THE EFFECT OF FIBROBLAST GROWTH FACTOR-2 ON CONNEXIN 43 PHOSPHORYLATION AND DISTRIBUTION IN THE NORMAL AND ISCHEMIC HEART

BY

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ABSTRACT

Background: Fibroblast growth factor-2 (FGF-2) confers acute, pre-conditioning–like cardioprotection against ischemic injury in a protein kinase C (PKC)-dependent fashion. In fact, PKC activation is a central mediator of acute cardioprotection. Our lab has shown previously that the gap junction protein connexin-43 (Cx43) is a target of PKC, as well as FGF-2-triggered signal transduction, in neonatal cardiomyocytes in culture. Cx43, while predominantly phosphorylated in normal adult heart, is rapidly dephosphorylated upon the onset of ischemia, an event that precedes its eventual degradation. Loss or abnormalities in Cx43 can contribute to ischemia-induced pathologies including arrhythmias and ventricular dysfunction. It is thus possible that cardioprotective treatments exert effects at the level of connexin-43. The overall objective of my studies was to determine whether FGF-2 treatment will affect Cx43 levels, distribution and PKC-mediated phosphorylation in the adult non-ischemic and ischemic heart. I hypothesized that: (1) FGF-2 stimulates Cx43 phosphorylation at PKC sites, in situ; (2) administration of FGF-2 prior to ischemia prevents the ischemia-induced Cx43 dephosphorylation and re-distribution.

Results: The ex vivo perfused adult rat heart model was used. Cx43 levels, phosphorylation, and distribution were examined by western blotting and immunofluorescence of tissue sections, (a) after FGF-2 administration but prior to ischemia, and, (b), after 30 minutes of global ischemia of the FGF-2-treated or non-treated hearts. In (a), FGF-2 was found to upregulate Cx43 levels and stimulate the phosphorylation of Cx43 at the PKC target sites serine (S) 262, and

S368, at intercalated disks. FGF-2 treatment therefore created a new cardiac state, characterized by 'extra' phosphorylation of Cx43 at PKC target sites. In (b), FGF-2 pre-treatment prevented the ischemia-induced Cx43 dephosphorylation and re-distribution away from intercalated disks, while maintaining elevated levels of Cx43 phosphorylation at S262, and S368, at intercalated disks. Cx43 phosphorylation at PKC sites therefore maintained a 'memory' of the FGF-2 pre-treatment even after 30 minutes of ischemia.

Conclusion: The FGF-2-stimulated 'extra' phosphorylation of Cx43 at PKC target sites S262 and S368 may sign-post a protected or pre-conditioned cardiac phenotype and contribute to the mechanism of FGF-2-induced cardioprotection against ischemia-reperfusion injury.

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aa	Amino acid
ANOVA	Analysis of variance
ADP	Action potential duration
AMP	Adenosine-5'-monophosphoate
ATP	Adenosine Triphosphate
AV nodes	Atrioventricular nodes
BSA	Bovine Serum Albumin
BCA	Bicinchonic Acid
ºC	Degree Celsius
Ck1	Casein kinase 1
Cx	Connexin
DAG	Diacylglycerol
DP	Dephosphorylation
FGF-2	Fibroblast Growth Factor-2
FGFR1	FGF- Receptor 1
GJs	Gap Junctions
GJIC	Gap Junction Intracellular Communication
HRP	Horseradish Peroxidase
ICDs	Intercalated disks
IPC	Ischemic preconditioning
K-H	Krebs-Henseleit solution
kDa	Kilodalton

m.AB	Monoclonal antibodies
MAPK	Mtiogen activated protein kinases
mg	Microgram
min	minute
Mito K_{ATP}	Mitochondria ATP-sensitive potassium channels
mM	Millimolar
NADH	Nicotinamide adenine dinucleotide
P	Phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PIC	Protein Inhibitor Cocktail
PKA	Protein kinase A
PKC	Protein kinase C
PKC ε	Protein kinase C-epsilon subtype
PKG	Protein kinase G
PLC	Phospholipase C
PMA	Phorbal 12 Myristate 13-acetate
PPIC	Phosphatase Inhibitor Cocktail
PVDF	Polyvinylidene difluoride
ROS	Reactive Oxygen Species
SA node	Sinoatrial node
SDS	Sodium Dodecyl Sulfate
S262	Serine 262
S368	Serine 368

TBST	Tris Buffered Saline with TWEEN-20
TPA	12-0-Tetradecanoyl-phorbol-13-Acetate
Tyr	Tyrosine
Tyrk	Tyrosine kinase
VDAC	Voltage-dependent anion channel
µM	Micromolar

REVIEW OF LITERATURE

1. ISCHEMIC HEART DISEASE

1.1. Introduction

Ischemic heart disease is a major cause of mortality and morbidity worldwide. Myocardial ischemia is an imbalance between the oxygen provided by the blood supply and the oxygen demand of the working heart. It is caused by compromised arterial blood flow and depressed ventricular function, disruption of aerobic respiration, presence of anaerobic glycolysis, depletion of metabolic substrates, accumulation of catabolites, and altered electromechanical activity, which is revealed by conductance disturbances and arrhythmias [Carmeliet, E 1984, Jennings, R B, et al. 1991b, Reimer, K A, et al. 1987]

1.2 Irreversible and reversible ischemia

"Reversible ischemia" is defined as a complete recovery of cardiomyoctes from the damage due to ischemia, which occurs in less than 20 minutes after reperfusion [Ip, J H, et al. 1988]. Reversible ischemia leads to mild abnormalities of the ion channels [Shaw, R M, et al. 1997] and contractile dysfunction or myocardial stunning [Opie, L H 1996]

Prolonged periods of ischemia (more than 20 min), known as "irreversible heart injury", cause activation of proteases and phosphatases leading to dephosphorylation and degradation of proteins including gap junctions, sarcomeres and intermediate filaments that resulting in cell death and

development of lethal arrhythmias [Ganote, C E, et al. 1987, Huang, X D, et al. 1999, Steenbergen, C, et al. 1985]

1.3 Pathogenesis of myocardial ischemic injury

1.3.1 Alterations in metabolism of ischemic myocardium

In the few seconds of abrupt arrest of cardiac flow, there is a change in the physiological mechanism to produce energy in the cardiomyocytes. Instead of producing energy via fatty acid oxidation in the mitochondria, the ischemic cardiomyocytes changes its production of energy toward anaerobic glycolysis by using glucose as the main energy [Ip, J H, et al. 1988]. This metabolic transition leads to a decrease in the levels of ATP by 65% within 15 minutes, and 90% within 40 minutes [Opie, L H 1990a, Opie, L H 1990b]. The fall of ATP production is associated with the reduction of intracellular glycogen production as glucose supply [Gwilt, M, et al. 1993].

In severe ischemia, the accumulation of metabolic wastes such as hydrogen ions, inorganic phosphates, NADH and lactic acid, leading to a decrease in intracellular pH, inhibition of glycolytic enzymes, and a further decrease in ATP production. Since ATP is required for the cardiac contractile process, the decrease in cardiac contractility could be observed with ATP deficiency. Furthermore, the rapid reduction of intracellular pH is correlated with decreased binding of Ca^{2+} to the contractile proteins in myocardial cells thereby reducing the myosin-ATPase activity at low pH. The reduction in ATP and rapid decrease in the intracellular pH are the cause of cardiac contractile dysfunction

that occurs shortly after the onset of ischemia [Opie, L H 1999, Taegtmeyer, H 2004].

1.3.2 Alterations in action potential profile in ischemic myocardium

The depletion of ATP and fall of intracellular pH directly affect changes in the action potential profile [Opie, L H 1996]. During acute ischemia, a shortening of action potential duration (APD) was observed. The APD shortening is attributed to an increase in K^+ outward current and decrease in K^+ inward current resulting in the loss of intracellular K^+ from cardiomyocytes and increase extracellular K^+ [Antzelevitch, C, et al. 1991]; [Kleber, A G 1984]. Alterations of intracellular and extracellular K^+ levels are mediated by: (1) decrease in ATP levels, which mediate the opening of the K^+_{ATP} channels thus decreasing APD; (2) an inhibition of Na/K pump due to reduced local ATP levels. [Dilly, S G, et al. 1988]; [Friedrich, M, et al. 1990]; [Winslow, R L, et al. 1999]. Therefore, with the increased conductance of extracellular K^+ membrane potential away from the voltage required for optimal opening of the Na+ channels, resulting in a decrease in the APD [Blanchard, E M, et al. 1984].

1.3.3 Alterations in ultrastructure of ischemic myocardium

The accumulation of catabolic waste and ATP depletion during ischemia affects the structural integrity of the cell. Enzyme denaturation, membrane disruption, and increased intracellular osmolarity, lead to cell swelling [Buja, L M, et al. 1993], [Jennings, R B, et al. 1991a, Jennings, R B, et al. 1991b]. Membrane disruption is one of the major characteristics in myocardial ischemic injury [Buja,

L M, et al. 1993, Jennings, R B, et al. 1991b, Wang, D, et al. 1996]. The three causes of membrane disruption in the ischemic hearts are firstly, the changes in ionic transport systems, especially K^+, Na^+, Mg^{2+}, and Ca^{2+}; secondly, elevated permeabilities of the phospholipid bilayer; and thirdly, physical disruption of the membrane [Buja, L M, et al. 1993]. This membrane damage is related to ATP depletion, changes of ion fluxes, and increased intracellular Ca^{2+} concentration [Barry, W H, et al. 1993, Barry, W H, et al. 1987, Jennings, R B, et al. 1991b, Reimer, K A, et al. 1987]. Ca^{2+} influx leads to the activation of phospholipases and proteases. Na+ and water influx contribute to the cell swelling and cause an impairment of the cell membrane with detachment of gap junction channels at cell-cell contact areas, leading to irreversible ischemic damage [Buja, L M 1998, Buja, L M, et al. 1998].

2. GAP JUNCTIONS

2.1 Introduction

Normal development, growth, and proper functioning of cells in various tissues and organs rely on intercellular communication. Cells can directly communicate with each other via unique communication junctions, termed "gap junctions" (GJs). GJs are specialized plasma membrane intercellular channels, which connect the cytoplasm from one cell to adjacent cells and provide a low-resistance pathway for cell-to-cell communication [Goodenough, D A, et al. 1996]. GJs are non-ion selective channels that allow the passage of ions and small metabolites by passive diffusion [Beblo, D A, et al. 1995, Beyer, E C, et al. 1995, Trexler, E B, et al. 1996, Veenstra, R D, et al. 1995]. GJs are responsible for electrical and metabolic coupling in a variety of cell types, including neurons [Dermietzel, R, et al. 1989], vascular endothelium cells, smooth muscle cells [Christ, G J, et al. 1996], lens cells [White, T W, et al. 2000], liver cells [Ren, P, et al. 1998], and cardiomyocytes [Beyer, E C, et al. 1991]; [Kanter, H L, et al. 1992] with the exception of mature skeletal cells, spermatocytes and blood cells [Simon, A M, et al. 1998]. The propagation of action potentials via GJs as well as passage of small metabolites less than 1 kDa, such as amino acid, short peptides, ions and second messengers (ATP, IP_3, cAMP) permit the coordinated and synchronized signal transmission within organs, such as heart, and brain, that is essential for proper cellular functions in both excitable and non-excitable cells [Goodenough, D A, et al. 1996];[Valiunas, V, et al. 2002]. In normal cardiac tissue, the electrical transmission via gap junctions allows the myocyte cells to

act as a syncytium. Alterations in GJ expression and/or distribution have contributed to lethal arrhythmias [Kanter, H L, et al. 1992] [Luke, R A, et al. 1991]. GJs abnormalities are believed to cause sudden arrhythmias and death in transgenic mice with cardiac-restricted inactivation of Cx43 [Gutstein DE et.al.2001]. Malfunction of GJs are associated with several diseases, including heart disease, cancer, skin disease, cataracts, hereditary deafness and some forms of neuropathy [Willecke, K, et al. 2002].

2.1.1 Gap junction structure

GJs are composed of six protein subunits called connexons. Each connexon is formed by six connexins, which are arranged in a hexameric structure, as shown in Figure 1. [Morley, G E, et al. 1997]. Connexons from one cell are connected noncovalently to connexon of adjacent cells via the extracellular loops (E1 and E2) of the connexins [Dhein, S 1998] to form a complete gap junction channel. The length of gap junction is 2-3 nm between neighboring cells and is less than 2.5 nm at its widest point of the central pore.

Each connexin contains four hydrophobic transmembrane domains, two extracellular domains, and three cytoplasmic domains comprising of the amino (N)-terminal, carboxy (C)– terminal, and a loop between transmembrane domains 2 and 3, as shown in Figure 2 [Harris, A L, et al. 2001, Sosinsky, G E, et al. 2000] [Goodenough, D A, et al. 1996]; [Yancey, S B, et al. 1989]. The extracellular domains are responsible for the interaction between connexons of the adjacent cells [Hennemann, H, et al. 1992]. The intracellular domains, which

include both of N- and C-terminals, are responsible for the regulation of channel activity.

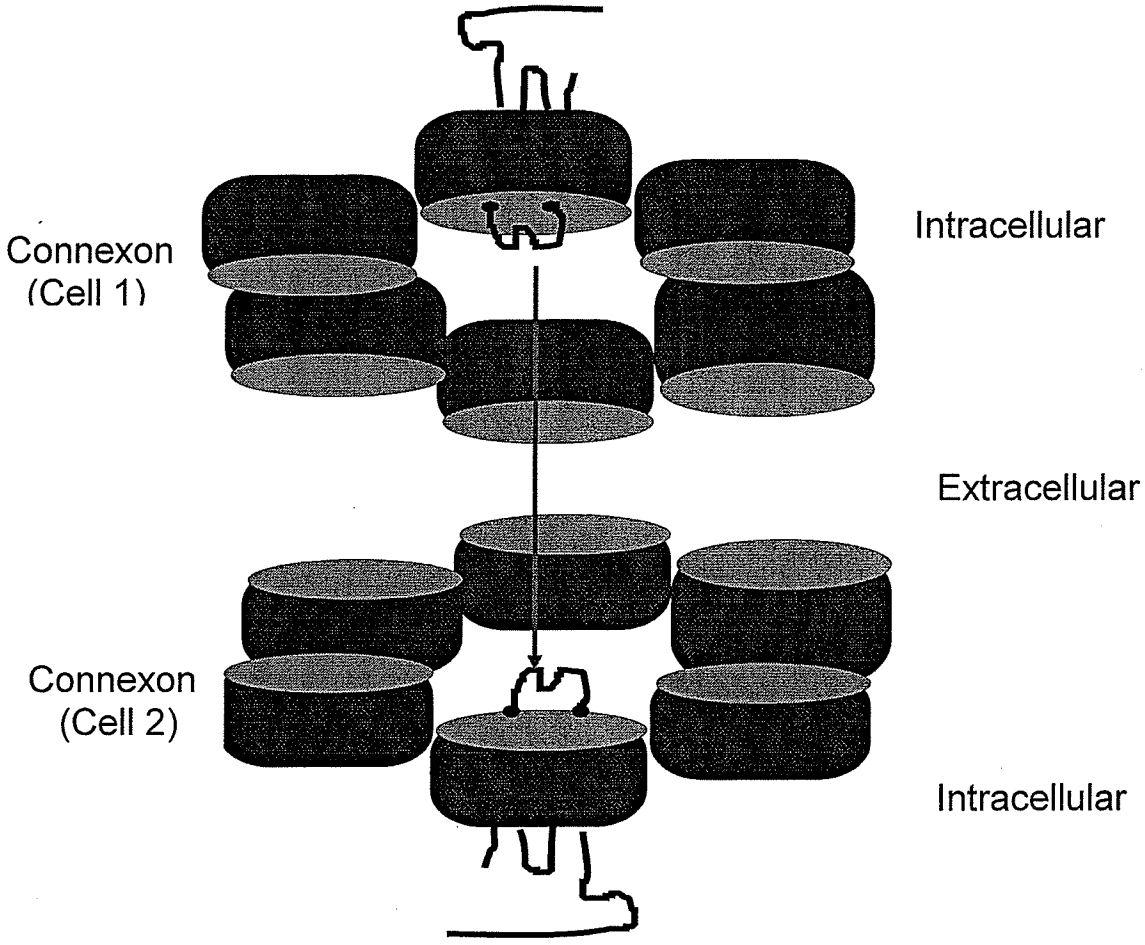


Figure 1: Structure of a gap junction channel. Gap junctions are formed by the connexons from neighboring cells. Each connexon is made up of six connexin proteins. The red arrow indicates the channel pore.

