INSULIN REGULATES MYOCARDIAL PROTECTION AND HEAT SHOCK PROTEINS THROUGH MULTIPLE SIGNALING PATHWAYS

by

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學無止境

Never stop learning

A Chinese Traditional Philosophy

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ABSTRACT

In cardiac surgery, insulin is used to improve heart function before and after cardioplegia or ischemic injury. In my experiments I examined several possible molecular mechanisms for the insulin induced myocardial protection. Rat hearts were examined for function and expression of heat shock proteins at 1 and 6 hrs after a single bolus of insulin injected intramuscularly. Indeed, the contractile recovery of hearts after 30 min of global ischemia was significantly improved at 1 and 6 hrs after insulin treatment. While insulin induced expression of Hsp70, this expression was not through the myocardial nitric oxide-cyclic guanosine monophosphate signaling pathway. The insulin-induced myocardial protection at 1 hr after insulin treatment was associated with phosphorylation of Hsp27. Treatment of rats with SB203580, an inhibitor of p38 MAPK, blocked the insulin induced phosphorylation of Hsp27 and the improved myocardial contractile recovery. This suggests that at 1 hr after insulin treatment, the phosphorylation of Hsp27 and the improved myocardial contractile recovery is mainly through insulin stimulation of the p38 MAPK pathway. At 6 hrs after insulin treatment, the heat shock transcription factor (HSF1) was activated, binding to the heat shock element of the promoter region of HS genes. At 6 hrs after insulin treatment, Hsp70 was detected at elevated levels in the hearts and was co-localized with dystrophin to cardiomyocytes membranes. This latter finding is in contrast to the localization of Hsp70 to small blood vessels after heat shock treatment. These results suggest that at 1 and 6 hr after insulin treatment the myocardial protection may be due to stimulation of more than one signaling pathway, and possibly localization of Hsp70 to vulnerable areas of cardiomyocytes. It may be that inducible endogenous myocardial protection as seen after insulin treatment (and after heat shock treatment) is temporally due to multiple signaling pathways and mechanisms.

LIST OF ABBREVIATIONS USED

In this thesis I have used abbreviations as listed in Dorland's Illustrated Medical Dictionary 31st edition (2007). Other abbreviations are listed below.

Akt	protein kinase B, also as PKB
aPKCζ	atypical protein kinase C ζ
±dp/dt	first derivative of the left ventricular pressure
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
elK	Ets-like transcription factor
ERK	extracellular signal-regulated protein kinase
FA-CoA	fatty acyl-Coenzyme A
FOXO1	forkhead transcription factor O1
FRAP	FKBP12-rapamycin-associated protein (mTOR)
G6Pase	glucose-6-phosphatase
GIK	Glucose – insulin – potassium cocktail solution
GLUT4	glucose transporter 4
Grb2	growth factor receptor bond protein 2
GSK3	glycogen synthase kinase 3
hr(s)	hour(s)
HR	heart rate
Hsc70	constitutive heat shock protein 70 kDa
HSE	heat shock element

HSF1	heat shock transcription factor 1
HSF-HSP	heat shock transcript factor – heat shock protein complex
HSPs	heat shock proteins
Hsp25	heat shock protein 25 kDa (Hsp27 homolog in mouse)
Hsp27	heat shock protein 27 kDa
HSP70	heat shock protein 70 kDa family
Hsp70	inducible heat shock protein 70 kDa
i.m.	Intramuscular
i.p.	Intraperitoneal
IRS	insulin receptor substrate
L-NAME	L-nitro arginine methyl ester
LVDP	left ventricular developed pressure
LVESP	left ventricular end systolic pressure
LVW	left ventricular work
JNK/SAPK	c-jun N-terminal kinase /stress-activated protein kinases
MAPKAPK	mitogen-activated protein kinase activated protein kinases
MEK	mitogen-activated protein kinase kinase
MNK	MAPK interacting kinase
NO	nitric oxide
NOS	nitric oxide synthase
P70S6K	p70 S6 ribosome kinase
P90RSK	p90 ribosome kinase (mitogen-activated protein kinase activated protein kinase -1)

PDK	phosphoinositide-dependent kinase
PEPCK	phosphoenolpyruvate carboxykinase
PH	pleckstrin homology
pHsp27	phosphorylated heat shock protein 27 kDa
PI3K	phosphatidylinositol 3-kinases
PIP_3	phosphatidylinositol-3,4,5-phosphate
$PI3,4P_2$	phosphatidylinositol-3,4-phosphate
$PI4,5P_2$	phosphatidylinositol-4,5-phosphate
PB	phosphate buffer
PBS	phosphate buffered saline
PBST	phosphate buffered saline / Triton-100
PTEN	phosphatase and tensin homolog
Raf	Raf proto-oncogene serine/threonine-protein kinase
Ras	A family of proto-oncogenes, a GTP-binding protein with GTPase activity, resembles regulatory G-proteins
Rheb	small G-protein Ras homolog enriched in brain
ROS	reactive oxygen species
SH2	src homology 2
SH3	src homology 3
SHIP	SH2-containing inositol 5'-phosphatase
Shc	SH2-containing collagen-related protein
SNP	sodium nitroprusside

SOS	son-of-sevenless, guanine nucleotide change factor, a GTPase activating protein
TBS	Tris buffered saline
TBST	Tris buffered saline / Tween 20
TC10(α/β)	Rho-related GTP-binding protein RhoQ (Ras-related GTP-binding protein TC10
TSC	tuberous sclerosis complex

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CHAPTER 1:

INTRODUCTION

Introduction

Insulin is a hormone that was first discovered and used as a treatment for diabetes mellitus more than 85 years ago. Now insulin is understood to be much more than a hormone regulating glucose levels. Insulin is a crucial metabolic hormone that controls organismal and cellular physiological activities by initiating intracellular signals via various signaling pathways. Insulin modulates cellular functions by regulating synthesis and storage of carbohydrates, and synthesis of proteins and lipids. Since 1962 insulin has been used to improve cardiac contractile function in acute myocardial infarction (Sodi-Pallares et al., 1962), but the mechanisms by which insulin improves heart function are not fully understood. In this thesis, the focus will be on insulin and its effects on the heart. In particular, I will examine whether insulin modulates expression of heat shock proteins that are known to improve recovery of the heart from ischemic injury.

Discovery and Milestones of Insulin

Insulin was initially discovered in pancreas by two Canadian researchers, Banting and Best (1922) at University of Toronto. For this discovery, Banting and Best received the Noble Prize in 1923. Purified insulin was used to treat a patient with type 1 diabetes and the patient's blood glucose levels were successfully controlled (Banting, 1925; Bliss, 1982). In 1955, the amino acid sequence of bovine insulin was announced (Sanger et al., 1955). The first chemical synthesis of biologically active crystalline bovine insulin was achieved by chemists in China in 1965 (Kung et al., 1965; Institute of Biochemistry Academia Sinica et al., 1966). With progress in molecular biology, production of human insulin by cloning recombinant DNA in *Escherichia coli* was accomplished (Frank and Chance, 1983).

Chemistry and Biosynthesis

Human insulin consists of 51 amino acids arranged in two chains, an A chain (21 - residues) and B chain (30 - residues) that are linked by two disulfide bonds, at positions A7 – B7 and A20 – B19. The biosynthesis of insulin is in β-cells within the islets of Langerhans of the pancreas. The human insulin gene is located in the short arm of chromosome 11 (Bell et al., 1980; Robbins et al., 1984). Insulin is synthesized as preproinsulin (about 110 amino acids), that is assembled in rough endothelial reticulum and includes 23 amino acids of "signal sequence" (Sims and Calles-Escadon, 1997; Dodson and Steiner, 1998). Preproinsulin moves to the Golgi apparatus and is cleaved to proinsulin, a single-chain 86 amino acid peptide. In the Golgi apparatus of the β-cell, proinsulin is processed and packaged into granules. Proinsulin is then cleaved into insulin and C-peptide, a 31 - amino acid connecting peptide. Both insulin and C-peptide are secreted into the portal vein from the β-cell upon stimulation from glucose and other insulin releasing factors, on an equimolar basis.

The Regulation of Insulin Secretion

The regulation of insulin secretion is well established (Vander et al., 1994; Sims and Calles-Escadon, 1997) and several regulator pathways are apparent. Firstly, the most important and basic control of insulin secretion is the blood glucose concentration. Blood glucose regulation of insulin secretion is independent of neural or other hormonal regulation. Secondly, elevated plasma concentration of amino acids, and succinate (Attali et al., 2006), regulates increased insulin secretion. Thirdly, autonomic neurons to the islets of Langerhans regulate insulin secretion. Sympathetic stimulation inhibits insulin secretion and parasympathetic stimulation increases insulin secretion. Fourthly, other hormones,

such as glucose-dependent insulinotropic peptide secreted by the gastrointestinal tract, regulates insulin secretion. Finally, nitric oxide (NO) is involved in the control of insulin secretion (Kahn et al., 2000; Henningsson et al., 2002; Nakata and Yada., 2003).

Physiological Actions of Insulin

Insulin has multiple physiological actions (Vander et al., 1994; Moule and Denton, 1997; Sims and Calles-Escadon, 1997): 1) Insulin controls metabolism by regulating membrane transport of glucose and amino acids in muscle (including cardiac muscle), adipose tissue and liver. 2) Insulin increases storage of glycogen in liver and muscle and controls triacylglycerol synthesis. 3) Insulin also regulates protein synthesis by stimulating transcription and translation.

Insulin Signaling Transduction Pathways

The multiple physiological actions of insulin are regulated by intracellular signal transduction pathways. These insulin stimulated intracellular pathways and linked actions are summarized in Figure 1.1. Insulin binds to the insulin receptor, a tetrameric protein, that consists of two extracellular α–subunits and two intracellular β–subunits. The insulin receptor was the first receptor found with endogenous tyrosine kinase activity. When insulin binds to its receptor, the intracellular β-subunit phosphorylates the insulin receptor substrate (IRS) (Shemer et al., 1987; Komori et al., 1989). In this section I will review the established signaling pathways stimulated by insulin.

Figure 1.1. Insulin signaling pathways. 1. PI3K – Akt pathway (Blue); 2. PKC pathway (Gray); 3. TC10 pathway (yellow); 4. NO pathway (rose); 5. MAPK pathway (light purple). Inhibitors of PIP_3 are in red and transcription factors/translation regulators are in green. For definitions of abbreviations see the text and list of abbreviations.

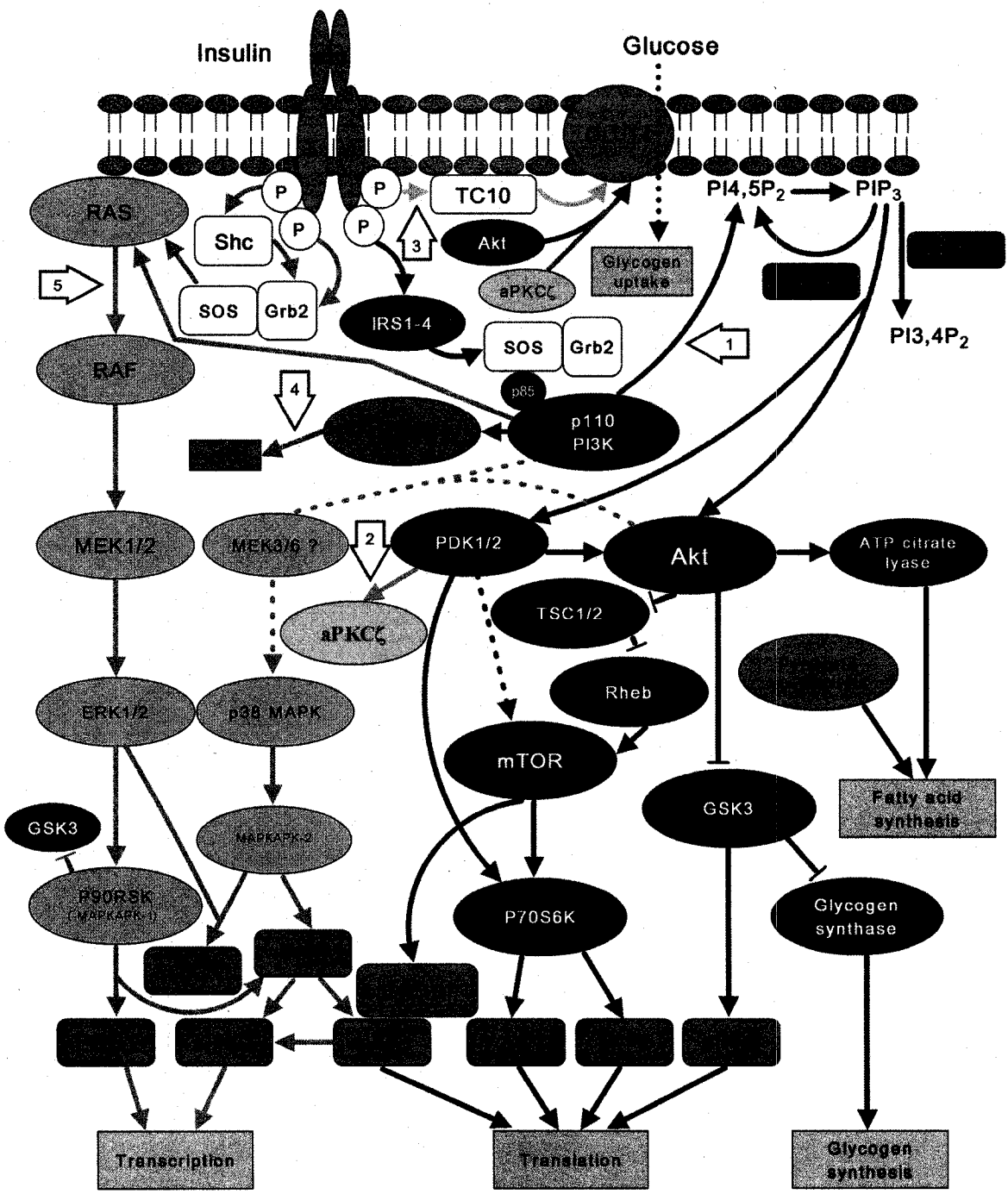
Insulin
Glucose
RAS
Shc
P
TC10
Akt
aPKCζ
Glycogen uptake
PI4,5P2
PIP3
PI3,4P2
SOS
Grb2
IRS1-4
RAF
p85
p110 PI3K
MEK1/2
MEK3/6 ?
PDK1/2
Akt
ATP citrate lyase
TSC1/2
aPKCζ
Rheb
ERK1/2
p38 MAPK
mTOR
GSK3
Fatty acid synthesis
MAPKAPK-2
P90RSK (MAPKAPK-1)
P70S6K
Glycogen synthase
Transcription
Translation
Glycogen synthesis

Figure 1.1

The Phosphatidylinositol 3-Kinase (PI3K) - Protein Kinase B (PKB/Akt) Pathway

PI3K is in the key regulator of the insulin signaling pathways. The insulin induced PI3K - PKB/Akt signaling pathway is conserved throughout eukaryotic evolution (Engelman et al., 2006). In the past decade, much has been done to find the target of the insulin stimulated PI3K - PKB/Akt pathway, and the relationship of this pathway with various diseases (Shepherd et al., 1998; Cantley, 2002; Engelman et al., 2006; Shaw and Cantley, 2006; Taniguchi et al., 2006).

When insulin binds to its receptor on the cellular membrane, endogenous tyrosine kinase of the intracellular β-subunit phosphorylates the insulin receptor substrate (IRS) on several tyrosine residues (Shemer et al., 1987; Komori et al. 1989). Once phosphorylated, IRS-1 binds many proteins containing SH2 (Src-homology region 2) domains (Sun et al., 1992). In 1995, a protein with similar structure as IRS-1 was identified as IRS-2 (Sun et al., 1995). IRS-2 mediates insulin-induced glucose transporter GLUT4 translocation in adipocytes and plays a role in mediating the effects of insulin in muscle and liver (Kerouz et al., 1997; Zhou, et al., 1997; Hei, 1998). IRS-3 and IRS-4 were also discovered at about the same time (Lavan et al., 1997a, 1997b). Recent evidence showed IRS-3 mediates insulin-induced glucose uptake in differentiated IRS-2(-/-) brown adipocytes (Escribano et al., 2006). The IRS-1 - 4 have tissue-specific functions in different tissues and organs (Taniguchi et al., 2006). IRS-1 - 4 in complexes with adaptor proteins such as growth factor receptor bound protein 2 (Grb2) and a guanine nucleotide exchange factor Son-of-Sevenless (SOS) phosphorylates PI3K.

PI3K is not only the key mediator of insulin regulated metabolic activities (Moule and Denton, 1997; Hei, 1998; Shepherd et al., 1998; Cantley, 2002; Engelman et al., 2006;

Taniguchi et al., 2006), but is also a key mediator for other signaling transduction pathways, such as to growth factors and cytokines (Fruman et al., 1998; Cantley, 2002; Engelman et al., 2006). There are three classes of PI3K (I - III). Class IA PI3K is a heterodimer consisting of a p110 catalytic subunit, and a p85 regulatory subunit. P110 has a p85 binding domain and Ras binding domain. Insulin and insulin-like growth factor receptors use the IRS family of adaptor proteins to engage class IA PI3K. Binding of PI3K through its SH2 domain to IRS-1 results in the activation of the enzyme and the increase of phosphatidylinositol-3,4,5-triphosphate (PIP_3) (Moule and Denton, 1997).

