitatively similar properties to those of S2, however they are less potent (E.Kardami, unpublished observations) than S2, so they need to be used at higher concentrations (1:500, 1:1000 for immunofluorescence). The guinea-pig anti-FGF-2 antibodies were used to localize FGF-2 when rabbit IgG were used to localize other antigens (double-labeling).

3.2.3.2 Other marker antibodies and nuclear staining

Monoclonal antibodies against vinculin [Sigma Chemical Co. St. Louis, MO] and phosphotyrosine residues [4G10; no. 05-321, Upstate Biotechnology Inc.; Lake Placid, NY.] were used at 1:40 dilution and 1:10, respectively.

Polyclonal antibodies against PKCα(1μg/ml), PKCδ1 (4μg/ml), PKCε (1μg/ml), PKCζ (1μg/ml),PLCß3 (1μg/ml), PLCγ1 (1μg/ml) and βARK1 (1μg/ml), src (1μg/ml), c-fos (2μg/ml) and their corresponding immunizing peptides were purchased from Santa Cruz Biotechnology, Inc. [Santa Cruz, CA.] and polyclonal antibodies against MAPK, dually phosphorylated-MAPK and c-jun were purchased from New England Biolabs, Inc. [Missisauga, ON.]. All these polyclonal antibodies were used at dilutions specified by the manufacturer.

The secondary antibodies used were the following: Fluorescein-conjugated anti-rabbit IgG [Amersham Corp.; Arlington Heights, IL] raised in donkey and used at 1:20 dilution; Texas Red-conjugated anti-mouse IgG [Amersham Corp.], raised in goat and

used at 1:20 dilution; Biotinylated - anti-rabbit IgG [Amersham Corp.] used at 1:50 dilution; and Fluorescein-Streptavidin [Amersham Corp.] used at 1:50 dilution.

Nuclear counterstain used was Bisbenzimide Hoechst dye 33342 [Boehringer Manhiem Diagnostics; La Jolla, CA] at a concentration of 1 μg/ml.

3.2.3.3 Detection of FGF-2 by immunofluorescence

Cardiac tissue was obtained from the apex and middle portion of the heart immediately after perfusion with or without FGF-2, frozen in a dry ice/ethanol bath and used immediately for cryosectioning. Transverse sections, 7 μm thick were routinely obtained using a Leitz Kryostat [E. Leitz Inc.; Wetzlar, FRG]. The sections were collected onto gelatin-coated slides and placed in humid chambers. The sections were then incubated overnight at 4^0C with the rabbit anti-[1-24] FGF-2, at 1:2000 dilution, and with one of the mouse monoclonal antibodies in 1% (w/v) bovine serum albumin (BSA; Sigma Chemical Co.; St. Louis, MO) and 0.01% (w/v) sodium azide [Sigma Chemical Co.] in phosphate buffered saline (PBS). The PBS used consisted of 26.82 mM potassium chloride, 0.59 M sodium chloride and 81 mM sodium phosphate dibasic. Sections were then washed gently with cold PBS (3 times) and incubated with the appropriate combination of secondary antibodies. Sections were then incubated for 1 hr, 20^{O}C, with biotinylated anti-rabbit IgG and Texas Red conjugated-anti-mouse IgG at 1:20 and 1:30 dilution, respectively, in 1% BSA-PBS. Sections incubated with biotinylated anti-rabbit antibodies required a third incubation with Fluorescein-Streptavidin. Non-specific fluorescence was tested by routinely incubating sections with pre-immune rabbit serum at identical dilutions as the anti-FGF-2 sera used. A fluorescent image was considered to be

FGF-2-specific when it was obtained with the anti-FGF-2 but not with the non-immune sera.

After extensive washing with cold PBS, all sections were fixed in cold 95% ethanol [Commercial Alcohols Ltd.; Toronto, Ontario] for ten minutes, washed with PBS and immersed for thirty seconds in 1μM Bisbenzimide Hoechst dye 33342. After washing, sections were mounted in glycerol-PBS (9:1) containing 1mg/ml p-phenylenediamene) [Eastman, Kodak Co., Rochester, N.Y.]. Coverslips and slides were sealed with colorless nail varnish and stored at -20^{O}C until observation.

3.2.3.4 Photography

A Nikon Labophot microscope [Diaphot:Nikon Inc.; Garden City, NY] equipped with epifluorescence optics and appropriate filters (B-2A for Fluorescein, G1-B for Texas Red, and DM4400 for Hoechst 33342), plus phase contrast optics was used for specimen observation. The UFX-IIA Nikon system and TX-400 black and white film (Kodak) were used to photograph selected fields.

3.2.4 Tissue Extraction

3.2.4.1 Extraction for FGF-2, and heparin-sepharose affinity chromatography

All procedures were performed at 4^{O}C unless otherwise specified. Immediately after perfusion, hearts were weighed and ventricular muscles were minced with scissors and homogenized briefly with a polytron homogenizer [Brinkman Instruments Co.; Rexdale, Ontario] at low setting in three volumes over mass of extraction buffer (0.15M ammonium sulfate [Mallinckrodt Inc.] pH 4.5, 1mM phenylmethylsulphonyl fluoride, 5 μ g/ml leupeptin and 5 μg/ml pepstatin). Residual tissue was removed by ultracentrifugation

at 195,000 x g for 60 min. at 4°C [ultracentrifuge and 42.1 rotor head: Beckman Instruments; Palo Alto, CA]. The supernatant collected and protein concentration was then determined using a Bradford colorimetric assay [Bio-Rad Laboratories; Richmond, CA], according to manufacturer's instructions.

Heparin-sepharose affinity chromatography was used for FGF-2 isolation from cardiac extracts. 100 µl of packed heparin-sepharose beads [prepared according to the manufacturer's instructions] were used per 100 mg of extracted protein. The concentration of the crude extracts were made to 0.6 M NaCl by adding solid NaCl. Extracts (6-8 mg/ml) in 0.6 M NaCl, 10 mM Tris-HCl, pH 7.0 (column buffer) were absorbed to heparin-sepharose beads (1 mg protein/µl settled heparin beads; Pharmacia Fine Chemicals, Uppsala, Sweden). After extensive washing with column buffer, acidic FGF-like peptides were eluted with 1.1 M NaCl, 10 mM Tris-HCl, pH 7.0. The beads were then equilibrated in 0.1 M NaCl in 10 mM Tris-HCl, pH 7.0, and heparin-bound peptides eluted by boiling directly into sample buffer. The sample buffer used consisted of 10% mercaptoethanol [Biorad] 10% glycerol [Mallinckrodt], 1% SDS [Biorad] and 0.05M Tris at pH 6.8. After boiling of the beads in two volumes (over mass) of SDS/Page sample buffer, the whole suspension was loaded onto the wells of the gel.

3.2.4.2 Extraction for analysis of tyrosine phosphorylation

After rat hearts were injected with 2 µg of recombinant FGF-2 in 50 µl of 1mM sodium orthovanadate/PBS and removed from the animal, they were placed immediately in ice-cold 1 mM $NaHCO_3$ containing 5 mM EDTA, 1mM phenylmethylsulfonyl fluoride, 0.2 trypsin inhibitor units aprotonin/ml (Sigma Chemical Co.), 10 µM leupeptin (Sigma

Chemical Co.) and 1 mM sodium orthovanadate. Control hearts were treated similarly except no FGF-2 was present in the 1mM sodium orthovanadate/PBS solution. After thorough rinsing, tissues were homogenized briefly in 10 volumes of 1mM $NaHCO_3$ solution with a homogenizer (Polytron; Brinkmann Instruments Co., Rexdale, Ont.) at low setting at $4^{o}C$ to a obtain a uniform dispersion. Aliquots were removed for protein determination, and the remainder was stored in small 50 μg aliquots at $-70^{o}C$. Protein concentration was determined using a Bradford colorimetric assay [Biorad Laboratories; Richmond, CA], according to manufacturer's instructions. Then 10-20 μg of extracts were placed into 2 volumes of sample buffer consisting of 10% mercaptoethanol [Biorad] 10% glycerol [Mallinckrodt], 1% SDS [Biorad], and 0.05M Tris at pH 6.8 and boiled for 5 min. and loaded onto a well for SDS-PAGE electrophoresis.

3.2.4.3 Isolation of Sarcolemmal Membranes and Cytosolic Fractions

Hearts were perfused with buffer in the presence or absence of FGF-2 for 10 minutes. Subsequently, hearts were frozen in liquid nitrogen with a freeze-clamp technique [22], stored briefly (<3hr) at -80°C, pooled (3hearts per preparation) and processed according to the method described by Pitts [23] to prepare sarcolemma-enriched membranes. All isolation procedures were carried out at 4°C. Ventricular tissue from the 3 pooled hearts (5 ml buffer/ g tissue) was finely minced by hand in a 0.6M sucrose 10 mM imidazole. The pieces were homogenized with a Polytron 3000 homogenizer (Kinematica AG, Switzerland) at 13000 RPM for 6 x 15 seconds. The resulting homogenate was then centrifuged at 12,000 g for 30 min. and the pellet was discarded. A 500μl supernatant aliquot was centrifuged at 100,000 g for 60 min. in a Beckman TL-100 Ultracentrifuge to

remove any membrane fractions. The resulting cytosolic fraction was divided, frozen in liquid nitrogen and stored at -80°C until later use. The remaining supernatant was diluted with 140mM KCL, 20 mM 3-(-N-morpholino)-propanesulfonic acid (MOPS), pH 7.4 (5 ml buffer/ g tissue) and centrifuged at 100,000 g for 90 min. in a Beckman swinging bucket rotor (SW-28) the band at the sucrose-buffer interface was taken and diluted with 3 volumes of 140 mM KCL, 20 mM MOPS, pH 7.4. The pellet from this final centrifugation at 100,000 g for 30 min. was resuspended in 0.25 M sucrose, 10mM histidine, pH 7.4 (225μl/ g tissue). This sarcolemmal enriched fraction was divided into aliquots, frozen in liquid nitrogen and stored at -70°C until later use. The purity of the sarcolemmal membrane prepared by this method has been evaluated previously [24-26] by assaying for the activity of marker enzymes for sarcolemma, mitochondria, sarcoplasmic reticulum and endothelial cells. Protein concentration was determined using a Bradford colorimetric assay [Biorad Laboratories; Richmond, CA], according to manufacturer's instructions. Then 10-20 μg of membrane and cytosolic fractions were placed into 2 volumes of sample buffer consisting of 10% mercaptoethanol [Biorad] 10% glycerol [Mallinckrodt], 1% SDS [Biorad] and 0.05M Tris at pH 6.8 and boiled for 5 min. and loaded onto a well for SDS-PAGE electrophoresis.

3.2.5 SDS-PAGE, Western blotting and autoradiography

3.2.5.1 SDS-PAGE

7.5-12.5% polyacrylamide gels were used [15]. Each gel contained molecular weight markers (10-100 kDa or 10-200 kDa; Bio-Rad Laboratories depending on the specific proteins). To examine changes in FGF-2 levels in FGF-2-treated and control hearts, human recombinant FGF-2 was used as a reference marker, at 5 ng/lane.

To compare FGF-2 levels in FGF-2-treated and control hearts before ischemia and after global ischemia/reperfusion, heparin bound fractions (3μg of protein) from equivalent amounts of heart extracts (40 mg of extracted protein) were analyzed in 12.5% polyacrylamide gels, as described [6,13,16]

To analyze changes in the protein profile of phosphorylated tyrosine residues in FGF-2-treated versus that of control hearts, equivalent amounts of cardiac extracts (30μg protein) from control (injected with orthovanadate-PBS) hearts and FGF-2-treated (injected with FGF-2-PBS-orthovanadate) hearts were analyzed in 7.5% polyacrylamide gels, as described [6,13,16].

To analyze signal transduction proteins (PKCα,δ,ζ, ε, PLC β_3, SRC, MAPK, and MAPK-P) in FGF-2-treated versus control hearts, equivalent amounts of sarcolemmal membranes and cytosolic fractions (25μg protein/lane) from control and FGF-2-perfused hearts were analyzed in 7.5% polyacrylamide gels, as described [6,13,16].

3.2.5.2 Western blotting

Proteins analyzed by SDS-PAGE were transferred electrophoretically onto Immobilon-P membranes (Millipore, Ontario, Canada). All subsequent steps are as described previously [6,13,16].

For FGF-2 and phosphorylated tyrosine residue analysis, after protein transfer was completed, immobilon-P membrane was blocked either in 1% BSA in PBS for 30 min. at room temperature for FGF-2 or with 5% milk in PBS for 1 hr at room temperature. These blocking agents served to saturate the non-specific protein binding sites. Afterwards, the blocking agent was poured off and either a solution of anti-FGF-2 (S2) [1:10,000] or anti-phosphorylated-tyrosine antibodies (1:1000) in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween [Biorad Laboratories]) was added to the blot and incubated for 25 hr. at 4^0C under gentle shaking motion. Several TBST washes 15 min./each were followed to remove any non-specifically bound antibodies. Antigen-antibody complexes were then visualized by incubating the membrane with 0.1 μCi/ml of ^{125}I-protein A [Amersham Corp.; Oakville, Ontario] in TBST wash buffer for one hour at room temperature. Afterwards, the blot was washed 5 times with TBST and with PBS. The blots were then dried and packaged in plastic seal-a-meal bags.

Once Western blot transfer and immunoblotting was completed, the sealed blots were placed, face up, between two intensifying screens [Lightning plus model; Dupont Cronex Co.]. An X-OMAT film {Eastern Kodak Co.; Rochester, NY] was placed on top of the blot in the dark and the autoradiographic cassette was then incubated at -70^0C for 7

days. Autoradiograms were then developed and scanned with a LBK 2202 Ultrascan laser densitometer [Sweden], as per manufacturer's instructions.

Visualization of antigen-antibody complexes was also achieved using a chemiluminescence kit (Pierce, Rockford, IL.) as per manufacturer's instructions, for the analysis of signal transduction proteins (PKCα,δ,ζ, ε, PLC β_3, SRC, MAPK, and MAPK-P) from sarcolemma and cytosolic fractions of FGF-2-perfused and control hearts. In this instance, immobilon P membranes were incubated with a 1:10,000 dilution of goat anti-rabbit IgG (H +L)-HRP conjugated (Bio-Rad Inc.), followed by incubation with a Chemiluminescence Blotting Substrate as per manufacturer's instructions. X-ray film was subsequently exposed to these blots for 10 sec to 30 min., depending on the intensity of protein bands.

3.2.6 Densitometer

Densitometery was performed using the Bio-Rad Model GS-670 imaging densitometer and the computer program Molecular Analyst/PC 700 Densitometry Version 3.11 [Bio-Rad, Mississauga,Ont.]. Values were plotted as arbitrary units of value.

3.2.7 Assay for Protein Kinase C Activity

The enzyme activity present in 5µl of cytosolic or membrane cardiomyocyte fractions was assayed using MARCKS peptide (Calbiochem) as the substrate [19]. To measure calcium-dependent activity, the assay was conducted in a reaction mixture (total volume of 50 µl) containing 25 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.1 mg/ml phosphatidylserine, 0.5 µg/ml diacylglycerol, 1.2 mM $CaCl_2$ and 10 µM peptide [20]. The reaction mixture used to assay calcium-independent activity was identical except for the

absence of $CaCl_2$. The reaction was initiated by the addition of [^{32}P]-ATP (4 X 10^6 cpm) and the samples were incubated at 30°C for 10 min. A 40 µl aliquot was transferred to P81 paper which were subsequently washed 5 times in 150 mM phosphoric acid and once in ethanol. Radioactivity incorporated into the peptide substrate was quantified by scintillation counting. All values were corrected for background incorporation using a complete reaction (including sample) minus the substrate. Specificity for PKC was confirmed by conducting parallel experiments in the presence of 40 µM chelerythrine. Similar data were obtained with reactions using 0.12 mM neurogranin$_{(28-43)}$ [21] instead of MARCKS peptide as the substrate.

3.2.8 Phospholipase C Assay

The assay for PLC activity was conducted on sarcolemma and cytosolic fractions as previously described by Meij et al.[68,69]. Exogenous substrates were prepared by mixing an aliquot of [^{3}H]PIP_2, [^{3}H]PIP, [^{3}H]PI (Dupont/NEN, Mississauga, ON) with an aliquot of the respective non-labeled substrate (Sigma Chemical Co., St. Louis, MO). A stream of N2 gas was used to subsequently dry this mixture. Mixture was then redissolved in 0.1 g/ml (232 mM) sodium cholate(Sigma Chemical Co., St. Louis, MO). This substrate solution was stored in the presence of N2 gas overnight at 4°C and was diluted to 112 mM Na-cholate shortly before addition to the incubation mixture. For the reaction, mixture consisted of 30 mM HEPES-Tris (pH 7.0), 100 mM NaCl, 2mM EGTA, 3.13mM CaCl2, ~15 µg cytosolic or SL proteins, 14 mM Na-cholate and 20 µM ^{3}H-labeled substrate (400-500 dpm/µl) in a final volume of 40 µl. These reactions were carried out at 37°C and terminated after 2.5 min. by the addition of 144 µl ice-cold $CHCl_3/CH_3OH/HCl$

(1:2:0.2, v/v/v), followed by 48 µl 2M KCl and 48 µl $CHCl_3$. In addition, blanks were treated identically to experimental samples, except that protein was added after the reaction was stopped. Phase separation was achieved by mixing and centrifugation of the reaction mix. The resulting aqueous upper phase was aspirated and applied to a column of 500 µl Dowex AG 1-X8 slurry (Bio-Rad Laboratories, Mississauga, ON). Inositol mono-, bis-, and trisphosphate were eluted each with 1 ml 0.1 M formic acid containing 0.2 M, 0.4M and 1,0 M NH_4-formate, respectively [68,69]. Quantitation was done by liquid scintillation counting in 10 ml of CytoScint™. Inositol triphosphate is the primary product of PIP_2 hydrolysis[68,69]. Unless otherwise indicated, data represent the sum of the three inositol phosphates[68,69].

3.2.9 Calculation and Statistical Analysis

All calculations and statistical analysis were performed using the Microsoft Excel Analysis Tool Pac version 5.0 (Microsoft Corporation, Cambridge, MA) and the T-ease statistical calculation software. Utilizing a student t-test for statistical significance (as determined by the degree of standard error).

3.3 Studies on potential mechanism of FGF-2 induced cardioprotection

3.3.1 Localization of exogenous FGF-2 in perfused hearts

Sections from hearts perfused with buffer or buffer supplemented with 10μg FGF-2 were processed for FGF-2 localization by indirect immunofluorescence staining. Results are shown in Fig. 3.1. Control heart sections displayed near background levels of anti-FGF-2 staining (Fig. 3.1d). Treated hearts displayed intense anti-FGF-2 staining in association with blood vessels (Fig. 3.1a and c, large straight arrows) and capillaries (Fig. 3.1b and c, curved arrows). In addition, strong anti-FGF-2 labeling was detected around cardiomyocytes near FGF-2 rich blood vessels (Fig. 3.1a,b and c), in a distribution resembling that of basement membranes [27]. The pattern of anti-FGF-2 staining was similar in hearts sectioned before ischemia (Fig. 3.1) or after ischemia and reperfusion (not shown). Entry of FGF-2 into the myocardium, occurred, presumably, via the capillaries.