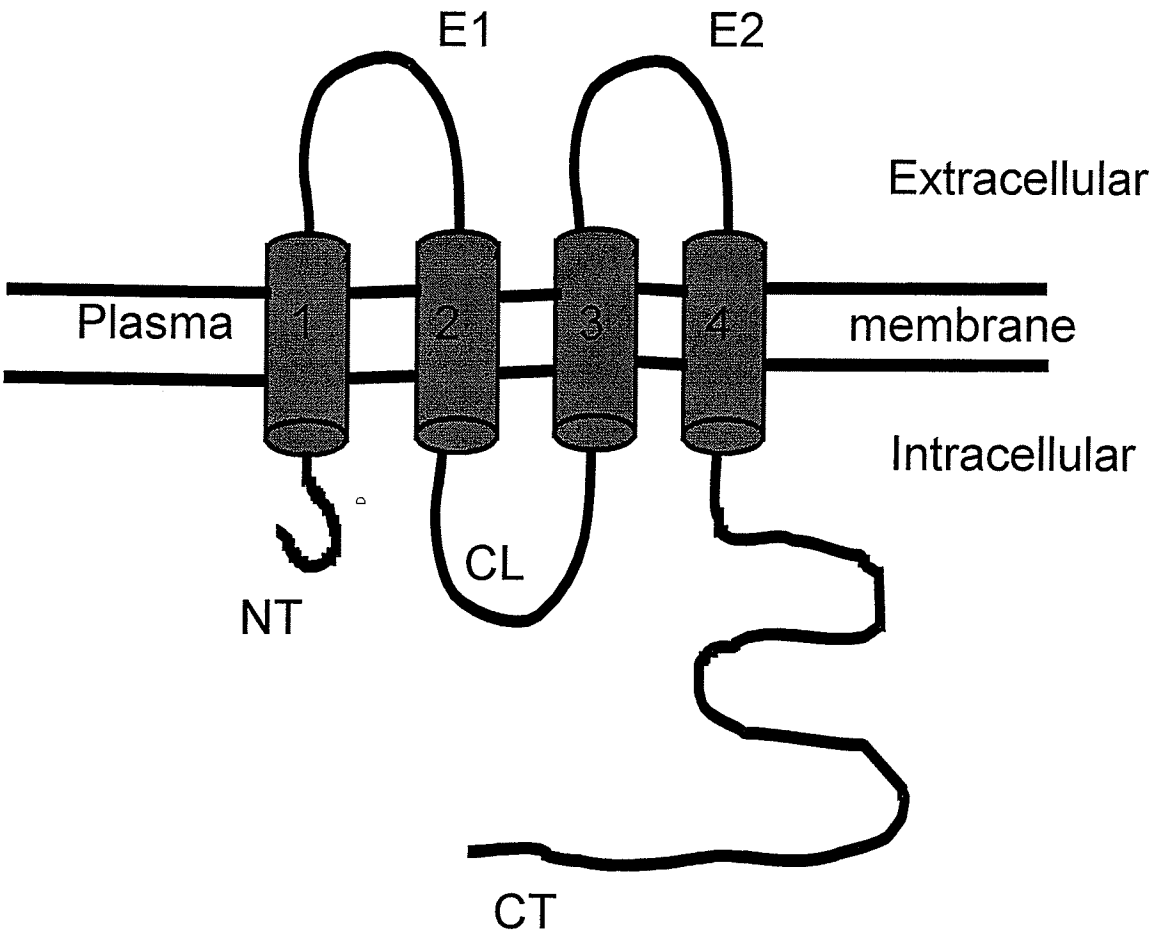


Figure 2: Connexin structure. The abbreviations are as follows: NT, N-terminus; CL, cytoplasmic loop; CT, C-terminus; E1 and E2, extracellular loops 1 and 2; 1-4, transmembrane segments 1-4.

To date, the multi-gene family of connexin (Cx) consists of 20 isoforms in mice [Willecke, K, et al. 2002], and 21 isoforms in humans [Sohl, G, et al. 2003]. There are presently two systems for nomenclature of connexins. The most widely used is based on the predicted molecular mass (in kilodaltons) of the polypeptide connexin [Beyer, E C, et al. 1987, Beyer, E C, et al. 1990] ie., Cx43 has a molecular mass of 43 kDa. An alternative system is based on primary amino acid sequence homology and uses α and β nomenclature, such as α_1Cx (Cx43), β_1Cx (Cx32), β_2Cx (Cx26) [Kumar, N M, et al. 1992].

2.2 Cardiac gap junctions

2.2.1 Cardiac gap junction proteins

The major connexin isoform in the mammalian heart is Cx43, although other Cx isoforms are expressed in less quantities [Jalife, J, et al. 1999];[Blackburn, J P, et al. 1995, Severs, N J 1995a, Severs, N J 1995b]. Different cardiac areas are composed of diverse Cx combinations [Kanter, H L, et al. 1993a, Kanter, H L, et al. 1993b, Kanter, H L, et al. 1992]. Cx37 is abundant in cardiac endothelial cells [Davis, L M, et al. 1994, Davis, L M, et al. 1995]. Cx40 is predominantly expressed in the atrial myocytes, cells of His-Purkinje system, the sinus (SA) nodes and atrioventricular (AV) nodes. Cx45 is mainly expressed in cells of the atria, ventricular, the SA nodes and AV nodes [Davis, L M, et al. 1994, Davis, L M, et al. 1995] . Cx43 is mainly detectable in the working ventricular myocardium, vascular smooth muscle cells and fibroblasts, with the exception of the conduction system and the SA and AV nodes [Bastide, B, et al. 1993] [Gourdie, R G, et al. 1993a, Gourdie, R G, et al. 1993b] [Gros, D, et al.

1994]; [Vozzi, C, et al. 1999]. Cx43 in the heart plays a key role in maintaining normal cardiac electrical and metabolic coupling and its absence results in arrhythmic death [Danik, S B, et al. 2004]. The expression of Cx43 can be altered by different factors, including FGF-2, which stimulates Cx43 expression in cardiac fibroblasts [Doble, B W, et al. 1995] and parathyoid hormone and thyroid hormone, which can regulate transcription of Cx43 gene [Mitchell, J A, et al. 2001, Stock, A, et al. 2000].

With the advancement of molecular and cellular biology approaches, transgenic animal models have been developed to analyze the functions of different Cxs. Cx43-null mice die shortly after birth, due to pulmonary outflow obstruction, and it was impossible to measure the properties of cardiac conduction in these mice [Reaume, A G, et al. 1995]. Nevertheless, the heterozygous knockout Cx43 mice (Cx43+/-) were used to study the role of Cx43 for normal cardiac conduction. These Cx43+/- mice appeared essentially normal, reproduced normally (were fertile), and had no significant differences in conduction properties when compared to wild-type Cx43 mice [Guerrero, P A, et al. 1997]; [Morley, G E, et al. 1999, Thomas, S A, et al. 1998a, Thomas, S A, et al. 1998b]. However, the activation of epicardial conduction was slowed in the ventricle, indicating ventricular conduction interruption [Guerrero, P A, et al. 1997]; [Thomas, S A, et al. 1998a]. The effect of density and distribution of gap junctions on conduction velocity in the Cx43+/- mice were studied using computer simulation. The results suggest that cell dimension is more important than gap

junction distribution, since ~40% reduction in total GJ content has only moderate impact on the conduction velocity [Jongsma, H J 2000, Spach, M S, et al. 2000].

Cardiac tissue-specific Cx43 knockout mice have also been developed. These mice were born with normal cardiac structure and contractile function, but they died of spontaneous lethal ventricular arrhythmia at approximately 2 months of age [Gutstein, D E, et al. 2001a, Gutstein, D E, et al. 2001b], suggesting the Cx43 mutation plays a vital role the development of lethal arrhythmias [Paznekas, W A, et al. 2003]. Cx43 therefore is an important cellular target in various cardiac diseases that cause lethal arrhythmias.

2.2.2 Function of cardiac gap junctions

Under normal physiological conditions, cardiac GJs play an essential role in cardiac function by mediating the propagation of electrical impulses that ensure the synchronized contraction of the cardiac muscle [Kanter, H L, et al. 1993b]. GJ channels also mediate cellular metabolic coupling to adjacent myocytes, a process that may be associated with signal transduction of cell growth, differentiation, and resistance to injury [Doble, B W, et al. 2000]; [Schulz, R, et al. 2004] . Differences in size, abundance and distribution of gap junctions in different cardiac areas contribute to the properties of electrical conduction [Jalife, J, et al. 1989]; [Luke, R A, et al. 1991, Peters, N S, et al. 1993] [Davis, L M, et al. 1994]. Moreover, GJ channels composed of different connexin isoforms have different conductance and gating activities [Davis, L M, et al. 1994].

GJs are predominantly located at the specialized regions of intercellular contact, known as the intercalated disk (ICD) areas of the myocytes. GJs that

exist at the ICD, are related to the functional coupling between adjacent cells [Saez, J C, et al. 1993, Saez, J C, et al. 1997]; [Jongsma, H J 2000]. Each ventricular cardiomyocyte is connected to approximately nine other myocytes by gap junctions [Luke, R A, et al. 1989]. Myocytes can be coupled through gap junctions in end-to-end or side-to-side orientation, which can influence the conduction of the impulse from the atria to the ventricles [Davis, L M, et al. 1994, Davis, L M, et al. 1995]. Ventricular myocytes are well connected to the adjacent cells by GJs, leading to a large communication system with other cardiomyocytes. The velocity of longitudinal conduction along the cardiomyocytes in the ventricle is three-folds higher than the transverse conduction [Delmar, M, et al. 1987];[Oosthoek, P W, et al. 1993a, Oosthoek, P W, et al. 1993b].

In addition, the extensive connection of the Purkinje fibers by GJs forms a fast conducting network, suggesting a rapid impulse propagation from Purkinje fibers to ventricles.

2.2.3 Regulation of connexin 43 and gap junction channels

GJs are dynamic membrane structures with rapid turnover rates of less than 1.5 hours in cultured cardiac myocytes [Beardslee, M A, et al. 1998] and less than 2 hours in heart tissue [Musil, L S, et al. 1990b]; [Crow, D S, et al. 1990]; [Laird, D W, et al. 1991]; [Lampe, P D 1994];[Beardslee, M A, et al. 1998]. Assembly and turnover of GJs is likely to exert control of intercellular communication. Permeability and conductance of GJs can be regulated by effects at the cytoplasmic domains of Cx43 [Swenson, K I, et al. 1989];[Beyer, E

C, et al. 1989] as well as the types of connexin forming the channels [Veenstra, R D 1996a, Veenstra, R D 1996b]. GJs can be composed of different types of connexin that exhibit different ranges of permeability heteromere [Bevans, C G, et al. 1998, Niessen, H, et al. 2000]. Heteromeric GJs show different conductance properties than the respective homomeric GJs [Koval, M, et al. 1995]; [Berthoud, V M, et al. 2000]. Signal transduction via GJ channels can be regulated by a variety of molecules and physiologic stimuli, including a reduction in cytosolic pH, changes in intercellular voltage, increases in intracellular Ca^{2+} concentration, and phosphorylation of connexins [Ramon, F, et al. 1986]; [Dermietzel, R, et al. 1990, Spray, D C, et al. 1990] [Luque, E A, et al. 1994]. Phosphorylation of Cx43 can reduce the conductance and permeability of GJ channels [Takens-Kwak, B R, et al. 1992a, Takens-Kwak, B R, et al. 1992b], whereas dephosphorylation of Cx43 increases conductance [Saez, J C, et al. 1993]; [Burt, J M, et al. 1988a, Burt, J M, et al. 1988b];[Lau, A F, et al. 1991].

2.2.4 Phosphorylation of connexin 43

The majority of connexins (Cx31, Cx32, Cx37, Cx40, Cx43, Cx45, Cx46, Cx50, and Cx56) with the exception of Cx26 are phosphoproteins. Phosphorylation of connexins is implicated in regulating all known Cx43 properties [Asamoto, M, et al. 1991], [Saez, J C, et al. 2003].

Cx43 exists mostly in a phosphorylated state in the normal healthy heart. Phosphorylation affects electrophoretic mobility of Cx43; increasing phosphorylation events result in decreasing electrophoretic mobility. Cx43 exhibits various electrophorectic mobilities with apparent molecular weights

ranging from 41-45 kDa and even > 45 kDa, when examined by polyacrylamide gel electrophoresis (SDS-PAGE). The faster mobility band correspond to non-phosphorylated or dephosphorylated (DP or Po, 41 kDa) Cx43. Slower mobility isoforms are partially (43 kDa) or highly (45 kDa) phosphorylated Cx43. Phosphorylated 43-45 kDa Cx43 species can be changed to Po by phosphatase treatment, indicating that phosphorylation is the modification being detected by SDS-PAGE analysis [Crow, D S, et al. 1990]; [Musil, L S, et al. 1990a, VanSlyke, J K, et al. 2000a, VanSlyke, J K, et al. 2000b];[Saez, J C, et al. 1997];[Hertzberg, E L, et al. 2000]. However, electrophorectic mobility may be insufficient to discriminate between non-phosphorylated and minimally phosphorylated (S368) Cx43, migrating at 41 kDa [Solan, J L, et al. 2003].

Cx43 is phosphorylated primarily on multiple serine residues, but can also be phosphorylated on threonine or tyrosine residues [Laird, D W, et al. 1991]. Cx43 has two serine residues in its amino (N-) terminal and 21 serine residues in the C-terminal. However, there is currently no report that provides any evidence for the phosporylation of serine residues on the N-terminal [Shin JL et.al.2001; Lampe et.al; 2004]. The C-terminal of Cx43 is the primary phosphorylated domain [Berthoud, V M, et al. 1997]; [Lampe, P D, et al. 2004, Solan, J L, et al. 2005]. The sites phosphorylated by various kinases are summarized in Table 1.

Cx43-mediated intercellular communication is regulated by various kinases, including protein kinase C, A (PKC, PKA), tyrosine kinase (such as src), and mitogen-activated protein kinase (MAP kinase) [Kwak, B R, et al. 1995b]; [Darrow, B J, et al. 1996]; [Doble, B W, et al. 2000]; [Polontchouk, L O, et al.

2002]. In cardiomyocytes, active PKA increases the GJ mediated intracellular communication [Darrow, B J, et al. 1996]. GJ channel conductance of neonatal cardiac myocytes is increased by PKC and decreased by PKG [Kwak, B R, et al. 1995a]. It is important to note that PKC increases electrical coupling but decreases metabolic coupling between cells [Kwak, B R, et al. 1995a, Kwak, B R, et al. 1995b]. The effects of protein kinases on GJ permeability and conductance in cardiomyocytes and non-cardiomyocytes are summarized in Table 2 and 3.