PI3K phosphorylates PIP_3, a second messenger, to initiate signal transduction. In turn, PIP_3 is negatively controlled by "phosphatase and tensin homology" (PTEN) and SH2-containing inositol phosphatase (SHIP). Akt is either directly phosphorylated or activated by increased intracellular PIP_3 (Sable et al., 1998; Taniguchi et al., 2006) or by PIP_3 activated 3'-phosphoinositide-dependent kinase 1 (PDK1, Alessi et al., 1997). In cells, Akt is the most important target of PI3K for signal transduction. Activated Akt further inhibits glycogen synthase kinase-3 (GSK3), blocks the forkhead (FOX) family of transcription factors, and activates the FK506 binding protein (FKBP) 12 -rapamycin-associated protein/mammalian target of rapamycin (FRAP/mTOR) complex and glucose transporter-4 (GLUT4) (Withers et al., 1997). Inhibition of GSK3 upregulates glycogen synthase and increases glycogen synthesis (MacAulay et al., 2003). Activated FRAP/mTOR further promotes dissociation of eIF4E-BP1 and p70 ribosomal protein S6 kinase (P70S6K) to initiate protein synthesis (Engelman et al., 2006, Taniguchi et al., 2006). Akt is required for insulin-stimulated translocation of GLUT4 to the plasma membrane (Thong et al., 2005; Gonzalez and McGraw, 2006).

PI3K is required for insulin stimulated ERK1/2 activation in several cancer cell lines. Blockade of PI3K with wortmannin and/or LY294002 diminishes insulin activated phosphorylation of ERK1/2 and Akt. This insulin-induced PI3K-dependent phosphorylation of ERK1/2 requires Ras activation (Liu et al., 2006).

The Atypical Protein Kinase C Pathway

PI3K, through the phosphorylation of PIP_3 and activation of PDK1, also activates atypical protein kinase Cζ (aPKCζ), and aPKCλ/τ, that are a group of Ca^{2+}-independent PKC (Bandyopadhyay et al., 1997; Le Good et al., 1998; Standaert et al., 1999; Patel et al., 2005). While there are more than 10 members in the aPKC family, only aPKCζ appears to be directly involved in GLUT4 translocation and insulin-stimulated glucose uptake. However, insulin-induced aPKCζ activation may be tissue specific. Recently, in perfused rat hearts, insulin activated PKB/Akt 16 times the level of untreated control hearts (Mouton et al., 2007). In these hearts no change in phosphorylation state of aPKCζ was observed. In contrast, in isolated rat epididymal adipocytes, insulin activated Akt 30 times the level of untreated control epididymal adipocytes, a 50% increase in aPKCζ phosphorylation was detected (Mouton et al., 2007).

The Glucose Uptake Signaling from Lipid Rafts - TC10 pathway

Another proposed insulin signaling pathway is the TC10 pathway that is independent of the PI3K – Akt pathway (Chang et al., 2004, Zhou et al., 2004). Insulin receptors (at least some) located on microdomains of the cellular membrane stimulate, through multiple phosphorylation steps, activation of TC10 (a small G protein), that in turn stimulates membrane translocation of GLUT4 and glucose uptake.

The Nitric Oxide Pathway

Nitric oxide (NO) is an important biochemical molecule. Nitric oxide synthase (NOS) catalyzes the synthesis of NO from L-arginine. There are three isoforms of NOS: neural NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The PI3K pathway and ERK pathway may regulate eNOS induction at the transcription level (Wu, 2002). The abundance of eNOS is upregulated by increased binding of the transcription factor Specificity protein 1 (Sp1) to its cognate site on eNOS promoter/enhancer region via the action of protein phosphatase A2 (PPA2).

Insulin stimulates various cells to produce nitric oxide (Steinberg et al., 1994; Sinha et al., 1999; Montagnani et al., 2001; Gao et al., 2002; Sundell and Knuuti, 2003). NO is proposed as the "second messenger" of insulin (Kahn et al., 2000). This action of insulin depends on PI3K (Gao et al., 2002), Akt phosphorylation at $Serine^{1179}$ and eNOS activation (Montagnani et al., 2001). There is a dose response relationship between insulin and the production of NO (Sinha et al., 1999; Kahn et al., 2000; Montagnani et al., 2001; Sundell and Knuuti, 2003). Insulin-induced NO is anti-apoptotic and provides myocardial protection against ischemia/reperfusion injury (Gao et al., 2002). Insulin may induce eNOS activation and production of NO in endothelium, that then releases NO into smooth muscles, that causes vascular relaxation and maintains vascular tone (Mather et al., 2001).

The Mitogen-Activated Protein Kinase (MAPK) Pathways

The MAPK pathways (Figure 1.2) can be described as three sub-pathways: ERK/MAPK, p38 MAPK, and JNK/SAPK. Mitogen-activated protein kinase (MAPK) induced by insulin was initially named as microtubule-associated protein-2 (MAP2) kinase and also abbreviated as MAP kinase (Ray and Sturgill, 1987). Boulton and his colleagues first designated this protein as extracellular signal-regulated kinase 1 (ERK1) in 1990. This protein kinase was characterized by its ability to phosphorylate MAP2 and myelin basic protein (MBP). ERK1 was thought to play a pivotal role in the transduction of signals from many receptors in response to their ligands (Boulton et al., 1990, 1991). The ERK1/2 also called as p44 and p42 MAPK by their molecular mass (Denton and Tararé, 1995).

ERK/MAPK pathway - Insulin can directly stimulate the ERK/MAPK pathway. The insulin receptor endogenous tyrosine kinase promotes Ras activation through enhanced exchange of GTP for GDP (Avruch, 1998; Murphy and Blenis, 2006). Ras is the founding member of the superfamily of small G - proteins and is located normally attached to the inner surface of the plasma membrane by lipid interaction (Valencia et al., 1991). Ras has 189 amino acids and molecular mass of 21 kDa. Ras activation results from binding GTP. The GTP-dependent reconfiguration of the Ras effector loop creates a high affinity binding site for protein kinase known as Raf (Zhang et al., 1993). The interaction of Ras with Raf requires other proteins. Normally, Raf is localized in the cytosol in association with Hsp90, Hsp50 and an emerging family of cytosolic adaptor proteins called 14-3-3 that bind to serine/threonine-phosphorylated residues. These heat shock proteins and 14-3-3 appear to stabilize Raf, to facilitate interaction with Ras, and to translocate Raf to the plasma

Figure 1.2. Summary of MAPK pathways.

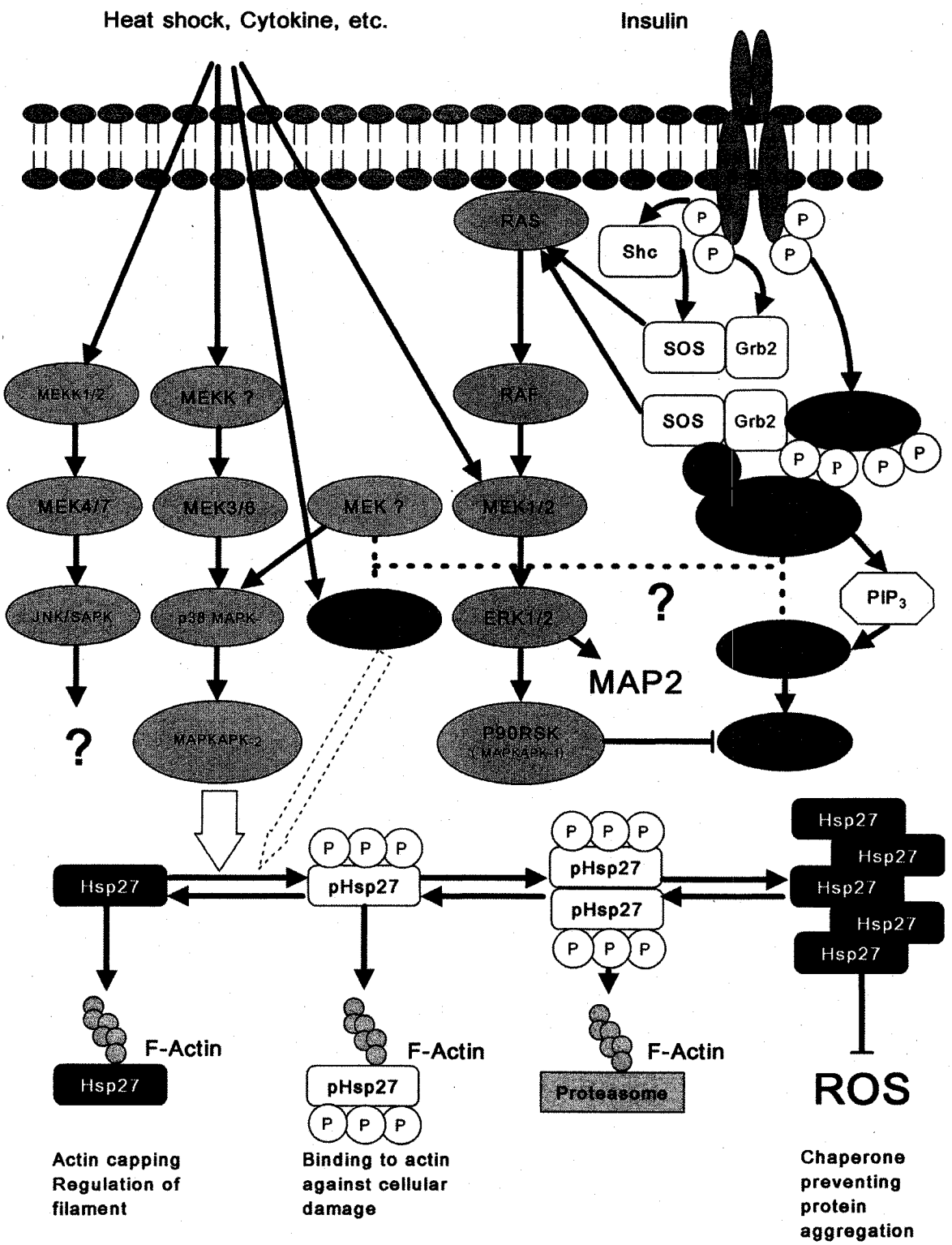
Heat shock, Cytokine, etc.
Insulin
RAS
Shc
P
SOS
Grb2
MEKK1/2
MEKK ?
RAF
MEK4/7
MEK3/6
MEK ?
MEK1/2
JNK/SAPK
p38 MAPK
ERK1/2
?
PIP3
MAP2
MAPKAPK-2
P90RSK
Hsp27
pHsp27
F-Actin
Proteasome
ROS
Actin capping
Regulation of
filament
Binding to actin
against cellular
damage
Chaperone
preventing
protein
aggregation

Figure 1.2

membrane (Shulte et al., 1995; Denhardt, 1996; Baccarini, 2005). All activated Raf isoforms can phosphorylate and activate MAPK kinases (mitogen-activated ERK-activating protein kinases, MEK 1-7) (Yan et al., 1994). MEK1/2 is a dual-specificity kinase that phosphorylates the threonine 183 and tyrosine 185 residues on ERK1/2 required for activation (Ray and Sturgill, 1987; Boulton et al., 1990, 1991; Cobb, 1999). Several other ERK isoforms have been discovered including: ERK3 (Boulton et al., 1991), ERK4 (Boulton et al., 1991), ERK5 (Zhou et al., 1995), ERK6 (p38γ, Lechner et al., 1996), ERK7 (Abe et al., 1999), and ERK8 (Abe, et al., 2002). There are numerous downstream substrates of ERK/MAPK, in the nucleus, cytosol, cytoskeleton and membrane, such as p90 ribosome protein kinase (P90RSK, MAPKAPK-1), p70 S6 kinase (P70S6K), ets-like transcription factor (elK), MAPK interacting kinase (MNK), and glycogen synthase kinase 3 (GSK3) (Roux and Blenis., 2004; Kolch, 2005).

p38 MAPK pathway - Insulin not only activates ERK1/2 but also phosphorylates and activates p38 MAPK through PI3K activation (Kayali et al., 2000; Shi and Gaestel, 2002; Niu et al., 2003; Thong et al., 2003; Baruine et al., 2004; Han and Lee, 2005). Insulin-induced phosphorylational activation of p38 MAPK appears to be through MEK3/6 (Niu et al., 2003). The biological actions of p38 MAPK are through its downstream effector, MAPK-activated protein kinase 2 (MAPKAPK-2) (Rouse et al., 1994). MAPKAPK-2 activates MNK1 (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997), that in turn phosphorylates eIF4E that initiates translation. MNK1 also activates eIF4G that promotes initiation of transcription (Gingras et al., 1999). Most interestingly MAPKAPK-2 also phosphorylates small heat shock proteins (sHsps) such as Hsp27 (Rouse et al., 1994; Venkatakrishnan et al., 2006). p38 MAPK is activated by insulin in embryonic stem cells

(Han and Lee, 2005), and in cardiac myocytes p38 MAPK may be an inducer of GLUT4 (Montessuit et al., 2004), however this is still unclear.

JNK/SAPK pathway - The Jun-N-terminal kinases/Stress-activated protein kinases (JNK/SAPK) include JNK1, JNK2, and JNK3. JNK is the downstream effector of PI3K. PI3K activity is necessary and sufficient for growth factor-stimulated activation of JNK (Klippel et al., 1996). Insulin phosphorylates JNK and mediates insulin-induced phosphorylation of IRS-1 (Müssig et al., 2005). Insulin and p85α regulatory subunit of PI3K activate JNK via cell division cycle 42 (cdc42, GTP binding protein, 25 kDa) and MEK4 (Taniguchi et al., 2007). Generally, effects of extracellular stimulators induce JNK mediated – phosphorylation, that leads to induction of AP-1 transcript factor (c-jun), to activate transcription. JNK has roles in numerous cellular processes, such as, cell survival, cell differentiation, cell proliferation, and inflammation.

Insulin and Myocardial Protection

Cardiovascular disease continues to be among the leading causes of death and emergency hospital admissions in Canada. Between 2002 and 2004, about one out of every nine people (11.1%) admitted to hospital with a new heart attack died in hospital within 30 days (CIHI, 2006). Comparison of patients enrolled in Global Utilization of Streptokinase and tissue plasminogen activator (t-PA) for Occluded Coronary Arteries study, five-year mortality rate was 19.6% among U.S.A and 21.4% among Canadian patients (Kaul et al., 2004). In cardiovascular surgery, protecting the heart from ischemic injury is the challenge that persists. To protect myocardium from lethal ischemia/reperfusion injury is one of the major challenges in clinical cardiology and cardiovascular surgery. In this section, I will

summarize current knowledge on myocardial ischemia/reperfusion injury, and the possible benefits of insulin treatment.

Pathophysiology in Myocardial Ischemia - Reperfusion Injury

Metabolism in Normal Heart

The mammalian heart is a highly oxygen-dependent organ. At a resting pulse rate, the heart consumes approximately 8–15 ml O_2/min/100 g tissue. This is significantly more than that consumed by the brain (approximately 3 ml O_2/min/100 g tissue) and can increase to more than 70 ml O_2/min/100 g myocardial tissue during vigorous exercise (Giordano, 2006). Normally, the heart cannot stop working for even one minute during a human life time. Therefore, to meet the demand for energy the cardiac muscle consumes many metabolic substances, with the capacity to oxidize fatty acids, carbohydrates and also amino acids (in certain circumstances).

Bing's pioneering work on arterial and venous levels of various substrates in the human heart delineated the contribution of each substrate to cardiac energy metabolism. The myocardial extraction and usage of glucose, lactate and pyruvate were measured in fifty-three patients with and without cardiac failure (Bing et al., 1953). In the 1960s, much work was done in the isolated perfused rat heart to identify the regulation of energy production from various substrates. The finding of high concentrations of fatty acids suppressing glucose oxidation, gave rise to the concept of a "glucose-fatty acid-cycle" (Randle et al., 1963; Opie, 1965). In 1980, this concept was modified with the discovery of the suppression of free fatty acid (FFA) oxidation by glucose in a working perfused isolated rat heart (Taegtmeyer et al., 1980). For a given physiologic environment, the heart selects the most efficient substrate for energy production. For example, the heart switches

from FFA to carbohydrate oxidation during acute increase in work load (Goodwin et al., 1998). More recently, the transcriptional regulation of glycolysis has been investigated with the identification of glucose responsive transcription factors such as carbohydrate response binding protein (C*h*EBP) (Uyeda et al., 2002) and sterol regulatory element binding protein (SREBP) (Girard et al., 1997).

Metabolism in Ischemic Heart

Myocardial ischemia happens when blood supply is partially or fully blocked in the coronary arterial system. Theoretically, myocardial ischemia is a very simple pathophysiologic process. Lack of oxygen and metabolic substrates rapidly decreases the available energy in the cell, and leads to reversible or irreversible cell injury. Practically, the process is complex. Various factors affect the severity of ischemia, such as, the ischemia status (global or partial), the initiation of the ischemia (acute or chronic), and changes in metabolic and physical environment. This complex situation in ischemia leads to a complex pathophysiology. For example, glucose uptake in myocardial cells in low-flow ischemia (1-5% of normal flow) may be partially maintained due to membrane translocation of GLUT4, but in global ischemia glucose uptake is stopped, and the glucose production by glycolysis is from pre-ischemic stored glycogen only (Opie and Sack, 2002). When myocardial ischemia happens, cells convert from aerobic FFA metabolism to anaerobic carbohydrate metabolism resulting in the formation of metabolites toxic to the myocardium. During ischemia, high levels of FFA and their fatty acyl-CoA (FA-CoA) derivatives accumulate in the cells. FA-CoA inhibits insulin-stimulated phosphorylation of GSK3 and Akt (Schmitz-Peiffer, 1999). FA-CoA also directly inhibits hexokinase (Thompson et al., 2000).