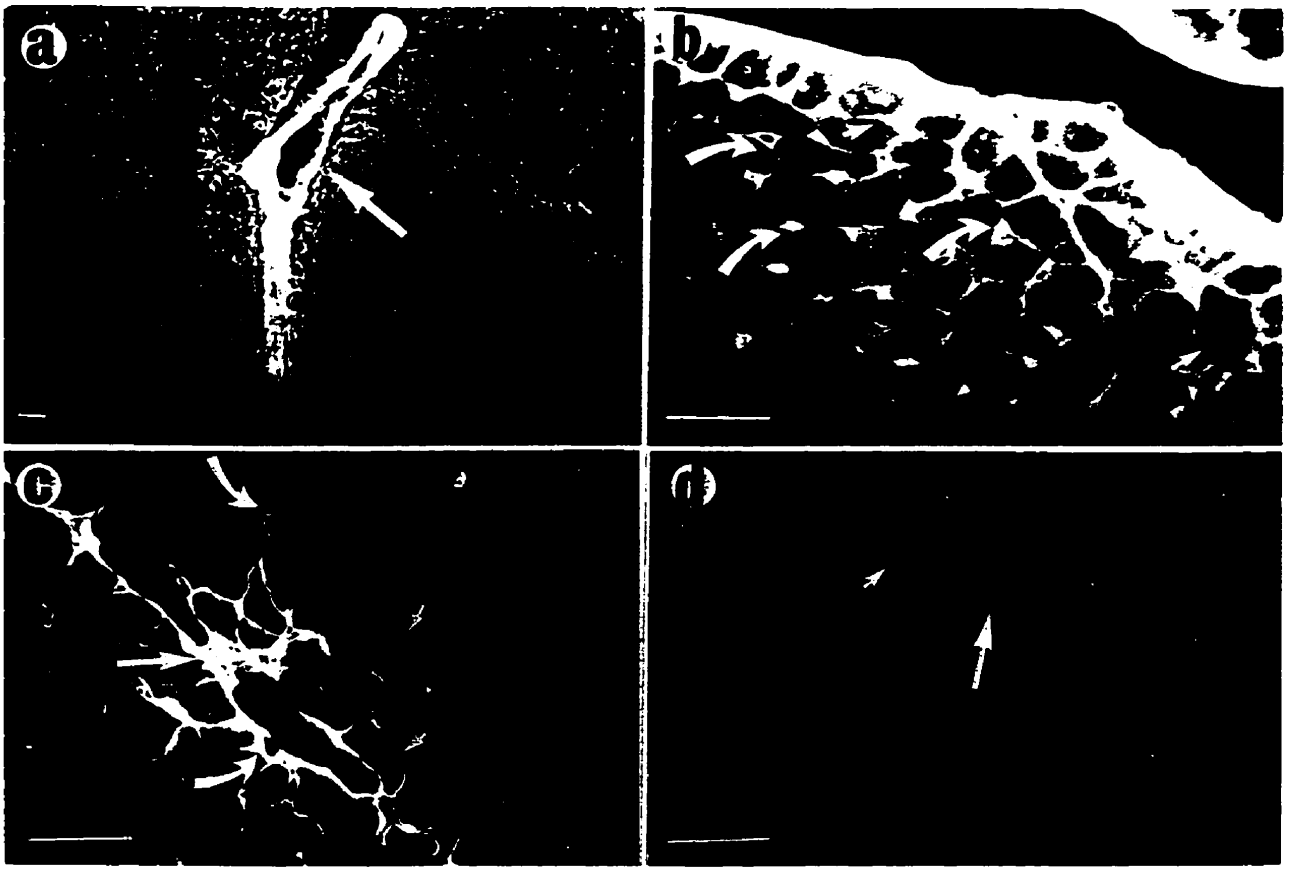


Figure 3.1: Micrographs of indirect immunofluorescence for FGF-2 on tissue sections from perfused hearts. (a), (b), and (c), sections from hearts perfused with 10 μg of FGF-2. (d) hearts perfused with buffer. Large straight arrows point to blood vessels, curved arrows to capillaries, and, small arrows to cardiomyocytes. (Size bar = 50 μm).

3.3.2 Relative levels of FGF-2 in perfused hearts

Extracts were obtained from age-matched isolated perfused ventricles that were subjected to (i), perfusion with FGF-2, (ii), perfusion with FGF-2 followed by 60 min. of global ischemia and 20 min. of reperfusion, and, (iii), perfusion with medium, in the absence of FGF-2. Protein yields were similar in extracts from control as well as FGF-2-treated hearts (42±2 mg protein per g wet weight of tissue). Equivalent amounts of these extracts (i.e. containing exactly the same amount of extracted protein) were fractionated by heparin-sepharose affinity chromatography and analyzed for FGF-2 content by immunoblotting. A characteristic set of results is shown in Fig. 3.2. Recombinant human FGF-2 used as a positive control migrates with a mobility corresponding to an apparent molecular mass of 18 kDa (Fig. 3.2, lane 4). Hearts perfused with FGF-2 displayed a clear increase in FGF-2 levels (Fig. 3.2, lane 1) compared to controls (Fig. 3.2, lane 3). Extracts from FGF-2-treated hearts displayed a 4-fold increase in the relative intensity of the 18 kDa FGF-2 band compared to that of controls.

Increased FGF-2 content over controls (3.5-fold) was also evident in hearts perfused with FGF-2 and subjected to global ischemia and reperfusion (Fig. 3.2, lane 2), indicating a strong association with cardiac tissue, in agreement with a recent paper reporting that intravenous administration of this factor resulted in rapid deposition within organs including the heart [28].

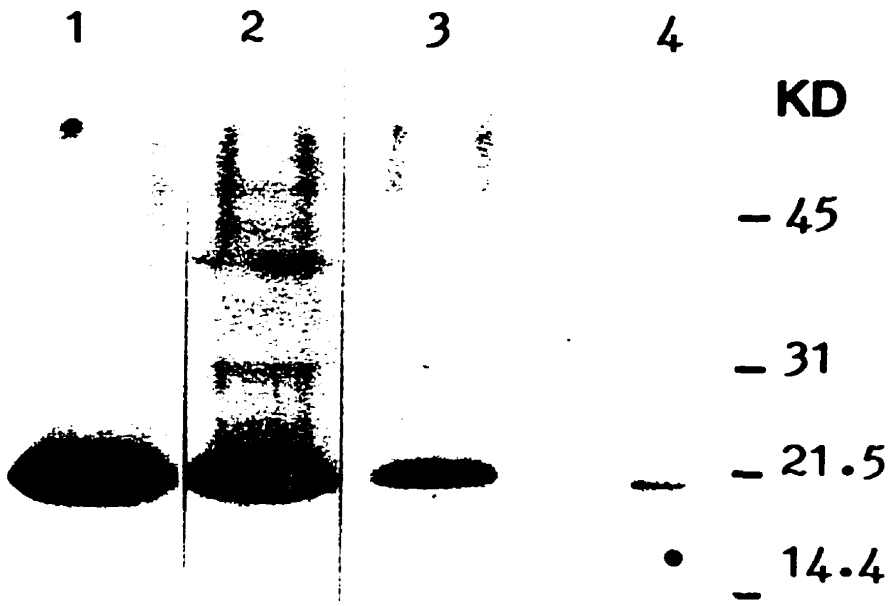


Figure 3.2: Analysis of FGF-2 in cardiac heparin-binding fractions by immunoblotting. Lane 1, hearts perfused with 10 μg of FGF-2, lane 2, hearts perfused with 10 μg FGF-2 and subjected to ischemia and reperfusion, lane 3, hearts perfused with buffer, lane 4, 5 ng of human recombinant FGF-2. Relative mobility of molecular weight markers is indicated in kilodalton (kDa).

3.3.3 Localization of phosphorylated tyrosine residues after FGF-2 administration in isolated perfused hearts

Because FGF-2 high-affinity receptors are tyrosine kinases [1], we examined whether FGF-2 can induce tyrosine phosphorylation in the adult myocardium. To this end we therefore examined anti-phosphotyrosine staining in cardiac sections from control and FGF-2-perfused hearts within 10 min. from the initiation of FGF-2 perfusion (Fig.3.3). Control hearts, i.e., hearts perfused with buffer and expressing basal levels of FGF-2, displayed faint anti-phosphotyrosine staining in areas of intercalated disks, in agreement with previous reports [29] (Fig. 3.3B). Intensity of anti-FGF-2 staining in these sections was barely above background, because we used a high dilution of the anti-FGF-2 serum (Fig. 3.3A). Myocytes in sections from FGF-2-perfused hearts displayed increased anti-FGF-2 (Fig. 3.3C) as well as increased anti-phosphotyrosine staining (Fig. 3.3D). The increase in anti-phosphotyrosine staining was evident at the intercalated disk regions but also in association with lateral surfaces of cardiomyocytes (Fig. 3.3D). Patterns of anti-phosphotyrosine staining identical to those described above were detected in sections that had not been costained for FGF-2.

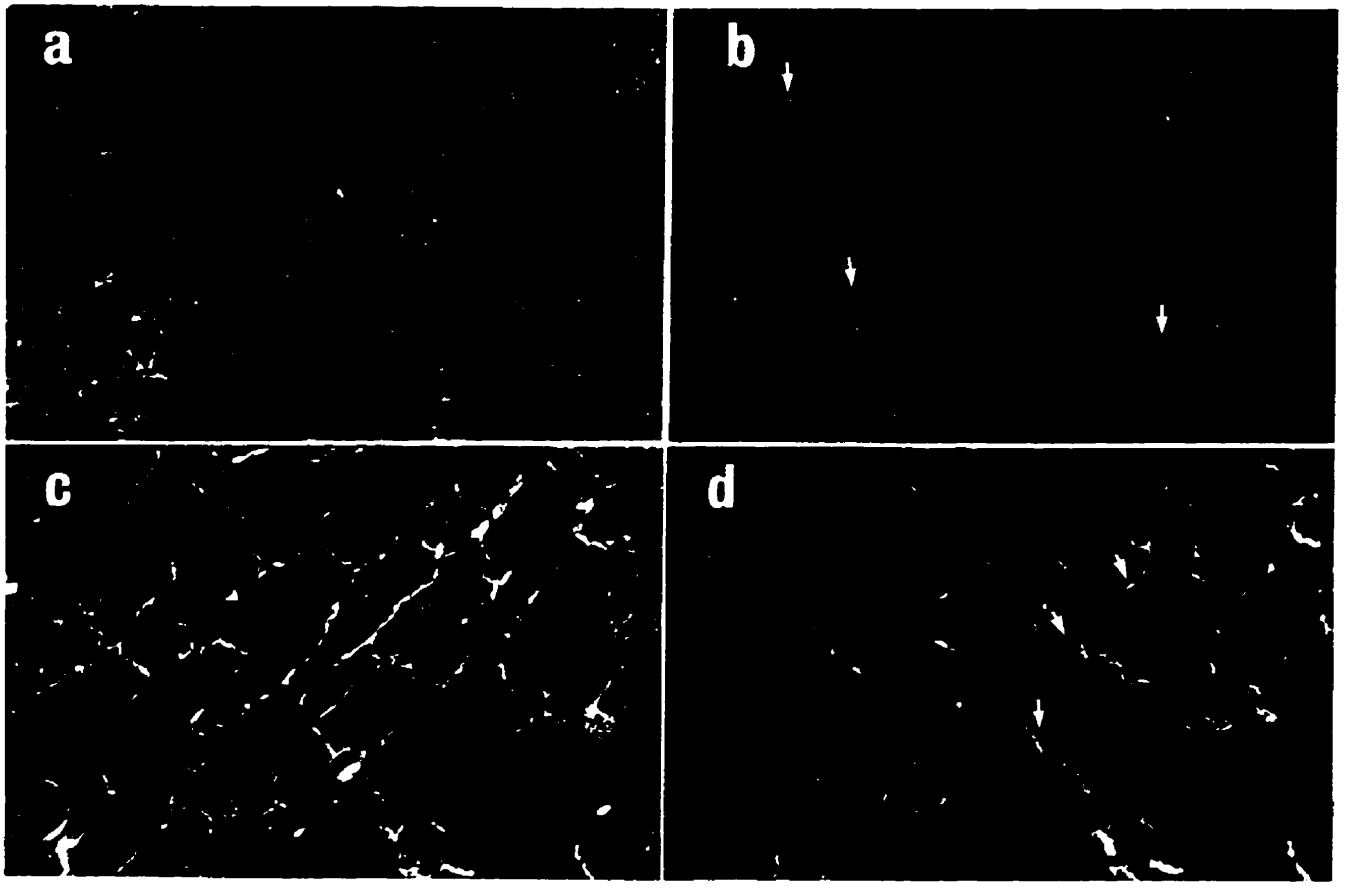


Figure 3.3: FGF-2 induced stimulation of tyrosine phosphorylation in adult cardiomyocytes *in vivo*. Micrographs of indirect immunofluorescence for phosphotyrosine-containing proteins and/or FGF-2 on tissue sections from perfused hearts. a and b: double immunofluorescence labeling of ventricular sections for FGF-2 and phosphotyrosine, respectively, in hearts perfused with buffer only. c and d: double imminofluorescence labeling for FGF-2 and phosphotyrosine, respectively, in hearts perfused with FGF-2 (10 μg/heart). Arrowheads, areas of phosphotyrosine labeling. Bar, 50 μm.

3.3.4 Examination of tyrosine phosphorylation of proteins in cardiac extracts after administration of FGF-2 to rat hearts in vivo

We investigated the effect of FGF-2, administered by direct injection to the heart apex, on tyrosine phosphorylation pattern and levels as assessed by western blotting with phosphotyrosine-specific antibodies. Results are shown in Fig.4. Injection of FGF-2 into the myocardium resulted in changes in tyrosine phosphorylation levels of several proteins, compared to control hearts injected with buffer alone. Tyrosine phosphorylation was stimulated in proteins of more than 200 and about 98, 83, 72, 59 and 42 kDa (Fig. 3.4, lane 2). Decreased tyrosine phosphorylation after FGF-2 treatment, however, was seen in proteins of about 123 and 66 kDa, which appeared to have higher phosphotyrosine content in the controls (Fig. 3.4, lane 1). Net anti-phosphotyrosine immunoreactivity was increased by about 50% in FGF-2-treated cardiac tissue, as estimated by densitometry of the immunoblot shown in Figure 3.4, indicating that the immunoreactive bands seen after FGF-2 treatment cannot be accounted for by degradation of immunoreactive bands of control tissue. At any rate, protein staining of duplicate gels did not reveal any differences in protein band composition and intensity between control and FGF-2-treated heart lysates, indicating that no major changes in proteolysis occurred as a function of FGF-2 treatment. In addition, levels and pattern of phosphotyrosine phosphorylation in extracts from non-injected areas of the heart were similar to those of controls shown in Figure 3.4 (lane 1), indicating that neither injection per se nor the use of orthovanadate could account for the changes in phosphotyrosine observed in the FGF-2-injected cardiac tissue. The

observed decrease in tyrosine phosphorylation of certain proteins indicated that FGF-2 treatment may have induced specific phosphotyrosine phosphatase activation in vivo.

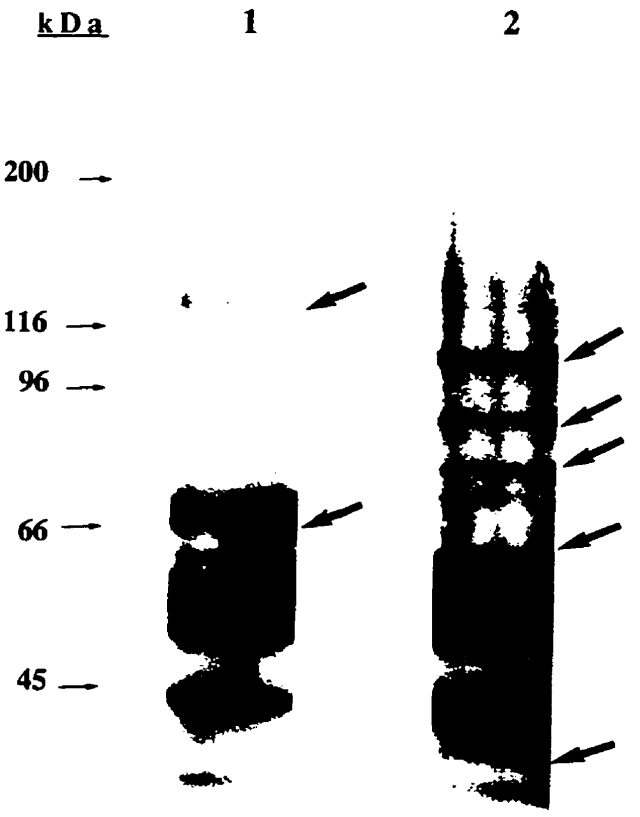


Figure 3.4: Effects of FGF-2 injection on phosphotyrosine phosphorylation. Cardiac extracts (30 μg protein) obtained form hearts injected with FGF-2 or vehicle were analyzed for phosphotyrosine containing proteins by western blotting. Lane 1, extract from control (injected with orthovanadate-PBS) hearts; lane 2, extract from experimental (injected with FGF-2-PBS-orthovanadate) hearts. Arrows indicate bands for which the intensity differs between control and experimental hearts. Migration of molecular weight markers are indicated on the left, in kDa.

3.3.5 Examination of Phospholipase-C activity (PLC) activity from FGF-2-perfused and control hearts

Figure 3.5 shows total inositol phosphate produced (nmol InsP mg/min) as a result of the total PLC activity in the sarcolemma and cytosolic fractions. FGF-2-treated hearts showed a 11.5% increase in total inositol phosphate produced over that of control perfused hearts in the cytosol. Similarly, in sarcolemma fractions, FGF-2 treated hearts showed a 21.8% increase in total inositol phosphate produced over that of control perfused hearts.

3.3.6 Examination of Protein Kinase C activity (PKC) activity from FGF-2-perfused and control hearts

PKC activity of sarcolemmal fractions was measured in the presence of diacylglycerol and phosphatidylserine to fully activate PKC. Thus, this assay represents a means of detecting levels of PKC at the sarcolemmal fraction and does not evaluate endogenous activation of PKC per se. The substrate utilized in this assay was the MARCKS peptide since it has been previously proven to be highly specific for PKC [19]. As summarized in Table 1, samples from FGF-2 treated hearts exhibited substantially increased activity, tested in the absence as well as in the presence of calcium. This FGF-2-induced activity was inhibitable by chelerythrine (40 μmol/L), when included in the reaction medium (data not shown), providing additional confirmation that PKC activity was actually being measured. Similar results were obtained when neurogranin was used as an alternate PKC substrate (data not shown).

In PKC assays conducted in the presence of EGTA, FGF-2 administration in the absence of chelerythrine elicited a nearly 5-fold increase in sarcolemmal PKC compared to control untreated hearts, while FGF-2 administered in the presence of chelerythrine resulted in sarcolemmal PKC activity levels virtually indistinguishable to those of control hearts, and only slightly (1.4-fold) higher than those seen in hearts treated with chelerythrine alone. Chelerythrine administration itself appeared to reduce, compared to control values, sarcolemmal PKC activity.

In PKC assays conducted in the presence of calcium, FGF-2 administration in the absence of chelerythrine elicited an over 2-fold increase in sarcolemmal PKC activity, while no such increase was detected when FGF-2 was given in the presence of chelerythrine; if anything, a decrease in sarcolemmal PKC activity to levels below those of control or chelerythrine treated hearts was seen in the latter case; the reason for this finding is unclear at present. Chelerythrine itself did not affect sarcolemmal PKC activity, assayed in the presence of calcium.