Table 1: Cx43 residues phosphorlated by active protein kinase

Phosphorylated residues	Kinases responsible	References
Tyr 247	TyrK (Src)	[Lin, R, et al. 2001]
Ser 255	MAPK	[Lampe, P D, et al. 1998]; [Kanemitsu, M Y, et al. 1998]; [Lau, A F, et al. 1996, Warn-Cramer, B J, et al. 1996]
Ser 257	PKG	[Kwak, B R, et al. 1995b]
Ser 262	Cyclin B/p34^{cdc2}, PKC	[Kanemitsu, M Y, et al. 1998]; [Doble, B W, et al. 2004] [Doble, B W, et al. 2000]
Tyr 265	Tyrk (Src)	[Lin, R, et al. 2001]
Ser 272	MAPK	[Warn-Cramer, B J, et al. 1996]
Ser 273	MAPK	[Warn-Cramer, B J, et al. 1996]
Ser 279	MAPK	[Lau, A F, et al. 1996, Warn-Cramer, B J, et al. 1996]
Ser 282	MAPK	[Lau, A F, et al. 1996, Warn-Cramer, B J, et al. 1996]
Ser 325	CK1	[Cooper, C D, et al. 2002]
Ser328	CK1	[Cooper, C D, et al. 2002]
Ser 330	CK1	[Cooper, C D, et al. 2002]
Ser 364	PKA	[Shah, M M, et al. 2002]; [TenBroek, E M, et al. 2001] [Lau, A F, et al. 1996, Warn-Cramer, B J, et al. 1996]
Ser 365	PKA	[Yogo, K, et al. 2002]
Ser 368	PKC	[Lampe, P D, et al. 2000]
Ser 369	PKA	[Yogo, K, et al. 2002]
Ser 372	PKC	[Saez, J C, et al. 1997]
Ser 373	PKA	[Yogo, K, et al. 2002]

Abbreviations: Tyr: tyrosine; Ser: Serine; Tyrk: Tyrosine kinase; MAPK: Mitogen activated protein kinases; Ck1: Casein Kinase1; PKA: Protein kinase A; PKC: Protein kinase C

Table 2: Effects of protein kinases and phosphatase on GJ permeability and conductance in cardiomyocytes

Kinases	GJ permeability to metabolites	GJ conductance	References
PKA	n/a	increased	Burt JM et.al. 1988 ; De Mello WC et.al.1996
PKC	decreased	increased	Kwak BR et.al.1995; Kwak BR et.al.1996
PKG	decreased	decreased	Taken-Kwak BR et.al.1992; Burt JM et.al.1988
PTK	decreased	n/a	Doble BW et.al.2000; Doble BW et.al.1996
MAPKs	n/a	increased	Polontchouk L et.al.2002
Alkaline PP	increased	decreased	Taken-Kwak BR et.al. 1992

Abbreviations:

PKA: Protein kinase A; PKC: Protein kinase C; PKG: Protein kinase G; PTK: Protein tyrosine kinases; PP: Protein phosphatases; MAPKs:Mitogen activated protein kinases

Table 3:

Effects of protein kinases on GJ permeability and conductance in non-cardiomyocytes

Kinases	GJ permeability to metabolites	GJ conductance	References
PKA	increased	n/a	TenBroek EM et.al.2003; Paulson AF et.al.2000; Lampe PD et.al.2001
PKC	decreased	decreased	Lampe PD et.al.2000;Cruciani V et.al.2001; Ruch RJ et.al.2001
PTK	decreased	decreased	Toyofuku T et.al.1999; Lin R et.al.2001; Conttrell GT et.al.2003; Postma FR et.al 1998
MAPKs	decreased	decreased	Conttrell GT et.al.2003; Cameron SJ et.al.2003 Warn- Cramer BJ et.al.1998; Kim DY et.al.1999
CK 1	n/a	decreased	Cooper CD et.al.2003

Abbreviations:

PKA: Protein kinase A; PKC: Protein kinase C; PKG: Protein kinase G;

PTK: Protein tyrosine kinases; CK1: Casein kinase 1; MAPKs: Mitogen activated protein kinases

Phosphorylation of Cx43 by protein kinase C (PKC)

The involvement of PKC in the phosphorylation of cardiomyocyte Cx43 has been shown by using known PKC activators such as PMA (phorbol 12-myristate 13-acetate) or FGF-2 [Doble, B W, et al. 2000]; [Doble, B W, et al. 2001]; [Doble, B W, et al. 1996]. PMA increases Cx43 phosphorylation with a subsequent decrease in GJ intercellular dye-coupling.

Cx43 has several potential PKC target sites. Of these, Doble *et al.* has demonstrated that S262 becomes phosphorylated in response to FGF-2, or PMA stimulation, within the cell environment [Doble, B W, et al. 2004]. Additional PKC specific phosphorylation sites on Cx43 are S372, and S368 identified in studies *in vitro* [Shah, M M, et al. 2002]; [Lampe, P D, et al. 2000]; [Saez, J C, et al. 1997]. Both PKC sites can be phosphorylated in response to PMA treatment [Lampe, P D, et al. 2004]. S368 appears to be a major PKC site of phosphorylation *in vivo* and can cause the GJ uncoupling after PMA treatment; expression of a S368A mutant resulted in resistance to PMA-induced uncoupling effect [Lampe, P D, et al. 2000]; [Liu, T F, et al. 1999].

Protein kinase C (PKC) belongs to a large family of serine/threonine kinases that consists of at least 12 members, divided in three groups: classic, novel, and atypical. The classic PKC isoforms (α, β1, β2, γ) are activated by phorbol esters, calcium and diacylglycerol (DAG). The novel PKC isoforms (δ,ε,η,θ) are activated by phorbol esters and DAG but are not dependent on calcium [Baines CP et.al.,1999]. The atypical PKC isoforms (ξ,ι,λ,μ) are activated

by 3'-phosphoinositides but not by calcium, phorbol esters , nor DAG [Baines, C P, et al. 1999]; [Murphy, S, et al. 2005].

Of these isoforms, PKCα and PKCε have been shown to co-localize and interact directly with Cx43 in cardiomyocytes. Our laboratory has studied Cx43 phosphorylation by PKC over several years. Using dominant-negative forms of PKCε, Doble *et al.* demonstrated that PKCε is required for Cx43 phosphorylation in response to FGF-2 [Doble, B W, et al. 2001, Doble, B W, et al. 2000], and that S262 phosphorylation is required to reduce the 'hyper' phosphorylated Cx43 (>45 kDa) in response to PMA. This PKC-mediated phosphorylation at S262 was also shown to regulate the growth-inhibiting activity of C43 [Doble, B W, et al. 2004]. Phosphorylation of Cx43 via PKCα activation has been implicated in increased GJ conductance of adult guinea pig cardiomyocytes in response to treatment with the anti-arrhythmic peptide AAP10 [Weng, S, et al. 2002].

2.3 Gap junctions and connexin 43 in cardiac ischemia, arrhythmias and hypertrophy

2.3.1 Gap junction-mediated intercellular communication during ischemia

Most cardiac disorders, such as ischemic heart disease, arrhythmias and heart failure, are associated with alterations of Cx43 expression and distribution [Lo, C W 2000]; [Jongsma, H J 2000]. Ischemia causes Cx43 dephosphorylation, followed by Cx43 degradation [Beardslee, M A, et al. 2000]; [Huang, X D, et al. 1999].

Closure of cardiac GJs during acute myocardial ischemia was first proposed in 1979 [McCallister, L P, et al. 1979]. Early studies showed a reduction of GJ diameter pore following 30 minutes of ischemia [Ashraf, M, et al. 1978]. It has also been shown that 30 - 60 min of hypoxia result in the separation of intercalated discs (ICDs) membrane, and a 40-45% decrease in GJ surface density [Hoyt, R H, et al. 1990]; [Gourdie, R G, et al. 1991]. Morphological studies have shown that the density of GJ in ventricular myocardium is diminished in the post ischemic heart or in chronic hypertrophy [Kieval, R S, et al. 1992]; [Peters, N S, et al. 1993]. The number of myocytes attached to ICDs is decreased from 11 to 6-7 cells in the canine infarcted area [Luke, R A, et al. 1991]. The re-distribution of Cx43 away from the ICD area to the lateral surfaces of cardiomyocytes is also observed in the damage tissue [Peters, N S, et al. 1994]; [Severs, N J 1995b]. These results have suggested that GJ uncoupling could be a result of an alteration of GJ channel conformation and distribution.

A reduction of electrical propagation during acute myocardial ischemia is correlated with ATP depletion as well as an elevation of intracellular Ca^{2+} and H^{+} concentrations and accumulation of cellular metabolites [Kleber, A G 1987]; [Riegger, C B, et al. 1989];[Rodriguez-Sinovas, A, et al. 2003];[Padilla, F, et al. 2003]. The decline of electrical myocardial coupling after ischemia has been proposed to be a result of Cx43 dephosphorylation, leading to the translocation of Cx43 from the cell membrane to the cytosol [Beardslee, M A, et al. 2000]; [Lerner, D L, et al. 2000].

In contrast, other studies report that dephosphorylation of Cx43 opens the channels of GJs leading to increased conductance and permeablilty of GJs, and is associated with the propagation of ischemia or ischemia-reperfusion injury to adjacent myocytes [Garcia-Dorado, D, et al. 1997]. Using a dye-coupling technique to assess metabolic coupling, it was demonstrated that dye coupling persists in severely ischemic myocytes and was associated with the propagation of ischemic injury and a putative death factor via GJs [Cotrina, M L, et al. 1998, Lin, J H, et al. 1998]. Down-regulation of Cx43 levels is a feature of end-stage heart failure [Peters, N S, et al. 1993].

Arrhythmias and hypertrophy are additional ischemia-related pathologies linked to changes in cardiac GJs channels. Arrhythmia is defined as a uni-directional block of cardiac impulse transmission and the slowing of conduction leading to re-entry of cardiac electrical pathway [Hoffman, B F, et al. 1987], which is associated with GJs abnormalities. The irregularities of GJs play a significant role in arrhythmogenesis since resting membrane potential is contributed to by

ions that pass through the GJs channel. GJs remodeling and the alteration of Cx43 expression level are important causes of developing arrhythmias during ischemia [Peters, N S, et al. 1995]. Cardiac specific knockout of Cx43 in mice, which have normal heart structure and contractile cardiac function at birth, develop sudden lethal ventricular arrhythmias by 2 months of age [Gutstein, D E, et al. 2001a]. Correspondingly, the incidence of ventricular arrhythmias is raised in Cx43 deficient mice during ischemia [Lerner, D L, et al. 2000], suggesting that abnormalities of Cx43 and GJs contribute to the arrhythmogenesis.

Cardiac hypertrophy is described as a compensatory response to pressure or volume overload, which is associated with GJs remodeling. Although Cx43 levels are elevated in the first phase of hypertrophy, a significant reduction of Cx43 expression, and alterations in density and organization of GJs are noted in chronically hypertrophied hearts [Saffitz, J E, et al. 1994]; [Emdad, L, et al. 2001].

3. CARDIOPROTECTION

3.1 Introduction

Loss of heart muscle due to cell death (by either necrosis and/or apoptosis) is a common feature of many cardiovascular pathologies, such as ischemic heart disease, myocardial infarction, ischemia-reperfusion injury, cardiomyopathy and heart failure. Interventions that can raise cardiac, and cardiomyocyte resistance to injury ('cardioprotection') have been the subject of intense interest over several years since they offer the promise of protecting and even salvaging the myocardium under adverse conditions. New therapeutic strategies can furthermore be developed by understanding the signal transduction and subcellular mechanisms associated with development of an injury-resistant heart. Detailed reviews on the topic can be found in [Murphy, E 2004]; [Kardami E, et.al. 2006]; [Schulz, R, et al. 2001a]; [Cohen, M V, et al. 2000].

Established and powerful experimental procedures for achieving cardioprotection include ischemic or pharmacological preconditioning. Ischemic preconditioning, ie subjecting the heart to brief periods of ischemia and reperfusion, protects the heart from cell death and contractile dysfunction induced by subsequent prolonged ischemia. Other stress stimuli, and various pharmacological agents (detailed in [Cohen, M V, et al. 2000]) can have similar effects. More recently, *post-conditioning* (brief intermittent episodes of ischemia and reperfusion, at the onset of reperfusion *after* prolonged period of ischemia) was also shown to protect hearts from ischemia-reperfusion associated injury

and cell death [Hausenloy, D, et al. 2004]. While multiple signal transduction pathways have been implicated in generating a protective response, all seem to converge in the requirement for protein kinase C (PKC) activity, downregulation of GSK-3 activity through its phosphorylation by PKC [Juhaszova, M, et al. 2004] and the engagement of key mitochondrial entities. Acute cardioprotection is dependent on post-translational modifications of proteins such as phosphorylation, thus, proteins becoming phosphorylated downstream of PKC are of major interest as likely mediators of protection.

Diverse signaling pathways that cause cardioprotection meet at the mitochondria. Key mitochondria-associated entities, proposed to act as end-effectors or cardioprotection include the putative ATP-sensitive mitochondrial potassium channel, mitoK_{ATP} [O'Rourke, B 2004];[Gross, G J, et al. 2003], the mitochondrial permeability transition (MPT) pore, and members of the Bcl-2 family of proteins [Murphy, E 2004]. Opening of the mito K_{ATP} channel during ischemic preconditioning is proposed to be protective by causing a non-lethal transient (low conductance) opening of the mitochondrial permeability transition (MPT) pore at the inner mitochondrial membrane, and by stimulating reactive oxygen species (ROS) production [Hausenloy, D, et al. 2004]. Mitochondrial-dependent ROS can act as a second messenger to activate kinases such as PKC. Activation of PKC, however, can also occur upstream of, and promote, mitoK_{ATP} opening [Murphy, E 2004]. In contrast to the transient, low conductance MPT opening during preconditioning, the prolonged, high conductance, opening of the MPT pore occurring during the reperfusion phase, after ischemia,

depolarizes the matrix, stopping energy production and releasing cytochrome C to the cytosol, thus inducing cell death [Juhaszova, M, et al. 2004]; [Murphy, E 2004]. A wide variety of agents, acting via distinct upstream mechanisms have been shown to raise cardiac resistance to injury by limiting the induction/opening of high conductance MPT [Juhaszova, M, et al. 2004]. The balance of pro- and anti-apoptotic members of the Bcl-2 family of proteins are thought to determine formation of the votage-dependent anion channel (VDAC), that releases cytochrome C and promotes cell death.

Diverse triggers of cardioprotection require the activity of PKC. Of the several PKC isoforms identified to-date, the calcium-independent PKCε is widely accepted to be of major importance in cardioprotection. Upon activation PKCε translocates to its sites of action; it can form multimolecule-signaling modules [Vondriska, T M, et al. 2001] (thus integrating several signals) and is found at several subcellular sites, at cell-cell contact areas (intercalated disks), but also mitochondrial membranes [Baines, C P, et al. 2002]; [Baines, C P, et al. 2003]. One of the subcellular targets, and an interacting partner of PKCε, is Connexin - 43 (Cx43) [Doble, B W, et al. 2000].

3.2 Cardioprotection and FGF-2

Fibroblast growth factor-2 (FGF-2) is a member of a large family (FGF-1 to FGF-23) of heparin binding growth factors that share 50-70% of structural sequence homology [Yamashita et.al. 2000]; [Ornitz, D M 2001]; [Ornitz, D M, et al. 2001]. FGF-2 is synthesized and expressed in a variety of tissues, including astrocytes, neuronal cells, muscle cells, chondrocytes, platelets, keratinocytes, and macrophages [Abraham, J A, et al. 1986] ;[Gospodarowicz, D, et al. 1986b]; [Gospodarowicz, D, et al. 1986a]; [Basilico, C, et al. 1992]; [Baird, A 1994]. In the heart, FGF-2 is expressed in multiple cell types, such as myocytes, vascular cells, and fibroblasts, at all developmental stages [Cummins, P 1993]; [Kardami, E, et al. 1995].

FGF-2 is multifunctional. FGF-2 is a mitogen and has effects on differentiation, adhesion, migration, motility, and apoptosis [Szebenyi, G, et al. 1999]. FGF-2 has been recognized as a growth and survival factor in normal cells from different tissues, such as vascular endothelial cells, cardiac myocytes, vascular smooth muscle cells, fibroblasts, astrocytes, Schwann cells, retinal pigmented epithelial cells, gonadal cells, ovarian cells, gastric cells and hepatic cells [Chen, C H, et al. 2004]; [Conklin, B S, et al. 2004]. FGF-2 is an anti-apoptotic agent in endothelial and neural cells [Schweigerer, L, et al. 1987]. The ability of FGF-2 to act as an angiogenic factor to stimulate the growth of new blood capillaries in tissue that has undergone ischemia has been well established [Saksela, O, et al. 1987]; [Klagsbrun, M, et al. 1988]; [Folkman, J, et al. 1988].

FGF-2 exerts its biological functions by interacting with specific cell surface tyrosine kinase receptors. FGFR1 is the predominant FGF-2 receptor in the heart [Szebenyi, G, et al. 1999]. FGFR1 is activated by FGF-2, inducing FGFR1 dimerization and consequent transphosphorylation, which initiates the FGF-2-FGFR1 signaling complex, as shown in Figure 3. FGF2- FGFR1 results in activation of several signal transduction pathways including the PKC pathway, which is involved in cardioprotection. FGF-2 regulation of PKC activation results in a reduction of cell-cell permeability and may be associated with opening of K_{ATP} channels, all of which contribute to cardioprotection.