Oxygen-depletion during ischemia leads to energy loss and ion channel dysfunction. With the reperfusion and re-introduction of oxygen, ATP level and ion channel function on cellular membranes increase transiently and then decline (oxygen paradox). The overload of intracellular calcium induced by the oxygen paradox has been considered as one of the main reasons for reperfusion injury following ischemia. Recent evidence suggests that cardiac dysfunction due to calcium overload may be associated with apoptosis (Xu et al., 2006). Reactive oxygen species (ROS), such as the superoxide anion radical (dioxide or O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), peroxynitrite anion ($ONOO^-$) and nitric oxide (NO), are derived from oxygen after ischemia during reperfusion. Because molecules of ROS are chemically unstable and highly reactive, they are toxic and cause molecular damage, cardiac dysfunction and cell death. NO reacts differently compared to other oxygen free radicals. NO does not readily react with most bio-molecules despite its unpaired electron. On the other hand it easily reacts with other free radicals, generating less reactive molecules, such as peroxyl and alkyl radicals. Thus, NO functions as a free radical scavenger. A postulated model for ROS damage in ischemia/reperfusion is that: ischemia/reperfusion causes a large increase of O_2^- and activates NOS to produce NO. The large amount of O_2^- predominantly reacts with NO to produce peroxynitrite, which is highly cytotoxic (Beckman and Koppenol, 1996). As NO bio-availability declines, its normal physiological function also declines. Increased peroxynitrite induces apoptosis and production of proinflammation factors within the myocardium, and leads to myocardial necrosis (Di Filippo et al., 2006). Peroxynitrite also initiates membrane lipid peroxydation, that alters ion channel configuration and ion influx (Giodano, 2006). Cardiac dysfunction-induced distress causes adrenal catecholamine release, that inhibits insulin release,

increases heart rate, and myocardial contractility, that in turn, increases the requirement of oxygen and energy supply.

After ischemia, reperfusion is required for tissue survival, but reintroduction of molecular oxygen causes severe cell injury. With reperfusion there is a sudden increase of intracellular calcium in the cytosol and mitochondria that has a profound effect. This high calcium opens the mitochondrial permeability transition pore (proton exchange channel for ATP and ADP) during reperfusion. The opening of this pore leads to permanent loss of ATP and thus cell death (Halestrap, et al., 2004). Multiple inflammatory products and apoptosis are induced by ischemia/reperfusion injury in the heart during cardiac surgery (Anselmi et al., 2004). Two major forms of cell death are recognized in the pathology of myocardial ischemia/reperfusion injury, namely, necrotic cell death and apoptotic cell death. The exact contributions of necrosis and apoptosis in myocardial cell death are unclear (Kunapuli et al., 2006).

In the last 20 years, the concept of endogenous myocardial protection has been developed. Mild stress enhances myocardial recovery from subsequent severe injury. Myocardial ischemic preconditioning (Murry et al., 1986) or heat shock (Currie et al., 1988) protects the heart from ischemia/reperfusion injury.

Myocardial preconditioning has been classified into two windows of protection. The first window is 5 - 10 minutes after a brief ischemia and lasts about 2 to 3 hours. The second window of protection is delayed or late preconditioning, from about 24 - 96 hours after brief ischemia. This delayed preconditioning can be induced by many stimuli, including heat shock. Heat shock proteins are involved in this delayed myocardial protection. The period between the first window and the second window is thought to be

an unprotected window (Cornelussen et al., 2003; Yellon and Downey, 2003; Eisen et al., 2004). The intracellular mechanisms of the myocardial protection against ischemia/reperfusion injury include several possible mechanisms: Adenosine receptors and mitochondrial ATP-sensitive potassium channels (Oldenburg et al., 2002); nitric oxide and nitric oxide synthase (Cohen et al., 2006), anti-oxidants (Currie et al., 1988; 1993; Zweier and Talukder, 2006); "survival kinases" (Garrido et al., 2001; Housenloy and Yellon, 2006) MAPK pathway and anti-inflammation (Vinten-Johansen et al., 2007), and heat shock proteins (Marber et al., 1993, 1995; Plumier et al., 1995).

Glucose – Insulin – Potassium Cocktail Solution (GIK) for Cardiac Ischemia

Insulin treatment during acute myocardial ischemia is still controversial after 45 years in clinical practice. Insulin in a cocktail of glucose - insulin - potassium (GIK) cocktail was first used to treat acute myocardial infarction in 1962 (Sodi-Pallares et al., 1962). The GIK improved electrical stability (ECG changes) in the heart, so was also referred to as a "polarizing solution". Many studies suggest the beneficial effects of GIK are through changes in energy metabolism (Chain et al., 1969; Opie, 1965, 1971, 1975; Oliver and Opie, 1994). During ischemia, the components of GIK favour a shift from the anaerobic FFA metabolism with generation of toxic FA-CoA to the less toxic anaerobic glucose-dependent metabolism, and thus may be considered as a metabolic intervention. Mechanisms by which GIK may protect against myocardial ischemia (Janiger and Cheng, 2002) are: 1) Enhanced glycolysis and increased cellular glucose uptake for anaerobic metabolism; 2) Reduced cytosolic free fatty acid concentrations and reduced direct myocardial toxic effects; and 3) Reduced cytosolic calcium ion concentrate by activating sodium-potassium adenosine triphosphatase and reduced arrhythmias.

Clinical Trials

The changes in energy metabolism induced by GIK continue to promote its clinical use. Most clinical studies suggest GIK reduces both morbidity and mortality (Malmberg et al., 1995; Fath-Ordoubadi and Beatt; 1997; Diaz et al., 1998; van der Horst et al., 2003; Langley and Adams, 2006). However, some studies show that GIK is of minimal benefit (Ceremuzynski et al., 1999; Mehta et al., 2005).

Laboratory Evidence

In animal studies GIK has consistently improved myocardial contractile function. Whether GIK or insulin infusion, both protect cardiac contractile function when given before and immediately following ischemia. The benefits of insulin are thought to be through insulin intracellular mechanisms or signaling pathways, such as: ATP-sensitive potassium channel (K_{ATP}) (LaDisa et al., 2004); anti-inflammation (Chaudhuri et al., 2004; Celkan et al., 2006); NO pathway for myocardial blood flow (Ma et al., 2006); and "survival pathway" for anti-apoptosis (Zhang et al., 2004; Ma et al., 2006). Several of these insulin stimulated mechanisms and pathways may be in common with survival pathways induced by ischemic preconditioning (Hausenloy and Yellon, 2006) and heat shock.

Insulin and Heat Shock Protein

Ritossa (1962) first reported that elevated temperature induced a new puffing pattern on the chromosomes of *Drosophila larvae* salivary glands. He concluded that the altered puffing pattern was indicative of changes in gene expression. The pioneering observation of Ritossa was followed by the identification of seven newly synthesized "heat shock proteins" (HSPs) in the salivary glands of *Drosophila lavae* following heat shock

treatment (Tissières et al., 1974). This heat shock response has been found universally in all organisms examined (Craig, 1985).

Expression of heat shock proteins is regulated developmentally, by changes in the cell environment, or pathophysiologically (Lindquist, 1986, 1992; Lindquist and Craig, 1988; Morimoto et al., 1990, 1994). While Hsps were discovered and named after heat shock, it is clear that many noxious stimuli trigger their expression. Interestingly with elevated expression of Hsps, cells also acquire resistance (tolerance) to subsequent injury (Dokladny et al., 2006). After heat shock, cells have cytoprotection against ischemia/reperfusion injury (Benjamin and McMillan, 1998; Currie et al., 1988; Karmazyn et al., 1990; Plumier et al., 1995; Plumier and Currie, 1996; Snoeckx et al., 2001), pro-apoptotic stimuli (Garrido et al., 2001; Latchman, 2001) and pro-inflammatory stimuli (Chen et al., 2004a, 2004b, and 2006).

The severity of heat shock is dependent on the maximum temperature and the length of exposure to the elevated temperature. For mammalian tissues, 42 °C approaches lethal temperature. At such temperatures, there are many changes in cellular functions. Generally, these changes include: 1) preferential synthesis of Hsps while normal transcription and translation are inhibited; 2) inhibition of progression through the cell cycle; 3) increased denaturation, aggregation and degradation of proteins; 4) disruption of cytoskeletal architecture; and 5) alteration of cellular metabolism and change of membrane permeability (Lindquist, 1986; Hightower, 1991; Kuhl and Rensing, 2000; Sonna et al., 2001).

Heat shock proteins are classified into several families according to approximate molecular mass, amino acid sequence, and function. Generally, the families are HSP110,

HSP90, HSP70, HSP60 and small heat shock proteins (sHsps) (Lindquist and Craig, 1988; Morimoto et al., 1994; Plumier and Currie, 1996; Benjamin and McMillan, 1998; Kiang and Tsokos, 1998; Latchman, 2001; Snoeckx et al., 2001). Specific Hsps are discussed below.

Heat Shock Transcription Factors

The regulation of heat shock gene expression in eukaryotes is mediated by the conserved heat shock transcription factors (HSFs). Multiple HSFs have been identified from plants to mammals (reviewed by Pirkkala et al., 2001). HSF1, HSF2 and HSF4 exist in human, mouse and chicken, but HSF3 is only found in chicken (Morimoto, 1998). HSF1 is conserved among human, mouse and chicken (92.6 %). HSFs are normally in the cytoplasm maintained in an inactive form by binding with Hsp70/Hsp40 and Hsp90. With stress and denaturation of proteins, Hsp90 dissociates from the inactive HSF - Hsp90 complex and is replaced by Hsp70/Hsp40 (Morimoto, 1998; Shi et al., 1998). With accumulation of denatured protein, Hsp70 and Hsp40 are recruited to the damaged protein freeing HSF. Free HSF is phosphorylated at Ser230 and is trimerized. Trimerized HSF is translocated to the nucleus, and binds to the promoter region of heat shock genes.

HSF1 and HSF2 have different binding sites on human HS genes. Activated and trimerized HSFs bind to a regulatory upstream promoter heat shock element (HSE) on heat shock genes (Holmgren et al., 1981, Pelham, 1982). HSE has inverted repeats of the pentameric sequence 5'-nGAAn-3', located at various distances from the site of transcription initiation (Amin et al., 1988; Perisic et al., 1989). In higher eukaryotes only the G residue remains highly conserved, such as 5'-nGGGn-3' and 5'-nGAGn-3' (Abravaya et al., 1991; Cunniff et al., 1991). Activated and trimerized HSF1 binds to all

five pentameric sites to initiate transcription, but HSF2 fails to interact with the first site (5'-nGAAn- consensus) (Kroeger et al., 1993).

HSF1 is also regulated by GSK3β. Specific inhibition of GSK3 increased the levels of HSF1-HSE binding and transcription of heat shock genes and delayed the inactivation of HSF1 during recovery. In contrast, the overexpression of GSK3β resulted in significant reduction in heat-induced HSF1 activities. Activation of HSF1-HSE binding under non-heat shock condition was also observed after inhibiting GSK3β with LiCl (LiCl mimics insulin inhibition of GSK3β) (Xavier et al., 2000).

HSF1 not only binds Hsp genes but also other proinflammatory genes and anti-oxidative genes. While HSF1 increases the expression of Hsp genes, HSF1 represses the expression (Trinklein et al., 2004) of some pro-inflammatory genes such as tumor necrosis factor α (TNFα) (Singh et al., 2000), prointerleukin-1β (IL1β) (Cahill et al., 1996, Xie et al., 2002), and anti-oxidative genes such as copper/zinc superoxide dismutase (Yoo et al., 1999). DNA-binding by HSF1 is not only activated by heat shock but is also activated by hypoxia (Benjamin et al., 1990). Depletion of intracellular ATP plays an important role in triggering the HSF1-DNA binding activity in ischemia (Benjamin et al., 1992).

The 70 kDa Family of Heat Shock Proteins

The 70 kDa family of heat shock proteins consists of various members that differ depending on the organism. For the human 70 kDa family of Hsps, Tavaria et al (1996) provided a hitchhiker's guide to the Hsp70 constellation of Hsps. In human, as many as 11 proteins are evident in protein data bases and 12 members are classified by their gene loci (Table 1.1). Their molecular masses range from about 70 kDa to 94 kDa. In rat, there are 8 proteins and 8 gene loci for the 70 kDa family of Hsps (Table 1.2).

Table 1.1. Human HSP70 nomenclature and gene location.

SwissProt/TrEMBL[1] Entry Name	Access No	OMIM[2]	Gene Name	Alternative Gene Name	Chromosome Loci[7]	Protein Name	MW (kDa)	REF
HSP71_HUMAN	P08107	140550	HSPA1A	HSPA1	6p21.3	Heat shock 70 kDa protein 1A	70.1	Milner et al., 1990
HSP71_HUMAN	P08107	603012	HSPA1B	HSPA1	6p21.3	Heat shock 70 kDa protein 1B		Milner et al., 1990
HSP70L_HUMAN	P34931	140559	HSPA1L		6p21.3	Heat shock 70 kDa protein 1L	70.4	Milner et al., 1990
HSP72_HUMAN	P54652	140560	HSPA2		14q24.1	Heat shock 70 kDa protein 2	70.0	Bonnycastle et al., 1994
		140570	HSPA3[3]		21	Heat shock 70 kDa protein 3	...	Harisson et al., 1987
HSP74_HUMAN	P34932	601113	HSPA4	APG2, HSP70RY	5q31.1-q31.2	Heat shock 70 kDa protein 4	94.3	Fathallah, et al., 1993
HS74L_HUMAN	O95757		HSPA4L[4]	APG1, OSP94	4q28	Heat shock 70 kDa protein 4L	94.5	Nonoguchi et al., 1999
GRP78_HUMAN	P11021	138120	HSPA5	GRP78, BiP	9q33-q34.1	Heat shock 70 kDa protein 5	72.3	Ting & Lee, 1988
HSP76_HUMAN	P17066	140555	HSPA6	HSP70B'	1q22	Heat shock 70 kDa protein 6	71.0	Brzustowicz et al., 2002
HSP77_HUMAN[6]	P48741	140556	HSPA7	HSP70B	1q23.1(1pter-qter)	Heat shock 70 kDa protein 7	26.9	Leung et al., 1992
HSP7C_HUMAN	P11142	600816	HSPA8	HSPA10, HSC70, HSP73	11q24.1	Heat shock 70 kDa protein 8	70.9	Tavaria et al., 1995
GRP75_HUMAN	P38646	600548	HSPA9B	HSPA9, GRP75, PBP74	5q31.1	Heat shock 70 kDa protein 9B	73.7	Kaul et al., 1995

1. SwissPro/TrEMBL: Swiss-Prot protein knowledge database/TrEMBL protein database; 2. OMIM: Online Mendelian Inheritance in Man (Johns Hopkins University); 3. only listed in OMIM, and without a related protein in human; 4. only listed in Swiss/TrEMBL, and initially classified in HSP110 family by Nonoguchi (1999); 6. maybe fragment sequence. 7. all gene locus updated as in database

Table 1.2. Rat HSP70 nomenclature and gene location.

SwissProt/TrEMBL[1]		NCBI[2]							
Entry Name	Access No	GENE ID	Gene Name	Alternative Gene Name	Chromosome Loci	Protein Name	Alternative Protein Name	MW (kDa)	REF
HS70L_RAT	P55063	24963	Hspa1l	Hsp70-3	20	Heat shock 70 kDa protein 1L	Heat shock 70 kDa protein 1-like, Hsp70.3	70.549	Walter et al., 1994
HSP71_RAT	Q07439	24472	Hspa1a	Hsp70-1, Hspa1	20p12	Heat shock 70 kDa protein 1A/1B	Hsp1A/1B, Hsp70.1/2, Hsp72	70.185	Walter et al., 1994
		294254[3]	Hspa1b	Hsp70-2, Hspa2	20p12		Hspa2,		
HSP72_RAT	P14659	60460	Hspa2	Hst70	6q24	Heat shock-related 70 kDa protein 2	HST, hsp70.2	69.528	Wisniewski et al., 1990
HSP74_RAT	O88600	266759	Hspa4	Irp94	10q22	Heat shock 70 kDa protein 4	Ischemia responsive 94 kDa protein, hsp110, Hsp70	94.057	Yagita et al., 1999
HS74L_RAT	P83581[4]		Hspa4l	Apg1, Hsp4l, Osp94		Heat shock 70 kDa protein 4L[5]	Osmotic stress protein 94	27.96	Itoh, Tashima, et al., 1990
GRP78_RAT	P06761	25617	Hspa5	Grp78	3p11	Heat shock 70 kDa protein 5[6]	78 kDa glucose-regulated protein, Bip	72.347	Munro & Pelham, 1986
HSP7C_RAT	P63018	24468	Hspa8	Hsc70, Hsc73	5q36	Heat shock 70 kDa protein 8	Heat shock cognate 71 kDa protein, Hsp73	70.871	Sorger & Pelham, 1987
GRP75_RAT	P48721	291671	Hspa9	Grp75, Hspa9a	18p12	heat shock 70kDa protein 9A	PBP74, MTHSP70, mortalin	73.858	Webster et al., 1994

1. SwissPro/TrEMBL: Swiss-Prot protein knowledge database/TrEMBL protein database; 2. NBCI (GENE): National Center for Biotechnology Information; 3. only listed in NBCI (GENE); 4. only listed in Swiss/TrEMBL; 5. Fragment sequence, and 6. Precursor.

In this thesis, Hsp70 is the highly inducible member of approximately 71 kDa in rat and 72 kDa in human. Hsc70 is the constitutive member of 73 kDa in rat and human. Grp78 (also called Bip) is glucose regulated and localized in the endoplasmic reticulum and has a molecular mass of approximately 78 kDa in rat and human. Grp75 is glucose regulated mitochondrial protein of ~75 kDa in rat and human. This nomenclature is similar to the traditional classification according to molecular mass and amino acid sequence (Kiang and Tosokos, 1998; Snoeckx et al., 2001). The 70 kDa family of heat shock proteins is highly conserved and exhibits a 60 - 78% amino acid sequence identity among eukaryotic cells, and a 40 - 60% amino acid similarity between the inducible Hsp70 and *Escherichia coli* DnaK (Lindquist, 1986; Kiang and Tsokos, 1998).