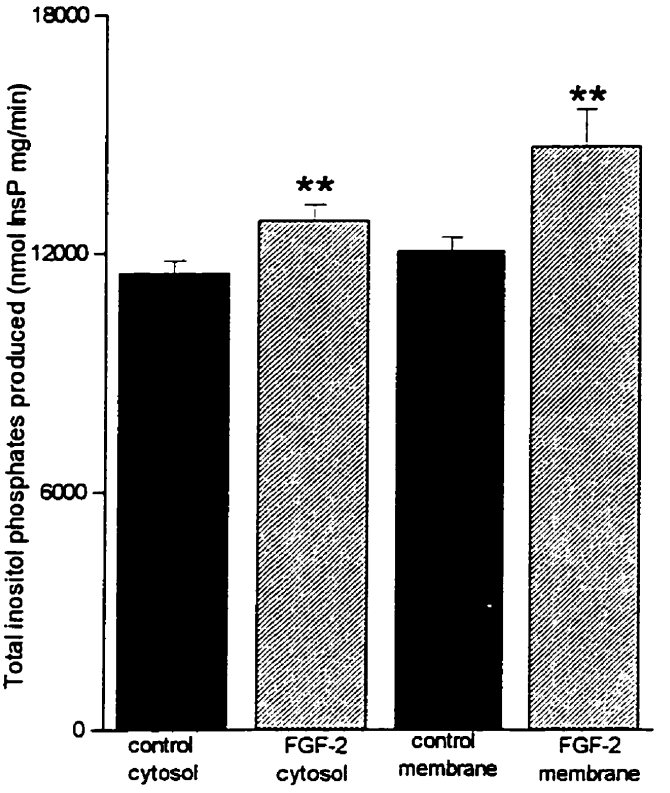


Figure 3.5: Examination of Phospholipase-C (PLC) activity from FGF-2-perfused and control hearts. PLC activity was measured as a function of the amount of total inositol phosphate produced. Solid columns represent cytosolic and membrane fractions from control perfused hearts. Hatched columns represent cytosolic and membrane fractions from perfused hearts treated with 10 μg FGF-2. Absolute values for the total inositol phosphates produced (nmol InsP mg/min.) in control and FGF-2-treated cytosolic fractions were 11486.34±313.22 and 12806.10±389nmol InPs/mg.min, respectively. ** denotes significant difference from control values at $P \leq 0.05$. Statistical analysis was performed using an unpaired, one-tailed student t-test.

TABLE 3.1: Effect of FGF-2 Administration on Sarcolemmal PKC Activity.

Treatment	Activity/EGTA pmol/min/mg protein	Activity/Calcium pmol/min/mg protein
Control	0.032±0.002	0.065±0.006
+FGF-2	0.153±.006	0.144±0.009
+Chl	0.021±0.000	0.061±0.002
+Chl/FGF-2	0.030±0.001	0.049±0.001

3.3.7 Examination of PKC-α,PKC-δ, PKC-ζ and PKC-ε subcellular localization

Studies using cultured cells and cell lines have shown that FGF-2 activates PKC [30,31], although the effect of FGF on PKC localization in cardiomyocytes is not known. Activation of PKC by other effectors (e.g. adenosine [32], stretch/rennin-angiotensin [33] and noradenaline [34]), produces a translocation of the enzymes to the sarcolemma compartments of the stimulated cell, including the sarcolemma of cardiomyocytes. Hearts treated with FGF-2 prior to ischemia were thus examined for an indication that similar effects occurred with adult cardiac myocytes *in situ*. Subcellular distribution of the various PKC isoforms was examined by western blotting of sarcolemmal fraction and by immunolocalization on tissue sections, whenever possible. Representative results from one series of western blot analyses are shown in Fig. 3.6A. Cardiac sarcolemmal samples contained bands recognized specifically by antibodies to the PKC subtypes -α, -δ,-ε,-ζ, and migrating with an apparent molecular mass of 85,78,90 and 72 kDa, respectively. These bands are eliminated when antibodies are incubated with their corresponding immunizing peptides (data not shown). In each instance, administration of FGF-2 resulted in increased levels of PKC in association with sarcolemma. This would indicate translocation and presumably activation, of all four subtypes. A quantitative estimate of relative sarcolemmal PKC levels is shown in Fig. 3.6B. Measurements represent the average of three separate determinations (Western blot analysis followed by densitometry) from the same pooled samples. The complete experimental series (sarcolemmal preparations from control and FGF-treated hearts, followed by western blotting and densitometry) has been repeated four times with very similar results.

Administration of chelerythrine alone resulted in decreased levels of PKC-ε and PKC-ζ in association with the sarcolemma, compared to untreated control samples, while levels of subtypes -α and -δ and did not change substantially. FGF-2 administration to chelerythrine-treated hearts resulted in increased sarcolemmal PKC levels (all subtypes) compared to those from chelerythrine-alone treated hearts. On the other hand, administration of FGF-2 to chelerythrine-treated hearts resulted in all cases, in reduced levels of PKC associated with the sarcolemma compared to samples from FGF-2-only treated hearts.

Immunolocalization of PKC-α indicated diffuse cytoplasmic, bright nuclear and weak sarcolemma-associated staining in cardiomyocytes of control heart sections (Fig. 3.7). FGF-2 administration led to no perceptible change in the immunostaining of the cardiomyocytes however, increased vascular endothelial and/or smooth muscle staining was observed with FGF-2 treatment as indicated in Fig. 3.7.

Immunolocalization of PKC-δ indicated a diffuse cytoplasmic and weak sarcolemma-associated localization in cardiomyocytes of control heart sections (Fig. 3.8). FGF-2 administration led to increased plasma membrane-associated anti-PKC-δ staining compared to controls, confirming the results from Western blotting analysis. Immunolocalization of PKC-ε and PKC-ζ on cardiac tissue sections did not show any obvious changes in localization patterns between controls and FGF-treated hearts (data not shown).

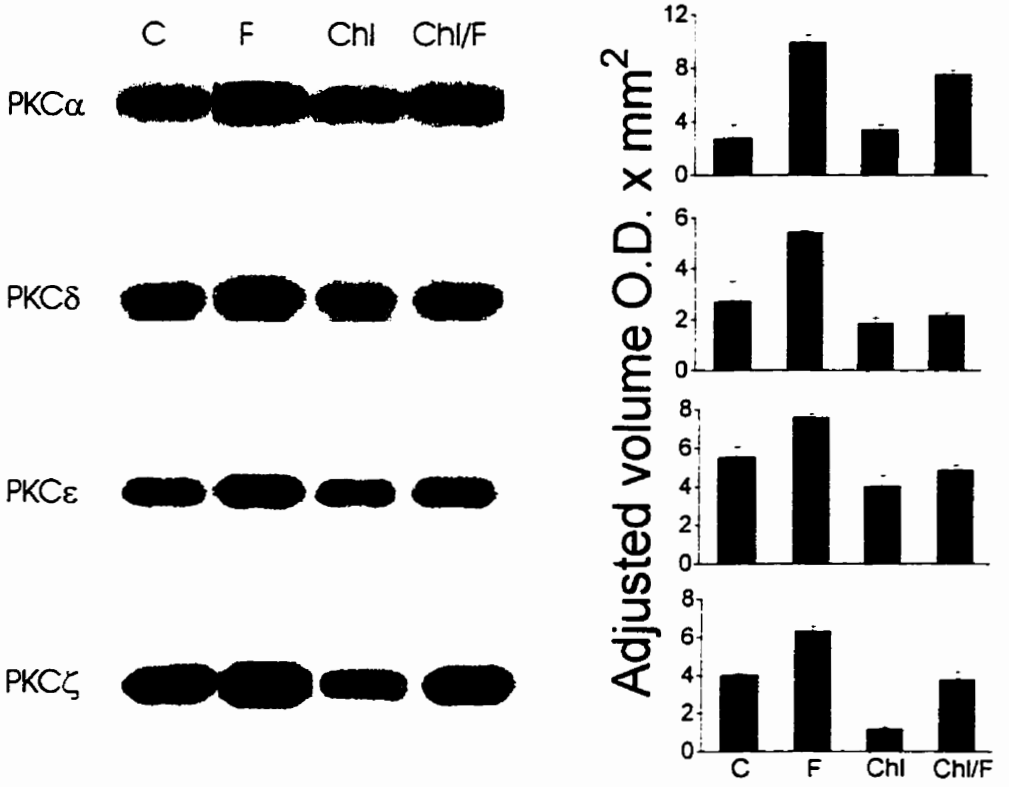


Figure 3.6: Effect of FGF-2 on PKC distribution to sarcolemmal fractions (A): representative micrographs from western blot analysis of cardiac sarcolemmal fractions (20 g/lane) obtained from control (C), FGF-2-treated (F), chelerythrine-treated (Chl) and chelerythrine-FGF-treated (Chl/F) hearts and probed for PKC , PKC , PKC and PKC as indicated. Immunoreactive bands migrating at 82 kDa (PKC), 78 kDa (PKC), 90 kDa (PKC), 72 kDa (PKC are indicated. (B) Quantitative (densitometric) analysis of PKC (PKC , PKC PKC and PKC as indicated) subtype levels in cardiac sarcolemmal fractions obtained before and after FGF-2 administration and analyzed by western blotting (n=3). Statistcal analysis was performed using an unpaired, two-tailed student t-test.

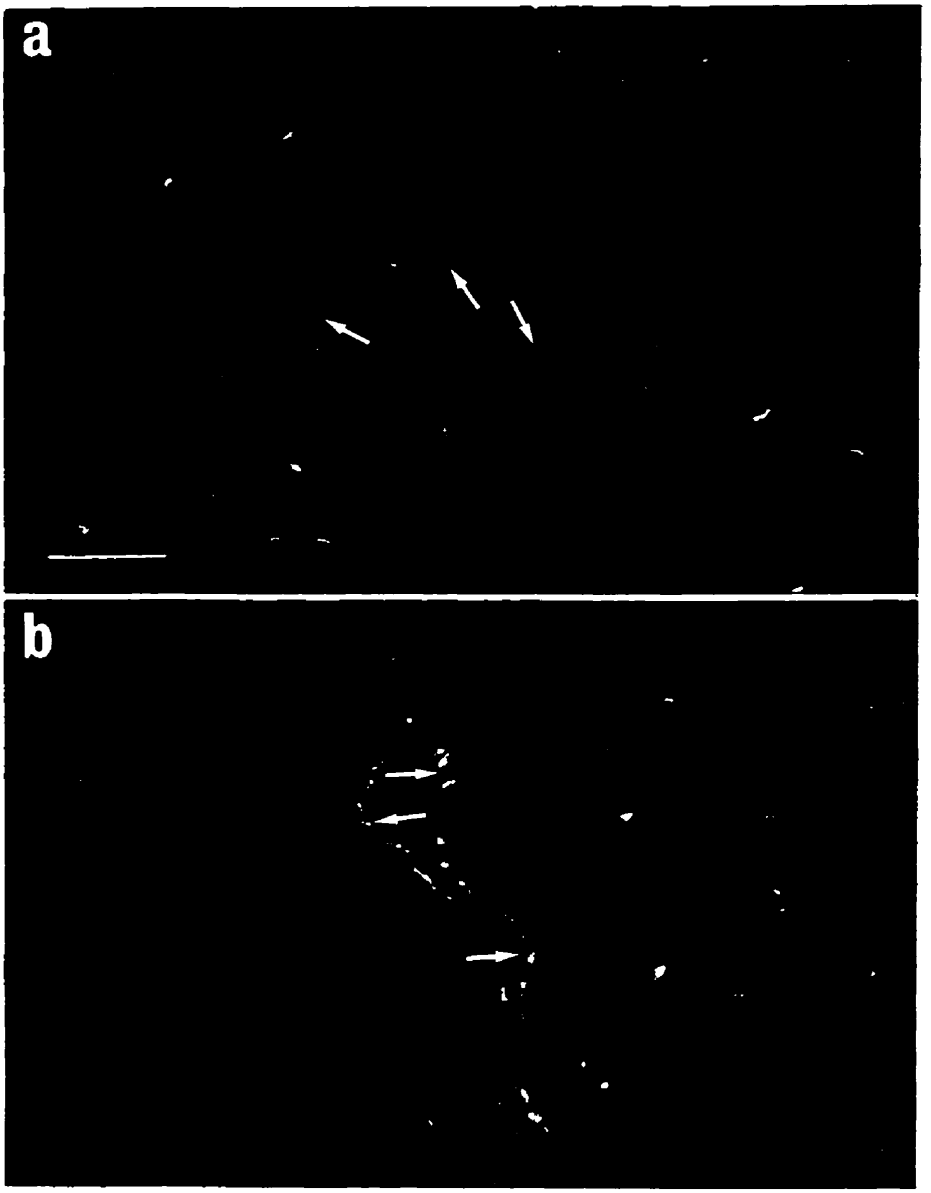


Fig. 3.7: FGF-2 effect on PKCα localization. Micrographs of transverse sections of hearts perfused with (b) or without (a) 10 μg of FGF-2, and stained for PKC-α by immunofluorescence. Arrows in (a) indicate normal PKC-α staining along the endothelial cell region of the coronary vessel. Arrows in (b) indicate increased PKC-α staining along the endothelial cell region of coronary vessels from hearts treated with exogenous FGF-2. (bar = 20 μm).

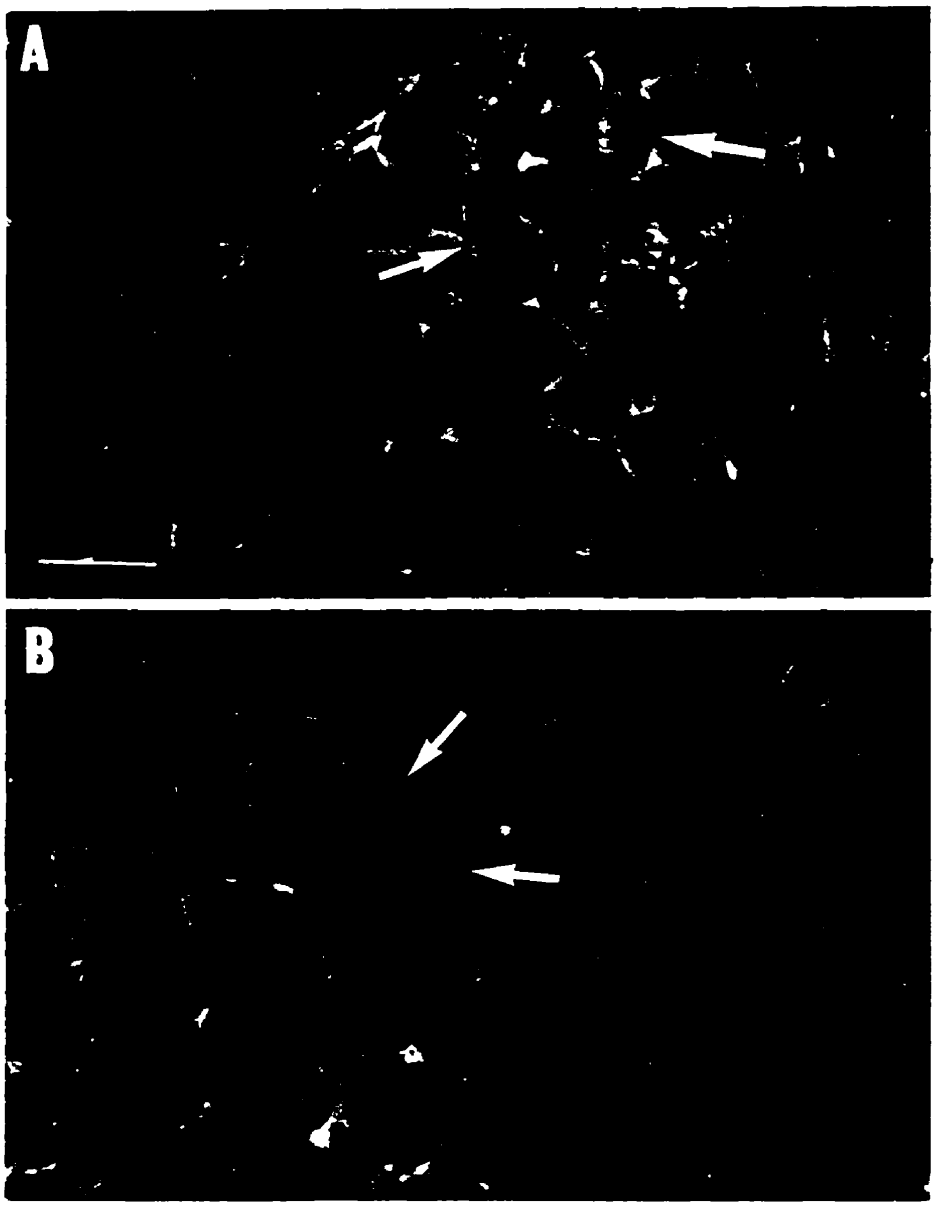


Fig. 3.8: FGF-2 effect on PKCδ localization. Micrographs from immunofluorescence staining of cardiac tissue sections for PKCδ. (A), Staining in sections from FGF-treated hearts. (B), Staining in sections from control hearts. Arrows point to areas in cardiomyocyte periphery. Bar = 50 μm.

3.3.8 Examination of PLC-γ1,PLC-δ1 and PLC-ß3 subcellular localization

Studies using cultured cells and cell lines have shown that FGF-2 activates PLC [35-39], although the effect of FGF on PLC activation and localization in cardiomyocytes is not known. Activation of PLC by other effectors produces a translocation of the enzymes to the membrane compartments of the stimulated cell [40]. Stimulation of PLC in the heart is considered to contribute to cardiac growth and hypertrophy [41-44]. In addition PLC activation induces the production of DAG, a potent activator of PKC [45,46] and a possible mediator of ischemic preconditioning [47].

Hearts treated with FGF-2 prior to ischemia were thus examined for any indication that similar effects occurred in adult cardiac myocytes *in situ*. Subcellular distribution of the various PLC isoforms was examined by western blotting of sarcolemmal fraction and by immunolocalization of tissue sections, whenever possible. Representative results from one series of western blot analyses are shown in Fig. 3.9A. Cardiac sarcolemmal and cytosolic samples contained bands recognized specifically by antibodies to the PLC subtypes -γ1, -δ1,-ß3. These bands were at 148 kDa for PLC-γ1, at 87 and 110 kDa for PLC-δ1, and at 110 and 145 kDa for PLC-β3. These bands were eliminated when the antibodies were incubated with their corresponding immunizing peptides (data not shown).

In the case of PLC-β3, administration of FGF-2 induced negligible changes in the levels of the 145 kDa band at the sarcolemma but a significant decrease of this same band in the cytosol. FGF-2 induced a significant increased in the 110 kDa band at the sarcolemma (Fig. 3.9 A and B).

In the case of PLC-δ1, FGF-2 did not induce any changes in the levels of the 87 kDa band at either the cytosol of sarcolemma. FGF-2 induced a significant increase in the levels of the 110 kDa band at the sarcolemma and a significant decrease of the same band in the cytosol.