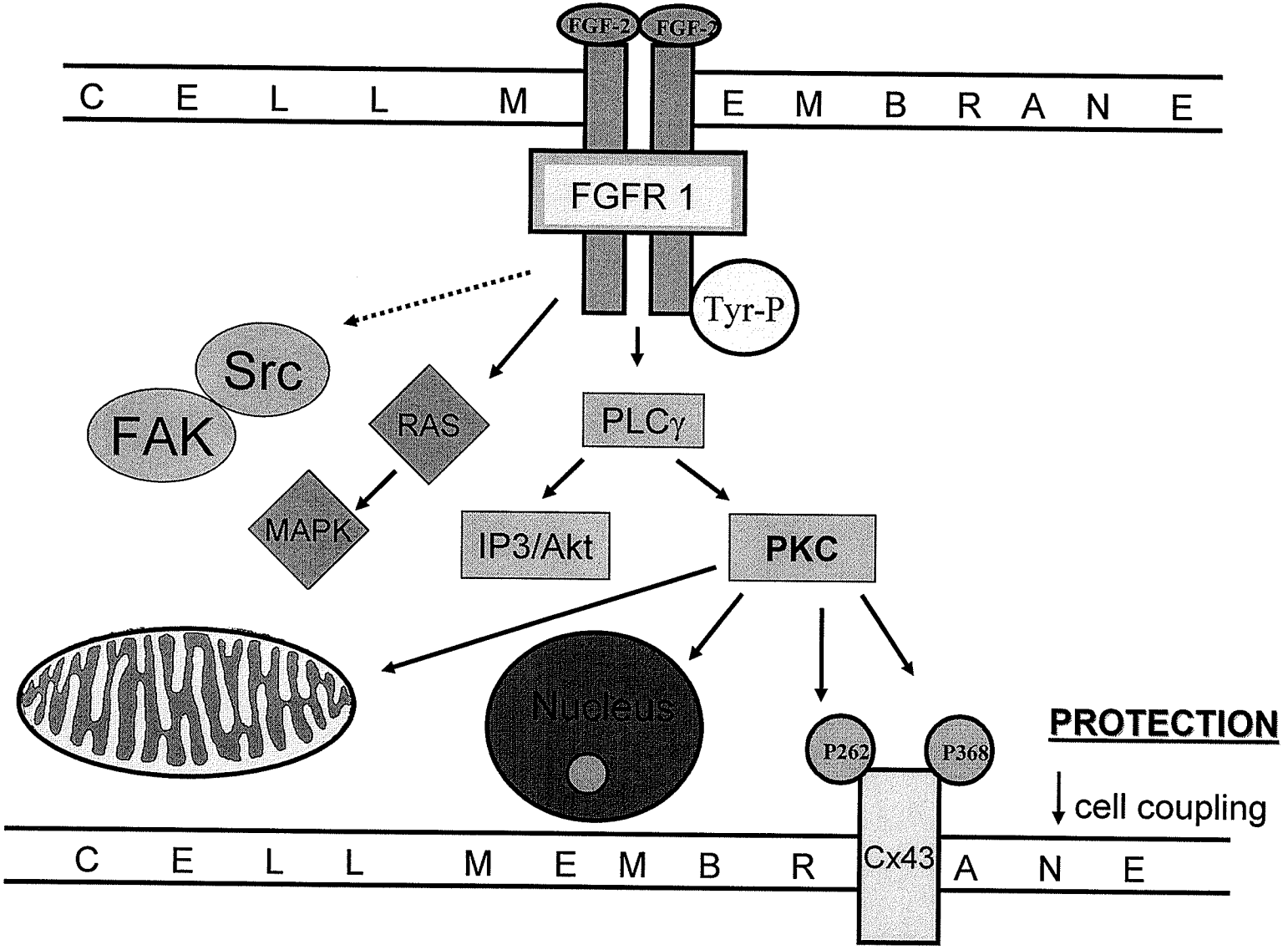
FGF-2 exerts substantial beneficial effects in the heart, acutely as well as long term. While the long term benefits of FGF-2 are mediated by its angiogenic activity, as reviewed recently by Dettilieux et al. [Detillieux, K A, et al. 2004], acute benefits are derived from direct cytoprotective effects on the myocardium [Kardami E, et.al.2006].

FGF-2 exerts cardioprotection in a pre-conditioning as well as post-conditioning like fashion. Administration of FGF-2 prior to ischemia protects the heart from tissue injury and contractile dysfunction resulting from ischemia-reperfusion [Padua, R R, et al. 1998, Padua, R R, et al. 1995]. FGF-2, given to the heart during reperfusion after 30 minutes of ischemia remains cardioprotective [Jiang, Z S, et al. 2002, Jiang, Z S, et al. 2004]. Injection of FGF-2 directly into the ischemic myocardium during irreversible coronary occlusion confers acute protection from tissue loss and functional decline [Jiang, Z S, et al.

2002, Jiang, Z S, et al. 2004]. Chronic FGF-2 overexpression in transgenic mouse hearts raises resistance to ischemic injury [Sheikh, F, et al. 2001].

FGF-2 cardioprotection is associated with the activation of several PKC isoforms, including PKCε and PKC∀, requires binding to its tyrosine kinase receptors and is abolished when PKC is inhibited by chelerythrine [Padua, R R, et al. 1998]; [Jiang, Z S, et al. 2002]. Chelerythrine also blocks the FGF-2 induced Cx43 phopshorylation [Doble, B W, et al. 2000].

Figure 3: Signaling pathways involved in FGF-2 and Cx43 phosphorylation



3.3 Cardioprotection and Cx43

It is becoming increasingly evident that Cx43 is actively involved in the regulation of cellular and tissue vulnerability to ischemic injury. Work from non-muscle systems has pointed to Cx43 mostly as a cytoprotective molecule [Nakase, T, et al. 2003]. In the heart, Schwanke *et al.* demonstrated that the Cx43 (+/-) mice, expressing ~50% of the normal Cx43 levels, are incapable of cardioprotection by ischemic preconditioning and thus proposed that Cx43 is essential for the development of resistance to injury [Schwanke, U, et al. 2002]. In apparent contrast, another group used the same mouse model and reported that Cx43-defficiency caused smaller infarcts upon coronary ligation, and concluded that Cx43 is actually increasing vulnerability to injury [Kanno, S, et al. 2003]. However Cx43 can also promote vascular growth [Walker, D L, et al. 2005] ,the decreased infarcts in the Cx43 (+/-) hearts may be an indirect effect of reduced re-vascularization.

The role of intercellular communication (GJIC) *per se* in cytoprotection is not well understood, although there is some evidence that cardioprotection by ischemic preconditioning requires decreased GJ channel permeability [Miura, T, et al. 2004]. GJ have been implicated in the propagation of injurious stimuli and the spread of contracture in the ischemic heart [Garcia-Dorado, D, et al. 2004], thus the protective effects of volatile anesthetics has been attributed to their ability to decrease GJ permeability. In addition to studies implicating the channel-forming ability of Cx43 as mediating its ability to regulate response to

injury, there is also evidence that Cx43 can induce cardioprotection in the absence of intercellular communication [Li, X, et al. 2004]

As mentioned, Cx43 is relatively abundant at cardiomyocyte ICDs. There is now evidence that Cx43 may be also found at other cellular locations as well. Studies using endothelial cells first reported that oxidative stress upregulated Cx43 expression, and induced translocation of Cx43 from the plasma membrane to an intracellular compartment, identified as the mitochondria [Li, X, et al. 2004]. Subsequently, a series of elegant and convincing studies by Heusch and colleagues [Boengler, K, et al. 2005] demonstrated that (i) Cx43 can also be found at the inner mitochondrial membrane of cardiomyocytes, (ii) ischemic preconditioning is accompanied by translocation of Cx43 to mitochondria [Dhein, S 2005]; [Boengler, K, et al. 2005], and (iii) there is a functional role for mitochondria-Cx43. Cx43 is needed for diazoxide-induced mitochondrial reactive oxygen species, (ROS), generation [Heinzel, F R, et al. 2005]. These authors proposed that since ROS generation is required for ischemic preconditioning, a defect in ROS production, caused by Cx43 defficiency, could explain lack of cardioprotection by ischemic preconditioning in Cx43 (+/-) mice.

4. RATIONAL FOR PROPOSED STUDIES

Acute cardioprotection by FGF-2 and other triggers is dependent on PKC activation and PKC-mediated post-translational modification of proteins. Identification of downstream targets of PKC activation in the adult heart therefore will improve understanding of the mechanism of cardioportection. The objective of the current study was to investigate whether Cx43 is such a target in the adult heart.

To address these objectives, we examined the following hypotheses:

(1) FGF-2 will stimulate Cx43 phosphorylation at PKC target sites (S262, S368) in the adult heart

(2) FGF-2 administered prior to ischemia will prevent ischemia-induced Cx43 dephosphorylation and redistribution away from ICDs.

MATERIALS AND METHODS

1. MATERIALS

1.1 Animal models:

Male Sprague-Dawley rat (200-250g) provided by the Central Animal Care Facility at the University of Manitoba were used in all experiments. Animals were used according to guidelines of the Canadian Council of Animal Care, in agreement with the Local Animal Care Committee of the National Research Council of Canada.

1.2 Antibodies:

All antibodies have been fully characterized. The following antibodies were used: (1) Rabbit polyclonal anti-Cx43 antibody, raised against aa 367-382 (#p.AB), has been characterized by [Doble, B W, et al. 2000]. (2) Mouse monoclonal anti-Cx43 antibody (#m.AB), raised against aa 250-270, was purchased from Transduction Laboratories (CA) and was characterized by Doble *et al.* [Doble, B W, et al. 1996]. (3) Mouse monoclonal antibody (#13-800), raised against amino acid 360-376, and recognizing only dephosphorylated or recognizing the dephosphorylated or minimally phosphorylated Cx43 [Nagy, J I, et al. 1997]; [Cruciani, V, et al. 1999] was purchased from Zymed Laboratories (CA). (4) Rabbit polyclonal anti-P262 antibody (#Anti-P262-Cx43), recognizing the phosphorylated form of S262 on Cx43, was purchased from Santa Cruz Biotechnology (CA). (5) Rabbit polyclonal anti-P368 antibody (#Anti-P368-Cx43), recognizing the phosphorylated isoform of S368 on Cx43, was purchased from Chemicon Laboratories (CA). This has been characterzed by Lampe PD *et al.*

[Lampe, P D, et al. 2004, Lampe, P D, et al. 2000] (6) Anti rabbit- and (7) anti mouse- horse radish peroxidase (HRP) secondary antibodies were purchased from Bio-Rad (CA).

1.3 Chemicals and solutions:

Krebs-Henseleit (K-H) perfusion solution (118 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 246.5 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 180.2 mM glucose) was filtered (1.2 µm, Millipore) before use. The K-H perfused solution pH 7.4 was constantly gassed using 95%O_2 and 5% CO_2, and maintained at a temperature of 37 °C.

2. METHODS

2.1 Source of Fibroblast growth factor-2 (FGF-2)

Recombinant wild type (the 18 kDa, AUG-initiated isoform) FGF-2 was produced in *Escherichia coli* bacteria and purified to homogeneity in our laboratory according to standard protocols as published previously [Jiang, Z S, et al. 2002]. It was used within one month of preparation.

2.2. Whole heart perfusion using Langendorff preparation

Male adult Sprague-Dawley rats (200-250 g) were anesthetized, using Ketamine and Xylazine, and sacrificed by decapitation. Hearts were rapidly removed, washed in cold buffer, and the atria, extraneous fat and connective tissue were trimmed off. A short cannula was inserted in the aorta and hearts perfused with oxygenated K-H solution at a constant pressure of 80 mmHg under non-recirculating conditions. FGF-2 (10 μg) was dissolved in 12 ml of K-H solution and infused at the position of entry to the heart via retrograde perfusion by a peristaltic pump. FGF-2 was used at a concentration inducing a cardioprotective effect against ischemia-reperfusion-induced contractile dysfunction and tissue damage, as documented by our laboratory in several previous publications [Padua, R R, et al. 1995];[Padua, R R, et al. 1998];[Jiang, Z S, et al. 2002]; [Jiang, Z S, et al. 2004].

2.3 Experimental design

2.3.1 FGF-2 treatment.

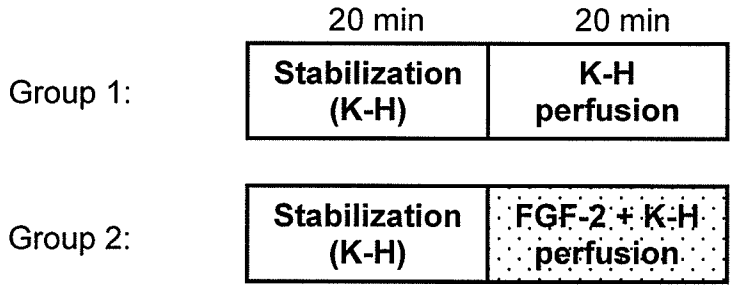
Two groups of perfused hearts (n=6/group) were used in the first set of experiments. Both groups were subjected to a stabilization period, consisting of 20 min perfusion with K-H. Hearts from group 1 were continued to be perfused by K-H alone, while the hearts from group 2 were perfused with FGF-2 in K-H, for 20 min. Hearts were then processed for extraction and immunofluorescence (Figure4).

2.3.2 FGF-2 pretreatment followed by ischemia.

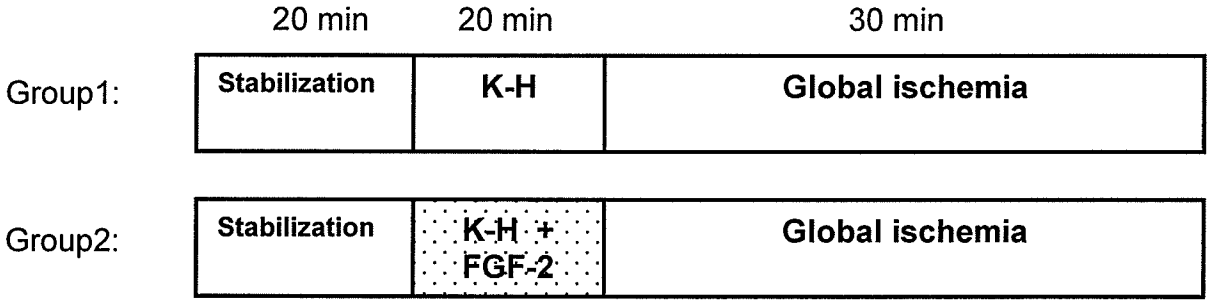
The second series of experiments followed exactly the same experimental design as in the first series of experiments. Hearts from both groups (n=7/group) were then subjected to 30 min of global ischemia. Hearts were then processed for extraction and immunofluorescence (Figure 4).

Figure 4: Scheme of experimental protocol

2.3.1 FGF-2 treatment .



2.3.2 FGF-2 pretreatment followed by ischemia.



2.4 Cardiac tissue extraction

Immediately after perfusion, hearts were frozen in liquid nitrogen and stored at -80°C. All subsequent procedures were done at 4°C. Ventricular tissue (50 mg) was powdered using a mortar and pestle with liquid nitrogen then transferred to a 15 ml tube containing 500 µl (approximate 10 volumes/ weight) cold TNM buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 300 mM sucrose, 2 mM $MgCl_2$, 1% thiodiglycol, 60 mM β-glycerophosphate, and 10mM NaF) with 0.25% NP40. Protein Inhibitor Cocktail (PIC; Sigma # P8340), and Phosphatase Inhibitor Cocktails 1 and 2 (PPIC1; Sigma #P2850; PPIC2; Sigma#P5726) at dilutions of 1:100 were added to all buffers. Following homogenization with a glass homogenizer, the homogenate was filtered using Nitex in a Swinnex filter to remove large insoluble material. An equal volume of 2X SDS buffer (20% glycerol, 100mM Tris-HCl pH 6.8, 2% SDS, 60 mM β-glycerophosphate, 5mM EDTA, 5mM EGTA, 2mM NaOV, 1mM NaF) with inhibitors was added and the samples sonicated at 40 Hz for 3x5 seconds. The extract was boiled for 5 minutes and centrifuged at 21000 g for 15 min at 4°C. The supernatant was collected and stored at -80 °C for further analysis. The pellet was discarded. Protein concentration was determined using the BCA assay.