Transcription of Hsp70 depends on the activation of HSF1. As early as 1980, Hightower suggested that intracellular accumulation of abnormally folded proteins induced by heat shock or other stresses could be the trigger of increased synthesis of Hsps (Hightower, 1980). This hypothesis was further linked to HSF1 regulation and Hsp transcription (Morimoto, 1992, 1993, 1998; Morimoto et al., 1992).

In addition to heat shock, Hsp70 can be induced by many stimuli, such as anoxia, oxidative stress, some antibiotics, heavy metals, and other physical stimulations. Once heat shock proteins are induced, cells, tissues and organisms acquire a remarkable resistance to subsequent injury. In cell cultures, the Hsps protect human monocytes from hydrogen peroxide-induced toxicity (Polla et al., 1987). Similarly, Hsps protects cultured cells from apoptosis (Samali and Cotter, 1996), and from hypoxia/reoxygenation injury (Kiang et al., 1996).

In rats, heat shock and transient ischemia induce expression of Hsp70 in tissues including the heart and brain (Currie and White, 1981). After 6 hours of occlusion of the left anterior descending coronary artery of the dog, Hsp70 mRNA was detected in ischemic myocardium but not in normal myocardium (Dillmann et al., 1986). Similarly, ischemia induces expression of Hsp70 mRNA in the rat heart (Plumier et al., 1996). Once Hsp70 protein is induced and expressed in the heart, it is detectable for more than 2 weeks (Currie and White, 1983; Karmazyn et al., 1990; Pantos et al., 2007).

Induced thermal resistance in cells was first described in 1975 by Gerner and Schneider. In hearts, resistance to ischemic injury was seen in rats 24 hrs after whole-body heat shock at 42 °C for 15 min (Currie et al., 1988). Hsp70 was elevated and the isolated hearts were resistant to ischemia/reperfusion injury. Numerous studies have been done examining the role of heat shock proteins on the myocardial protection by heat shock and other stimuli in various animals, including rat, rabbit, pig and dog (Currie, 1987; Currie et al., 1988; Knowlton et al., 1991; Sharma et al., 1992; Marber et al., 1993; Hutter et al., 1994; Guisasola et al., 2006). In fact, there is a direct correlation between whole body temperature elevation (39 - 42 °C), the amount of Hsp70 induced and the reduction in infarct size in the rat heart (Hutter et al., 1994). Directly heating the rat heart to 42.5 °C to 43.5 °C for 15 min with 4 hrs of recovery also induces Hsp70 and significantly reduces infarct size (Gowda et al., 1998). After heat shock, the myocardial protection is not immediate and is only evident at about 24 hrs recovery following heat shock (Cornelussen et al., 1998, Guisasola et al., 2006). The contribution of Hsp70 to myocardial protection was confirmed in transgenic mice overexpressing human Hsp70 (Plumier et al., 1995) and rat Hsp70 (Marber et al., 1995) and in knockout mice deficient in Hsp70 expression (Kim

et al., 2006). Overexpression of Hsp70 confers significant protection to the heart after ischemia during reperfusion.

The Family of Small Heat Shock Proteins

The family of small heat shock proteins (sHsps) includes a diverse group of proteins that all share an α-crystallin domain. The α-crystallin domain is about 90 amino acid residues and is located toward the C-terminus of the protein. In mammals there are 10 members (HspB1 - HspB10) in this family of Hsps (Kappé et al., 2003; Franck et al., 2004). The members of the small heat shock protein family have four common structural and functional features: 1) a small monomeric molecular mass between 12 - 43 kDa; 2) the formation of large oligomeric complexes; 3) the presence of a conserved central region, the α-crystallin domain; and 4) molecular chaperone function (MacRae, 2000; Narberhaus, 2002). The classification of sHsps has been changed and is now limited to those proteins that contain an α-crystallin domain. Hsps without an α-crystallin domain such as Hsp32 (human heme oxygenase) and Hsp40 are no longer considered to be members of the sHsps (Kappé et al., 2003).

In mammals, the best-know members in small heat shock protein family are Hsp27 (also named Hsp25 in murine) and αB-crystallin. There is a tissue specific distribution of Hsp27 and αB-crystallin in rat. αA-crystallin and αB-crystallin are major structural proteins in vertebrate lens (de Jong et al., 1993). αB-crystallin is also constitutively high in heart (Inaguma et al., 1995). In unstressed adult rat, Hsp27 is constitutively high in skeletal muscle, heart, brain stem and spinal cord (Inaguma et al., 1995; Plumier et al., 1997; Leger

et al., 2000). At 16 hrs and 24 hrs following heat shock, expression of Hsp27 is only moderately increased by about double in heart (Inaguma et al., 1995; Leger et al., 2000).

Increased expression of Hsp27 in response to heat shock was first observed in Hela cells in 1982 (Hickey and Weber, 1982). The gene encoding Hsp27 has been identified for human (Hickey et al., 1986), mouse (Gaestel et al., 1993), and rat (Uoshima et al., 1993). The human Hsp27/HspB1 gene is located in chromosome 7, initially detected at 7q11.23 and later at 7p12.3 (Kappé et al., 2003). The Hsp27/Hspb1 gene in rat is located at 12q12 (NCBI GeneID: 24471). In mammals, transcription of the Hsp27 gene is regulated by activation of HSF1 and its binding to the HSE in the promoter region of the gene. Rat Hsp27 consists of 206 amino acid residues with an apparent molecular mass on SDS gels of approximately 27 kDa (Hickey and Weber, 1982; Leger et al., 2000) and has a calculated molecular mass of 22.9 kDa (UniProtKB/Swiss-Prot entry P42930). The position of the highly conserved α-crystallin domain of Hsp27 in rat is from amino acid 91 to 188 (MacRae, 2000; Sun and MacRae, 2005a) and in human from amino acid 88 - 188 (Lambert et al., 1999). Hsp27 localizes predominantly within the nucleus shortly after heat shock treatment and with increasing recovery time after heat shock, the majority of Hsp27 was found within the cytoplasm of cells (Arrigo and Welch, 1987). Similarly, in heat shocked cells Hsp27 is localized near the center of the cells in perinuclear structures while in unstressed cells, Hsp27 is localized near the cell cortex at the apical face of cells (Lavoie et al., 1995).

Hsp27 has two characteristic features: oligomerization and phosphorylation. Like other sHsps in cells, Hsp27 forms large oligomers (de Jong et al., 1988; Welsh and Gaestel, 1998; Lambert et al., 1999; MacRae, 2000) as large as 200 - 800 kDa (Arrigo et al., 1988;

Kato et al., 1994; Lavoie et al., 1995). The N-terminus of the protein may provide an essential stabilizing force to keep sHsps oligomerized (Carver et al., 1994). Phosphorylation of Hsp27 at Ser90 may weaken the N-terminal interactions and result in the dissociation of Hsp27 into dimers (Lambert et al., 1999). The status of Hsp27 phosphorylation and oligomerization may modulate its interaction with actin. There are two proposed possibilities. Firstly, Ser90 phosphorylation leads to the N-terminal domain becoming exposed and dissociation of Hsp27 oligomers and then Ser15 phosphorylation modulates Hsp27 interaction with actin (Lambert et al., 1999). Secondly, the oligomerization of Hsp27 in cells is determined by its phosphorylation and dephosphorylation, and the rapid dissociation of the aggregated form of Hsp27 by phosphorylation might be involved in a cellular defense mechanism for protection against stress (Kato et al., 1994). The oligomer size of Hsp27 appears to be directly related to its chaperone function. The large oligomers of the sHsps are necessary for chaperone function and resistance against oxidative stress while phosphorylation dissociates sHsp complexes to tetramers and down-regulates these activities (Rogalla et al., 1999).

Phosphorylation of Hsp27 occurs at Ser15 and Ser85 and gives several phosphorylated isoforms of Hsp27 (Arrigo and Welch, 1987). Phosphorylation of Ser15 and Ser85 of Hsp27 facilitates actin remodeling and provides cellular protection to injury (Venkatakrishnan et al., 2006). Phosphorylation of Hsp27 has been suggested to improve cardiac function after ischemic injury (Pantos et al., 2007).

The expression of Hsp27 enhances the survival of mammalian cells when exposed to environmental and physiological stressors (Landry et al., 1989; Mehlen et al., 1993; Rollet et al., 1992; Wissing and Jäättelä, 1996). Hsp27 also seems to have more than one

function. Specifically, Hsp27 acts as a molecular chaperone (Jakob et al., 1993; Ehrnsperger et al., 1997; Rogalla et al., 1999; Diaz-Latoud et al., 2005), inhibits actin and intermediate filament polymerization (Lavoie et al., 1993a, 1993b; Perng et al., 1999; Wieske et al., 2001), reduces oxidative stress related to tumor necrosis factor α (TNFα)-mediated cell death (Mehlen et al., 1995a, 1995b) and suppresses signaling events leading to apoptosis (Mehlen et al., 1996a, 1996b; Bruey et al., 2000; Charette et al., 2000; Pandey et al., 2000; Concannon et al., 2001; Gaibai and Sherman, 2002; Arrigo, 2005; Beere, 2005). In mitochondrial-mediated apoptosis Hsp27 blocks cytochrome C release and activation of caspase-3 and procaspase-9 (Garrido et al., 1999; Bruey et al., 2000; Pandey et al., 2000; Beere, 2001) by sequestering cytochrome C and procaspase-3 (Concannon et al., 2001; Paul et al., 2002).

The members of sHsp family also function as anti-ischemia agents (Sun and MacRae, 2005a, 2005b; Taylor and Benjamin, 2005; Pantos et al., 2007). Transgenic overexpression of Hsp27 is also protective. Transgenic overexpression of Hsp27 in mice suppressed kainic acid induced excitotoxicity and hippocampal neuronal cell death (Akbar et al., 2003). Similarly, hearts from these Hsp27 transgenic mice were resistant to global ischemia and had less injury (Efthymiou et al., 2004).

Pharmacological Agents and Heat Shock Proteins

Pharmacological agents can be used to induce expression of Hsps and the heat shock response. The drugs used to induce the heat shock response may be divided into four classes:

1) Geldanamycin and its derivatives, including geldanamycin, herbimycin A, and ansamycins. Drugs in this class block the N-terminal pocket of Hsp90 and inhibit Hsp90

function (Zou et al., 1998; Blagosklonny, 2002). Herbimycin A induces Hsp70 and indicates the possibility of a pharmacological approach to Hsp70 induction and cardiac protection (Morris et al., 1996). Genistetin inhibits herbimycin A-induced Hsp70 expression because it decreases HSF1 production (Kiang, 2003).

2) Non-steroidal anti-inflammatory drugs, such as indomethasone, sodium salicylate, and dexamethasone. Sodium salicylate (Jurivich et al., 1992) and indomethasone (Lee et al., 1995) induce HSF1-HSE binding. Indomethasone is not sufficient to induce Hsp70 mRNA expression; however, it lowers the threshold for heat shock induced HSF1-HSE binding, and increases cell survival (Lee et al., 1995).

3) Immunosuppressants, such as cyclosporine and FK506. Rats pretreated with low-dose cyclosporine (3 mg/kg) or FK506 (0.3 mg/kg) at 6 hrs before ischemia had elevated levels of Hsp70 and reduced ischemia/reperfusion injury in their kidneys (Yang et al., 2001). Also, subcutaneous injections of FK506 (2 or 10 mg/kg) for 2 weeks markedly increased Hsp70 immunostaining in sensory neurons, motor neurons, Purkinje cells, and other regions of the brain. The ability of FK506 to increase Hsp70 expression may underlie its neuroprotective action (Gold et al., 2004).

4) Hormones, such as adrenalin and insulin. Rats exposed to a cat express elevated levels of Hsp70 in response to release of adrenal hormones. The Hsp70 expression is blocked or attenuated by adrenalectomy (Fleshner et al., 2004). Rats treated with norepinephrine (3.1 μMol/kg, i.p.) had upregulated expression of c-fos and Hsp70 in the myocardium and protection against postischemic myocardial dysfunction in the isolated rat hearts 2 or 24 hrs after norepinephrine injection (Meng et al., 1996). The induction of both cardiac Hsp70 expression and the cardioprotection by norepinephrine appears to be

mediated by α1- adrenoceptors (Meng et al., 1996). In human hepatoma Hep3B/T2 cells, the inducible Hsp70 gene is induced by insulin. Hsp70 mRNA is expressed at 2 - 6 hr after insulin treatment (Ting et al., 1989). The signal transducing pathway of insulin may play a role in the modulation of gene expression and particularly that of Hsp70. Chick embryo derived (pro) insulin modulates Hsc70 levels and prevents apoptosis (de la Rosa et al., 1998). In rat skeletal and smooth muscle, insulin stimulation phosphorylates Hsp20 (Wang et al., 1999).

There is plenty of evidence to suggest that Hsps have the potential to be used therapeutically to protect against ischemia. Whether insulin-induced expression of Hsps is involved in myocardial protection is an intriguing question.

Rationale

Cardiovascular disease continues to be among the leading causes of death and emergency hospital admissions in Canada. In cardiac surgery, the heart is often subjected to global and/or regional ischemia. During open heart surgery to repair valves or congenital defects, the aorta is clamped and the heart is stopped to provide the surgeon a bloodless and immobile field. Life support is provided by extracorporeal circulation. The most technically "successful" surgery is lost if the heart does not function immediately upon restoration of blood flow. Protecting the heart from ischemic injury reduces morbidity and mortality associated with open-heart surgery. Various solutions of glucose, insulin and potassium (GIK) are used to protect the heart during and after ischemia even though their use and effectiveness remain controversial. Insulin may be the key component in myocardial protection, through the insulin-activated "survival pathway". In addition, insulin is a general protein transcription and translation modulator and it activates Hsp70

mRNA expression in hepatoma cells. Other evidence suggests that heat shock proteins, and especially Hsp70 and Hsp27, have a role in myocardial protection. Therefore, it seems reasonable to **hypothesize** that insulin may induce elevated expression of heat shock proteins and these Hsps may contribute to insulin-induced myocardial protection. Insulin and heat shock may share a common intracellular pathway for induction of Hsps.

The **objectives** of this thesis are:

1. Examine whether insulin *per se* induces a preconditioning and provides protection to the heart against ischemia/reperfusion injury.
2. Examine whether insulin induces Hsps in adult rat heart via nitric oxide pathway.
3. Examine whether insulin modulates the localization of Hsps in cardiac tissue, and compare to the localization of heat shock induced Hsps.
4. Examine the time course of insulin induced Hsps and then possible role in myocardial protection.
5. Examine whether insulin regulates phosphorylation of Hsp27, and the relation with myocardial protection.
6. Examine whether insulin induces phosphorylation of Hsp27 through p38 MAPK or ERK1/2 pathways.

Overview of the Research Work

In this thesis, I am reporting on the insulin induced changes in expression of Hsps in heart and myocardial protection from ischemia/reperfusion injury.

Six hrs after insulin treatment Hsp70 was increased in cardiac tissue, as seen in enzyme immuno assay, Western blot and confocal microscopy. Heat shock induced a stronger expression of Hsp70 in cardiac tissue, and heat shock combined with insulin induced the strongest expression of Hsp70 in the heart (Chapter 2).

It appears that insulin induced Hsp70 expression is not via the myocardial nitric oxide-cyclic guanosine monophosphate (NO-cGMP) signaling pathway. L-NAME-blocked the NO-cGMP pathway but did not affect insulin-induced Hsp70 levels in cardiac tissue (Chapter 2). However, insulin activates HSF1 binding to HSE, initiating transcription of the Hsp70 gene (Chapter 3).

Six hrs after insulin treatment, isolated and perfused hearts were protected from ischemic injury and had enhanced contractile function during reperfusion. This protection may be associated with elevated levels of Hsp70. At 6 hrs after insulin treatment, Hsp70 is distributed to the plasma membrane of cardiomyocytes. Membrane bound Hsp70 was detected by Western analysis of fractionated cardiac tissue and by confocal microscopy showing Hsp70 immunoreactivity double labeled with dystrophin on plasma membranes. After heat shock, no myocardial protection was evident and localization of Hsp70 was in microvessels and in perivascular areas (Chapter 3).

At 1 hr after insulin treatment, isolated and perfused hearts were protected from ischemic injury and had enhanced contractile function during reperfusion. This protection may be associated with elevated phosphorylation levels of Hsp27. Heat shock had no

effect on myocardial protection or abundance of Hsps at this time. Insulin induced phosphorylation of Hsp27 and the protection of myocardial contractile function was blocked by SB203580, an inhibitor of p38 MAPK (Chapter 4).

CHAPTER 2:

INSULIN POTENTIATES EXPRESSION OF MYOCARDIAL HEAT SHOCK PROTEIN 70

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Introduction

Induction of the heat shock response and subsequent synthesis of heat shock protein 70 (Hsp70) renders the heart resistant to ischemia-reperfusion injury (Currie et al., 1988). The optimal synthesis of Hsp70 in the heart following heat shock treatment is dependent upon a transient up-regulation of the myocardial nitric oxide–cyclic guanosine monophosphate (NO–cGMP) signaling pathway (Malyshev et al., 1995). Administration of the non-specific nitric oxide synthase (NOS) inhibitor N(omega)-nitro-L-arginine to heat shock treated rats abrogated the transient increase in NO and significantly reduced myocardial Hsp70 synthesis (Malyshev et al., 1995). Furthermore, administration of NO-donor compounds (Katori et al., 2000) to rats markedly enhanced myocardial Hsp70 synthesis and inhibition of NOS abolished the infarct-reducing effect of heat shock (Arnaud et al., 2001). Thus, there is a relationship between heat shock and the myocardial NO-cGMP signaling system.