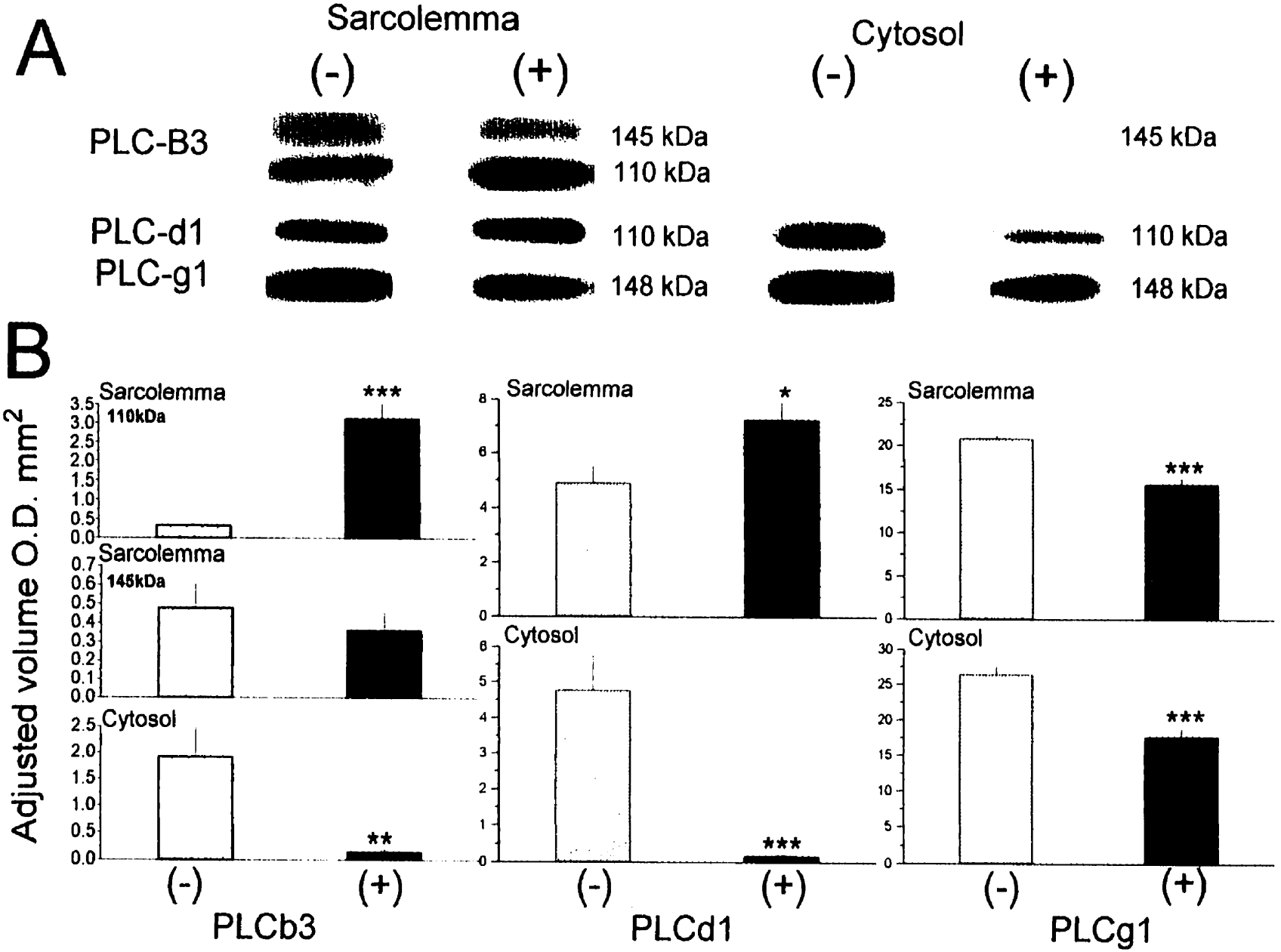
In the case of PLC-γ1, FGF-2 induced a significant decrease in the levels of the 148 kDa band at both sarcolemma and cytosol.

A quantitative estimate of relative sarcolemmal and cytosolic PLC levels is shown in Fig. 3.9B. Measurements represent the average of three separate determinations (Western blot analysis followed by densitometry) from the same pooled samples. The complete experimental series (sarcolemmal preparations from control and FGF-treated hearts, followed by western blotting and densitometry) has been repeated three times with very similar results.

Immunolocalization of PLC-β3 is shown in Figure 3.10. Immunolocalization of PLC-γ1 and PLC-δ1 on cardiac tissue sections did not show any obvious changes in localization patterns between controls and FGF-treated hearts (data not shown). Transverse sections from control (Fig. 3.10a and b) and FGF-2-perfused (Fig. 3.10c and d) hearts were co-stained with anti-PLC-β_3 (Fig. 3.10a and c) and Hoechst nuclear stain (Fig. 3.10b and d). In sections from control hearts (Fig. 3.10a) PLC-β3 immunostaining was observed faintly around pericellular regions of myocytes (arrows). The corresponding nuclear stain of the section depicted in Fig. 10a is shown in Fig. 10b. In FGF-2-perfused hearts (Fig. 3.10c), PLC-β3 immunostaining was increased around myocytes (arrows). The corresponding nuclear stain of this section is depicted in Fig. 3.10d. This increased pericellular staining likely represents the increased association of the 110 kDa and 145

kDa PLC-β3 with sarcolemma which was detected by western blotting

Fig. 3.9: Effect of FGF-2 on PLC levels at sarcolemmal and cytosolic fractions (A): representative micrographs from western blot analysis of cardiac sarcolemmal and cytosolic fractions (20 μg/lane) obtained from control (-) and FGF-2-treated hearts(+) and probed for PLCβ3, PLCδ1, and PLCγ1 as indicated. Immunoreactive bands migrating at: 110 and 145 kDa (PLCβ3); 110 kDa (PLCδ1); and 148 kDa (PLCγ1) are indicated. (B) Quantitative (densitometric) analysis of PLC (PLCβ3, PLCδ1, and PLCγ1 as indicated) subtype levels in cardiac sarcolemmal and cytosolic fractions obtained before and after FGF-2 administration and analyzed by western blotting(n=3).


A
Sarcolemma
Cytosol
(-)
(+)
(-)
(+)
PLC-B3
145 kDa
110 kDa
145 kDa
PLC-d1
110 kDa
110 kDa
PLC-g1
148 kDa
148 kDa
B
Adjusted volume O.D. mm2
Sarcolemma
110kDa
***
Sarcolemma
145kDa
Cytosol
**
Sarcolemma
*
Cytosol
***
Sarcolemma
***
Cytosol
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(-)
(+)
PLCb3
PLCd1
PLCg1

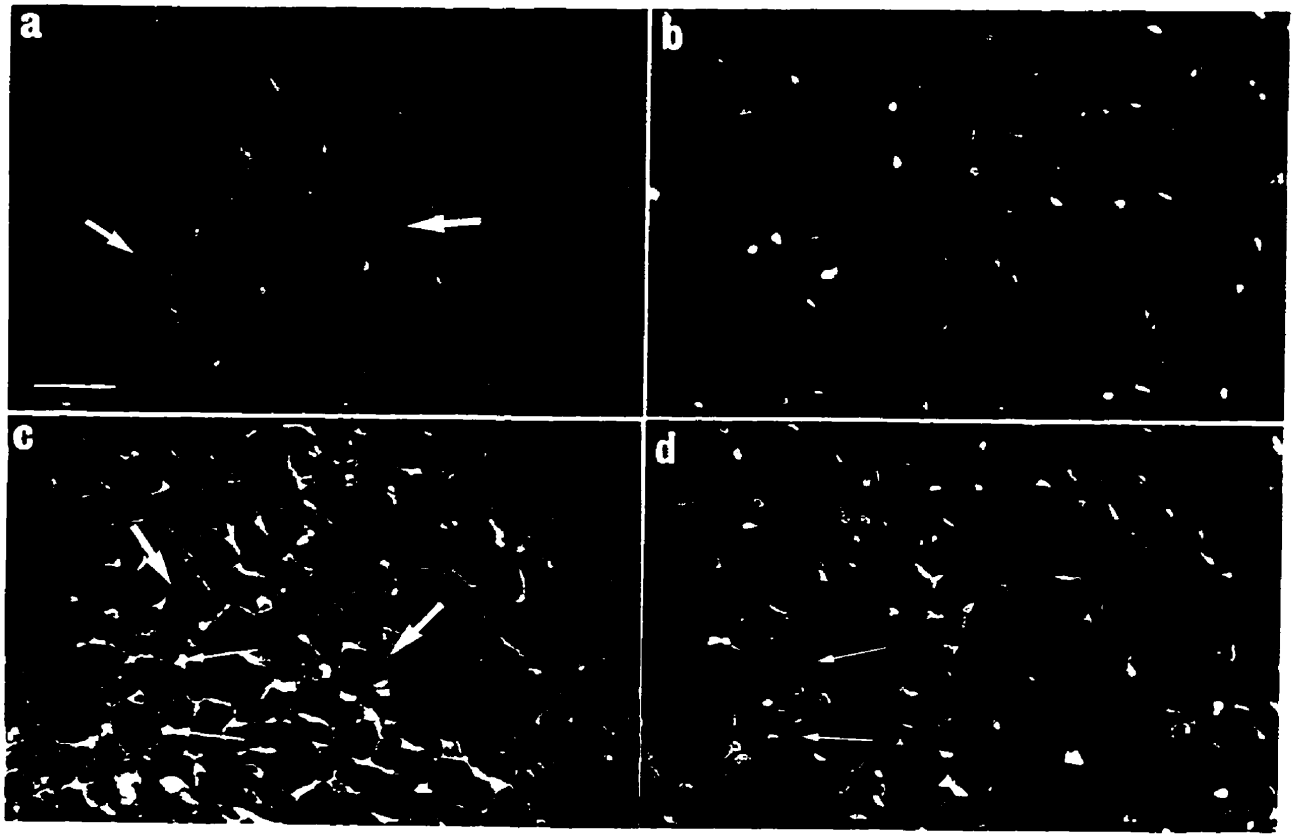


Fig. 3.10: FGF-2 effect on PLCβ3 localization. Micrographs of transverse cardiac sections of hearts perfused with (c,d) or without (a,b) 10 μg of FGF-2. Sections were stained by double fluorescence labeling for PLC-β3 (a and c) and nuclear DNA (b and d). Thick arrows in (a) and (b) indicate normal myocytes double labeled for PLC-β3 and nuclear DNA, respectively. Thick arrows in (c) indicate myocytes perfused with exogenous FGF-2 and displaying enhanced pericellular PLC-β3 staining. Thin arrows in (c) and (d) indicate possible capillaries perfused with exogenous FGF-2 and displaying enhanced PLC-B3 staining. Lack of nuclear staining in these regions (d) argue against the presence fibroblast cells. Bar=50μm.

3.3.9 Examination of MAPK and phosphorylated-MAPK subcellular localization

Studies using cultured cells and cell lines including cardiac myocytes have shown that FGF-2 signaling results in phosphorylation and activation of MAPK [48-51], although the effect of FGF on MAPK localization in cardiomyocytes is not known. In the heart, the predominate MAPK isoforms are the P42 and P44 [50,54]. Hearts treated with FGF-2 prior to ischemia were thus examined for MAPK activation in cardiac myocytes *in situ*. Subcellular distribution of the various MAPK and phosphorylated MAPK isoforms were examined by western blotting of sarcolemmal fraction and by immunolocalization on tissue sections, whenever possible. Representative results from one series of western blot analyses are shown in Fig. 3.11A. Cardiac sarcolemma and cytosolic fractions contained bands recognized specifically by antibodies to the p42 and p44 MAPK and dually phosphorylated (and therefore activated) MAPK p42 and p44.

Administration of FGF-2 resulted in increased levels of the p42 and p44 MAPK and phosphorylated MAPK in association with sarcolemma. In the cytosolic fractions, FGF-2 induced decreased p42 and p44 MAPK but increased dually-phosphorylated p42 and p44 MAPK (Fig. 3.11A). These results indicate that translocation, and activation of both subtypes is taking place. A quantitative estimate of relative sarcolemmal and cytosolic p42 and p44 MAPK and phosphorylated MAPK levels is shown in Fig.10B. Measurements represent a typical determination (Western blot analysis followed by densitometry) from the same pooled samples. The complete experimental series (sarcolemmal and cytosolic preparations from control and FGF-treated hearts, followed by western blotting and densitometry) has been repeated three times with very similar results.

Since MAPK has been reported to be activated downstream of PKC in cardiac myocytes, we examined the effect of the PKC inhibition by chelerythrine on MAPK activation at the sarcolemma under our conditions.

Fig. 3.12 depicts western blot results and their respective densitometer measurements of sarcolemma fractions from perfused hearts treated with: (i) control; (ii) FGF-2; (iii) chelerythrine; and (iv) FGF-2 in the presence of chelerythrine. Blots were probed for phosphorylated-MAPK. As expected, we observed a significant increase in the levels of dually phosphorylated p42 and p44 with FGF-2 treatment. FGF-2 continued to stimulate increases in the levels of phosphorylated MAPK even in the presence of chelerythrine. Chelerythrine alone induced a 2-fold increase in the levels of the phosphorylated p42, which is rather small compared to the 8-fold increase induced by FGF-2 alone or with chelerythrine. Overall, our results suggest that FGF-2-induced activation of MAPK at the sarcolemma may be independent of PKC activation.

Immunolocalization of MAPK and phosphorylated MAPK on cardiac tissue sections did not to show any obvious changes in localization patterns between controls and FGF-treated hearts (data not shown).

Fig. 3.11: Effect of FGF-2 on MAPK and phosphorylated MAPK levels at sarcolemmal and cytosolic fractions (A): representative micrographs from western blot analysis of cardiac sarcolemmal and cytosolic fractions (20 μg/lane) obtained from control (-) and FGF-2-treated hearts(+) and probed for MAPK and dually-phosphorylated MAPK as indicated. Immunoreactive bands migrating at: 42 and 44 kDa are indicated. (B) Quantitative (densitometric) analysis of p42 and p44 MAPK and phosphorylated MAPK levels in cardiac sarcolemmal and cytosolic fractions obtained before and after FGF-2 administration and analyzed by western blotting(n=3).

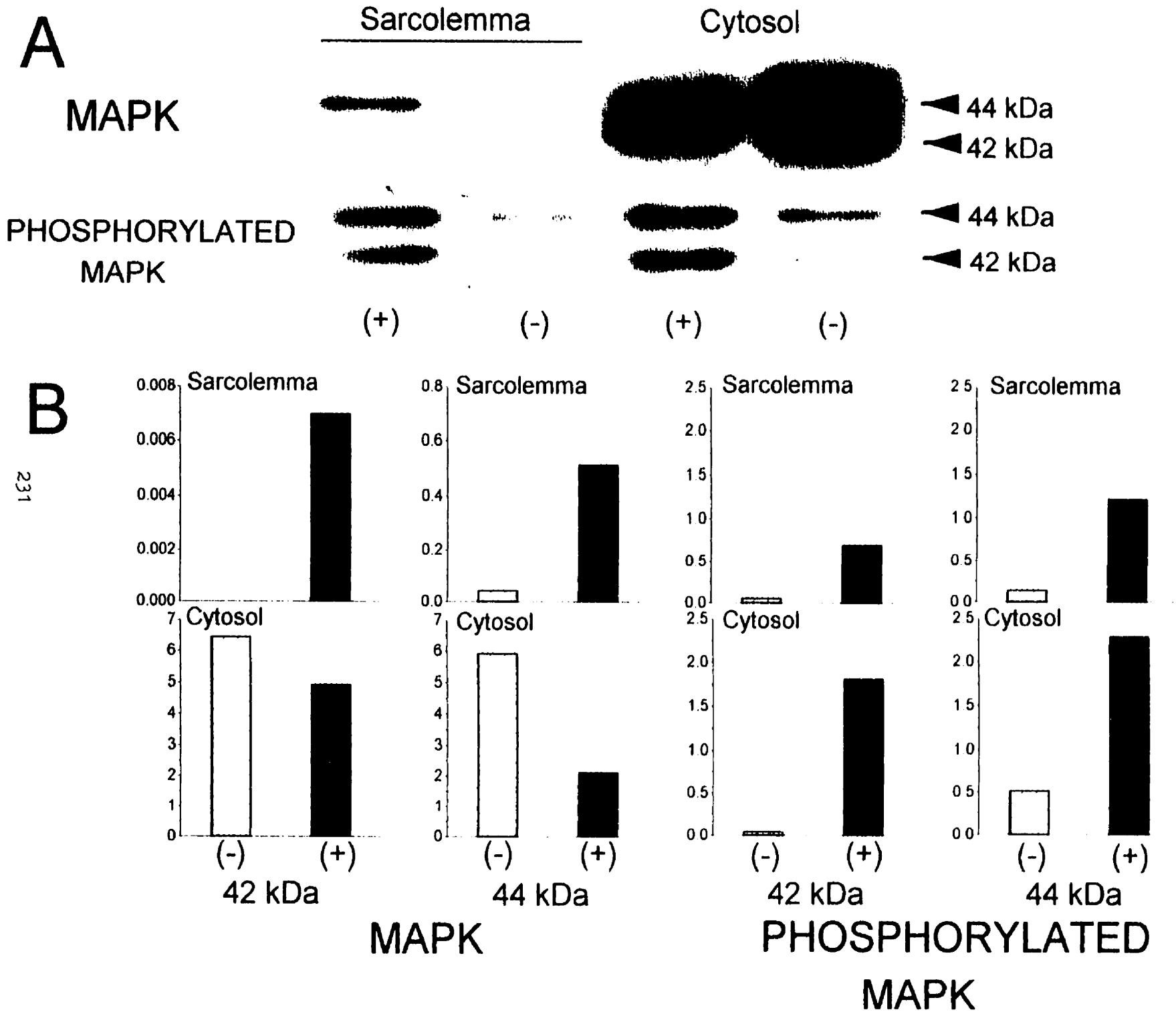
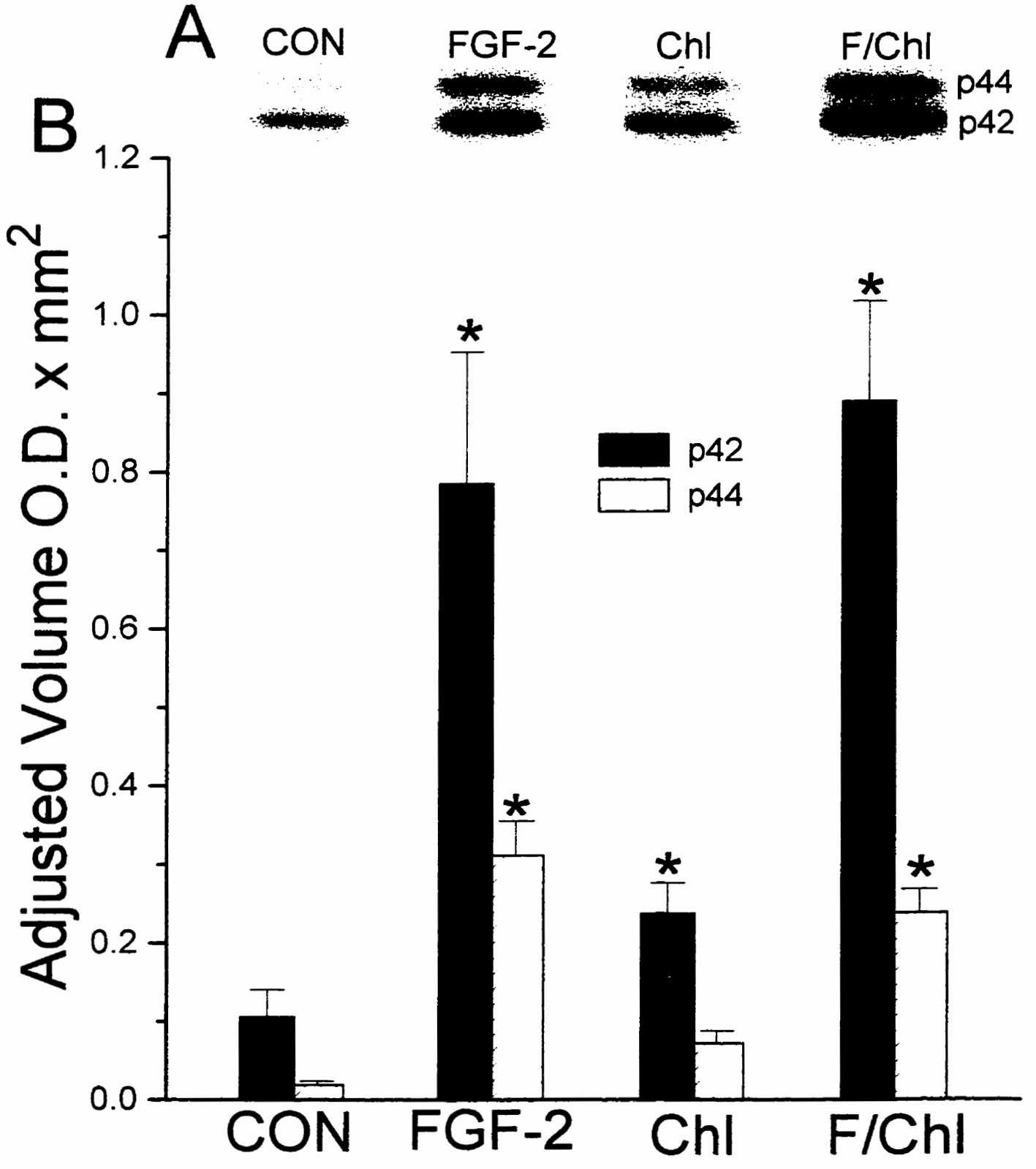