2.5 SDS-PAGE gel electrophoresis, western blotting

Total cardiac extracts were analyzed on 10 % polyacrylamide gels at 30 µg/µl of protein per lane with molecular weight markers from BioRad (broad range: 6.5- 200 kDa) and Invitrogen benchmark (6.0-181.8 kDa). Following electrophoresis, protein was transferred to PVDF (Polyvinylidene difluoride) membrane (Roche) and stained with Ponceau Red for 5 min to confirm effective transfer and also to evaluate equivalent loading.

For immunodetection, all steps were done at room temperature unless indicated (Table 3). To block non-specific protein binding sites, the membrane was incubated for 1 hour in 3% or 5% BSA (98% Albumin, bovine serum; Sigma) or 10 % skim milk in Tris buffered saline with TWEEN-20 (TBST), as shown in table 3. Following blocking, the membrane was rinsed briefly with TBST. Primary and secondary antibodies were used as indicate in table 4, 5. Finally, the membrane was rinsed twice and washed for 15 min, followed by 3x5 min of washing in TBST. Antigen-antibody complexes were detected by enhanced chemiluminescence (ECL$^+$ plus; Amersham Biosciences) and exposure to Kodak X-omat LS film.

Table 4: Primary antibodies used in western blotting

Antibodies	Blocking solution	Primary Ab Conc.	Incubation condition
# p.AB	10% milk	1:15000 in 1% milk	1 hour/ room temperature
# m.AB	10% milk	1:1000 in 1% milk	1 hour/ room temperature
# 13-800	10% milk	1:1000 in 1% milk	1hour/ room temperature
# Anti-P262-Cx43	3% BSA	1:2000 in 1%BSA	1 hour/ room temperature
# Anti-P368-Cx43	5% BSA	1:1000 in 5%BSA	overnight/ 4°C

Table 5: Secondary antibodies used in western blotting

Primary Antibodies	Secondary Antibodies	Secondary Ab Conc.	Incubation condition
# p.AB	Goat anti-rabbit HRP	1:10000 in 1%milk	1 hour/ room temperature
# m.AB	Goat anti-mouse HRP	1:10000 in 1% milk	1 hour/ room temperature
# 13-800	Goat anti-mouse HRP	1:10000 in 1% milk	1 hour/ room temperature
# Anti-P262-Cx43	Goat anti-rabbit HRP	1:10000 in 1%BSA	1 hour/ room temperature
# Anti-P368-Cx43	Goat anti-rabbit HRP	1:10000 in 1%BSA	1 hour/ room temperature

2.6 Densitometric analysis

Band density was determined using the Bio-Rad Model GS-800 densitometer with Molecular Analyst software (Bio -Rad).

2.7 Cardiac tissue sectioning and immunofluorescent labeling

The middle and apex portion of cardiac tissues obtained after Langendorff perfusion with or without FGF-2, were immediately frozen in OCT in a dry ice /ethanol bath and stored at -80°C. Transverse cryosections of 7 μm thickness were consistently obtained using a Microm HM550 cryostat. Sections on slides were fixed in freshly made cold 1% paraformaldehyde in PBS for 15 minutes immediately after sectioning, and washed extensively with PBS. Sections were placed in a humid chamber and incubated overnight with primary antibodies diluted in 1% BSA /PBS. Primary antibodies were used as shown in Table 5. Cardiac sections stained with primary antibodies were then washed gently 4x5 min with PBS and incubated for 1 hour at room temperature with biotinylated anti-rabbit IgG (Amersham Biosciences) at 1:20 dilution in 1% BSA/PBS followed by Strepavidin fluorescein (Amersham Biosciences) at 1:20 dilution. Sections stained with mouse primary antibodies were incubated with Texas Red conjugated-anti-mouse IgG (Jackson Laboratories) at 1:100 in 1% BSA/PBS. Nuclei were labeled with 2.5 mM Hoechst dye 33342 (Calbiochem) for 1 min and rinsed thoroughly. Cover slips were mounted using IgG Prolong antifade medium (Molecular Probes) and slides were stored at 4°C until observation.

Table 6: Antibodies used in immunofluoresecent labeling

Primary Antibodies	Detects	Primary Ab Conc.
# p.AB	Total Cx43	1:2000
# m.AB	Total Cx43	1:100
#13-800	DP-Cx43	1:200
#Anti-P262-Cx43	P262-Cx43	1:200
#Anti-P368-Cx43	P368-Cx43l	1:500

Abbreviations:
(p):Rabbit polyclonal
(m): mouse monoclonal
DP-Cx43: Dephosphorylated Cx43
P-262: Phosphorylation of serine 262
P-368: Phosphorylation of serine 368

2.8 Epifluorescence Microscopy

Cardiac sections were examined by epifluorescence microscopy (Zeiss Axiovert 3.0). Images were obtained using three filters (FITC: green, Texas Red: red and DAPI: blue) for reflected immunofluorescence. A series of images was obtained at 1 μm intervals of tissues depth with the aid of a mechanized stage and the AxioVision Program. To reduce inconsistency between measurements, standardized parameters for imaging, objective and exposure time were kept constant between sections.

2.9 Statistical analysis

All statistical data is presented as means ± SEM. using the Graph Pad Instat 3.0 statistical software program and Microsoft Excel. Differences between groups were compared using the student's t-test (unpaired) and ANOVA. $P < 0.05$ and $P < 0.01$ were considered significant.

RESULTS

1. The effect of FGF-2 on Cx43 in the perfused, non-ischemic heart.

1.1 FGF-2 and Cx43 abundance, phosphorylation status and distribution.

To determine whether FGF-2 could affect Cx43 in adult cardiomyocytes in the context of the whole isolated heart we administered this protein by perfusion, at 10 ug/heart, in K-H buffer, over a period of 20 minutes, following procedures established in our laboratory [Padua, R R, et al. 1995]. Control hearts were simply perfused with K-H, over the same time period. Hearts were then processed for cryosectioning and extraction/ western blotting.

Cx43 distribution in the different groups was observed using immunofluorescence with several different antibodies recognizing either total Cx43 (m.AB, p.AB), or DP-Cx43 (#13-800). As shown in Fig.5, staining with p.AB localized Cx43 to the intercalated discs (ICDs) producing the expect punctate pattern, in both untreated (Fig.5A) and FGF-2-treated (Fig. 5B) hearts. Staining of the FGF-2-treated hearts however appeared more intense compared to non-tretated hearts. As expected, #13-800 antibodies (DP-Cx43) did not stain ICDs in either the FGF-2-treated or control group (data not shown).

Western blot analysis of heart lysates is shown in Fig.6. Both FGF-2-treated and non-treated hearts contained p-Cx43 migrating at 44-45 kDa. The intensity of anti-Cx43 bands was however stronger in the FGF-2-treated group. Statistical analysis indicated a statistically significant increase by nearly 50% in Cx43 accumulated in the FGF-2, compared to the control, group. There was no detectable DP-Cx43 at 41 kDa.

1.2 FGF-2 and Cx43 phosphorylation at PKC sites

Previously, our laboratory has shown that neonatal myocytes respond to FGF-2 by increasing Cx43 phosphorylation at the PKC site S262 [Doble BW et.al. 2004]. We now asked whether adult cardiomyocytes in the perfused heart model would respond in a similar manner. Thus we probed sections from FGF-2-treated or control hearts with antibodies specific for P-S262-Cx43, or for P-S368-Cx43. Both S262 and S368 are PKC target sites. Results are shown in Fig.7 and Fig.9. Control hearts had weak staining for either P-S262- (Fig.7A), or P-S368-Cx43 (Fig.9A). In contrast, FGF-treated hearts displayed strong staining for both of these antibodies, indicating that FGF-2 stimulated the phosphorylation of Cx43 at S262 (Fig. 7B) and S368 (Fig. 9B).

Comparable results were obtained by western blotting. Staining of the control group was at background levels under the conditions of this experiment while the FGF-2-treated group clearly displayed anti-P-S262-Cx43 immunoreactive band(s) (Fig.8). The immunoreactive band(s) migrated at 45 kDa (by comparison to molecular weight markers), as we reported previously for neonatal myocytes [Doble, B W, et al. 2004]. As was the case for S262, FGF-2 upregulated relative levels of P-S368-Cx43 significantly (Fig.10). In this case the immunoreactive Cx43 band(s) migrated slightly slower than 41 kDa, but clearly below the 45 kDa mark.

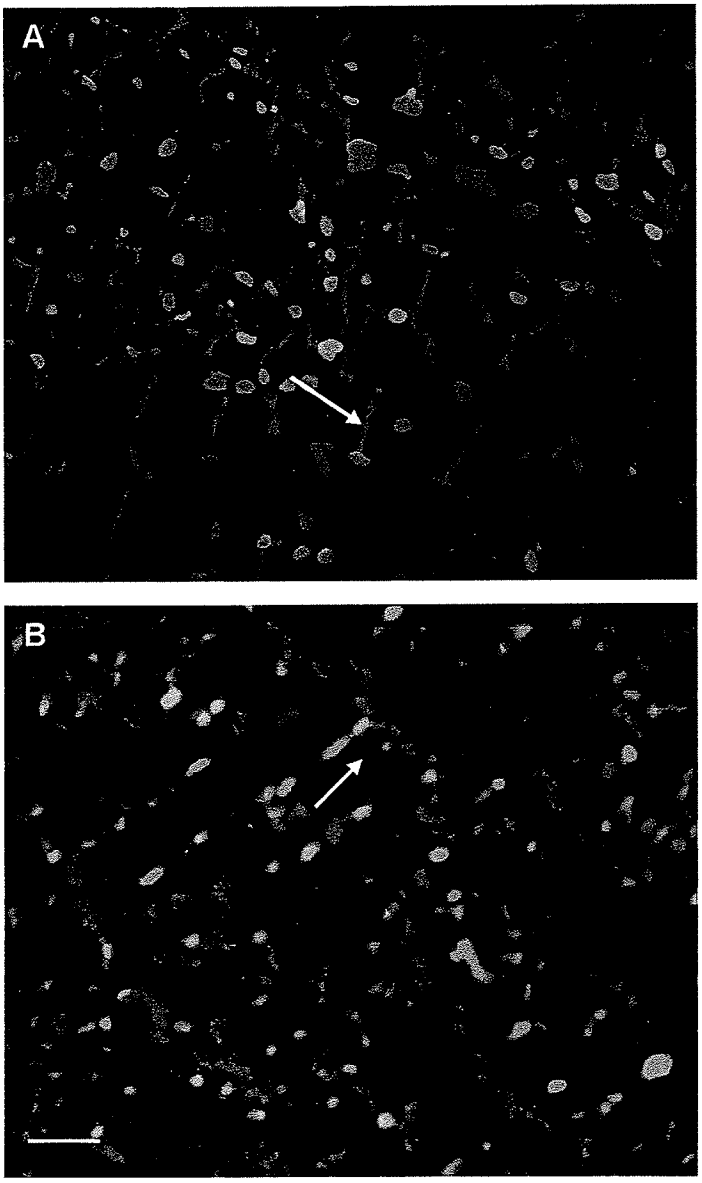


FIGURE 5: Total Cx43 localization in control and FGF-2 treated non-ischemic hearts. Localization of Cx43 in cardiac sections from control (A) and FGF-2 treated (B) hearts, using p.AB recognizing total Cx43 (green). Sections have been counter-stained with Hoechst 33342, to visualize nuclei (blue). Arrows point to ICDs.
Bar = 50 μm.

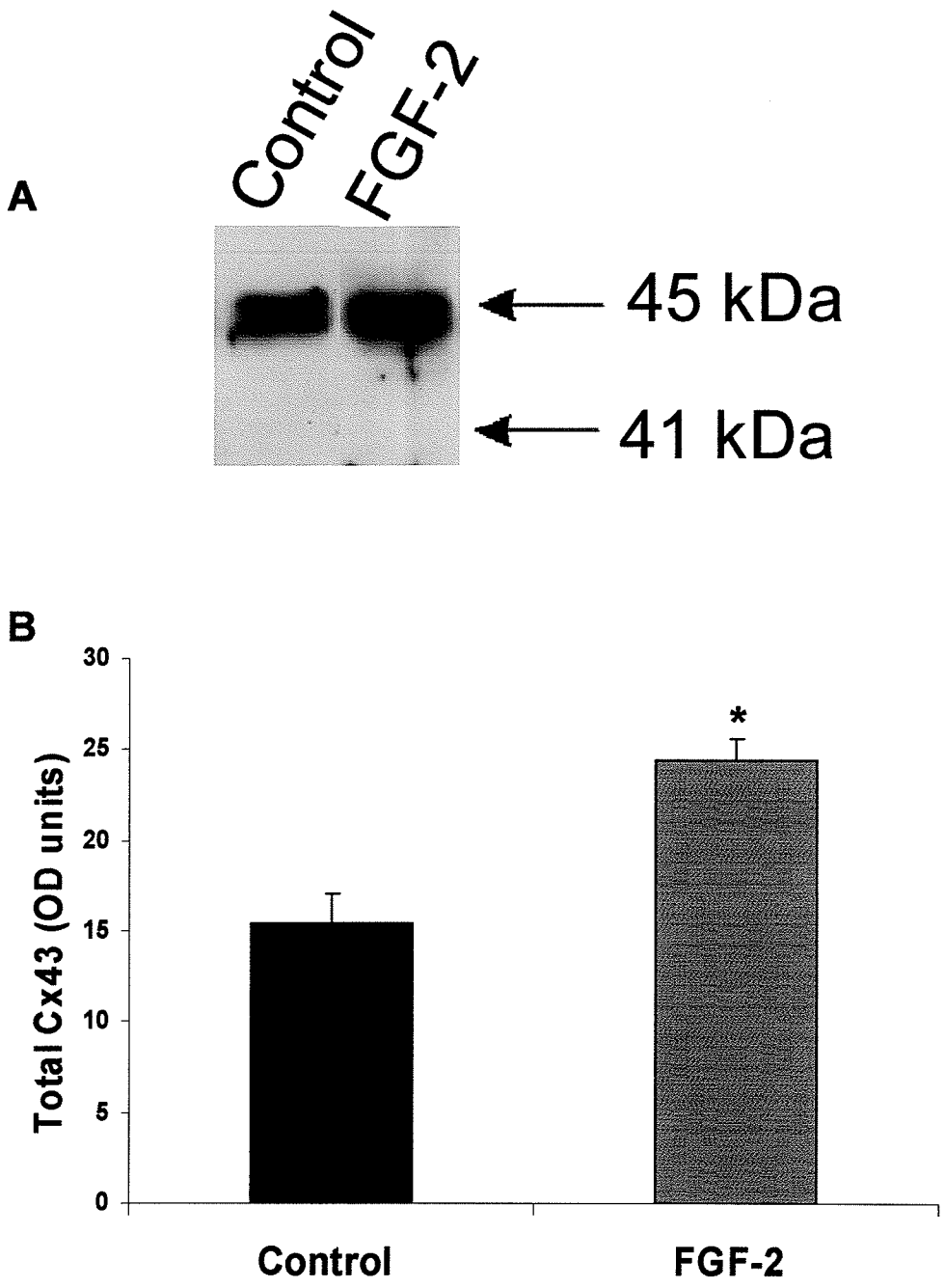


FIGURE 6: Effect of FGF-2 on Cx43 levels

Western blot of extracts from control or FGF-2 treated hearts were stained for total Cx43, using p.AB (A). Cx43 levels were compared by densitometry of the bands on western blots (B). Data are expressed as mean ± SEM. Asterisk (*) indicates statistically significant differences between control and FGF-2 treated hearts ($P<0.05$, $n=6$)

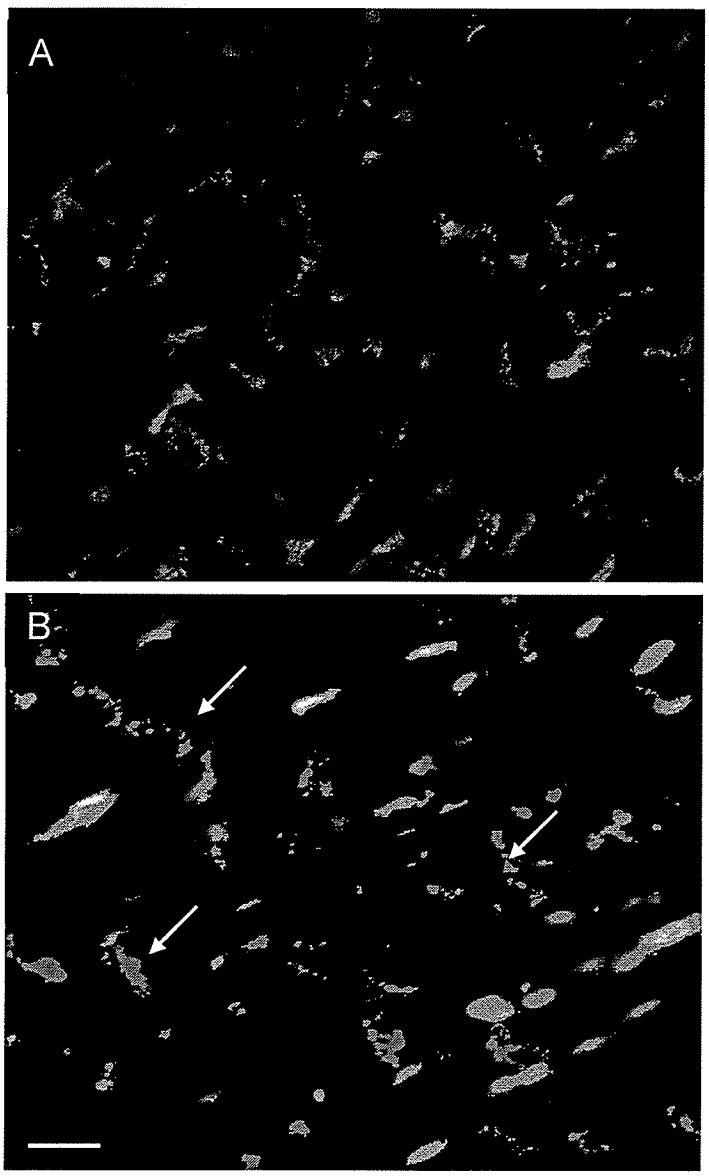


FIGURE 7: FGF-2 increases Cx43 phosphorylation at S262 in control and FGF-2 treated non-ischemic hearts. Localization of P-S262-Cx43 in cardiac sections from control (A) and FGF-2 treated hearts, using a polyclonal antibody recognizing P-S262-Cx43 (green). Sections have been counter-stained with Hoechst 33342, to visualize nuclei (blue). Arrows point to ICDs. Bar = 50μm.