Insulin, a metabolic modulator, is a potent cardioprotective agent. Insulin protects the ischemic-reperfused heart in animal models of myocardial infarction (Jonassen et al., 2001) and myocardial stunning (Saupe et al., 2001). Glucose–insulin–potassium solutions administered to humans with an evolving acute myocardial infarction significantly reduce mortality (Diaz et al., 1998). In patients undergoing coronary artery bypass grafting, insulin-supplemented cardioplegia accelerated myocardial metabolic and functional recovery (Rao et al., 2000). The cardioprotective effects of insulin have largely been attributed to its metabolic modulatory effects. More recently, however, insulin has been shown to modulate NO-cGMP signaling and increase NO production by endothelial cells via its ability to enhance endothelial NOS (eNOS) activity and expression (Kuboki et al.,

2000). Whether insulin modulates the myocardial heat shock response and alters myocardial Hsp70 synthesis is at present an open question.

Since a transient increase in NO following heat shock is required for optimal myocardial Hsp70 synthesis (Malyshev et al., 1995) and insulin stimulates NO production, we hypothesized that insulin would enhance myocardial Hsp70 synthesis by augmenting the NO signaling pathway. The purpose of our experiments was to determine the effect of a physiologic dose of insulin on myocardial Hsp70 content and localization in unstressed rats, and in rats following heat shock treatment. Our results suggest that insulin, at a physiologic dose, induces a low-level expression of Hsp70 in the naïve rat heart and also potentiates myocardial Hsp70 expression in heat shock treated rats independent of myocardial NO signaling.

Materials and Methods

Animals

Male Sprague-Dawley rats (250 - 325 gm, Charles River Inc. St Constant, Québec, Canada) were cared for in accordance with the *Guide to Care and Use of Experimental Animals* of the Canadian Council on Animal Care and experimental procedures were approved by the Dalhousie University Committee on Laboratory Animals.

Experimental Protocol and Groups

Rats were randomized into six groups, (1) control, CON; (2) insulin treated, INS; (3) heat shock treated, HS; (4) heat shock and insulin treated, HSINS, (5) L-nitro arginine methyl ester (L-NAME) and heat shock and insulin treated, L-NA; and (6) sodium nitroprusside (SNP) and heat shock and insulin treated, SNP. The experimental protocol and treatment groups are shown in Figure 2.1.

For all groups, rats were anesthetized with sodium pentobarbital (50 mg/kg) i.p. Control rats received an injection of sodium pentobarbital, but were not heated or injected with insulin. For insulin treatment, rats were injected intramuscularly in the thigh with 200 μU/gm body weight. For heat shock treatment, rats were placed on a temperature-controlled heating pad (50 °C) until core body temperature reached 42 °C, monitored with a rectal thermometer. Core body temperature was maintained between 42 and 42.5 °C for 15 min. For heat shock and insulin treatment, rats were subjected to the heat shock treatment first and at 10 min after the heat shock treatment, were injected with insulin (200 μU/gm body weight) intramuscularly in the thigh (Figure 2.1). L-NAME (10 mg/kg) was administered intravenously (penile vein) and SNP (8 μg/kg/min) was given as an

intravenous infusion over 10 min via the tail vein. Both L-NAME and SNP were administered prior to heat shock and insulin.

Measurement of Blood Glucose

Blood samples were collected from the tail vein of all rats at the times indicated in Figure 2.1 and glucose levels were determined with a glucose meter (AccuSoft Advantage, Roche Diagnostics, Québec, Canada).

Tissue Preparation and Assays

At 6 hr of recovery (Figure 2.1), rats were injected with sodium pentobarbital (50 mg/kg) and decapitated. For biochemical analysis, hearts were removed and immediately freeze clamped. The frozen tissue was stored in liquid nitrogen for later analysis of Hsp70 and total protein.

Western Analysis

Heart tissues were homogenized in 0.32 M sucrose. Protein concentration was determined by the method of Lowry et al. (1951). Samples containing 25 mg of protein were solubilized in sodium dodecyl sulfate (SDS) sample buffer, boiled for 10 min and loaded onto a polyacrylamide gel (2.5% upper gel, 7.5% running gel), according to previously described methods (Currie et al., 1988). Proteins were separated by electrophoresis and then electrotransferred onto a Millipore-P membrane. Membranes were incubated in bovine lacto transfer optomizer (Blotto) as previously described (Plumier et al., 1995) at 37 °C for 1 hr to block non-specific binding of primary antibody. Membranes were incubated at 4 °C overnight with primary mouse monoclonal anti-hsp70 antibody (1:500, catalogue number SPA-810, StressGen, Victoria, Canada) in fresh Blotto.

Next day, following three 20 min washes, membranes were incubated in secondary horse anti-mouse horseradish peroxidase conjugated antibody (1:500, catalogue number PI-2000, Vector Laboratories, Burlingame, CA, USA). After six 10 min washes, membranes were incubated in ECL™ solution (catalogue number RPN2109, Amersham Biosciences, U.K.) for 1 min, and then exposed on Hyperfilm ECL™ (catalogue number RPN2114, Amersham Biosciences, U.K.). Films were scanned on a HP6200 scanner to acquire digital imagines. Densitometric analysis was done with imaging software from Scion Co (Scion Imagine, Version 4.0.2, Scion Corporation, USA).

Enzyme Immunoassay for Hsp70

Hsp70 content of heart was determined by an enzyme immunoassay kit (StressGen, Victoria., Canada). Frozen powdered ventricular tissue (200 - 250 mg) was placed in 1.0 ml 1 X Hsp70 Extraction Reagent at 4 °C. One protease inhibitor cocktail tablet (catalogue number P2714, Sigma, St Louis, MO, USA) was used in every 10 ml 1 X Extraction Reagent. Tissue was homogenized with an electrical pestle at 3000 revolution/s at 4 °C for 30 s in a polypropylene tube. Homogenized samples were centrifuged at 21,000g for 10 min at 4 °C. Samples of supernatant (100 ml) and standards (100 ml) were added in duplicate to wells in the Hsp70 immunoassay plate, and then incubated at room temperature for 2 hrs. Wells were washed six times with 1 X wash buffer. Anti- Hsp70: Biotin (100 ml) was added to each well and incubated at room temperature for 1 hr. Wells were washed six times with 1 X wash buffer. Avidin-HRP conjugate (100 ml) was added to each well, covered and incubated at room temperature for 1 hr. Again, wells were washed six times with 1 X wash buffer. TMB Substrate (100 ml) was added to each well and incubated at room temperature for 10 min. Acid stop solution (100 ml) was added to

each well. The microplate was read at 450 nm wavelength with a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). Results were calculated and expressed as ng/mg protein.

Immunofluorescence Microscopy

Tissue Sectioning and Preparation

For immunofluorescence, fresh hearts were collected, rinsed of blood in saline, and then immersed and fixed in 2% paraformaldehyde at 4 °C overnight. Following fixation, tissues were cryoprotected in 30% sucrose at 4 °C. Tissues were cut into 20 μm thick sections with a freezing slicing microtome and sections were kept in Millonig's solution (pH 7.4) at 4 °C.

Hsp70 with Factor VIII and Phalloidin Immunofluorescence

To characterize the Hsp70 localization in heart and its relation to cardiomyocytes, sections were incubated with Hsp70 antibodies (mouse monoclonal anti-hsp70 antibody 1:500, catalogue number SPA-810, StressGen, Victoria, Canada), and either factor VIII antibodies (rabbit polyclonal anti-Factor VIII related antigen/vWF(Ab-1) 1:500, catalogue number PC313, Oncogene Research Products, USA) or phalloidin. For double labeling of Hsp70 and factor VIII, tissue sections were processed as previously described (Leger et al., 2000). For double labeling of Hsp70 and actin with phalloidin, tissue sections were first reacted for Hsp70 immunofluorescence. Next, the tissue sections were washed with PBS three times, and then fixed in 3.7% formaldehyde in PBS at room temperature for 10 min. Tissue sections were washed twice with PBS and extracted with 0°C acetone for 3 min. Sections were washed in PBS and incubated in a solution of phalloidin (1:40 in PBS, Alexa green 488 conjugated, catalogue number A12379, Molecular Probe Inc., USA) at 37 °C for

2 hrs. Finally, tissue sections were mounted onto gelatinized slides. In each batch of sections stained for fluorescence microscopy, some sections were incubated without primary antibody or phalloidin to serve as control for non-specific staining. A Carl Zeiss LSM510 laser-scanning microscope was used to scan sections for confocal imaging. Images were captured with LSM 510 software (Version 2.01, Carl Zeiss, Gemany). Captured images were edited adjusting only brightness and contrast and composed with Photoshop (Version 6.0, Adobe System Incorporated, USA).

Statistical Analysis

Standard curves for Hsp70 EIA were transformed to log–log plot and calculated with a linear model. Data were analyzed with ANOVA (SPSS Version 11.5, Chicago, IL, USA). If overall ANOVA was significant for a specific variable, Bonfferoni multi-comparison analysis was used to test the significance among the groups. For comparing the data of glucose levels before and after insulin injection or heat shock, the paired Student t-test was used. All data mentioned above were also confirmed with non-parametric statistical analysis after parametric analysis. The results are expressed as mean ± SEM. Significance was set at $P \leq 0.05$.

Results

Blood Glucose Determination

No significant differences were detected in blood glucose levels among the groups before and after insulin injection or heat shock treatment (Figure 2.2).

Western Analysis and Densitometry

Hsp70 in the insulin treatment group was at a detectable level by Western analysis (Figure 2.3A) while in the control group Hsp70 was not detectable. However, this apparent difference in Hsp70 was not significant ($P > 0.05$) following densitometric analysis (Figure 2.3B). Hsp70 in the heat shock treated group and in the heat shock and insulin treatment group was abundant by Western analysis and following densitometric analysis was significantly different ($P < 0.001$) compared to the control group (Figure 2.3B). Pretreatment with L-NAME or SNP did not abolish or further enhance, respectively, the ability of insulin to potentiate Hsp70 after heat shock (Figure 2.4A and B).

Hsp70 Immunoassay

Hsp70 in the insulin treatment group (15.5 ± 0.8 ng/mg protein) was not significantly different ($P > 0.05$) from that of the control group (12.9 ± 2.0 ng/mg protein, Figure 2.5). Hsp70 in the heat shock treated group (120.6 ± 16.8 ng/mg protein) and in the heat shock and insulin treatment group (164.4 ± 7.5 ng/mg protein) was significantly increased ($P < 0.001$) compared to the control group. In addition, Hsp70 in the heat shock and insulin treatment group (164.4 ± 7.5 ng/mg protein) was significantly greater ($P < 0.001$) from that of the heat shock group (120.6 ± 16.8 ng/mg protein, Figure 2.5). Pretreatment with the NOS inhibitor L-NAME did not abolish the ability of insulin to

potentiate Hsp70 in heat shocked animals (195.2 ± 13.4 ng/mg protein, P = 0.21; Figure 2.5). Furthermore, pretreatment with the NO donor compound SNP did not further enhance Hsp70 synthesis in the heat shock and insulin treated group (188.9 ± 8.2 ng/mg protein, P = 0.71; Figure 2.5).

Hsp70 and Factor VIII Immunofluorescence Microscopy

Confocal microscopy revealed little or no Hsp70 immunoreactivity (Hsp70-IR) in control hearts (Figure 2.6A). Interestingly, low level Hsp70-IR was evident in hearts from the insulin treated group (Figure 2.6B). Intense Hsp70-IR was evident in hearts from the heat shock treated group (Figure 2.6C) and the heat shock and insulin-treated group (Figure 2.6D). Factor VIII immunoreactivity (Factor VIII-IR) was evident in all hearts and was unaffected by the insulin or heat shock treatments (Figure 2.6A–D). Co-localization of Hsp70-IR and factor VIII-IR appeared to be in blood vessels (Figure 2.6B–D).

Hsp70 Immunofluorescence and Phalloidin Fluorescence Microscopy

Hsp70-IR was again not evident in control hearts (Figure 2.7A) and was evident at a low level in hearts from the insulin treated group (Figure 2.7B). Intense Hsp70-IR was evident in hearts from the heat shock treated group (Figure 2.7C) and the heat shock and insulin treated group (Figure 7D). Phalloidin fluorescence was evident in all hearts and was unaffected by the insulin or heat shock treatments (Figure 2.7A–D). No apparent co-localization of Hsp70-IR (in blood vessels) and phalloidin fluorescence (on actin in cardiomyocytes) was evident (Figure 2.7B–D).

Figure 2.1. Schematic representation of the six experimental groups and their treatments. All animals were anesthetized with sodium pentobarbital at the times indicated. Insulin (200 μU/gm body weight) was injected in the insulin group and the heat shock and insulin group at the times indicated. Blood samples were taken for glucose analysis from all animals 10 min before insulin injection or heat shock treatment and approximately 60 min later. L-NAME, 10 mg/kg intravenously injected; SNP, 8 μg/kg/min, intravenously injected; G, glucose testing; L, administration of L-NAME; S, administration of SNP.

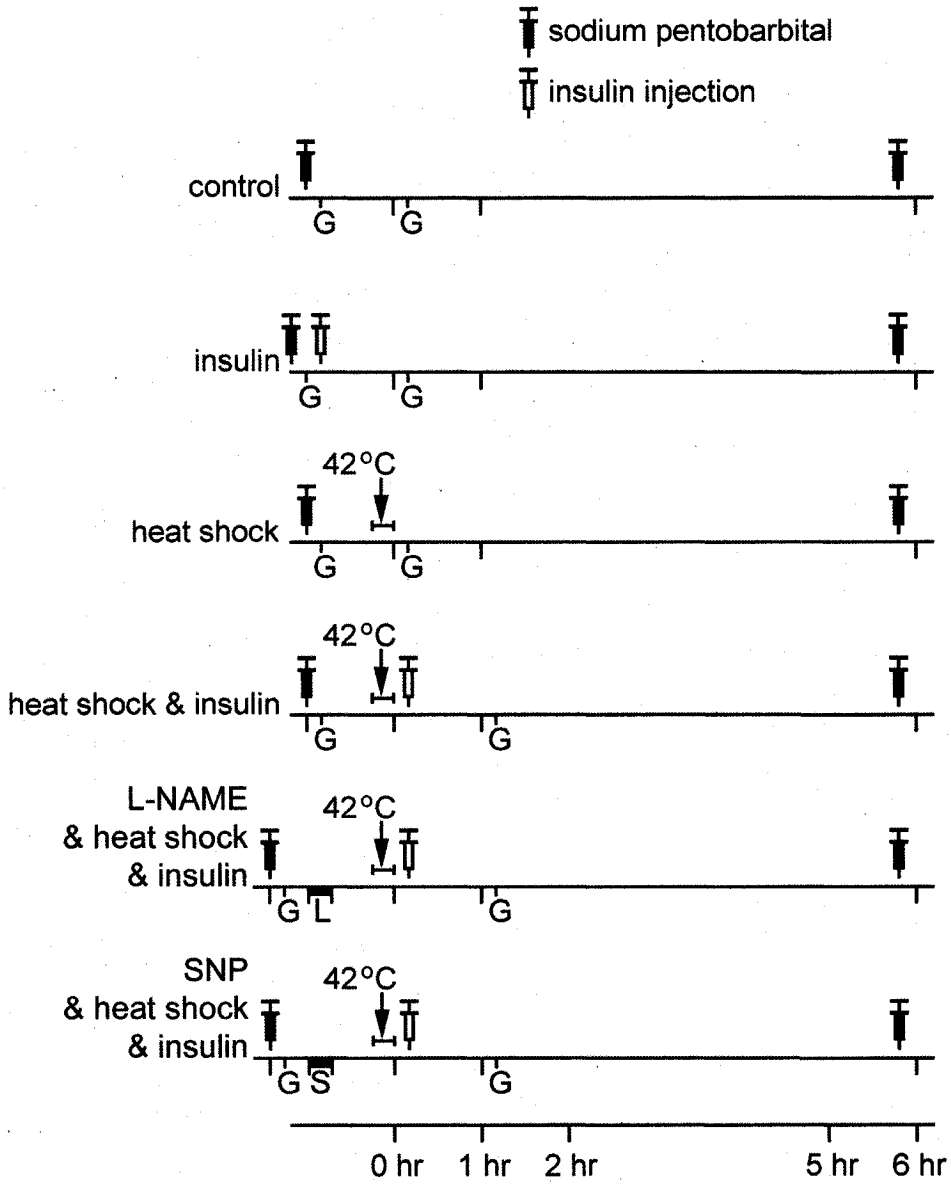
sodium pentobarbital
insulin injection
control
G
G
insulin
G
G
42°C
heat shock
G
G
42°C
heat shock & insulin
G
G
L-NAME
& heat shock
& insulin
42°C
G
L
G
SNP
& heat shock
& insulin
42°C
G
S
G
0 hr
1 hr
2 hr
5 hr
6 hr

Figure 2.1

Figure 2.2. Blood glucose levels before and after insulin treatment, heat shock treatment and heat shock and insulin treatment. There was no significant difference in glucose levels among the groups before and after insulin injection or heat shock treatment ($P > 0.05$).