A
Sarcolemma
Cytosol
MAPK
44 kDa
42 kDa
PHOSPHORYLATED MAPK
44 kDa
42 kDa
(+)
(-)
(+)
(-)
B
Sarcolemma
Cytosol
(-)
(+)
42 kDa
44 kDa
MAPK
PHOSPHORYLATED MAPK

Fig. 3.12: Effect of chlerythrine on FGF-2-induced phosphorylation and distribution of MAPK in sarcolemmal fractions (A): representative micrographs from western blot analysis of cardiac sarcolemmal fractions (20 μg/lane) obtained from: (I) control (con); (ii) FGF-2-treated hearts (FGF-2); (iii) chelerythrine (Chl) treated hearts; and (iv) FGF-2-treated hearts in the presence of chelerythrine (F/Chl). These blots were probed for phosphorylated MAPK as indicated. Immunoreactive bands migrating at: 42 and 44 kDa are indicated. (B) Quantitative (densitometric) analysis of p42 and p44 phosphorylated MAPK levels in cardiac sarcolemmal fractions obtained from control, FGF-2 treated, chelerythrine-treated and FGF-2/chelerythrine treated hearts and analyzed by western blotting (n=3). *P< 0.05 significant.


A
CON
FGF-2
Chl
F/Chl
p44
p42
B
Adjusted Volume O.D. x mm2
1.2
1.0
0.8
0.6
0.4
0.2
0.0
p42
p44
*
CON
FGF-2
Chl
F/Chl

3.3.10 Examination of beta adrenergic receptor kinase-1 (βARK1) localization

Subcellular distribution of the βARK1 was examined by western blotting of the cytosolic and sarcolemmal fractions. Representative results from one series of western blot analyses are shown in Fig. 3.13A. Cardiac sarcolemmal samples contained bands recognized specifically by antibodies to the βARK1 and migrating with an apparent molecular mass of 79 kDa. This band was eliminated when the antibodies were incubated with their corresponding immunizing peptides (data not shown). As shown in Fig. 3.12A, administration of FGF-2 resulted in increased levels of βARK1 in association with sarcolemma, indicating translocation and presumably activation. Similar results have been obtained with cultured myocytes in our laboratory. However, anti-βARK1 antibodies did not detect an immunoreactive band in the cytosolic fractions from control and FGF-2-treated hearts (data not shown). A quantitative estimate of relative sarcolemmal βARK1 levels is shown in Fig. 3.13B. Measurements represent the average of three separate determinations (Western blot analysis followed by densitometry) from the same pooled samples.

Immunolocalization of βARK1 on cardiac tissue sections did not show any obvious changes in localization patterns between controls and FGF-treated hearts (data not shown).

3.3.11 Examination of c-jun in sarcolemmal and cytosolic fractions from perfused hearts

Hearts treated with FGF-2 prior to ischemia were examined for changes in c-jun distribution *in situ*, by western blotting of sarcolemmal and cytosolic fractions.

Representative results from one series of western blot analyses are shown in Fig. 3.13A. Cardiac cytosolic fractions contained bands recognized specifically by antibodies to c-jun which migrates with an apparent molecular mass of 39 kDa. In Fig. 3.13A, administration of FGF-2 resulted in increased levels of c-jun in association with cytosol.

A quantitative estimate of relative cytosolic c-jun levels is shown in Fig. 3.13B. Measurements represent the average of three separate determinations (Western blot analysis followed by densitometry) from the same pooled samples. The complete experimental series (cytosolic preparations from control and FGF-treated hearts, followed by western blotting and densitometry) has been repeated three times with very similar results.

3.3.12 Examination of src-kinase in sarcolemmal and cytosolic fractions from perfused hearts

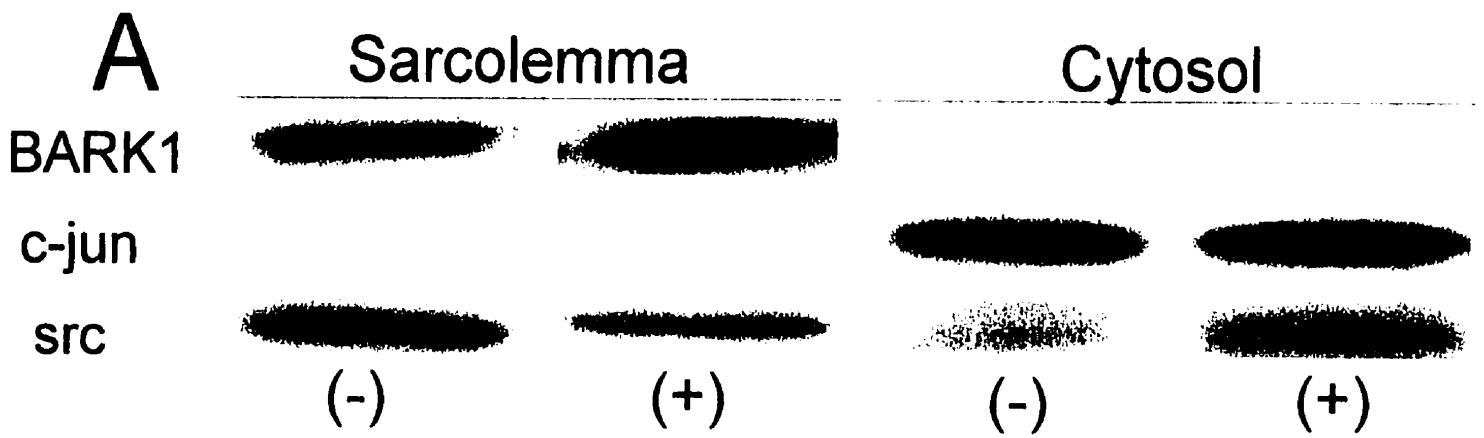
Activation of c-src by several effectors induces translocation [58,59] and autophosphorylation [60,61] of this enzyme to the membrane compartments of the stimulated cells. Subcellular distribution of src was examined by western blotting of the sarcolemmal and cytosolic fraction and by immunolocalization on tissue sections, whenever possible. Representative results from one series of western blot analysis is shown in Fig. 3.13A. Cardiac sarcolemma and cytosol were found to contain bands recognized specifically by antibodies to c-src. These bands were shown to migrate with an apparent molecular mass of 60 kDa. When anti-c-src antibodies were incubated with their corresponding immunizing peptides (data not shown), these 60 kDa bands were eliminated. Administration of FGF-2 resulted in decreased levels of src in association with

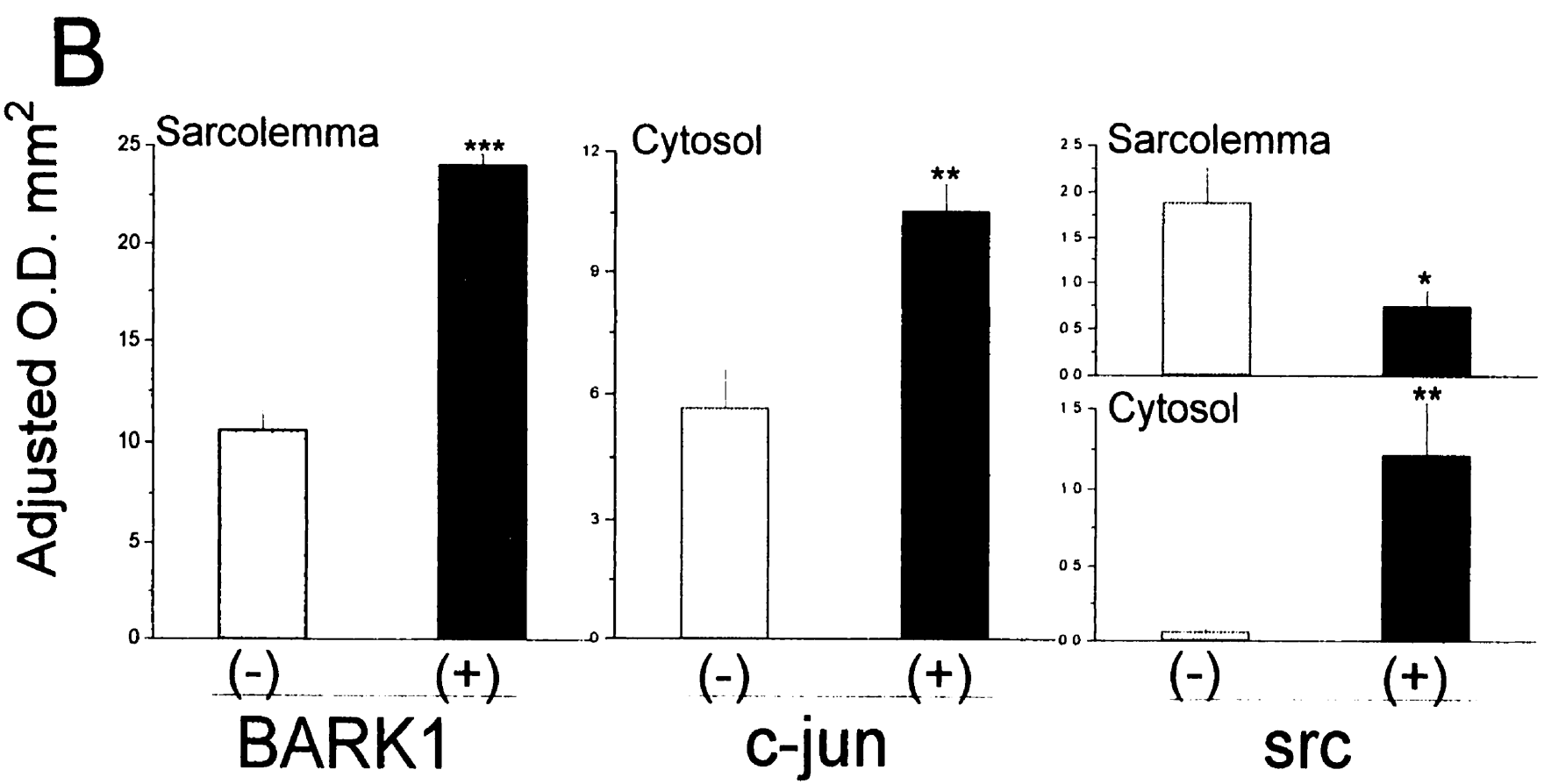
sarcolemma and increased src levels in cytosol. This src pattern of distribution would indicate that translocation to other subcellular fractions, degradation or down regulation of src at the sarcolemma is taking place with FGF-2 stimulation, at least in the time point examined.

A quantitative estimate of relative sarcolemmal and cytosol src levels is shown in Fig. 3.13B. Measurements represent the average of three separate determinations (Western blot analysis followed by densitometry) from the same pooled samples. The complete experimental series (sarcolemmal preparations from control and FGF-treated hearts, followed by western blotting and densitometry) has been repeated three times with very similar results.

Immunolocalization of src on cardiac tissue sections did not to show any obvious changes in localization patterns between controls and FGF-treated hearts (data not shown).

Fig. 3.13: Effect of FGF-2 on βARK1, c-jun and src levels at sarcolemmal fractions (A): representative micrographs from western blot analysis of cardiac sarcolemmal fractions (20 μg/lane) obtained from control (-) and FGF-2-treated (+) hearts and probed for βARK1, c-jun and src as indicated. Immunoreactive bands migrating at 79 kDa (βARK1), 39 kDa (c-jun), 60 kDa (src) are indicted. (B) Quantitative (densitometric) analysis of βARK1, c-jun and src levels in cardiac sarcolemmal and cytosolic fractions obtained before and after FGF-2 administration and analyzed by western blotting(n=3).


A
Sarcolemma
Cytosol
BARK1
c-jun
src
(-)
(+)
(-)
(+)


B
Adjusted O.D. mm2
Sarcolemma
***
Cytosol
**
Sarcolemma
*
Cytosol
**
(-)
(+)
(-)
(+)
(-)
(+)
BARK1
c-jun
src

3.3.13 c-fos

Activation of c-fos by other effectors, including PKC activation, induces nuclear translocation and increased c-fos expression in the stimulated cell, including the cardiomyocytes [55-57]. Hearts treated with FGF-2 prior to ischemia were thus examined for any indication of increased nuclear c-fos proteins in adult cardiac myocytes *in situ*. Representative results from one series of immunolocalization experiments is shown in Fig. 3.14.

Figure 3.14 shows transverse cardiac sections from control and FGF-2 perfused hearts double labeled with anti-c-fos (Fig. 3.14a and c), and Hoechst nuclear stain (Fig. 3.14b and d). Large arrows (Fig. 3.14c) point to the increased anti-c-fos myocyte nuclear stain in FGF-2-treated hearts. The large arrows in Fig. 3.14a point to regions in control perfused tissues where little to no anti-c-fos myocyte nuclear staining is observed. Further more, separate sections from FGF-2 treated hearts were stained only with the c-fos antibody (results not shown). Again, increased c-fos nuclear staining was seen in FGF-2-treated cardiomyocytes indicating that the anti-c-fos nuclear staining was specific and not due to carry-over of the Hoechst nuclear stain.

In addition, increased nuclear c-fos labeling was observed in nuclei of cells within coronary vessels of FGF-2-perfused hearts. In Fig 3.15c, large arrows indicate nuclear regions of a coronary vessel which exhibited increased c-fos staining compared to nuclear regions in coronary vessels of control perfused hearts (Fig. 3.15a, large arrows). Fig. 3.15d and b depicts the same section seen in Fig. 3.15c and a, respectively, double

immunolabelled with Hoescht nuclear stain to show that the c-fos staining was indeed nuclear in origin along the vessel walls.

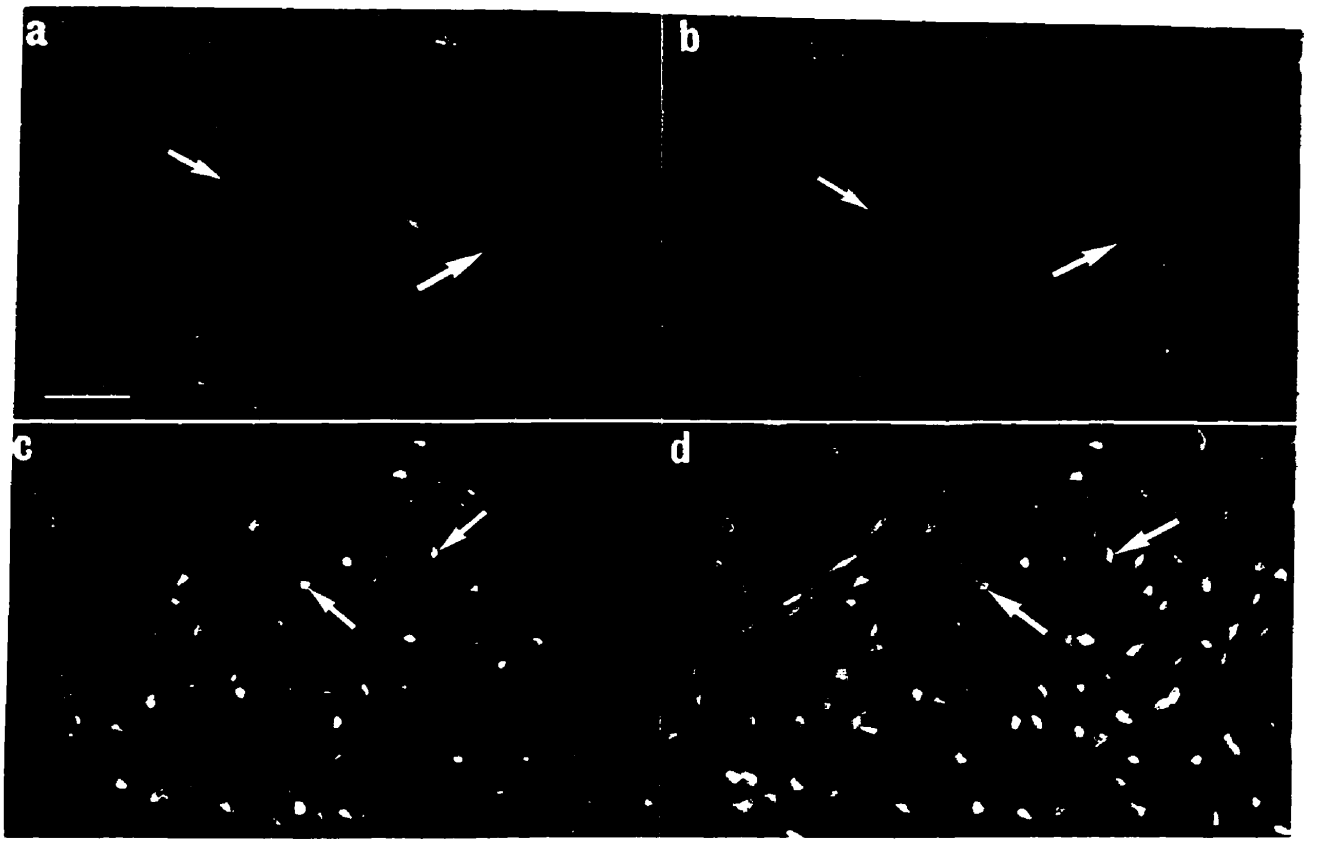


Fig. 3.14: Immunolocalization of c-fos in myocytes of transverse tissue sections from isolated perfused hearts. Micrographs of transverse sections of hearts perfused with (c,d) or without (a,b) FGF-2. These sections were stained by double immunofluorescence labeling for c-fos (a and c) and nuclear DNA (b and d). Arrows in (a) and (b) indicate nuclei of normal myocytes double labeled for c-fos and nuclear DNA, respectively. Arrows in (c) and (d) indicate nuclei of myocytes treated with exogenous FGF-2 and displaying enhanced c-fos staining. (bar = 50 μm).