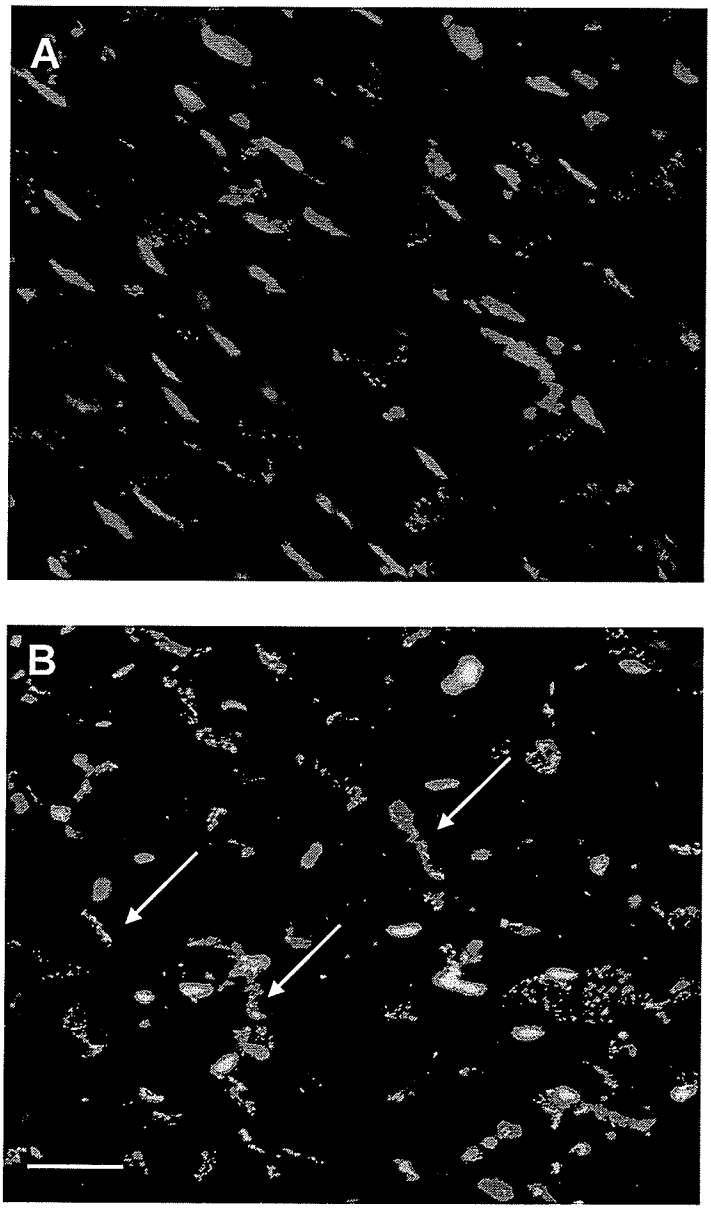


FIGURE 9: FGF-2 stimulates Cx43 phosphorylation at S368 in control and FGF-2 treated hearts. Localization of P-S368-Cx43 in cardiac sections from control (A) and FGF-2 treated hearts (B), using a polyclonal antibody to P-S368-Cx43. Sections have been counter-staining with Hoechst 33342, to visualize nuclei (blue). Arrows point to ICDs. Bar = 50μm.

2. The effect of ischemia on Cx43 in FGF-2 treated or untreated hearts.

2.1 Effect of ischemia (+/-FGF-2) on Cx43 levels distribution and phosphorylation.

Hearts pre-treated with K-H, or FGF-2, as in the previous section, were subjected to 30 min. of global normothermic ischemia. These groups will be referred to as I (-), or I (+), respectively. A third group was not subjected to ischemia (non-I), but was perfused with K-H for the duration of the experiment. Cx43 distribution, phosphorylation and abundance were examined subsequently, using the same antibodies as in the previous section. Results are shown in Figures 11-20.

As shown in Fig.11 and 12, Cx43, visualized either by p.AB or by m.AB. was distributed at ICDs in the non-I group (Fig.11A, Fig 12A). In contrast, the ischemic I (-) group presented Cx43 distribution not only at ICDs but also at lateral surfaces of cardiomyocytes (Fig.11C, Fig12B). The I (+) group on the other hand had Cx43 distribution restricted at ICDs (Fig.11E, Fig12C), in a manner similar to non-I hearts. FGF-2 treatment therefore prevented the ischemia-induced Cx43 redistribution at ICDs.

We also probed for DP-Cx43, with #13-800 antibodies. As expected, no staining was detected for the non-I group (Fig.11B). In contrast, the I (-) group displayed strong staining at ICDs as well as lateral surfaces (Fig 11D). Staining with this antibody was absent from the I (+) group (Fig.11F). FGF-2 therefore prevented the ischemia-induced Cx43 dephosphorylation at ICDs.

Western blotting was used to detect and quantitate total Cx43 as well as relative levels of P- and DP-Cx43 in the various groups. As shown in Fig.13, total levels of Cx43, assessed by either p.AB or m.AB were similar in all group (non-I, I (-), and I (+)). The p.AB detected mainly 44-45 kDa P-Cx43 in the non-I group , and mainly the 41-42 kDa DP-, or minimally phosphorylated, -Cx43 in the I(-) group. The I (+) group had mainly 44-45 kDa P-Cx43, as shown in Fig 14A. Similar results were also confirmed by m.AB (Fig14 B). We also used specific DP-Cx43 antibody, #13-800. As we expected, only the I (-) group was clearly detected by the anti-DP-Cx43 (Fig 14C). Taken together, these data indicated that Cx43 in the ischemic-FGF-2-treated group was phosphorylated (Fig 15). It also confirmed that FGF-2 prevented the ischemia-induced Cx43 dephosphorylation.

2.2 Effect of ischemia (+/-FGF-2) on Cx43 phosphorylation at PKC sites

We examined the effect of ischemia, in the presence or absence of FGF-2 pre-treatment, on Cx43 phosphorylation at S262, S368. Results are presented in Figures 16 and 17, respectively. Immunofluorescence of tissue sections showed that while I (-) hearts stained faintly for P-S262- (Fig.16A) or P-S368-Cx43 (Fig.17A), hearts in the I (+) group displayed strong staining for these antibodies at ICDs, as shown in Figures 16B :P-S262 and Figure 17B :P-S368-Cx43.

Western blots showed essentially similar results. Hearts in the I (-) group had minimal levels of P-S262- or P-S368-Cx43 (Figs 16C and 17C, respectively). On the other hand, hearts from the I (+) group had strong immunoreactivity for P-S262- or P-S368-Cx43 (Figs 16C and 17C respectively). P-S262-Cx43 migrated

at approximately 45 kDa, while P-S368-Cx43 migrated at approximately 41 kDa. The increased phosphorylation of Cx43 at PKC sites in response to FGF-2 is therefore maintained in the ischemic heart.

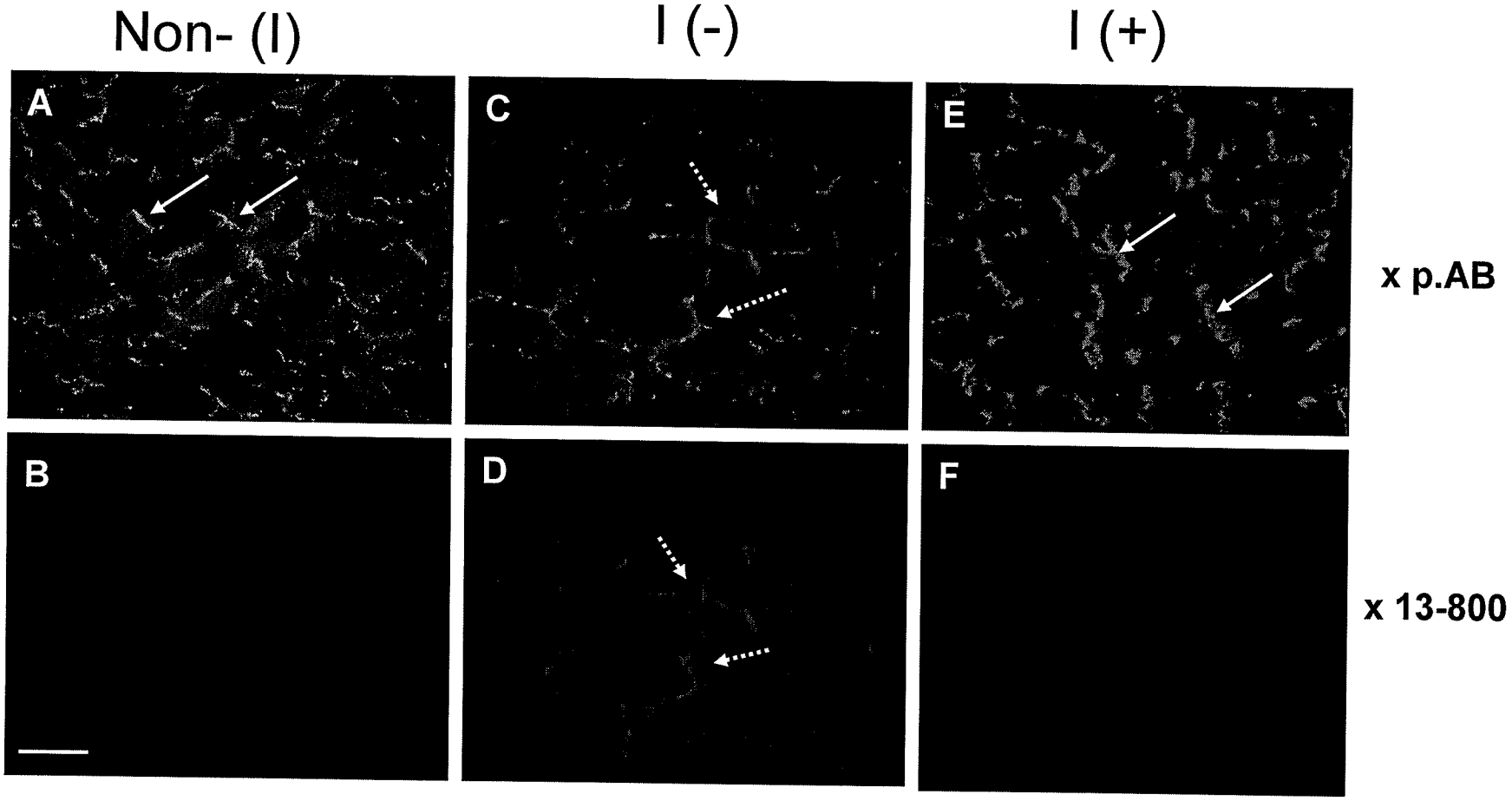


Figure 11:FGF-2 prevents ischemia-induced Cx43 dephosphorylation and redistribution away from ICDs.
Double immunofluorescence staining for total Cx43 (A,C,E) or Cx43 dephosphorylation (B,D, F), using p.AB, and #13-800, as indicated. (A,B), non-ischemic, non-FGF-2 treated hearts, (C,D), ischemic, non-FGF-2 treated hearts, (E,F), ischemic, FGF-2-treated hearts. Arrows point at ICDs. Broken arrow point at lateral myocyte surfaces. Bar=50μm

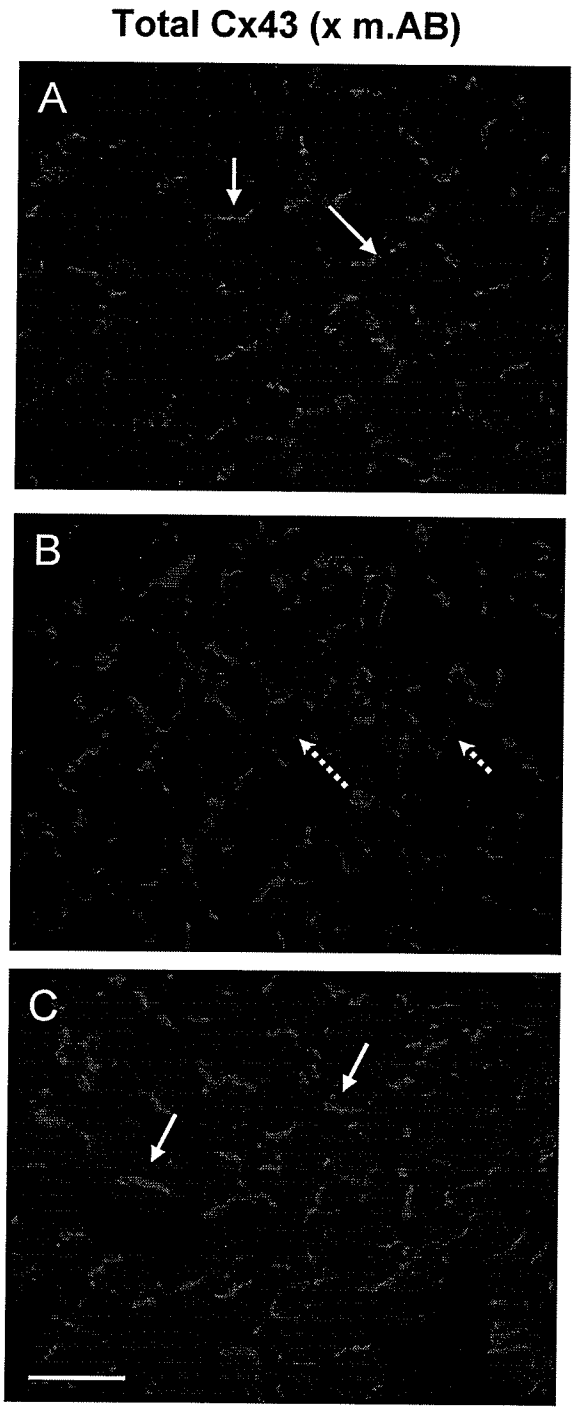


FIGURE 12: FGF-2 prevents ischemia-induced Cx43 redistribution away from ICDs. Immunofluorescence staining for total Cx43 using m.AB. (A), Non-ischemic, non-treated hearts, (B), ischemic, non-treated hearts, (C), ischemic FGF-2-treated hearts. Arrows point at ICDs. Broken arrows point at lateral myocyte surfaces. Bar=50 μm.