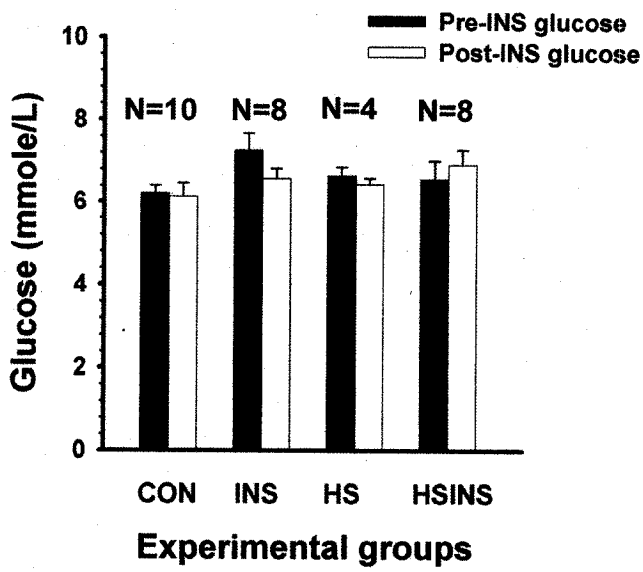
Pre-INS glucose
Post-INS glucose
Glucose (mmole/L)
10
8
6
4
2
0
N=10
N=8
N=4
N=8
CON
INS
HS
HSINS
Experimental groups

Figure 2.2

Figure 2.3. Hsp70 levels detected by Western analysis and densitometric analysis after Insulin, HS and HSINS treatments. (A) A representative membrane showing Hsp70 levels in two hearts in each of the four groups. (B) Relative densities (arbitrary units, mean ± SEM) of Hsp70 in each group. Asterisks indicate statistical differences ($p < 0.05$) versus control and insulin treated groups.

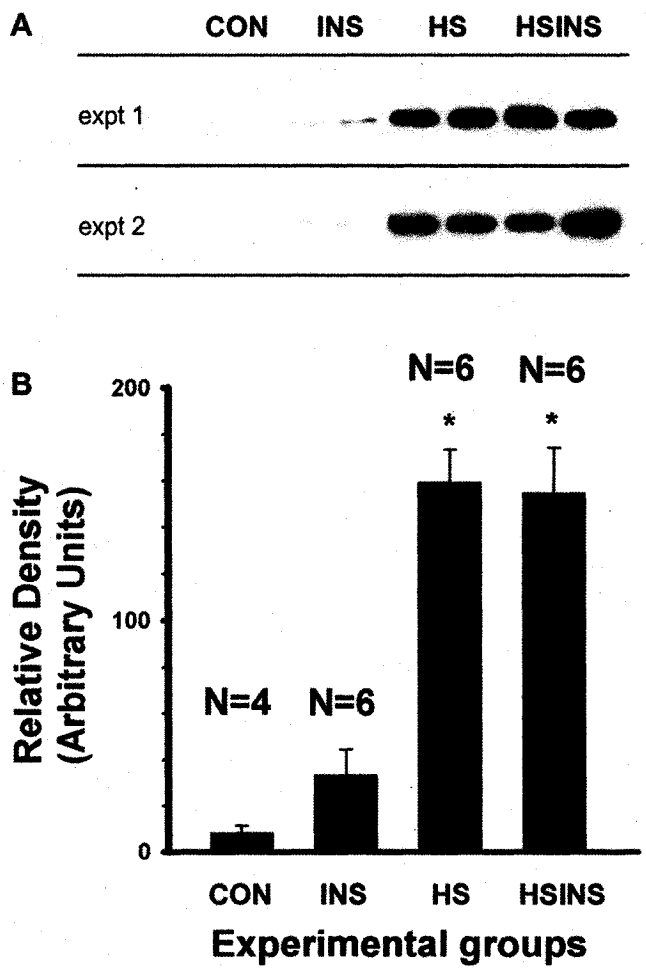
A
CON
INS
HS
HSINS
expt 1
expt 2
B
200
100
0
Relative Density
(Arbitrary Units)
N=4
N=6
N=6
N=6
*
*
CON
INS
HS
HSINS
Experimental groups

Figure 2.3

Figure 2.4. Hsp70 levels detected by Western analysis and densitometric analysis after L-NA and SNP treatments. (A) A representative membrane showing Hsp70 levels in two hearts in each of the four groups. (B) Relative densities (arbitrary units, mean ± SEM) of Hsp70 in each group. Asterisks indicate statistical differences ($P < 0.05$) versus control and heat shock groups.

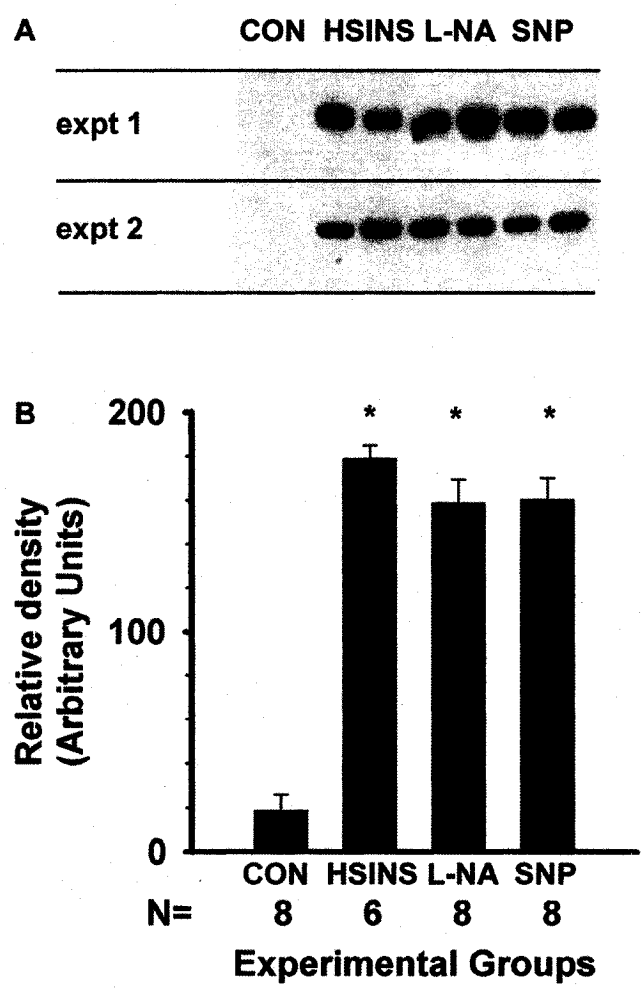
A
CON HSINS L-NA SNP
expt 1
expt 2
B
200
100
0
Relative density
(Arbitrary Units)
*
*
*
CON HSINS L-NA SNP
N= 8 6 8 8
Experimental Groups

Figure 2.4

Figure 2.5. Hsp70 levels measured by enzyme immunoassay in controls and at approximately 6 hrs in the six groups. * $P < 0.001$ for HS, HSINS, L-NA, SNP vs. CON and INS; ** $P = 0.01$ for HSINS, L-NA, SNP vs. HS; $P = 0.21$ for HSINS vs. L-NA; $P = 0.72$ for HSINS vs. SNP.

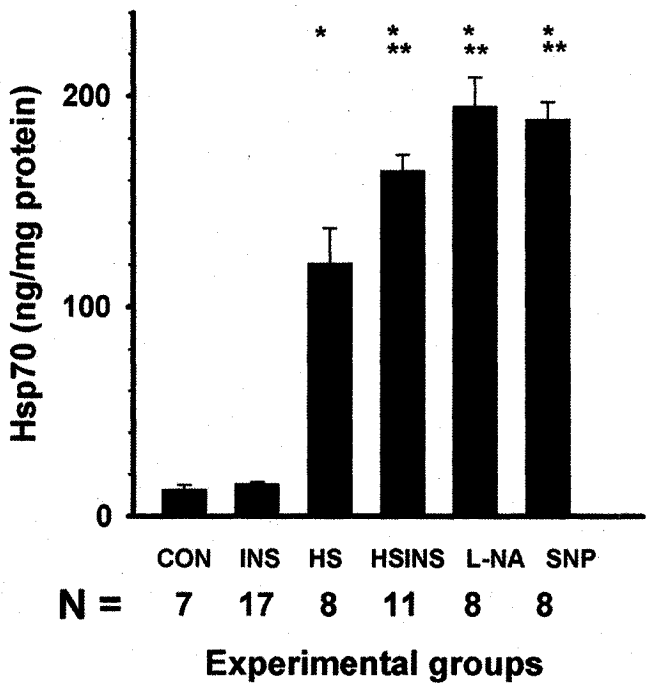
Hsp70 (ng/mg protein)
200
100
0
* * ** * ** * **
CON INS HS HSINS L-NA SNP
N = 7 17 8 11 8 8
Experimental groups

Figure 2.5

Figure 2.6. Confocal micrographs of ventricular sections double labeled with antibodies for Hsp70 and Factor VIII. Hsp70-IR (Cy3, red) and Factor VIII-IR (CY2, green). (A) Control, (B) insulin treated, (C) heat shock treated, (D) heat shock and insulin treated. Bar = 50 μm.

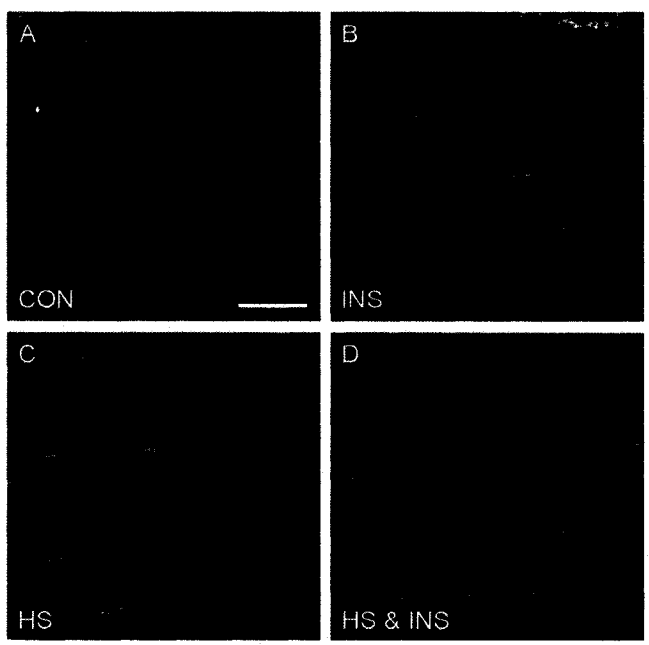

Figure 2.6

Figure 2.7. Confocal micrographs of ventricular sections double labeled with Hsp70 antibodies and phalloidin. Hsp70-IR (Cy3, red) and phalloidin (CY2, green). (A) Control, (B) insulin treated, (C) heat shock treated, (D) heat shock and insulin treated. Bar = 20 μm.

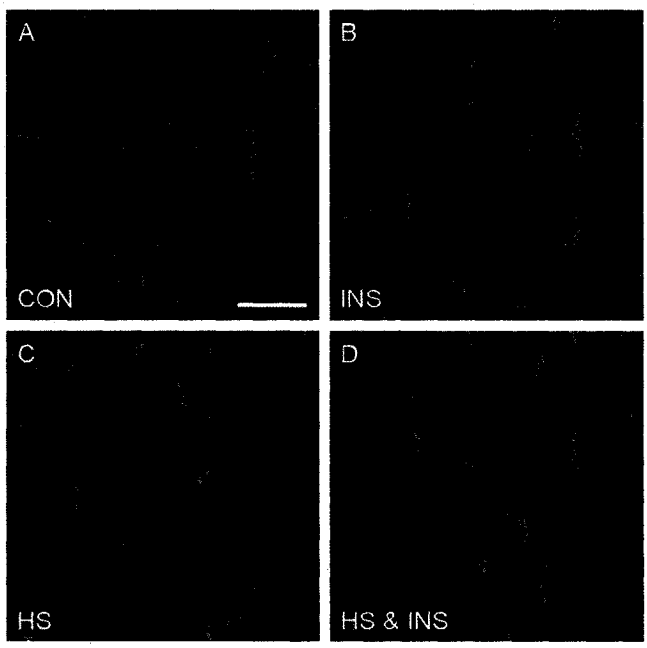

Figure 2.7

Discussion

This study demonstrates that insulin induces an apparent low-level expression of Hsp70 primarily in microvessels, of the naïve, non-ischemic heart. Interestingly, after heat shock treatment, insulin potentiates myocardial Hsp70 synthesis and this effect is neither abrogated by the NOS inhibitor L-NAME nor enhanced by the NO donor compound SNP. Thus, the insulin mediated potentiation of myocardial Hsp70 synthesis following heat shock appears to be independent of myocardial NO signaling.

Effect of Insulin on Hsp70 in Unstressed Heart

Insulin is a cardioprotective agent in myocardial ischemia-reperfusion injury. For example, insulin treatment in the isolated perfused heart significantly improves functional recovery following ischemic injury (Saupe et al., 2001). Similarly, insulin treatment *in vivo* results in significant infarct size reduction and this protective effect appears to be dependent upon insulin-stimulated NO production (Gao et al., 2002). The capacity of insulin to promote NO synthesis is considered paramount in its ability to mediate both metabolic and peripheral vascular actions.

In my study, the dose (200 μU/g body weight) and route (i.m.) of insulin administration was chosen based on this dose causing a nine-fold increase in basal plasma NO levels 30 min after injection in mice (Sinha et al., 1999). The insulin dose was physiological and did not significantly alter blood glucose levels in any of the experimental groups (Figure 2.2). This finding excludes the possibility that insulin altered myocardial Hsp70 expression secondary to a hypoglycemic effect. A low-level expression of Hsp70 was observed by Western analysis (Figure 2.3) and immunofluorescence (Figures. 2.6 and 2.7) in hearts in response to insulin treatment. However, this apparent low-level expression

of Hsp70 in insulin treated hearts did not reach statistical significance when semi-quantitated by optical densitometry (Figure 2.3B) or immunoassay techniques (Figure 2.5). Although we could not quantitatively demonstrate a statistically significant increase in Hsp70 in insulin treated hearts (Figure 2.3B and 2.5), we are confident that insulin is capable of modulating Hsp70 expression in the unstressed heart as evidenced by the mild but definite bands evident in the Western analysis (Figure 2.3A) and the immunofluorescence evident in Figures 2.6 and 2.7. Insulin is capable of inducing Hsp70 mRNA in the human hepatoma cell line Hep3B/T2 and this effect is mediated by the insulin receptor (Ting et al., 1989). Similarly, insulin induces a transient synthesis of Hsp70 in cultured fibroblasts and epithelial cells (Chelbi-Alix et al., 1994).

Effect of Heat Shock on Myocardial Hsp70

Induction of the heat shock response in rats is associated with a transient increase of NO synthesis in the heart, lung and liver (Malyshev et al., 1995). Administration of the NOS inhibitor N(omega)-nitro-L- arginine to heat shock treated rats abolishes the transient increase in NO and significantly attenuates myocardial Hsp70 synthesis (Malyshev et al., 1995). Administration of NOS inhibitors also abolishes the infarct-reducing effect of heat shock (Arnaud et al., 2001). These studies suggest that a transient stimulation of NOS and NO synthesis is crucial for expression of Hsp70 and the myocardial protection that results following heat shock.

Heat shock treatment of rats induces expression of Hsp70 and significantly improves contractile recovery of hearts following ischemic injury (Currie et al., 1988). Hearts of transgenic mice overexpressing the human Hsp70 (Plumier et al., 1995) show a significant resistance to ischemic injury and provide direct evidence for a protective role for

Hsp70 in myocardial ischemia reperfusion injury. As expected, heat shock treatment significantly increased myocardial Hsp70 content (Figures. 2.3 and 2.5).

Effect of Insulin on Myocardial Hsp70 in the Heat Shock Treated Rat

Insulin potentiated the expression of Hsp70 in the heart following heat-shock treatment. By the semi-quantitative Western analysis technique of optical densitometry, there was no apparent increase in Hsp70 when insulin was administered with heat shock treatment (Figure 2.3B), compared to the heat shock alone treatment group. However, by enzyme immunoassay, a more sensitive quantitative method, myocardial Hsp70 was significantly increased in the group administered insulin following heat shock treatment, as compared to the heat shock only treated group (Figure 2.5).

The potentiation of Hsp70 by insulin in the heat shock treated hearts was not abolished by pretreatment with the NOS inhibitor L-NAME and was not further amplified by pretreatment with the NO donor compound SNP (Figure 2.5). This suggests that insulin potentiates Hsp70 in heat shocked myocardium by mechanisms independent of its ability to stimulate NO signaling. The mechanism(s) responsible for the potentiation of Hsp70 synthesis by insulin following heat shock are at best speculative. The downstream signaling events occurring in response to insulin occupying its receptor are numerous and complex. In addition, the intracellular signaling cascades activated by heat shock are poorly understood. However, it is interesting to note that both insulin and heat shock may share common downstream signaling pathways. For example, insulin (Hermann et al., 2000) and heat shock (Bang et al., 2000) activate the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. This signaling pathway appears to be critical for inhibiting apoptosis (Matsui et al., 2001) and results in eNOS stimulation and NO production (Zeng

et al., 2000). Apoptosis in the heart following reperfusion is inhibited by heat shock and this may contribute to its infarct-reducing effect (Suzuki et al., 2000). The infarct-reducing effect of insulin *in vivo* is also related to an inhibition of apoptosis that is dependent upon NO production (Gao et al., 2002). Whether the stimulation of Hsp70 synthesis by insulin contributes to the latter's anti-apoptotic effect in the heart remains to be determined. The potentiation of Hsp70 synthesis by insulin with heat shock treatment observed in my study may be indicative of similar signaling pathways being activated by insulin and heat shock treatments.

Localization of Hsp70 Following Insulin and Heat Shock

Confocal microscopy demonstrates that Hsp70 appears to localize predominantly in blood vessels following both insulin and heat shock treatments (Figures. 2.6 and 2.7). This finding supports our previous results (Leger et al., 2000) and those of other investigators (Amrani et al., 1998) and highlights the importance of blood vessels and endothelium in myocardial protection. Coronary endothelial damage contributes to the poor contractile recovery of the isolated ischemic reperfused heart. Heat shock treatment completely prevents endothelial dysfunction in coronary arteries isolated from rats subjected to myocardial ischemia reperfusion injury and restores NO-mediated coronary artery vasorelaxation (Richard et al., 2002). The critical importance of the coronary endothelium in heat-shock mediated cardioprotection is also demonstrated by the lack of a protective effect of heat-shock on functional recovery following ischemia reperfusion injury in rat hearts which have had endothelium removed (Amrani et al., 1998). These findings suggest an association between Hsp70 in myocardial blood vessels and functional recovery during reperfusion after an ischemic injury. Furthermore, showing that insulin induces an

apparent low-level expression of Hsp70 in blood vessels of the heart and potentiates Hsp70 expression following heat shock raises the possibility that insulin may augment Hsp70 synthesis following other pathophysiological stimuli such as ischemia. If insulin is indeed capable of enhancing Hsp70 expression, some of the infarct-reducing capacity of insulin may be attributable to mechanisms quite independent of its metabolic modulatory effects.