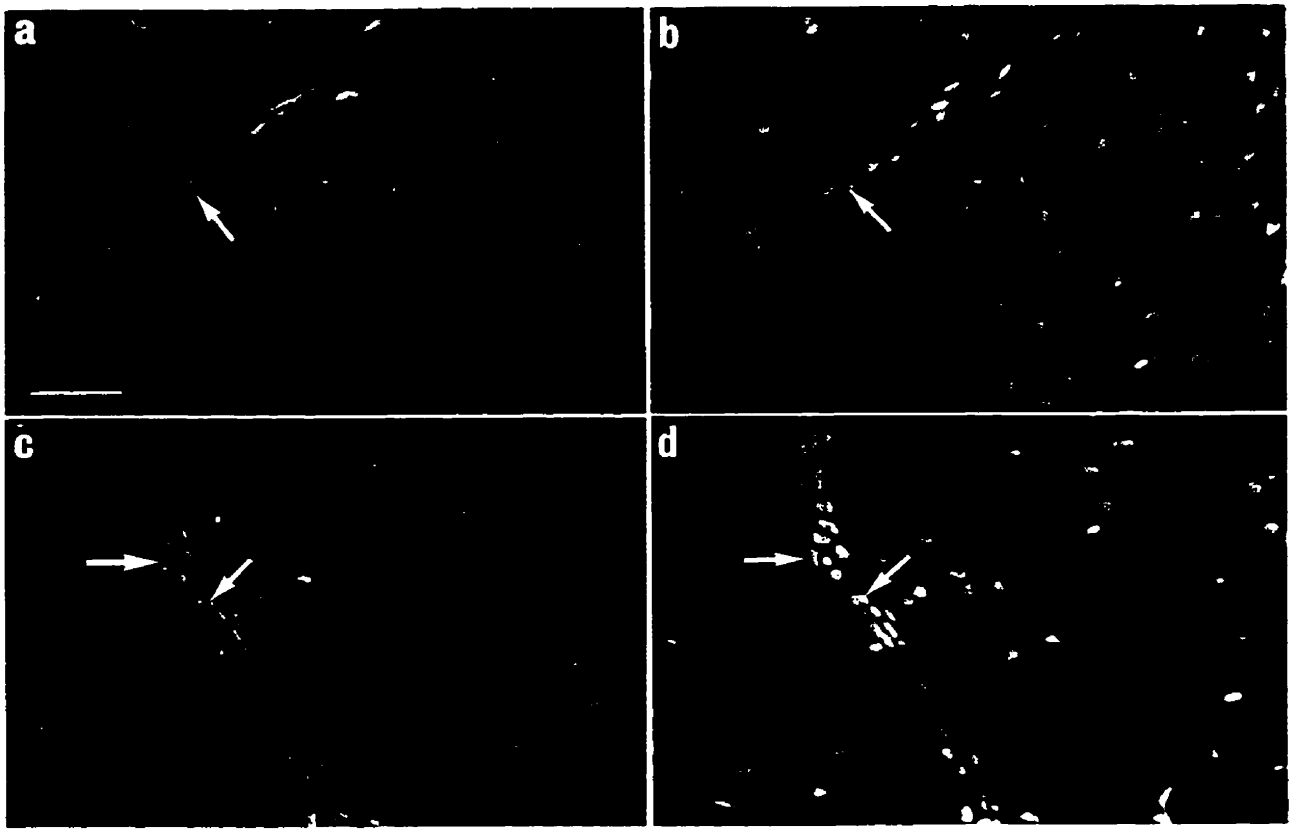


Fig. 3.15: Immunolocalization of c-fos in coronary vessels of transverse tissue sections from isolated perfused hearts. Micrographs of transverse sections of hearts perfused with (c,d) or without (a,b) FGF-2. These sections were stained by double-immunofluorescence labeling for c-fos (a and c) and nuclear DNA (b and d). Arrows in (a) and (b) indicate nuclei from normal coronary vessel cells double labeled for c-fos and nuclear DNA, respectively. Arrows in (c) and (d) indicate nuclei of coronary vessel cells treated with exogenous FGF-2 and displaying enhanced c-fos staining. (bar = 20 μm).

3.3.14 Effect of FGF-2 administration during reperfusion on the localization of c-fos and PLC-β3

Fig. 3.16 depicts typical transverse cardiac sections taken from isolated rat hearts treated with (Fig. 3.16 c and d) or without (Fig. 3.16a and b) FGF-2 during reperfusion, after ischemia. These sections were double immunostained with anti-c-fos (Fig. 3.16 a and c) and Hoechst nuclear stain (Fig. 3.16b and d). Arrows in Fig. 3.16a show endogenous c-fos immunostaining diffusely in myocyte nuclei. Arrows in Fig. 3.16b confirm that c-fos immunostaining in the myocytes depicted in Fig. 3.16a are indeed within nuclear regions. Nuclear anti-c-fos staining is brighter in FGF-2-treated compared to intreated hearts (compare Fig. 3.16c to Fig. 3.16a, respectively).

When coronary vessels were examined for c-fos immunostaining (Fig. 3.17), no c-fos nuclear staining was observed (arrows) in tissue sections of control-treated reperfused hearts (Fig. 3.17a). In contrast, in FGF-2-reperfused hearts (Fig. 3.17c) arrows show increased c-fos immunostaining along nuclear regions of the coronary vessel, as confirmed by nuclear double immunolabelling of this same section (Fig. 3.17d, arrows).

Fig. 3.18 depicts typical transverse cardiac sections taken from FGF-2-treated (Fig. 3.18c and d) or control (Fig. 3.18a and b) rat hearts after I-R. These sections were double immunostained with anti-PLC-β_3 (Fig. 3.18 a and c) and Hoechst nuclear stain (Fig. 3.18c and d). Increased PLC-β_3 immunostaining is detected around myocytes in FGF-2-treated hearts. In contrast, only faint PLC-β_3 immunostaining is seen around myocytes of control reperfused hearts. Our data indicate that FGF-2 induced translocation of PLC-β3 from the cytosol to the sarcolemma of cardiomyocytes during reperfusion.

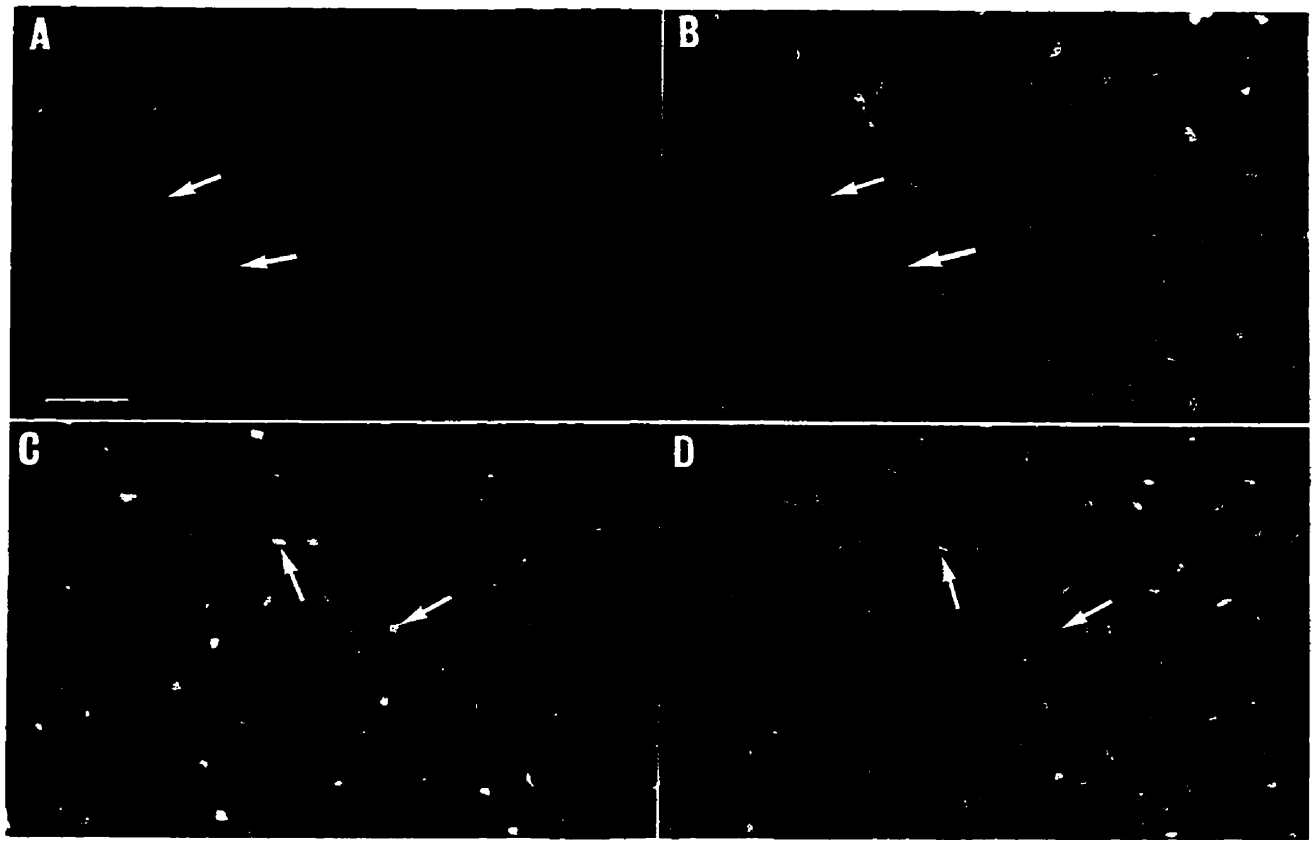


Fig. 3.16: Immunolocalization of c-fos in myocytes after I-R. Micrographs of transverse sections of hearts subjected to I-R with (C,D) or without (A,B) FGF-2 in the reperfusion medium. These sections were stained by double-immunofluorescence labeling for c-fos (A and B) and nuclear DNA (C and D). Arrows in (A) and (B) indicate nuclei of normal myocytes double labeled for c-fos and nuclear DNA, respectively. Arrows in (C) and (D) indicate nuclei of myocytes treated with exogenous FGF-2 and displaying enhanced c-fos staining. (bar = 50 μm).

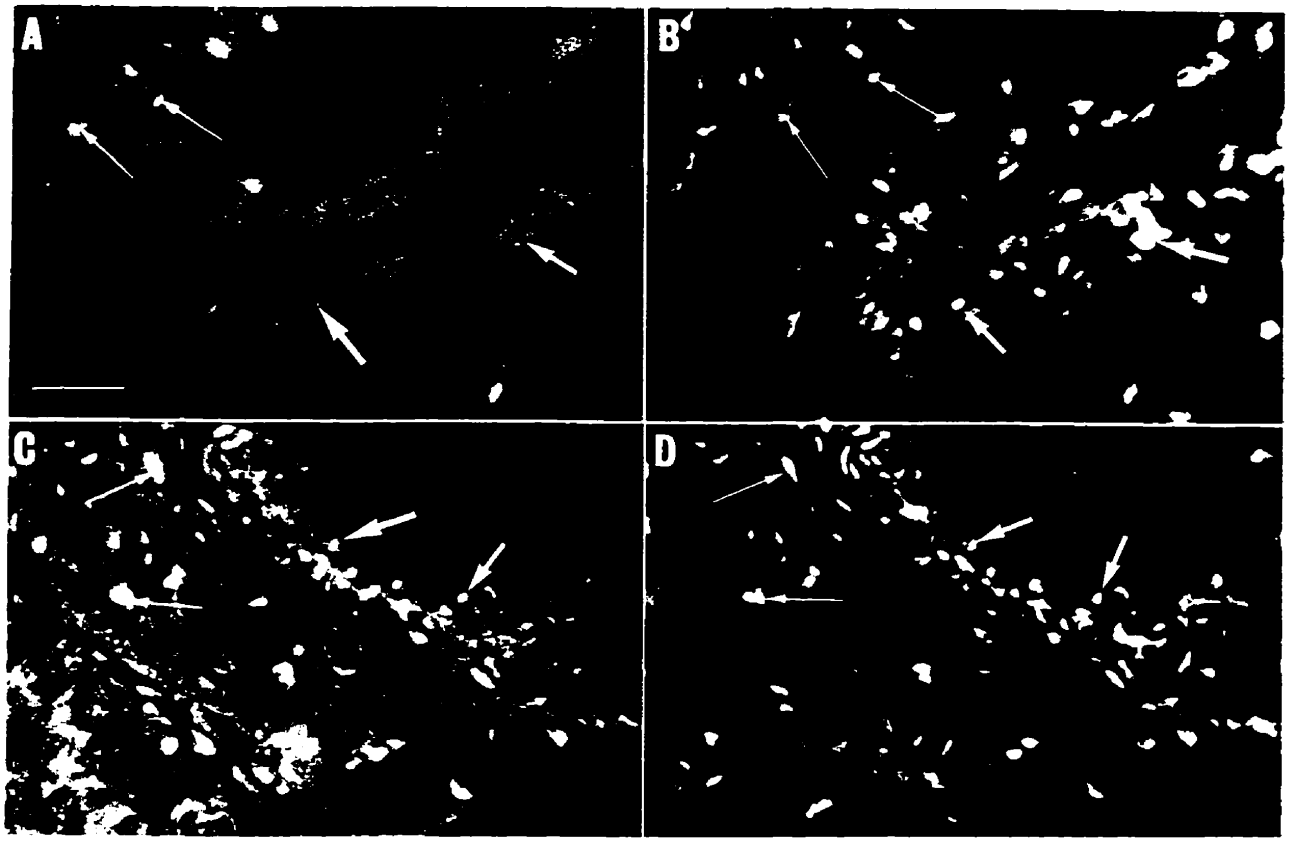


Fig. 3.17: Immunolocalization of c-fos in coronary vessels after I-R. Micrographs of transverse sections of hearts subjected I-R with (C,D) or without (A,B) FGF-2 in the reperfusion medium These sections were stained by double-immunofluorescence labeling for c-fos (A and C) and nuclear DNA (B and D). Arrows in (A) and (B) indicate nuclei from normal coronary vessel cells double labeled for c-fos and nuclear DNA, respectively. Arrows in (C) and (D) indicate nuclei of coronary vessel cells treated with exogenous FGF-2 and displaying enhanced c-fos staining. (bar = 50 μm).

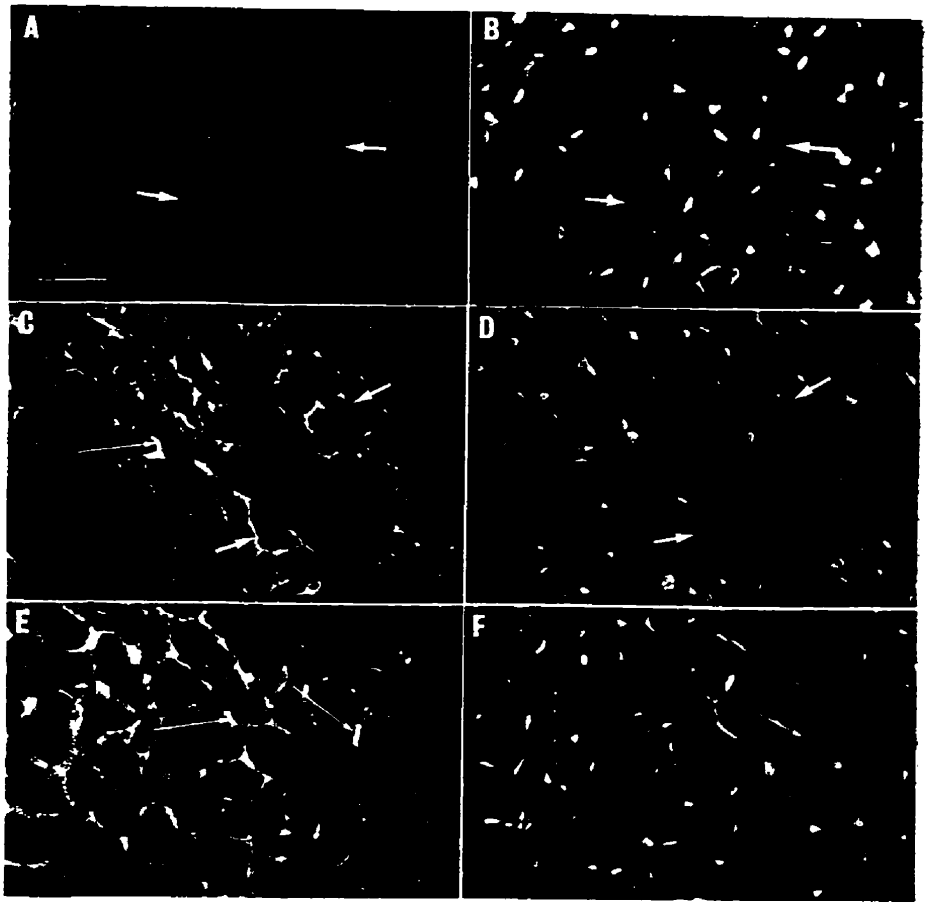


Fig. 3.18: Immunolocalization of PLC-β3 in myocytes of transverse tissue sections from isolated perfused hearts after I-R. Micrographs of transverse sections of hearts perfused with (C-F) or without (A,B) FGF-2. Sections were stained by double-immunofluorescence labeling for PLC-β3 (A,C,E) and nuclear DNA (B,D,F). Thick arrows in (A) and (B) indicate normal myocytes double labeled for PLC-β3 and nuclear DNA, respectively. Thick arrows in (C) and (D) indicate myocytes perfused with exogenous FGF-2 and displaying enhanced pericellular PLC-β3 staining. Thin arrows in (C,D) and (E,F) indicate capillaries perfused with exogenous FGF-2 and displaying enhanced PLC-B3 staining.(bar = 50 μm).

3.4 DISCUSSION

In this chapter, the mechanism by which FGF-2 may induce cardioprotection against I-R injury is explored by examining signal transduction pathways which are triggered when this factor is administered before or after ischemia.

3.4.1 Distribution of exogenous FGF-2 and in perfused hearts

Initially, to gain some insight as to potential direct targets of exogenous FGF-2 in the heart we investigated its localization immediately after perfusion of hearts for 10 min. with FGF-2. This was achieved by immunofluorescence localization performed using antibodies specific for FGF-2. As well, tissue extraction, immunoblotting, and densitometry was performed to determine levels of FGF-2 associated with control and FGF-2 perfused hearts.

We found that FGF-2 administration to perfused hearts, resulted in at least a four fold increase in extracted FGF-2 compared to controls [1]. This increase in FGF-2 content persisted even after ischemia and reperfusion, indicating that there is a strong association between FGF-2 and cardiac tissue. This observation is in agreement with a report which showed that administration of this factor intravenously results in a rapid deposition of FGF-2 within organs, including the heart [6].

In addition, previous research has shown that FGF-2 is bound to heparan-sulfate proteoglycans present in the matrix, basement membranes, and plasma membranes of most cell types [7,8]. Similarly, this pattern of FGF-2 distribution was seen in our immunolocalization experiments. In these experiments, increased amounts of FGF-2 was found associated with large and small blood vessels, around capillaries and around

cardiomyocytes near these vessels, in a basement membrane-like distribution [1]. High dilutions of the anti-FGF-2 antibodies were used in our immunolocalization experiments to minimize the contribution of endogenous anti-FGF-2 labeling. Thus, control hearts did not display any staining above background [1]. It is presumed that the increased levels of FGF-2 reflect retention of the exogenous FGF-2 protein. It is theoretically possible that exogenous FGF might have induced synthesis and/or release of endogenous FGF-2. Both possibilities are considered unlikely. Increases in heart-associated FGF-2 were observed within 10 min from starting the infusion, arguing against *de novo* synthesis. Stored FGF-2 furthermore would have become accessible to detection following the exhaustive tissue extraction procedures established in our laboratory [1,2,3]. Entry of FGF-2 into the myocardium, occurred, presumably, via the capillaries. This is likely the result of increased permeability of the cardiac capillary bed compared to that of other vascular beds such as that of the brain [4], although it may also represent a direct effect of FGF-2 on permeability of cellular junctions. In our experiments, the fact that we see increased amounts of FGF-2 around cardiomyocytes proximal to capillaries and blood vessels supports this conclusion.