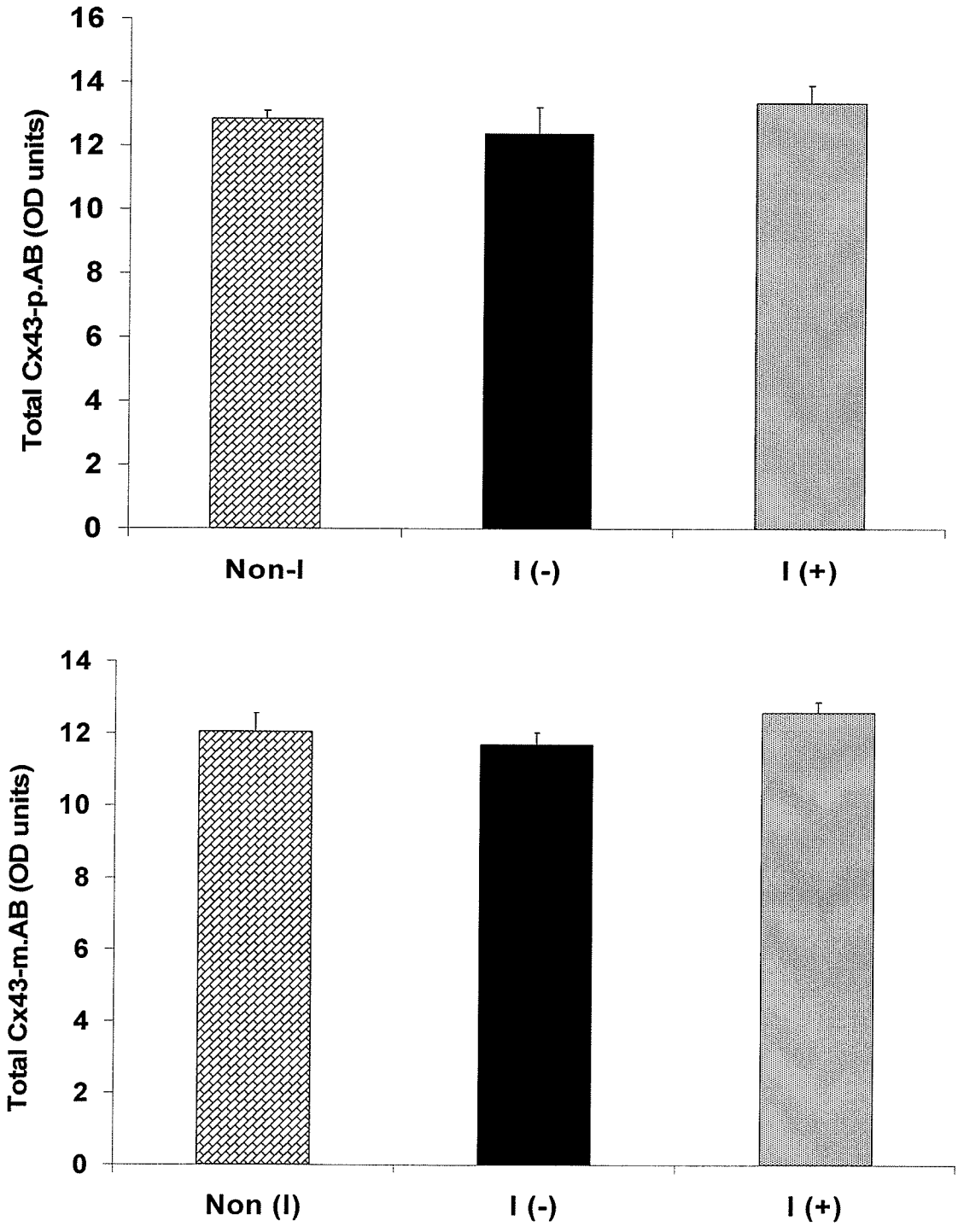


Figure 13: Ischemia (30 min) does not change Cx43 level. (A): Relative levels of total Cx43 were estimated by densitometry of p.AB probed western blots. (B): Relative levels of total Cx43 were estimated by densitometry of m.AB probed western blots.

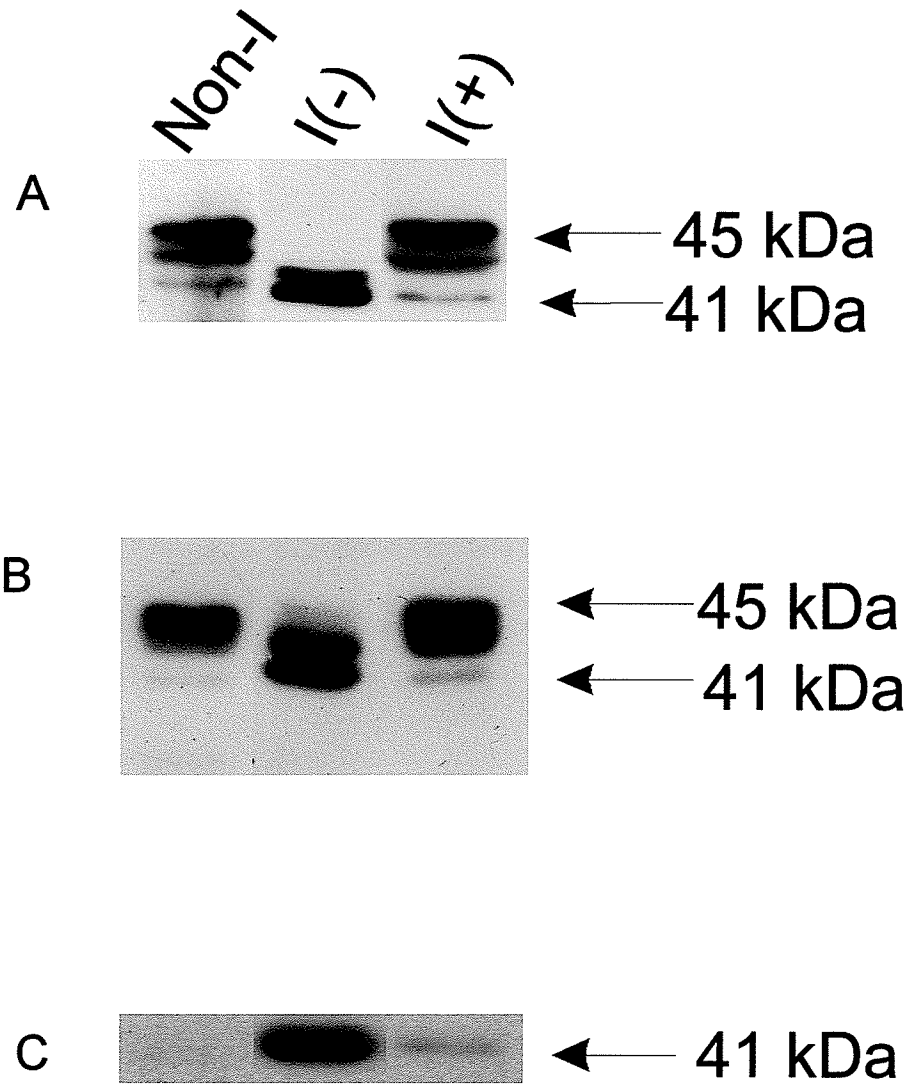


Figure 14: FGF-2 prevents ischemia-induced Cx43 dephosphorylation (i).
Western blottings were probed with (A):p.AB, (B):m.AB, (C): #13-800.

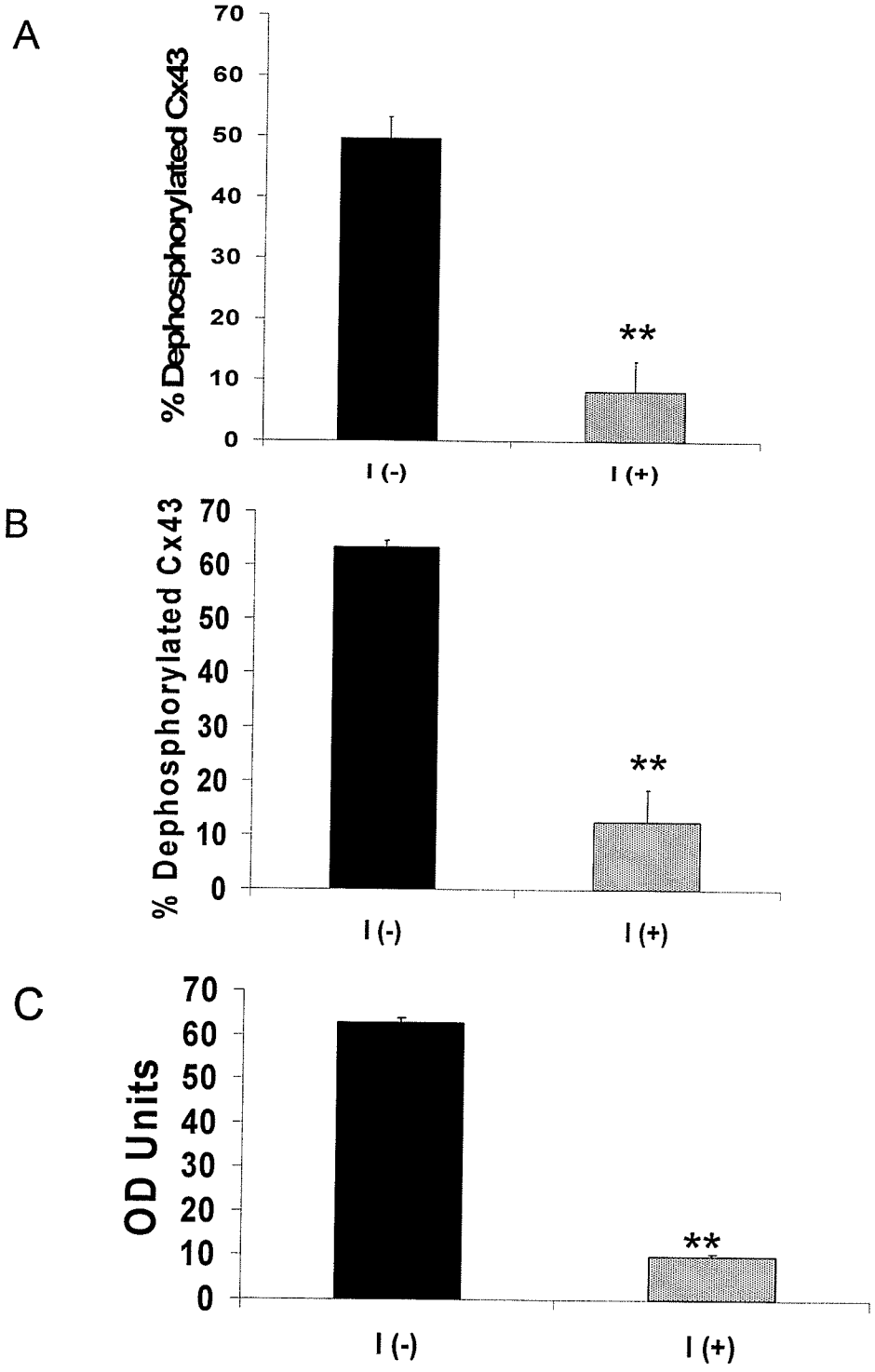


Figure 15: FGF-2 prevents ischemia-induced Cx43 dephosphorylation (ii)
Western blottings were probed with (A):p.AB, (B):m.AB, (C): #13-800. Data are expressed as mean±SEM. Asterisks (**) ($P<0.01$; n=7) indicate statistically significant differences between I (-) and I (+) hearts.

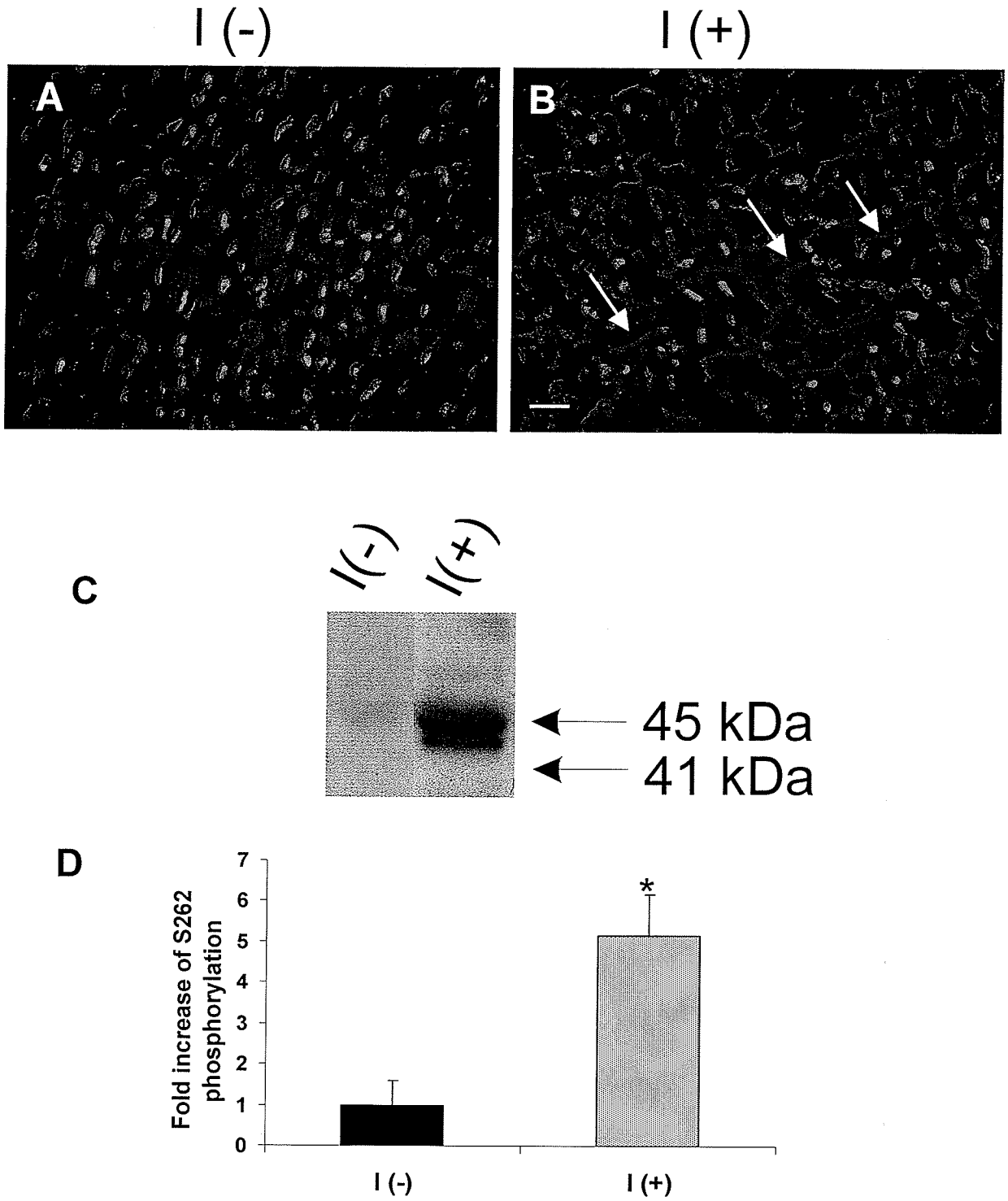


Figure16: Effect of ischemia (+/- FGF-2) on Cx43 phosphorylation at S262

Cardiac sections from I (-) (Fig A) and I(+) hearts (Fig B), using an anti-P262-Cx43 (green). Sections were stained with Hoechst 33342, to visualize nuclei (blue). Arrows point to ICDs. Bar= 50 μm. Western blotting from I(-) and I(+) were stained for P-S262-Cx43. Data expressed as mean± SEM. Asterisk (*) indicated statistically significant between I (-) and I (+) hearts ($P<0.05$, n=7).