Limitations

Our interpretation that insulin potentiates heat shock mediated myocardial Hsp70 synthesis independently of its ability to augment NO signaling is based on the fact that pretreatment with a NOS inhibitor (L-NAME) or NO donor (SNP) had no effect, respectively, in either abolishing or enhancing myocardial Hsp70 levels in animals treated with heat shock and insulin. However, this interpretation is limited by the fact that we have not actually measured NO. We could not demonstrate quantitatively a statistically significant increase in Hsp70 content of control hearts after insulin treatment alone, although an effect was clearly evident by the Western blot and immunohistochemistry. Only one dose of insulin was tested.

Conclusion

In summary, we have shown that a physiologic dose of insulin induces low-level expression of Hsp70 in the naïve, unstressed rat heart and significantly potentiates myocardial Hsp70 synthesis following heat-shock treatment. The latter effect is not altered by pretreatment with either L-NAME or SNP, suggesting that it is independent of insulin's known ability to stimulate NO signaling. Hsp70 localization appears to be in blood vessels predominantly following insulin treatment and heat shock. These findings suggest intriguing possibilities regarding the cardioprotective effects of insulin and heat shock.

CHAPTER 3:

INSULIN INDUCES MYOCARDIAL PROTECTION AND HSP70 LOCALIZATION TO PLASMA MEMBRANES IN RAT HEART

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Introduction

Insulin is a crucial regulator of metabolism and in cardiac muscle insulin induces glycogen and protein synthesis and in fat, lipid storage is favored. Insulin also mediates other diverse effects in a wide variety of cells and tissues (Myers and White, 1996). Sodi-Pallares et al. (1962) initially introduced the concept of infusing a metabolic cocktail of glucose, insulin, and potassium to improve recovery from myocardial infarction. Clinical studies suggest that this cocktail might have an important role in reducing in-hospital mortality following acute cardiac infarction (Donnelly et al., 1992; Fath-Oodoubadi et al., 1997; Diaz et al., 1998; Rao et al., 2000). Initially, two possible mechanisms for the effectiveness of the glucose, insulin, and potassium cocktail were suggested: 1) the promotion of cardiac glycolysis and 2) uptake of free fatty acids into adipocytes (Opie, 1970). More recently, insulin alone has been shown to be as effective as the glucose, insulin, and potassium cocktail at reducing myocardial ischemia/reperfusion injury (Jonassen et al., 2001). The cardioprotective effect of insulin may be mediated via phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt) and p70S6 kinase cell signaling (Sack et al., 2003; Pasupathy et al., 2005), or by increasing nitric oxide bioavailability (Gao et al., 2002). Nitric oxide is proposed as the "second messenger" of insulin. A physiological dose of insulin activates membrane-bound nitric oxide synthase (NOS). Injection of N^G-Nitro-arginine methyl ester (L-NAME) before the administration of insulin to mice inhibited not only the insulin-stimulated increase of NO in plasma but also the glucose-lowering effect of insulin (Kahn et al., 2000). Another mechanism of insulin activated cardioprotection may be related to the expression of heat shock proteins. Various heat shock proteins (Hsps) have cytoprotective functions (Jolly and Morimoto,

2000; Beere and Green, 2001; Garrido et al., 2001; Delagu et al., 2002). As molecular chaperones, Hsps regulate folding of nascent proteins, participate in refolding of misfolded or damaged proteins, stabilize structural proteins, and facilitate translocation of proteins across membranes among cellular compartments. Several Hsps prevent aggregation of proteins and target unstable or damaged proteins for degradation. Hsp70 and Hsp27 also suppress intracellular apoptotic signaling pathways (Mosser et al., 2000; Paul et al., 2002).

Hsps and particularly Hsp70 are associated with enhanced recovery of myocardial contractility after ischemic injury (Currie et al., 1988; Jolly and Morimoto, 2000). Transgenic overexpression of rat and human Hsp70 provided strong evidence for a direct role in protection of the mouse myocardium from ischemic injury (Marber et al., 1995; Plumier et al., 1995). Insulin induces Hsp70 in the Hepatoma cell line Hep3B/T2, and this effect is transient and occurrs between 4 to 8 hours after insulin treatment (Ting et al., 1989). We recently reported that the highly inducible 70 kDa heat shock protein (Hsp70) is indeed induced by insulin, albeit at a relatively low level, in rat heart. Insulin appeared to have an additive effect on the expression of Hsp70 following heat shock treatment (Figure 2.5 in this thesis, Li et al., 2004). While we have shown that after heat shock, Hsp70 is mainly localized to microvessels between cardiomyocytes in the rat heart (Leger et al., 2000), after insulin treatment the localization of Hsp70 was unclear.

In this study we show that insulin improves post-ischemic myocardial contractility and that expression of Hsp70 is elevated by activation of the heat shock transcription factor and we report the subcellular distribution and localization of Hsc70 and Hsp70 after insulin treatment and after heat shock treatment.

Materials and Methods

Animals

Male Sprague-Dawley rats (250 - 325 gm, Charles River Inc. St. Constant, Québec, Canada) were used in these experiments. All animal care, handling and experimental procedures on animals were in accordance with the *Guide to Care and Use of Experimental Animals* of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

Experimental Protocol and Groups

Rats were randomized into five groups, 1) naïve control, CON; 2) Sham control, injected with normal saline and recovery for 6 hours, SHAM; 3) insulin treated, INS; 4) heat shock treated, HS; 5) heat shock and insulin treated, HSINS. For all groups, rats were anesthetized with sodium pentobarbital (50 mg/kg) i.p. Naïve control rats received an injection of sodium pentobarbital, but were not heated or injected with insulin. For sham control rats, normal saline was injected intramuscularly, similar to the insulin treatment group. For insulin treatment, rats were injected intramuscularly in the thigh with 200 μU/gm body weight. For heat shock treatment, rats were placed on a temperature-controlled heating pad (50 °C) until core body temperature reached 42 °C, monitored with a rectal thermometer. Core body temperature was maintained between 42 °C to 42.5 °C for 15 minutes. For heat shock and insulin treatment, rats were subjected to the heat shock treatment first and at 10 minutes after the heat shock treatment, were injected with insulin (200 μU/gm body weight) intramuscularly in the thigh.

At six hours of recovery, rats were injected with sodium pentobarbital (50 mg/kg) and decapitated. For functional analysis, hearts were rapidly extirpated and mounted on a

Langendorff perfusion apparatus. For cell fractionation and subsequent Western analysis, hearts were removed, perfused briefly with cold (4 °C) normal saline to remove blood, and then homogenized. For electrophoretic mobility shift assay, hearts were rapidly extirpated and immediately freeze-clamped. The frozen tissue and cell fractions were stored in liquid nitrogen before protein content determination (Lowry et al., 1951), electrophoretic mobility shift assay, and Western blot analysis. For immunofluorescence microscopy, hearts were collected, perfused with a syringe and rinsed with cold saline (4 °C) until clean (about 1 minute), and then immersed and fixed in 2% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4 °C overnight.

Langendorff Perfusion and Cardiac Function

Hearts were perfused in the non-working Langendorff mode as described previously (Currie et al., 1988). Modified Krebs-Henseleit bicarbonate buffer (Aasum et al., 2002) (NaCl 118.5, Na_2HCO_3 25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, EDTA 0.5, Glucose 11 mM, pH 7.4) gassed with 95% O_2 - 5% CO_2 at 37 °C was perfused into hearts at a constant rate of 10 ml/min with a static roller pump. Hearts were equilibrated to the perfusion system for 30 min. Heart rate (HR) and perfusion pressure were continuously recorded with a Grass model 7 polygraph (Grass Instruments Inc, Quincy, MA, USA). A balloon connected to pressure transducer (P-23D, Gould Inc. USA), was inserted into the left ventricle through the mitral valve to measure left ventricular end-systolic pressure (LVESP) and left ventricular end-diastolic pressure (LVEDP) as well as the first derivative of the left ventricular pressure (±dp/dt). Maximal dp/dt was used in this study. The difference between LVESP and LVEDP, maximal left ventricular developed pressure (LVDP), was calculated. After 30 min of equilibration, all hearts except for SHAM were

subjected to 30 min no-flow global ischemia. SHAM hearts were continually perfused at 10 ml/min. Following ischemia, hearts were reperfused at 10 ml/min for 60 min. Physiological measurements were taken at 10 min before initiation of ischemia (time = -40 min) during pre-ischemic perfusion and at 5, 30 and 60 min of reperfusion.

Analysis of Left Ventricular Function and Pressure-Volume Relationship

Analysis of left ventricular function and calculation of pressure-volume relationship were done according to Li et al. (1998) and Burkhoff and Sagawa (1986) with minor modifications. In brief, analysis of left ventricular performance was derived from the measured LVESP and LVEDP data. The volume in the balloon was increased using 37 °C normal saline in 25 μl increments to a maximum volume of 200 μl. A linear regression analysis of LVESP versus volume was performed to estimate the left ventricular end-systolic pressure–volume relationship. To estimate left ventricular work (LVW), I calculated cumulative areas under the LVESP versus volume curve to produce the systolic work area (SWA), i.e., the area under the systolic regression curve and under the LVEDP versus volume curve to produce the diastolic work area (DWA), i.e., the area under the diastolic regression curve. SWA and DWA were inserted into the formula below to find LVW.

$$\text{LVW (mmHg·ml)} = \text{SWA} - \text{DWA}$$

Coronary vascular resistance (CR) was derived from constant perfusion flow (10 $ml \cdot min^{-1}$) and mean perfusion pressure (MAP) with the following formula.

$$\text{CR (mmHg·ml}^{-1}\text{·min}^{-1}\text{)} = \text{MAP (mmHg/10 ml·min}^{-1}\text{)}.$$

Preparation of Cell and Tissue Extracts for Electrophoretic Mobility Shift Assay

Cell and tissue extracts were prepared as described by Mosser et al. (1988) and Locke et al. (1995) with minor modifications. U937 human monocytic leukemia cells from American Type Culture Collection (ATCC) were grown in culture flasks in RPMI 1640 medium (95% air/5% CO_2) with L-glutamine (Hyclone, Logan Utah, USA), supplemented with 10% fetal bovine serum, penicillin 100 U/ml and streptomycin 100 µg/ml. For heat shock, the U937 cells (1 X 10^6 cells) were placed in an incubator at 42 °C for 30 min. After heat shock, cells in culture medium were centrifuged in 10 ml tubes at 1200 rpm, 4 °C for 5 min. Pellets were washed twice with 1X PBS, pH 7.4 (Sigma, USA). 100 µl of extract buffer [20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA (pH 8.0), 1 mM DTT, 0.5 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml antipain and 1 µg/ml aprotinin] was added to the washed pellets, homogenized and incubated on ice for 30 min. Homogenates were centrifuged at 20,000 X g (14,000 rpm) at 4 °C for 30 min. Supernatants were collected, frozen in liquid nitrogen and stored at -70 °C. Hearts from the five experimental groups and 0.5 hour post-heat shock rats were removed, freeze-clamped and stored at -70 °C until use. Extracts of cardiac tissues were prepared by methods similar to those used to preapre U937 cell extracts. About 150 – 200 mg of cardiac tissue was homogenized with a Polytron at 4 °C in 0.75 ml of extract buffer. The homogenate was centrifuged, and the supernatant collected and stored as described above. Protein concentration was determined by the method of Lowry et al. (1951).

Electrophoretic Mobility Shift Assay

The non-radioactive electrophoretic mobility shift assay (EMSA) kit for heat shock transcription factor 1 (HSF1) was obtained from Panomics Inc, (EMSA kit, catalogue

number AY1329, Panomics Inc, Redwood, CA, USA). Biotin-labeled or non-labeled heat shock element (HSE) oligonucleotide sequence (5'-CTGGAATATTCCCGACCTGGCAGCCTCATC) was provided with the kit (Benjamin et al., 1990). Our method followed the manufacturer's instructions. In brief, 10 µg protein samples from U937 cell extracts and 50 µg rat cardiac extracts were mixed with 2 µl of 5 X binding buffer, 1 µl of poly d(I-C), 1 µl of biotin-labeled HSE oligonucleotide and brought to 10 µl total volume by adding dH_2O. After 30 min incubation at room temperature, 1 µl of loading dye buffer was added to stop the reaction. Ten µl of this mixture was loaded onto a 6% natural gel [1 ml of 10 X TBE, 4 ml of 30% (29:1) acrylamide/bis-acrylamide, 625 µl of 80% glycerol, 14.375 ml of dH_2O, 300 µl of 10% ammonium persulfate, 20 µl of TEMED]. Gels were run for 15 min at 120 volts, the reaction mixture was loaded and resolved at 120 volts for about 55 min in 0.5 x TBE on ice in a Bio-Rad mini-gel electrophoretic unit. Gel reaction products were electro-transferred onto presoaked Byodyne B® membrane (catalogue number 60201, Pall Inc, USA). Reaction products were cross-linked to the membrane with an UV crosslinker for 3 min (UV Stratalinker 2400®, Stratagene, La Jolla, CA, USA). Membranes were incubated in 1 X blocking buffer for 15 min with gentle agitation and then for an additional 15 min with the addition of Streptavidin-HRP conjugate (1:1000). After 3 X 8 min washes in wash buffer, membranes were incubated in 20 ml of 1 X detection buffer for 5 min and then 2 ml of display buffer was added to each membrane for 5 min. Membranes were exposed to X-ray film (Hyperfilm ECL, catalogue number RPN2114k, Amersham Biosicences, Amersham, UK). The competition assay was done with 66 X non-labeled HSE oligonucleotide sequence added to the sample 10 minutes before addition of 1 X biotin-labeled HSE oligonucleotide

sequence. The supershift assay was done by adding rabbit polyclonal anti-HSF1 (1:50 μg/μg, SPA-901, StressGen, Victoria, Canada) to insulin treated heart extract and heat shock and insulin treated heart extract samples 15 min before adding EMSA reaction buffers.

Distribution of Hsp70/Hsc70 in the Myocardium

Plasma Membrane Separation

The commercially available membrane separation kit used in this study was from Bio-Rad (ReadyPrep™, Membrane I, Bio-Rad, Hercules, CA, USA). This kit is based on the method of Bordier (1981). In brief, and following the manufacturer's instructions, 50 mg of fresh cardiac tissue was collected and sonicated in a probe sonicator 4 X 10 seconds followed by 60 seconds of no sonication in 0.5 ml of membrane extraction buffer one (M1). The sample tubes were kept on ice during the procedure. Next, 0.5 ml of pre-chilled membrane extraction buffer two (M2) was added to the sample, mixed by vortexing and then incubated on ice for 10 min. Samples were transferred to a 37 °C heating block and incubated for 30 min with mixing 3 to 4 times. Samples were centrifuged at 16,000 g for 5 min at room temperature. The protease inhibitors Leupeptin, Pepstatin A and Approtinan were added at 1 μg/ml to buffer M1 and M2 just before use. Buffer M1 and M2 were kept on ice for 15 min before use. Aqueous phase, detergent-rich phase and homogenized tissue before phase separation were collected. The aqueous phase is enriched with soluble cytoplasmic proteins. The detergent-rich phase is enriched with proteins anchored to the membrane or proteins containing one or two transmembrane domains. The collected fractions were frozen in liquid nitrogen, and kept at -70 °C until analyzed. All fractions were assayed for membrane alkaline phosphatase assay as a membrane marker (Borregaard

et al., 1987, Morgan et al., 1997). Protein concentration in each fraction was determined by the RC DC method recommended by manufacturer of ReadyPrep™ Membrane I (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Western Analysis

Heart tissue samples containing 5 µg of protein were solubilized in sodium dodecyl sulfate (SDS) sample buffer, boiled for 10 min and loaded onto a mini-SDS-polyacrylamide gel (2.5% upper gel, 7.5% running gel), according to previously described methods with minor modifications (Chen et al., 2004). In order to do densitometric quantitative analysis, all samples on the gel were duplicated. Proteins were separated by electrophoresis at 75 volts for 20 min and then 125 volts for 45 min prior to electrotransfer onto PVDF membrane (Immobilon-P, Millipore, Billerica, MA, USA), using 100 volts for one hour in ice chilled transfer buffer. Membranes were incubated in 5% skim milk powder in 1X TBST (Tween 20, 0.1%) at room temperature for one hour to block non-specific binding of primary antibody as described previously (Chen et al., 2004). Membranes were incubated at 4 °C overnight with primary mouse monoclonal anti-hsp70 antibody (1:5,000, catalogue number SPA-810, StressGen, Victoria, Canada) or primary polyclonal rabbit anti-hsc70 antibody (1:5000, catalogue number SPA-816, StressGen, Victoria, Canada), and rabbit polyclonal anti-actin antibody (1:2,000, catalogue numberA-2066, Sigma, St Louis, USA) in 5% skim milk powder in 1X TBST. Next day, following two quick changes of 1X TBST and 3 x 5 min washes at room temperature, membranes were incubated in secondary horse anti-mouse horseradish peroxidase conjugated antibody (1:10,000, catalogue number PI-2000, Vector Laboratories, Burlingame, CA, USA) and goat anti-rabbit horseradish peroxidase conjugated antibody (1:10,000, catalogue number

PI-1000 , Victoria, Burlingame, CA, USA). After two quick washes and 3 X 5 min washes at room temperature, membranes were incubated in ECL Plus™ solution for horseradish peroxidase labeled antibody (1 ml per membrane, RPN2132, Amersham Biosciences, U.K.) for 5 min, and then washed quickly in distilled water. Chemiluminescence was detected in a STORM 840 scanner with a fluorescence setting at excitation of 430 nm, emission of 503 nm, Photo Manager Two (PMT) 600 Volts and Pixel size 100 micron (STORM 840, Molecular Dynamics, U.K.). Densitometric analyses for 1D gels were done with imaging software (ImageQuant TL V.2003, Amersham Bioscences, U.K.).