Of particular interest in our immunolocalization studies is the observation that administered FGF-2 may have some direct affect on the coronary vasculature. Since FGF-2 has been shown to induce vasodilation in arteries of rabbits [37] and coronary arteries of canines [37,5], the possibility exists that in our model, the exogenous FGF-2 localized on the coronary vasculature may similarly be inducing vasodilation. This vasodilatory effect may, in turn, lead to the cardioprotective effect we see with FGF-2 against I-R injury in a

way similar to SNP [36]. However, as previously mentioned, only a very mild decrease in coronary perfusion pressure was observed with FGF-2 administration suggesting that the vasodilatory effect by FGF-2 may play a minor role in cardioprotection. Another possibility could be that the localized FGF-2 may be acting to protect the coronary vasculature from I-R injury, thus preserving coronary vasculature function.

3.4.2 Localization of phosphorylated tyrosine residues after FGF-2 administration in isolated perfused hearts

The localization around cardiomyocytes suggests that administered FGF-2 may have direct effects on these cells such as protecting them against I-R injury, probably via a receptor mediated event. This notion is quite plausible in light of recent research in our laboratory showing the presence of high-affinity FGF-2 tyrosine kinase receptors in adult cardiomyocytes and the ability of FGF-2 [9] to protect cultured cardiomyocytes from H_2O_2 damage [12]. When FGF-2 binds to its high affinity tyrosine kinase receptor, a tyrosine phosphorylation cascade is induced leading to the biological response(s) [10]. Thus we investigated whether adult cardiac myocytes express functionally coupled FGF recptors [9] and if so, whether these receptors can be activated by FGF-2 in situ. We have demonstrated that FGF-2 can indeed stimulate the tyrosine kinase receptor in adult myocytes in culture [9]. To examine potential activation in situ, we looked and found increased protein tyrosine phosphorylations in cardiac myocytes in sections from control and FGF-2 treated hearts, using immunofluorescence with specific antibodies. Regions of the heart which displayed increased immunostaining for FGF-2 also had increased anti-phosphotyrosine staining consistent with a cause and effect relationship. Increased

phosphotyrosine staining was clearly associated with the intercalated discs region of myocytes. This region is significant since no other cell types are present, thus demonstrating the in situ stimulation of tyrosine phosphorylation by FGF-2 in cardiac myocytes. Our findings are consistent with the notion that adult cardiomyocytes possess high affinity tyrosine FGF-2 receptors capable of triggering a phosphorylation cascade that affects amongst other things areas of cellular contact. Some of these phosphorylation targets may include: cytoskeletal protein vinculin [12]; and connexin-43 (gap junction protein) in the intercalated disc area [11]. Indeed, Doble et al. [14] demonstrated in cultured neonatal rat cardiomyocytes that exogenously administered FGF-2 can induce phosphorylation of the serine residues on Cx43 and alter the metabolic coupling between cardiomyocytes.

One significant observation from these immunolocalization studies was the fact that blood vessels (which do accumulate FGF-2) did not stain for phosphotyrosine residues. One possibility for this phenomenon is that FGF-2 may not activate a tyrosine-kinase cascade in these cells under the conditions of these experiment. Alternatively sites containing the phosphotyrosine-containing amino acids may be masked from antibody-antigen interaction. One final possibility for lack of immunostaining of phosphotyrosine residues in blood vessels is that tyrosine phosphatases may also become activated by FGF-2 administration [11]. The following experiments lends support for this notion.

We looked for changes in the pattern of tyrosine phosphorylation, as assessed by western blotting, when FGF-2 is administered by direct injection to the apical region of the heart. Results showed that injection of FGF-2 into the myocardium resulted in increases in

the level of tyrosine phosphorylation of several proteins, compared to control hearts injected with buffer alone. Decreased tyrosine phosphorylation after FGF-2 treatment, however, was seen in proteins of about 123 and 66 kDa, which appeared to have higher phosphotyrosine content in the controls. Two interpretations are possible for these findings. Either FGF-2 stimulated a tyrosine phosphatase or reduced levels of the tyrosine-phosphorylated 66 and 123 kDa resulted from FGF-induced degradation. Densitometry of the immunoblot of FGF-2 treated hearts showed that the net anti-phosphotyrosine immunoreactivity was increased. This net increase suggests that the immunoreactive bands seen after FGF-2 treatment cannot be accounted for by degradation of immunoreactive bands of control tissue [12]. Furthermore, no significant difference in protein band composition and intensity was observed between control and FGF-2-treated heart lysates as revealed by protein staining of duplicate gels This lack of differences would indicate that no major changes in proteolysis levels occurred as a function of FGF-2 treatment [12]. The decrease in tyrosine phosphorylation of certain proteins is more in agreement with the activation of a phosphotyrosine phosphatase with FGF-2 treatment [12]. Indeed, an abundant phosphotyrosine phosphatase has been identified in the heart; this phosphatase is a target for protein tyrosine kinases such as growth factor receptors [11].

It must be noted that the tissue extracts examined contain both muscle and non-muscle cell proteins. Thus changes in phosphotyrosine phosphorylation observed by immunoblotting likely reflects the response of a mixed cell population. However, since approximately 80% of cardiac mass represents cardiomyocytes it is very likely that cardiomyocytes make a major contribution to this phosphotyrosine phosphorylation

phenomenon. Thus, FGF-2 elicits a complex, receptor-mediated response in the myocardium, which may be aimed at maintaining cardiac phosphotyrosine homeostasis [12]. Finally, it should be noted that FGF administration may also have indirect effects on cardiomyocytes by inducing for instance release of other factors which are acting on these cells. Studies in our laboratory however have shown that activation of signaling cascades in cardiac myocytes by FGF-2 in vitro is very similar to those seen in the perfused heart [113] and [12].

3.4.3 FGF-2 and cardiac PLC

Similarly to other tyrosine kinase receptors, activated FGFR has been linked to the stimulation of the ras-raf and MAPK pathways, a src-kinase pathway, and the phosphoinositide hydrolysis pathway via the binding and phosphorylation of PLC-γ1 [15,16,112]. In addition there are reports linking FGF-2 to activation of pertussis-sensitive G protein(s) via unknown mechanism(s) [71,108]. We therefore investigated activation of PLC and the phosphoinositide pathway by FGF-2 in *in situ*.

Phosphoinositide-specific PLC is an important signal-transducing enzyme which generates inositol triphosphate and diacylglycerol [13,22]. In the heart, PLC is involved in the inotropic and hypertrophic responses of the myocardium; it has also been linked to preconditioning [17-19,22]. Our enzymatic activity assays showed that FGF-2 treatment activated PLC and thus the phosphoinositide pathway at both sarcolemmal and cytosolic sites. This assay however does not provide information as to which PLC isoform (s) is responsible for the activity measured. As mentioned previously, there exist three groups of PLC isoforms; PLC-β and PLC-δ are coupled to receptor stimulation via G-protein

signaling, while PLC-γ binds directly to tyrosine kinase receptors such as FGFR [15,16,20-22,112]. FGF-2 signaling would be expected to stimulate primarily the PLC-γ group; because however there are reports of unknown G-proteins coupled to FGF-2 receptors, the other groups of PLCs are also potential candidates for stimulation.

We cannot at this point attribute the PLC activity measured to a specific PLC isoform. Predominant PLC isoforms identified in the adult rat heart are PLC-γ1, PLC-β3 and PLC-δ1 [28,106-108,23,111]. We have conducted an analysis of subcellular distribution of the various PLCs to obtain an indication as to how localization of molecules may be affected by FGF-2 signaling. It should be noted that, unlike the consensus for PKC isozymes [24], there is no straightforward relationship between PLC association with the particulate fraction and increased activation. Rather, changes in localization can be taken to signify an effect of FGF-2 signaling on subcellular distribution of these enzymes.

To study localization of the PLC isoforms we used commercially available antibodies. Specificity of these antibodies was ascertained by absorption with their immunizing peptides. The anti-PLC-γ1 recognized, at both cytosolic and sarcolemmal fractions, a band at approximately 148 kDa specifically, consistent with the known size of this enzyme [22]. The anti-PLC-β3 antibodies recognized specifically a band of approximately 145 kDa at both sarcolemmal and cytosolic sites; in addition they recognized specifically a 110 kDa band at the sarcolemma. This band likely represents a degradation product of PLC-β3 since PLC-β3 contains PEST sequences and has been shown to be susceptible to proteolysis by calpain [20,21]. Indeed, a 100 kDa

enzymatically active PLC-β3 has been reported, produced by calpain degradation of the larger form [20,21]. This truncated PLC-β3 was very susceptible to activation by the βγ-subunit of G-proteins [20,21]. Finally, the anti-PLC-δ1 antibodies recognized specifically an 87 and a 110 -kDa band at either sarcolemmal or cytosolic sites. It is considered that the 87 kDa band represent the conventional form [23], while the 110 kDa band likely represents a PLC-δ1-like enzyme.

Our data from Western blotting analysis of PLC isoform distribution demonstrated that FGF-2 induced changes in localization of all three types of PLCs. Levels of PLC-γ1 decreased at both cytosolic and sarcolemmal sites, suggesting translocation to another cellular site(s) or possibly degradation. Levels of the 145 kDa PLC-β3 decreased in the cytosolic compartment upon FGF-2 administration; this was accompanied by a pronounced increase in the 110 kDa form at the sarcolemma, while the 145 kDa band remained unchanged at the same site. The increased sarcolemma-associated anti-PLC-β3 staining seen by immunofluorescence after FGF-2 administration likely represents changes in the 110 kDa immunoreactive form and possibly activation of the latter. Finally, no changes were seen in the distribution of the 87 kDa immunoreactive PLC-δ1. The 110 kDa PLC-δ1-like band however appeared to translocate from the cytosol to the membrane upon FGF-2 treatment.

Our data point to some correlation between increased PLC activity at the sarcolemma and increased levels of PLC-β3 and/or PLC-δ1-like enzymes at that site, suggesting that FGF-2 may have stimulated the activity of these G-linked enzymes. Certainly, increased association of these PLCs with sarcolemmal membranes may reflect

coupling to the activating receptor and/or increased association with substrate. On the other hand, no correlation between activity and concentration could be seen in the cytosol, where enzymatic activity was elevated while levels of all three isoforms appeared to decrease compared to controls. It is possible that as yet unidentified or undetected PLC isoforms may have contributed to the cytosolic PLC activity; each major PLC isoform exists in several related variants (PLC-β1-3, PLC-γ1-2, PLC-δ1-4), not all of which have been examined here. A further complicating factor arises from the fact that measurements of enzymatic activity represent an expression not necessarily of local concentration but of local activation (such as the state of phosphorylation) of the particular enzyme.

To clearly demonstrate, therefore, that FGF-2 stimulates activity of the specific PLC isoforms it would be essential to examine activation of the individual isoforms. This was achieved in collaborative studies with Drs. P.Tappia and V.Panagia (Inst.Cardiov.Sciences, SBGH Res.Cntr).Although these experiments are not part of the present document (manuscript in preparation), it is important to summarize relevant information: Using immunoprecipitation with commercially available antibodies to PLC-γ1 and PLC-δ1 (there are no immunoprecipitation-competent antibodies to PLC-β3) it was found that FGF-2 clearly stimulated the activity of both PLC variants when added to fresh cultures of adult cardiomyocytes; FGF-2 also stimulated redistribution of all three PLC isoforms between cytosolic and particulate compartments, in a manner similar to our observations from the perfused hearts. These experiments demonstrate for the first time that FGF-2 is capable of G-linked signaling in cardiomyocytes.

Based on these observations we suggest that the redistribution of PLCs induced by FGF-2 in the perfused heart is linked to the activation of PLC-γ1, PLC-δ1 and possibly PLC-β3. Confirmation for our claim however awaits determination of the specific activities of the individual PLCs in the *in situ* setting. Changes in localization of all three isoforms in myocytes in culture and *in situ* demonstrate that they are direct or indirect targets of FGF-2 signaling.

3.4.4 Examination of PKC activity and subcellular localization of PKC isoforms -α, -δ, -ε and -ζ with FGF-2 administration (in the presence of chelerythrine) from FGF-2 perfused and controlled hearts

Activation of PKC is closely linked to its translocation to the membrane [24]. We therefore examined whether administration of FGF-2 to the heart causes any changes in PKC distribution and whether these changes are linked to the cardioprotective effects exerted by FGF-2. Certain variables were taken into account for these determinations. First, like PLC, PKC consists of a group of several isozymes. Thus, western blotting was employed to examine individual isoforms. Our studies extended to PKC subtypes -α, -δ, -ε and ζ, which have been consistently identified in rat hearts [24,25]. We did not examine PKC-ß, which has also been detected in the rat heart [26,98], since levels of anti-PKC-ß immunoreactivity at the sarcolemma fraction either in control or FGF-2 treated hearts were marginally above background (data not shown); a recent report localizes PKC-β to the perinuclear region [97]. Since there may still exist unidentified PKC subtypes, total PKC enzymatic activity was also determined as an additional measure of change. Second, like in our PLC studies, purified sarcolemmal membrane preparations were used

throughout, in order to examine the link to a particular cell type and a particular cellular compartment such as the cardiomyocyte plasma membrane. Although cardiac myocytes represent about 80% of cardiac mass and would be expected to be the major contributors in the total heart fractions, several other cell types (fibroblasts, endothelial, smooth muscle, neuronal cells , macrophages) which are likely responsive to FGF-2 in some fashion are present in the heart. Most importantly, crude cardiac membrane preparations do not differentiate between plasma membrane and other membrane compartments. It is not known whether PKC translocation/association involves all membrane compartments of a cell nor whether different cell types display the same PKC response to an agonist. In view of the distinct patterns of subcellular localization reported for various PKC isoforms [97,98], one might expect differences related to specific membrane targeting. The sarcolemmal preparations used for this investigation are highly enriched in myocyte sarcolemmal enzymes [99] and have minimal contribution from other cellular or subcellular compartments [99,100]. Finally, our studies were accompanied whenever possible by immunolocalization, to further ascertain the identity of affected cells as well as cellular sites. Having delimited our examinations thus, we could demonstrate that administration of FGF-2 to the heart resulted in increased levels of PKC-α, PKC-ε, PKC-δ and PKC-ζ to the sarcolemmal membrane fraction, *in situ*. This is presumed to reflect translocation from other cellular compartments, although we cannot exclude the possibility of strengthened association with the sarcolemmal membrane. In either case, increased presence at a membrane fraction is taken to indicate activation of the enzyme at that site. Other agonists in the heart have been demonstrated to similarly cause various PKC

isoform translocations. Phenylephrine, an α1 adrenergic agonist and ATP, which stimulates the purinergic receptor, are reported to cause translocation of the -δ and -ε but not the -α or -ζ subtypes [27] whereas the phorbol ester phorbol-12-myristate (PMA) [28,29] and 12-O-tetradecanoylphorbol-13-acetate (TPA) [30] caused translocation of the -α,-δ,-ε but not the -ζ subtypes to myocyte membranes[28-30]. Endothelin caused translocation of the -δ, -ε but not -α and -ζ PKCs [27,28]. Hypoxia increased membrane association of -α and -ε but decreased that of -δ subtype [31]. Ischemic preconditioning or high calcium simulated preconditioning induced, and was dependent on, translocation of PKC-α and PKC-δ to the myocyte sarcolemma [97]. The effects of FGF-2 treatment therefore on overall PKC redistribution in heart myocytes may be distinct from those of the above mentioned agonists or conditions, especially regarding the -ζ subtype. Changes in the latter isoform, however, may have been masked by the use of all membrane fractions for analysis in the above mentioned studies. Furthermore, FGF-2 administration to the whole heart may have resulted in release of other factors acting on myocytes, and therefore the PKC redistribution we detected may be the result of a combination of factors rather than FGF-2 alone; we think this scenario unlikely to be the major contributor to the changes we observed since FGF-2 elicited similar changes on PKC redistribution when cultured cardiac myocytes were examined [113].

Translocation of PKCs to cardiac sarcolemma as a result of FGF-2 administration was also indicated by the significant increase in PKC activity, assayed in the presence as well as in the absence of calcium at this cellular location. Furthermore, in one instance immunolocalization offered additional evidence for translocation of PKC-δ to the

sarcolemma of cardiomyocytes in situ after FGF-2 addition. A similar translocation of this isoform to the cardiomyocyte plasma membrane has been reported as a result of adenosine A1 stimulation [29] and of ischemic or calcium-induced preconditioning [32,97]. We were unable to detect differences in the distribution pattern of the other PKC isoforms by immunolocalization (data not shown). It is possible that antibody sensitivity was not adequate to detect small changes in local concentration of these isoforms or that translocation to a different site may have affected antibody recognition.

It is not at this point possible to discriminate whether some or all of the PKC subtypes affected by FGF-2 administration and examined here would act towards cardioprotection. Comparison with other manipulations which are cardioprotective and cause translocation of PKC-δ [29] and PKC-α [29,97] to the sarcolemma, however, strengthens the link between these subtypes and cardioprotection.

We have previously demonstrated that administered FGF-2 localizes to blood vessels, capillaries and around cardiomyocytes through the whole heart, in a basement membrane-like distribution [1]. Increases in sarcolemmal PLC and PKC activity after FGF-2 administration is certainly consistent with an effect of this factor on myocytes, as is increased sarcolemmal association of the PKC subtypes. Elimination of the FGF-2 induced increase in PKC activity at the sarcolemma, and decreased levels of sarcolemmal-associated PKC-ε and -ζ after chelerythrine treatment argue in favor of the notion that chelerythrine did reach these cells and successfully prevented or decreased FGF-2-induced PKC activation, *in situ*.

Translocation of PKC to the membrane is believed to require association with diacyl glycerol (DAG) which increases enzyme affinity for phosphatidylserine at the membrane; the latter, plus calcium binding for the calcium-dependent forms, results in full activation of PKC [33,34,104]. Chelerythrine is reported to affect the catalytic site of PKC [35]. Our data, showing that chelerythrine reduced the endogenous levels of PKC-ζ and possibly PKC-ε associated with sarcolemma, as well as the FGF-2 induced increases, suggest that this inhibitor may either interfere with translocation itself or weaken the interaction of PKCs with the membranes potentially leading to dissociation of PKCs during preparation of sarcolemma. It is interesting that chelerythrine, when administered alone, seemed to have marginal effects on control sarcolemmal levels of PKC-α and -δ but it clearly decreased control levels of PKC-ζ, introducing the notion that this inhibitor may affect membrane localization, and perhaps activation/inactivation, of different isoforms to a different degree. FGF-administration in the presence of chelerythrine resulted in clear increases in sarcolemma-associated subtypes-α and -ζ, compared to samples treated with chelerythrine alone. Under all circumstances, however, PKC levels at the sarcolemma of chelerythrine/FGF-2 treated samples were lower than those of FGF-2-treated samples. This reduction by chelerythrine of FGF-2-induced PKC association with the sarcolemma correlates well with this inhibitor's ability to eliminate the cardioprotective effect of FGF-2 after I-R, as well as the negative inotropic effect before ischemia.