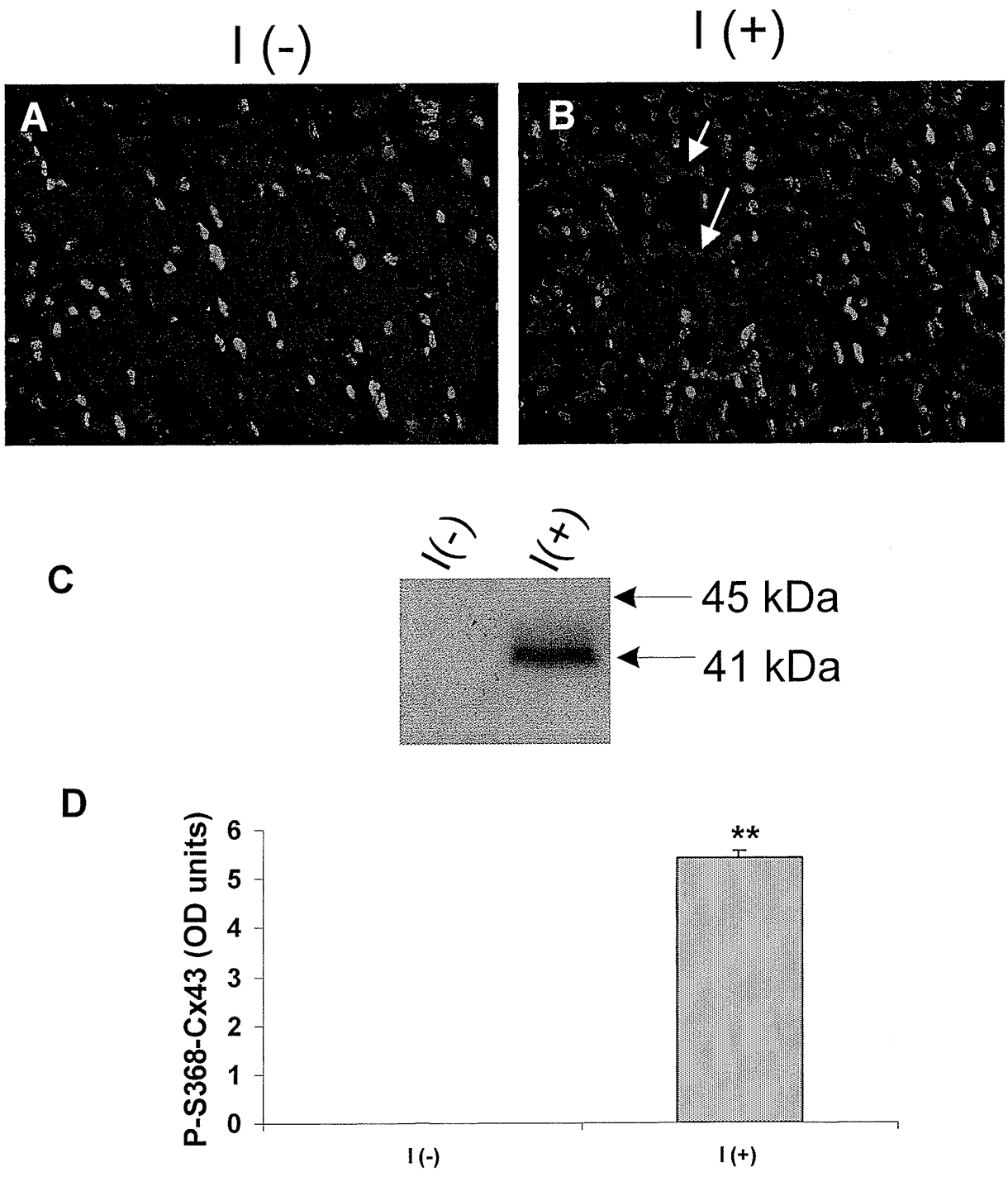


Figure17: Effect of ischemia (+/- FGF-2) on Cx43 phosphorylation at S368

Cardiac sections from I (-) (Fig A) and I(+) hearts (Fig B), using an anti-P368-Cx43 (green). Sections were stained with Hoechst 33342, to visualize nuclei (blue). Arrows point to ICDs. Bar= 50 μm. Western blotting from I(-) and I(+) were stained for P-S368-Cx43. Data expressed as mean± SEM. Asterisk (**) indicated statistically significant between I (-) and I (+) hearts ($P<0.01$, n=7).

Discussion

The overall objective of my studies was to investigate whether FGF-2 treatment will affect Cx43 level, distribution ,and PKC-mediated phosphorylation, in the adult non-ischemic and ischemic *ex vivo* heart.

1. FGF-2 affects Cx43 levels, phosphorylation and distribution in the normal heart.

FGF-2, a known cardioprotective agent, elicits acute, preconditioning–like resistance to ischemic injury in a PKC-dependent fashion [Padua, R R, et al. 1995]; [Padua, R R, et al. 1998]. Because acute cardioprotection is dependent on post-translational modifications of proteins such as phosphorylation, identification of proteins that become phosphorylated in response to FGF-2 treatment of the adult heart is an important step towards understanding the mechanism of cardioprotection. We chose to investigate if Cx43 is such a target of FGF-2-triggered, and PKC-dependent signaling, in a well-established experimental setting that was used previously to demonstrate FGF-2-induced cardioprotection from ischemia and reperfusion injury [Jiang, Z S, et al. 2002, Jiang, Z S, et al. 2004, Padua, R R, et al. 1998, Padua, R R, et al. 1995]. Cx43 was analyzed immediately after FGF-2 administration to the adult heart, to determine if changes could be detected in Cx43 prior to any subsequent stress stimulus.

One of our novel findings was that a 20 min exposure to FGF-2 significantly increased the total level of accumulated Cx43. Increased Cx43 levels were seen by both western blotting of heart lysates and by immunostaining of cardiac sections; in the latter case, more intense anti-Cx43 staining was

associated with ICDs indicating that the extra Cx43 was capable of assembly and targeting at sites of intercellular contact. Similar results were reported in simulated preconditioning of cultured rat neonatal cardiac myofibroblasts [Sundset, R, et al. 2004], and by FGF-2 treatment of cultured cardiac myofibroblasts [Doble, B W, et al. 1995]. On the other hand, our findings appear to contradict studies where ischemic preconditioning induced by 2 cycles of 5 min ischemia (by coronary ligation during open-chest surgery in a rabbit model) and 10 min reperfusion is reported to decrease Cx43 at ICDs [Daleau, P, et al. 2001]. One could argue that this reflects a difference between FGF-2-induced and ischemic preconditioning- induced cardiac effects. We do not think this is likely, since pilot studies in our lab indicated that ischemic preconditioning exerted similar effects on Cx43 as FGF-2 treatment. Differences between our findings and those of Daleau *et al.* [Daleau, P, et al. 2001] may reflect differences in the species used (rat versus rabbit); in the method used to produce ischemia (global versus regional); in antibodies used; and in the time interval between treatment and Cx43 analysis (zero in our case, but at least 30 minutes in theirs). In the case of antibody-based recognition, changed immunoreaction could reflect changes in exposure of specific Cx43 epitopes rather than changes in the amount of protein. We think this is unlikely in our system since similar results were obtained using antibodies recognizing epitopes within different Cx43 domains.

The mechanism by which FGF-2 treatment stimulated Cx43 accumulation is not known, but since it occurred within 20 minutes, it is unlikely to involve

effects on gene expression. Rather, FGF-2 may have influenced Cx43 turnover, possibly by preventing Cx43 degradation. Cx43 turnover in heart is quite fast (1.3-1.5 hour) [Beardslee, M A, et al. 1998], exerted by both the proteasomal and lysosomal pathways [Laird, D W 2005]. When both proteosomal and lysosomal pathways are inhibited in the isolated perfused hearts, there is an increase in Cx43 content seen by immunolabelling [Laing, J G, et al. 1997]. It would therefore be important to examine if FGF-2 can inactivate the degradation pathways of Cx43.

Another important and novel finding was that FGF-2 stimulated Cx43 phosphorylation at S262, and S368. Both of these serines are putative PKC target sites, and both become phosphorylated in response to PKC activation [Doble, B W, et al. 1996] [Doble, B W, et al. 2000] [Richards, T S, et al. 2004]. It follows that Cx43 phosphorylation at S262 and S368 is a marker of PKC activation, and confirms our previous results that FGF-2 administration to the perfused heart activates PKC [Padua, R R, et al. 1998]. It is also important to note that Cx43 phosphorylation at S262, S368 was detected at ICD sites, suggesting a possible consequence to gap junction-mediated coupling. Based on previous studies showing that phosphorylation at S368 is responsible for the PKC-induced closure of gap junctions [Lampe, PD, et.al.2000, Lampe, PD, et.al.2004], the FGF-2-induced phosphorylation at S368 is likely to decrease coupling between cardiomyocytes. The effect of S262 phosphorylation on gap junction coupling may be more subtle. Doble et al. have shown that preventing phosphorylation at S262 results in gap junction channels with enhanced dye-

coupling, and that phosphorylation at that site reduces, but does not eliminate, coupling [Doble, B W, et al. 2004]. Decreased channel coupling in response to Cx43 phosphorylation may contribute to cardioprotection against subsequent ischemia (and reperfusion) injury by preventing the spread of injurious metabolites.

Cx43 phosphorylated at S262 was found to represent 'hyper' phosphorylated Cx43 since it migrated at or above 45 kDa. Our data indicated that phosphorylation at S262 occurs on Cx43 that has already been phosphorylated at several other amino acid sites in the adult heart. These results are in agreement with previous studies in neonatal myocyte cultures [Doble BW et.al.2004]. In contrast to P-S262-Cx43, Cx43 that was phosphorylated at S368 migrated only slightly slower than 41 kDa, indicating that it represents minimally phosphorylated Cx43, in agreement with previous studies by Solan *et al.* [Solan, J L, et al. 2003]. The lack of common Cx43 bands reacting with both types of phosphospecific antibodies is intriguing, and puts forward the possibility that phosphorylation of Cx43 at these two PKC sites is mutually exclusive or they may exist in different subcellular pools of Cx43. The two types of phosphospecific antibodies used here produced broadly similar patterns of staining at ICDs, arguing against the latter possibility. Nevertheless, this merits further investigation, by conducting simultaneous staining for Cx43 phosphorylated at S262 or S368. It would also be interesting to determine if different PKC isoforms target different sites on Cx43. PKC has many isoforms, showing distinct patterns of subcellular localization [Mackay, K, et al. 2001]. Both

PKCα and PKCε have been linked to cardioprotection; they interact with, co-distribute and phosphorylate Cx43 [Doble, B W, et al. 2000];[Lampe, P D, et al. 2000]; these particular PKC isoforms therefore are prime candidates for targeting PKC sites on Cx43 in response to cardioprotective stimuli.

Overall, we have found that treatment with FGF-2 has created a novel cardiac state, characterized by increased phosphorylation at the PKC target sites S262 and S368. This state is distinct from that of the normal heart, and correlates with the FGF-2-induced, preconditioning like cardioprotection.

2. FGF-2 pre-treatment prevents the ischemia induced changes in Cx43.

To examine if FGF-2 pre-treatment could prevent the ischemia-induced changes in Cx43, treated and non-treated hearts were analyzed after 30 minutes of global ischemia. Two major Cx43-related events have been described upon global ischemia (20-30 min) of the perfused, non-preconditioned, heart: Cx43 becomes progressively dephosphorylated, and it redistributes away from intercalated disks, to lateral cardiomyocyte surfaces [Beardslee, M A, et al. 2000];[Schulz, R, et al. 2003];[Matsushita, S, et al. 2006]. These changes correlate, and have been proposed to cause, electrical uncoupling that occurs in the heart over approximately the same time period [Beardslee, M A, et al. 2000]. Similar changes in Cx43 were also seen in the non-FGF-2-treated hearts when subjected to 30 minutes of global ischemia in our experimental model. Pre-treatment with FGF-2 completely prevented both types of changes: Cx43 did not become dephosphorylated; and it did not redistribute to myocyte lateral surfaces. Prevention of ischemia-induced Cx43 changes is very likely to be part of the

mechanism of FGF-2-induced cardioprotection. Our findings are similar to those obtained in other models of cardioprotection (ischemic preconditioning), followed by ischemia, and include studies in isolated rat hearts [Miura, T, et al. 2004], rabbit hearts *in vivo* [Daleau, P, et al. 2001], and pig heart *in vivo* [Schulz, R, et al. 2001b].

The increase in total Cx43 levels elicited by FGF-2 pretreatment was no longer evident after 30 minutes of global ischemia: total Cx43 levels were similar between untreated and FGF-2-treated ischemic hearts, and at the same level as that of non-FGF-2-treated, non-ischemic, normal hearts. In other words, while FGF-2-treated hearts went into global ischemia with elevated total Cx43 levels, this 'Cx43 surplus' was lost during ischemia suggesting that any effect FGF-2 might have had on Cx43 accumulation/turnover prior to ischemia was no longer active under ischemic conditions.

Our most important as well as intriguing finding was that FGF-2-pretreated hearts had elevated levels of Cx43 phosphorylated at PKC target sites S262 and S368 even after 30 minutes of global ischemia, indicating that the FGF-2-induced Cx43 phosphorylation at PKC sites observed *prior* to ischemia was preserved during ischemia. Our lab has demonstrated that Cx43 dephosphorylation during ischemia requires the activities of serine/threonine protein phosphatases of the PP1 and PP2B groups [Jeyaraman, M, et al. 2003], which become activated when ATP levels decrease. It is possible that FGF-2-triggered signals may inactivate these phosphatases either directly, or indirectly, by preserving cellular ATP levels [Padua, R R, et al. 1995]. Inactivation of phosphatases would be

expected to prevent the dephosphorylation of PKC target sites such as S262 and S368, but also sites targeted by other kinases. We cannot exclude the possibility that the FGF-2 stimulus, which activates PKC and phosphorylates Cx43 was ongoing during the ischemic period, phosphorylating Cx43 at S262 and S368 *during* ischemia. After all, FGF-2 administered by perfusion prior to ischemia is absorbed and retained by the heart, distributes around cardiomyocytes, can be detected after ischemia and reperfusion [Padua, R R, et al. 1995], and would thus be expected to signal in a sustained fashion. Irrespectively however of how Cx43 phosphorylation at S262 and S368 remained elevated, our data show that Cx43, through its phosphorylation at PKC sites, retains a molecular 'memory' of FGF-2 pre-treatment (and PKC activation) after 30 minutes of global ischemia. FGF-2-pretreated hearts, therefore, would be expected, after 30 minutes of ischemia, to face reperfusion and the associated exacerbation of ischemic injury 'armed' with hyperphopshorylated Cx43. Our lab has previously demonstrated that rat hearts subjected to 30 minutes of global ischemia under constant pressure (as used here), show a 50% recovery in mechanical functional parameters after 60 minutes of reperfusion, and that FGF-2 pretreatment results in significant functional improvement (about 80% recovery), as well as reduced myocardial damage [Padua, R R, et al. 1995] [Padua, R R, et al. 1998]. Thus we suggest that sustained Cx43 phosphorylation at PKC sites may play an important role in preserving the heart from ischemia-reperfusion, possibly by preventing spread of contracture between cardiomyocytes.

In conclusion

We have shown for the first time that FGF-2 pretreatment of the perfused heart creates a new cardiac 'state' characterized by Cx43 phosphorylation at specific PKC sites and that this state is sustained even after relatively prolonged ischemia. We suggest that Cx43 phosphorylation at PKC sites S262 and S368 marks an injury-resistant heart, and that it may contribute to FGF-2-triggered cardioprotection

Future directions:

We have discovered a correlation between FGF-2 cardioprotection and phosphorylation of Cx43 at specific PKC sites. It would be important to determine whether other cardioprotective manipulations, ischemic preconditioning, or even post-conditioning, are also characterized by these changes in Cx43 phosphorylation.

It is well known that cardioprotection by ischemic or pharmacological preconditioning require the activation of PKC and involvement of mitochondria [Chen, W, et al. 1996]; [Liu, Y, et al. 1994]; [Ping, P, et al. 1997]; [Jiang, Z S, et al. 2002]. Furthermore there is evidence that Cx43 is localized at mitochondria, and that it translocates to mitochondria during preconditioning [Schwanke, U, et al. 2002]; [Boengler, K, et al. 2005]. However, there is no information about the phosphorylation status of mitochondrial Cx43 (mito-Cx43) at PKC sites in the injury-resistant heart. It would therefore be important to examine the mito-Cx43

phosphorylation status of S262 and S368 in the normal or protected heart, and to determine whether Cx43 phsophorylation affects mitochondrial function.

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