Thus, the data collected so far implies that increases in sarcolemma association of one, some, or all of the PKC isoforms studied here and achieved before ischemia by FGF-2 administration, is cardioprotective. This in turn would suggest that PKC is protective by

affecting the properties of specific sarcolemmal targets. One PKC target that has been strongly implicated in cardioprotection is the K_{ATP} channel [52,101,102]. Activation of this channel, as might occur by PKC phosphorylation, reduces action potential duration and decreases contractility [36] resulting in preservation of energy stores. It is therefore of interest that FGF-2 administration attenuated contractility and increased cardiac ATP stores before ischemia, suggesting that FGF-2 may produce a PKC-dependent modulation of K_{ATP} activity, leading thus to cardioprotection. A report that FGF-2 administered systematically affects vascular K_{ATP} channels [37] is consistent with the proposed scenario.

3.4.5 FGF-2 and MAPK in the heart

In the heart, the major isoforms of MAPK are the p42 and p44 [38,39]. Activation of these isoforms has been correlated with growth and hypertrophy [38,40]. In cardiac myocytes, MAPK has been shown to be triggered by various agonists including bradykinin [41], endothelin [42], aFGF [38], stretch [43] and phenylephrine [44]. Our laboratory has demonstrated that FGF-2 activates MAPK in freshly cultured adult cardiomyocytes. Although these agonists trigger MAPK to induce cardiac growth and hypertrophy, modes of MAPK activation differ [40]. For instance, whereas endothelin, bradykinin and phenylephrine activate MAPK to induce hypertrophy via the translocation and activation of nPKC (-δ and ε) [41], aFGF activation of MAPK is thought to be PKC independent [38].

Thus we examined whether MAPK may be involved in the FGF-2-induced cardioprotective effect. Western blot results showed FGF-2 administration causes an increase in both p42 and p44 MAPK levels at the sarcolemma suggesting the presence of

potential MAPK-targets at that location. Since activation of MAPK is dependent on its phosphorylation on both serine and tyrosine residues, by upstream MAPKK [40], we examined the distribution of dually phosphorylated MAPK in FGF-2-treated and control perfused hearts using specific antibodies. Western blot results show increased dually phosphorylated p42 and p44 MAPK isoforms at both cytosolic and sarcolemmal fractions with FGF-2 treatment. These results demonstrated that FGF-2 activated MAPK in cardiac myocytes *in situ*.

To examine whether MAPK is essential for the FGF-2-induced cardioprotection it will be important to investigate whether specific MAPK inhibitors block FGF-2 induced cardioprotection. The current investigation however has provided an indication that MAPK activation at least at the sarcolemma may not be important for the cardioprotective effect of FGF-2: Chelerythrine treatment which blocked FGF-2 induced cardioprotection completely as well as PKC activation, did not have any effect on FGF-2-induced MAPK which remained fully activated.

Other laboratories have reported that MAPK becomes activated downstream of PKC in cardiac myocytes, based on experiments where PKC is downregulated after prolonged treatment with tumor promoters [109]. Our data, however, indicate that there is no obvious link between PKC and MAPK activation in our setting. Similar data have been obtained in our laboratory from in vitro studies [113]. We suggest that tumor promoters may have PKC-independent long-term effects on myocytes influencing MAPK.

Activated MAPK translocates to the nucleus and phosphorylates transcription factors responsible for FGF-2 induced changes in gene expression [60]. Since the cellular

preparations examined consisted of purified perfused rat heart sarcolemma and cytosol, future experiments may concentrate on determining whether nuclear MAPK may play a role in FGF-2-induced cardioprotection.

3.4.6 FGF-2 and β-ARK1 in the heart

Studies in the heart have shown that βARK1 is activated by PKC [57]. Indeed, activation of βARK by PKC and other effectors produces a translocation of the βARK1 enzyme to the sarcolemma where it phosphorylates amd inactivates the agonist occupied β-adrenergic receptor by allowing β-arrestin to bind and uncouple the receptor from G-protein [45]. Various activators of PKC have been shown to induce a negative inotropic effect in the heart[58]. Thus, since FGF-2 has been shown by us to induce a negative inotropic effect as well as activate PKC [34,35], we investigated whether FGF-2 affected βARK1 translocation and thus activation in isolated perfused rat hearts.

Western blot analysis of purified sarcolemma and cytosol from FGF-2-treated and control hearts showed increased association of β-ARK1 to the cardiomyocyte sarcolemma consistent with translocation and activation. Assuming that βARK1 has indeed become activated, one would expect it to induce α1-adrenergic receptor desensitization and a decrease in the myocyte's contractile response to adrenergic stimulation. [45]. In the perfused heart, α1-adrenergic stimulation is thought to occur via the nerve fibers present in the ventricular tissues which contain and release catecholamines [114,115].

3.4.7 FGF-2 and c-fos, c-jun, c-src, in the heart

c-fos, along with c-jun are nuclear oncogenes coding for transcription factors which upon stimulation (phosphorylation), often by MAPK, interact and form the DNA-binding activator AP-1 complex [54]. The AP-1 complex mediates the action of various agonists and trophic factors, including: endothelin-1 [55,56]; norepinephrine [57,58]; angiotensin [55,56,59-62]; stretch receptors [58-60,63]; mechanical and emotional stress [57,64]; pressure overload [57,60]; hypertension and hypotension [65]; transforming growth factor-β [57,58]; hydrogen peroxide [66]; phenylephrine [67]; isoproterenol [68] and ischemia [69]. FGF-2 stimulates expression of c-fos and c-jun, accompanied by increased mitogenicity in fibroblasts [71]. In oligodendrocyte progenators FGF-2 induction of c-fos expression was correlated with protein kinase C activation and increased cell proliferation [83]. In cardiac muscle cells, FGF-2 was shown to upregulate c-fos expression. This expression was correlated with re-expression of fetal cardiac genes (such as skeletal α-actin, β-myosin heavy chain and atrial natriuretic factor [84].

Some biological effects of c-jun activation include: increased synthesis of messengers of the heat shock protein gene-HSP70 and cardiac hypertrophy [70]. During preconditioning, increased c-fos expression is correlated with an increased expression of various stress and anti-oxidant proteins [82].

As a first step in determining whether c-fos and c-jun are targets for acute, FGF-2-triggered signaling, we examined changes in their subcellular distribution by western blotting or immunofluorescence. The increased c-jun levels seen in the cytosol of FGF-2 treated hearts suggest translocation from another subcellular compartment. The

commercially available anti-c-jun antibodies used here were not effective in localizing this protein by immunofluorescence, at least under the conditions assayed, we do not therefore have any information about changes in its nuclear localization. Our subcellular fractions furthermore were obtained after removal of nuclei. Increased nuclear c-jun localization would be suggestive of stimulation of AP-1 dependent transcription, especially if accompanied by increased nuclear c-fos localization. Indeed, immunofluoresce analysis demonstrated increased nuclear localization of c-fos as a response to FGF-2 administration. Since this was observed within 10 minutes of treatment, it likely represents translocation to the nucleus rather than new synthesis of c-fos, although FGF-2 would also be expected to stimulate c-fos expression.

Overall our data offer evidence that c-fos and c-jun are targets of acute FGF-2 mediated signaling. It remains to be demonstrated whether the FGF-2 effects on c-fos and c-jun are mediating its acute cardioprotective effect.

Src is a peripheral membrane tyrosine kinase protein, which is involved in cellular signaling [72] for a variety of agonists such as thrombin [86], platelet derived growth factor [74], angiotensin II [75] and FGF-2 [76,77]. Very little is known about the role of c-src in the heart. Increased src activity appeared to stimulate the ANF promoter [78]. Since FGF-2 stimulates ANF expression, it may be expected to activate cardiac c-src. c-src activation has been linked to translocation [74]. To obtain therefore an indication as to whether FGF-2 affects c-src in our system we examined its subcellular distribution. FGF-2 administration was found to cause decreased levels of c-src at sarcolemmal but increased levels of c-src at the cytosol. Decreased c-src at the sarcolemma may represent

deactivation or degradation of this protein. It is also possible that initial c-src stimulation and sarcolemmal association is followed by deactivation and movement to the cytosol. Previous studies have shown that FGF-2 may activate or deactivate c-src, depending on the cell type. In lung capillary endothelial cells and murine fibroblasts, FGF-2 stimulation of FGFR1 led to an increase in autophosphorylation of various Src family members [79]. In human umbilical vein endothelial cells, activation of the fibroblast growth factor receptor 1 (FGFR1) is correlated with increased src tyrosine kinase activity and increased proliferation [80]. In contrast though, in porcine aortic endothelial cells and lung fibroblasts from Chinese hamster, activation of FGFR caused a reduction in the autophosphorylation of Src. In addition, these cells showed a lack of complex formation between Src and FGFR-1. This negative regulatory effect on Src activity by FGFR-1 was found to be mediated by an increase in PLC-γ and PKC activity [79]. FGF-2 may exert a stimulatory or inhibitory action on c-src depending on the particular tyrosine that is being phosphorylated: Phosphorylation of tyr-416 or -527 cause stimulation or inhibition of c-src activity, respectively [81].

FGF-2 induced changes in c-src subcellular distribution. Further studies are required to determine whether these changes reflect activation and/or inactivation of c-src, and whether this is relevant to cardioprotection. The same holds true for c-fos and c-jun. Our data however do show that all of these molecules which are important regulators of growth, differentiation and gene expression, are targeted, directly or indirectly, by FGF-2 signaling in the myocardium.

3.4.8 FGF-2 and signaling during reperfusion

Various treatments which prevent ischemic injury were found not to protect against reperfusion injury when administered after the ischemic insult (e.g. Ca^{2+} blockers [86] and activators of K_{ATP} channels [87]). Only a few treatments like administration of antioxidants [85] and Na^{+}/H^{+} exchange inhibitors [88], were found to be effective in reducing reperfusion injury. It is therefore of major importance to identify additional approaches which are cardioprotective when administered during reperfusion. As presented in chapter 2, FGF-2 added during reperfusion offered substantial protection against I-R injury, by promoting better recovery of the contractile parameters when compared to control hearts. It is also essential to define the mechanism by which FGF-2 induces cardioprotection when administered during reperfusion and compare it with the mechanism involved in pre-ischemic administration. These studies are currently in progress in our laboratory. Our preliminary data from immunolocalization of signaling molecules such as c-fos and PLC-β3, indicated that FGF-2- induced changes in the reperfused heart after ischemia are similar to those induced by this factor in the pre-ischemic heart.

3.4.9 Conclusion

FGF-2 administration to the isolated heart by perfusion can affect directly all cells it comes in contact with and affected cells presumably influence their neighbors. Therefore cardioprotection by FGF-2 most likely reflects a combined effect on several cell types originating both in the vasculature and the myocardium. We suggest that at least part of the protective effect represents a direct effect of FGF-2 on myocytes *in situ*, based on several points: (a) As mentioned, administered FGF-2 localizes around cardiomyocytes in

all areas of the heart [1]; these cells possess functional FGF-2 receptors and display increase tyrosine phosphorylation in situ after FGF-2 administration, a finding consistent with in situ activation of the FGF-2 receptor. (b) FGF-2 protects cultured myocytes against damage caused by serum deprivation and H_2O_2 [12]. A protective effect of FGF-2 against injury and degeneration is well established for neuronal cells in culture and in situ [89-93]. (c) Administered FGF-2 induced translocation of PKCs to cardiac myocyte sarcolemma, *in situ*. Similar results were obtained when FGF was added to neonatal cardiomyocytes in culture [94,103]. Activation of cardiomyocyte PKC *in situ* is consistent with the notion for a direct FGF-2, receptor-mediated effect on cardiac myocytes in the perfused heart. Blocking of FGF-2 induced PKC activation in cardiomyocytes by chelerythrine also blocked cardioprotection, pointing to a link between the effects on cardiac myocytes to the whole cardiac response. (d) Preconditioning is highly cardioprotective and this effect is linked to the activation/translocation of PKC in cardiac myocytes *in situ* [32,95]. The overall phenomenon of cardiac protection can thus be linked to stimulation of PKC in the cardiac myocyte. It has actually been postulated previously that agonists for receptors linked to PKC activation would be cardioprotective [50,95]. Data presented here are certainly in agreement with this postulate. Activation and involvement of PKC in both preconditioning-induced and FGF-2-induced cardioprotection against I-R injury, at least in the rat, leads to the speculation that local FGF-2 release and/or induction in the heart as a result of a brief injury could represent a component of the mechanism of preconditioning. FGF-2 release is believed to occur upon cellular injury [94] and increased levels of FGF-2 immunoreactivity have been reported in association

with damaged cardiomyocytes [96] *in situ*. One can hypothesize therefore that brief periods of ischemia cause increased release of FGF-2 which can then act on cardiomyocytes and other cell types in a manner equivalent to that obtained with administered FGF-2, leading to protection against a subsequent ischemic insult.

Since inhibitor studies have not been conducted on the other signal transduction mediators found to be affected by FGF-2 in our studies (i.e. PLC-δ1, PLC-γ1, PLC-β3, MAPK, β-ARK1, c-jun, c-src and c-fos), due to lack of specific inhibitors, it as yet not possible to determine whether these proteins mediate the cardioprotective effects of FGF-2 in cardiomyocytes.

Increased immunofluorescence staining observed with PKC-α and c-fos in the vascular wall in FGF-2-treated hearts do indicate FGFR1 mediated activation of these signal proteins in the vasculature. FGF-2 administration to dog [37] and rabbit [5] hearts does induce a vasorelaxing and hypotensive effects, respectively. However, as mentioned in Ch.2 discussion, only a small change in coronary back perfusion pressure was observed with FGF-2 administration to our perfused rat hearts making FGF-2-induced vasodilation a less likely mechanism for cardioprotection. Alternatively, since FGF-2 contains disulfide bonds in its cysteine residues [110], it may protect vascular cells during I-R by scavenging damaging free radicals. Thus, further investigation into the effects of FGF-2 on coronary vasculature cells during I-R is required.

In cardiomyocytes, possible mediators for the cardioprotective effects of FGF-2, upstream of PKC, may involve at least three isoforms of PLCs: PLC-δ1, PLC-β3 and PLC-γ1. The involvement of PLC-β3, in particular, indicates participation of the Gq

family of G proteins in the signal transduction pathway of FGF-2, which is a novel observation for cardiomyocytes, as is the observation that a form of PLC-δ1 is affected by FGF-2.

Other possible mediators of FGF-2 signaling include MAPK, β-ARK1, c-src and the nuclear oncogene proteins c-fos and c-jun. Activation of β-ARK1 by FGF-2 has not been reported before and is particularly important because it represents a possible mechanism for the negative inotropic effect, increased levels of high energy phosphate compounds and the cardioprotective effects seen with FGF-2 administration to perfused hearts. As well, since β-ARK1 is known to be activated by PKC, activation of β-ARK1 by FGF-2 may occur via the PKC pathway.

As a future direction it would be important to investigate whether cardiac proteins such as the K^{+}_{ATP} channel [52], heat shock proteins [82],antioxidants [85] which have been shown to participate in cardioprotection and which are associated with PKC [29,32,52], PLC[22] and nuclear c-fos[58] signaling, are targets of FGF-2 triggered signal transduction.

In addition, our previous findings in chapter 2 showed that FGF-2 can act as a cardioprotective against reperfusion injury. Whether PKC is involved in this type of protection is unknown. However, since our immunofluorescence results have shown PLC-β3 translocating to the membrane with FGF-2 treatment, it is likely various isoforms of PKC may become activated by DAG.

In view of data presented here FGF-2 can be considered as a candidate endogenous cardioprotective agent in the adult myocardium. Manipulations therefore

affecting cardiac FGF-2 expression and release stand to be of clinical relevance insofar as they affect cardiac resistance to injury and thus cardiac health.

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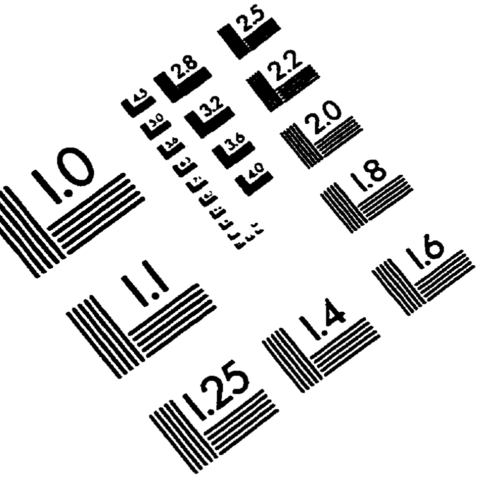
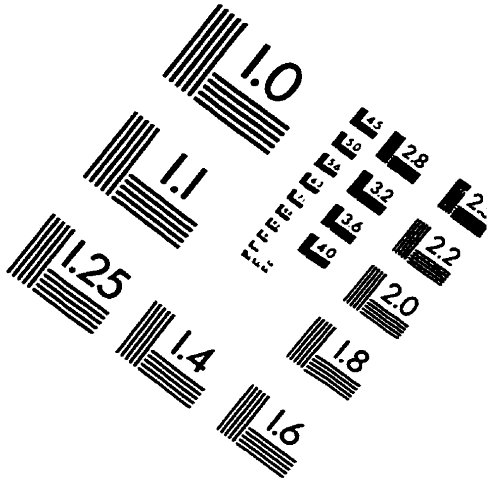
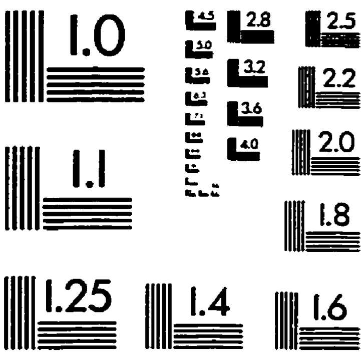
IMAGE EVALUATION TEST TARGET (QA-3)







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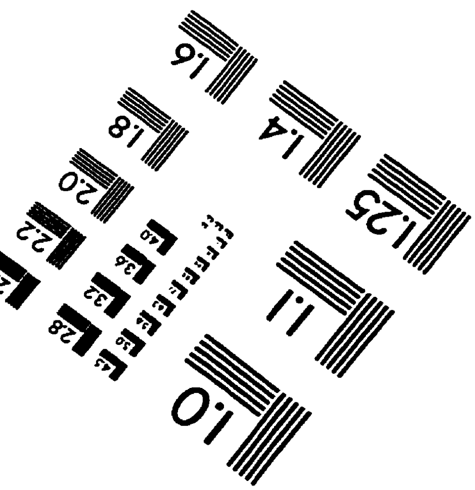

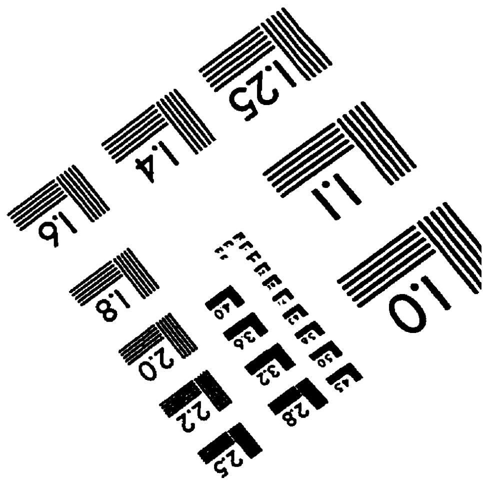

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