Confocal Immunofluorescence Microscopy

Tissue Preparation

Following overnight fixation in 2% paraformaldehyde in 0.1 M PBS (pH 7.4, 4 °C), tissues were cryoprotected in 30% sucrose in 0.1 M PBS (pH 7.4). Tissue sections were then cut at 20 μm with a freezing microtome and sections were kept in Millonig's solution at 4 °C.

Immunofluorescence

To characterize the localization of Hsp70 and Hsc70 in cardiac tissue and their relationship with components of the cell, sections were double labeled for either dystrophin, a cell membrane protein or α-tubulin, a cytoskeletal protein. Sections were incubated with primary rabbit polyclonal antibody against Hsp70 (1:1000, catalogue number SPA-812, StressGen, Victoria, Canada) or Hsc70 (1:1000, catalogue number SPA-816, StressGen, Victoria, Canada), and either mouse monoclonal anti-dystrophin, (1:100 in 0.01 M phosphate buffered saline with 0.1% Triton x-100, pH 7.4 (PBST) and 1% BSA, clone MANDRA, catalogue number D 8043, Sigma, St Louis, USA) (Gussoni et al., 1992),

mouse monoclonal anti-α-tubulin (1:1000 in PBST and 1% BSA, clone B-5-1-2, catalogue number T 5168; Sigma, St Louis, St Louis, USA) or mouse monoclonal anti-β-tubulin (1:1000 in PBST and 1% BSA, clone 2-28-3, catalogue number T 5293, Sigma, St Louis, USA) antibody (Decker et al., 2002). For double labeling of Hsp70 or Hsc70 and antibodies described above, tissue sections were processed as described previously (Leger et al., 2000, Li et al., 2004) with minor modifications. In brief, tissue sections were washed 3 x 10 min in PBST at room temperature, and then blocked with 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBST for one hour. Tissue sections were washed for 1 x 10 min in PBST. After the wash, sections were incubated in primary antibodies at the concentration mentioned above at 4 °C overnight. Next day, the tissue sections were washed with 0.01M phosphate buffered saline (PBS, pH 7.4) three times. Then tissue sections were incubated in secondary antibodies (1:400 in PBS and 1% BSA, Alexafluor 546 conjugated goat anti-rabbit IgG and Alexafluor 488 conjugated goat anti-mouse IgG, Molecular Probe Inc, USA) at room temperature for two hours. Finally, tissue sections were washed 3 x 10 min in 0.01M PBS and then mounted onto gelatinized slides. Sections were allowed to dry in the dark overnight at room temperature. Sections were cover-slipped with Prolong Gold mounting media (Molecular Probe, USA) and sealed. In each batch of sections stained for confocal fluorescence microscopy, some sections were incubated without primary antibody or secondary antibody to serve as control for non-specific staining. A Carl Zeiss Axiovert 200 laser-scanning microscope was used to Z section for confocal imaging at 1μm. Images were captured with a CCD camera and LSM 510 META software (Version 3.2, Carl Zeiss, Gemany). Captured images were edited

adjusting only brightness and contrast and composed with Photoshop (Version 7.0, Adobe System Incorporated, USA).

Statistical Analysis

Linear regression analysis was done to derive the constant and slope for the pressure-volume relationship (Borkhoff et al., 1986; Li et al., 2004). Cardiac function data were normalized to the pre-ischemic values. The normalized indexes were analyzed with the general linear model for repeated measures and Bonfferoni test for multi-comparison (SPSS, V13.0, SPSS Inc, Chicago, IL, USA). Values of Hsp70 and actin were calculated from densitometric data of Western blots. Hsp70 densitometric values of each treatment group were compared to the control group. Quantitative data were analyzed with one-way or two-way analysis of variance and Bonfferoni multi-comparison analysis. All statistical results were confirmed with non-parametric tests. The results are expressed as mean ± SEM. Significance was set at $p \leq 0.05$.

Results

Left Ventricular Function

Pre-ischemic values of cardiac function are presented in Table 3.1. No significant differences ($p > 0.05$) were observed in the pre-ischemic function between the experimental groups for heart rate (HR), LVDP, LVW, ± dp/dt, and CR (Table 3.1). SHAM hearts were not subjected to ischemia and their functional parameters did not change during 2 hours of perfusion (Figure 3.1 and 3.2). During the 30-min of no-flow global ischemia, contractility decreased quickly to zero for the CON, INS, HS, and HSINS groups. During reperfusion, there were no statistical differences ($p > 0.05$) between the groups for heart rate.

Following ischemia, during reperfusion, INS and HSINS hearts were not different from SHAM (non-ischemic) hearts for LVDP (Figure 3.1A). On the other hand, during reperfusion LVDP for CON and HS hearts was significantly different from SHAM ($p = 0.004$ and $p = 0.001$, respectively) and INS ($p = 0.026$ and $p = 0.008$, respectively) hearts.

INS and HSINS hearts had the highest level of recovery of LVW during reperfusion and were not different for SHAM hearts (Figure 3.1B). CON and HS hearts were significantly different for LVW at 5 min of reperfusion compared to SHAM ($p = 0.007$ and $p = 0.015$, respectively) and INS ($p = 0.012$ and $p = 0.025$, respectively) hearts. At 30 min of reperfusion CON hearts were significantly difference from SHAM, INS and HSINS hearts ($p < 0.001$, $p = 0.005$ and $p = 0.021$, respectively).

For +dp/dt (Figure 3.1C) INS hearts were not different from SHAM hearts during reperfusion. At 5 min reperfusion, CON, HS and HSINS hearts had significantly less recovery of +dp/dt compared to SHAM hearts ($p = 0.001$, $p < 0.001$ and $p = 0.023$,

respectively). Similarly, at 30 min of reperfusion, CON, and HS hearts had significantly less recovery of +dp/dt compared to SHAM hearts ($p = 0.003$ and $p = 0.044$, respectively).

At 5 min of reperfusion, –dp/dt (Figure 3.1D) was significantly different for SHAM hearts compared to CON, INS, HS and HSINS hearts ($p < 0.001$, $p = 0.042$, $p = 0.004$ and $p < 0.001$, respectively). At this time, INS hearts had significantly increased –dp/dt compared to HS hearts ($p = 0.015$).

Coronary vascular resistance (CR, Figure 3.2) was not different during reperfusion between SHAM, CON, INS or HSINS hearts. Interestingly CR was significantly elevated in the HS hearts compared to SHAM, CON and HSINS at 5 min of reperfusion ($p = 0.009$, $p < 0.001$ and $p = 0.063$, respectively). At 30 min of reperfusion, CR was significantly elevated in the HS hearts compared to SHAM, CON, INS and HSINS ($p = 0.003$, $p < 0.001$, $p = 0.024$ and $p = 0.007$, respectively). Similarly, at 60 min of reperfusion, CR was significantly elevated in the HS hearts compared to SHAM, CON, INS and HSINS ($p = 0.002$, $p < 0.001$, $p = 0.01$ and $p = 0.006$, respectively).

Electrophoretic Mobility Shift Assay for HSF1 Activity

HSF1 is considered active when it is competent to bind the HSE. HSF1-HSE binding is evident when the mobility of the HSE probe is retarded on electrophoretic migration. On our EMSA, HSF1-HSE complexes are evident as two bands (Figure 3.3). The upper band is considered to be inducible and trimerized HSF1 binding HSE, and the lower band is considered to be constitutive binding of HSF1-HSE (Locke et al., 1995, 1996). U937 cell extract showed strong HSF1-HSE binding and 0.5 hour post-heat shocked rat heart also showed HSF1-HSE binding. Addition of excess unlabeled oligonucleotide probe successfully abolished binding of the labeled probe. Addition of

HSF1 antibodies compromised the HSF1-HSE binding. At 6 hours after insulin injection, HSF1 was strongly activated compared to the CON and SHAM animals (Figure 3.3). HS also activated HSF1. The histogram (Figure 3.3) summarizes data for 3 animals in each group (normalized to the CON) and statistical analysis revealed no differences between groups (ANOVA, $p = 0.109$).

Hsc70 Western Blot and Densitometric Analysis

Homogenized heart samples were separated into aqueous phase (soluble proteins), detergent-rich phase (membrane bound proteins), and pellet (nuclear proteins). Hsc70 was detected in each of the various heart fractions and the experimental treatments had no apparent affect ($p > 0.05$) on the levels of Hsc70 (Figure 3.4).

Hsc70 and Dystrophin Immunofluorescence Microscopy

Confocal micrographs of ventricular sections, double labeled with primary anti-Hsc70 and secondary antibody (AlexaFluor 546 conjugated anti-rabbit IgG, red), and anti-dystrophin and secondary antibody (AlexaFluor 488 conjugated anti-mouse, green) are presented in Figure 3.5. Dystrophin immunoreactivity was clearly localized to plasma membranes in all experimental groups (Figure 3.5A, D, G, J, M). Hsc70 immunoreactivity was detectable in control (CON; Figure 3.5B, C) and sham (SHAM; Figure 3.5E, F) hearts and appeared to be localized diffusely throughout the cytoplasm and with some small concentrations in cardiomyocytes. Following insulin (INS; Figure 3.5H, I), heat shock (HS; Figure 3.5K, L), and heat shock and insulin (HSINS; Figure 3.5N, O) treatments, Hsc70 immunoreactivity was abundant and was localized throughout the cytoplasm and with some concentrations in cardiomyocytes. No obvious change in Hsc70 distribution was noted.

Hsc70 and α-Tubulin Immunofluorescence Microscopy

Confocal micrographs of ventricular sections, double labeled with primary anti-Hsc70 and secondary antibody (AlexaFluor 546 conjugated anti-rabbit IgG, red), and anti-α-tubulin and secondary antibody (AlexaFluor 488 conjugated anti-mouse, green) are presented in Fig 3.6. The α-tubulin immunoreactivity was localized throughout the cytoplasm and some concentration with microtubular networks in cardiomyoctes and small blood vessels in all experimental groups (Figure 3.6A, D, G, J, M). Hsc70 immunoreactivity was detectable in CON (Figure 6B, C) and SHAM (Figure 3.6E, F) hearts and appeared to be localized diffusely throughout the cytoplasm and with some small concentrations in cardiomyocytes. Following INS (Figure 3.6H, I), HS (Figure 3.6K, L), and HSINS (Figure 3.6N, O) treatments, Hsc70 IR was abundant and was localized throughout the cytoplasm and with some concentrations in cardiomyocytes. No obvious change in Hsc70 distribution was noted.

Hsp70 Western Blot and Densitometric Analysis

Little or no Hsp70 was detected by Western blot analysis in the heart fractions from CON and SHAM animals (Figure 3.7). In the INS treated animals, Hsp70 was detected in the homogenate and in the membrane fraction of hearts. In HS and HSINS treated animals, abundant levels of Hsp70 were detected in the homogenate and all fractions of hearts (Figure 3.7). Statistical analysis revealed differences in the homogenates between CON vs HS and HSINS, $p = 0.04$; cytosolic fractions between CON vs HS and HSINS, $p = 0.02$; membrane fractions between CON vs INS and HSINS, $p = 0.03$; and pellet fractions between CON vs HSINS, $p = 0.03$. It should also be noted that the magnitude of change in

the abundance of Hsp70 after INS, HS or HSINS treatments is many times greater than was seen for Hsc70.

Hsp70 and Dystrophin Immunofluorescence Microscopy

Confocal micrographs of ventricular sections, double labeled with primary anti-Hsp70 (AlexaFluor 546, red) and anti-dystrophin (AlexaFluor 488, green) are presented in Figure 3.8. Dystrophin immunoreactivity was clearly localized to plasma membranes in all experimental groups (Figure 3.8A, D, G, J, M) and appeared to be unchanged by the experimental treatments. Little or no Hsp70 immunoreactivity was detected in CON or SHAM hearts (Figure 3.8B, E). After INS, Hsp70 immunoreactivity was detected and localized mainly to plasma membranes (Figure 3.8H, I). Following HS, Hsp70 immunoreactivity was abundant in cardiac tissue, and appeared to be localized mostly in small capillaries and in peri-vascular compartments between the cardiomyocytes (Figure 3.8K, L). After HSINS, Hsp70 immunoreactivity was mostly localized to capillaries and peri-vascular compartments between the cardiomyocytes, however there also appeared to be some minor localization of Hsp70 immunoreactivity in the cytoplasm and on the cell membranes of the cardiomyocytes (Figure 3.8N, O).

Hsp70 and α-tubulin Immunofluorescence Microscopy

Confocal micrographs of ventricular sections, double labeled with primary anti-Hsp70 (AlexaFluor 546, red) and anti-α-tubulin (AlexaFluor 488, green) are presented in Figure 3.9. The α-tubulin immunoreactivity was localized to the microtubular networks in cardiomyocytes and possibly in vascular elements in all experimental groups (Figure 3.9A, D, G, J, M) and appeared to be unchanged by the experimental treatments. Little or no Hsp70 immunoreactivity was detected in CON or SHAM hearts (Figure 3.9B, E). After

INS, Hsp70 immunoreactivity was detected and localized mainly to cellular membranes (Figure 3.9H, I). Following HS, Hsp70 immunoreactivity was abundant in cardiac tissue, and appeared to be localized mostly in small capillaries and in peri-vascular compartments between the cardiomyocytes (Figure 3.9K, L). After HSINS, Hsp70 immunoreactivity was mostly localized to capillaries and peri-vascular compartments between the cardiomyocytes, however there also appeared to be some minor localization of Hsp70 immunoreactivity in the cytoplasm and on the cell membranes of the cardiomyocytes (Figure 3.9N, O).

Table 3.1. Pre-ischemia and sham values of cardiac function (Mean ± SEM)

Group (n)	HR (beat·min)	LVDP (mmHg)	LVW (mmHg•ul)	+dp/dt (mmHg/sec)	-dp/dt (mmHg/sec)	CR (mmHg/ml/min)
CON (7)	267 ± 14	71 ± 17	7377 ± 1886	2007 ± 490	1157 ± 262	5.9 ± 1.2
SHAM (8)	277 ± 9	97 ± 9	10038 ± 1370	2944 ± 392	1581 ± 167	4.8 ± 0.5
INS (10)	282 ± 6	73 ± 10	7035 ± 1254	2095 ± 338	1320 ± 199	5.8 ± 0.6
HS (7)	311 ± 15	107 ± 8	10394 ± 1342	3468 ± 469	1836 ± 203	6.2 ± 0.5
HSINS (7)	294 ± 15	87 ± 14	8560 ± 1711	2396 ± 370	1439 ± 239	8.4 ± 1.3

Pre-ischemia control values. There were no significant differences among the groups ($p > 0.05$).

Figure 3.1. Insulin treatment followed by 6 hrs improves cardiac function during post-ischemic reperfusion. Experimental group treatments are described in the Materials and Methods section under Experimental protocol and groups. Animals were recovered for 6 hrs before isolation and perfusion of hearts. Hearts were equilibrated to perfusion for 30 min at a flow rate of 10 ml/min. SHAM hearts were continually perfused at 10 ml/min (not subjected to ischemia). IS indicates the 30 min ischemic interval for the CON, INS, HS, and HSINS hearts. Hearts were reperfused at 10ml/min for 60 min starting at time = 0. Data are normalized to pre-ischemia values. A. Insulin improved LVDP during reperfusion. For LVDP insulin treated hearts (INS and HSINS) were not different from SHAM (non ischemia) hearts. Asterisk indicates significant differences as follows: at 5 min of reperfusion, SHAM and INS versus CON, $p = 0.004$ and $p = 0.026$, respectively; versus HS, $p = 0.001$ and $p = 0.008$, respectively, and at 30 min of reperfusion, SHAM and HSINS versus CON, $p = 0.006$ and $p = 0.012$, respectively; versus HS, $p = 0.018$ and $p = 0.037$, respectively. B. Insulin improved LVW during reperfusion. For LVW, insulin treated hearts (INS and HSINS) were not different from SHAM hearts. At 5 min of reperfusion LVW of SHAM and INS hearts were significantly greater than CON ($p = 0.007$ and $p = 0.015$, respectively) and HS hearts ($p = 0.012$ and $p = 0.025$). Similarly, at 30 min of reperfusion, LVW of SHAM, INS and HSINS were greater than CON hearts ($p < 0.001$, $p = 0.005$, $p = 0.021$). In addition, at 30 min of reperfusion, LVW of SHAM hearts was greater than HS hearts ($p = 0.04$). C. Insulin improved +dp/dt during reperfusion. For +dp/dt, insulin treated hearts (INS) were not different from SHAM hearts. At 5 min of reperfusion +dp/dt for SHAM hearts was greater than CON, HS and HSINS hearts ($p = 0.001$, $p < 0.001$ and $p = 0.023$, respectively) and at 30 min of reperfusion +dp/dt for SHAM hearts was greater than CON, and HS hearts ($p = 0.003$ and $p = 0.044$, respectively). D. Insulin improved –dp/dt during reperfusion. At 5 min of reperfusion, while –dp/dt for SHAM hearts was greater than CON, INS, HS and HSINS hearts ($p < 0.001$, $p = 0.042$, $p = 0.004$ and $p < 0.001$, respectively), –dp/dt for INS hearts was greater than HS hearts ($p = 0.015$).

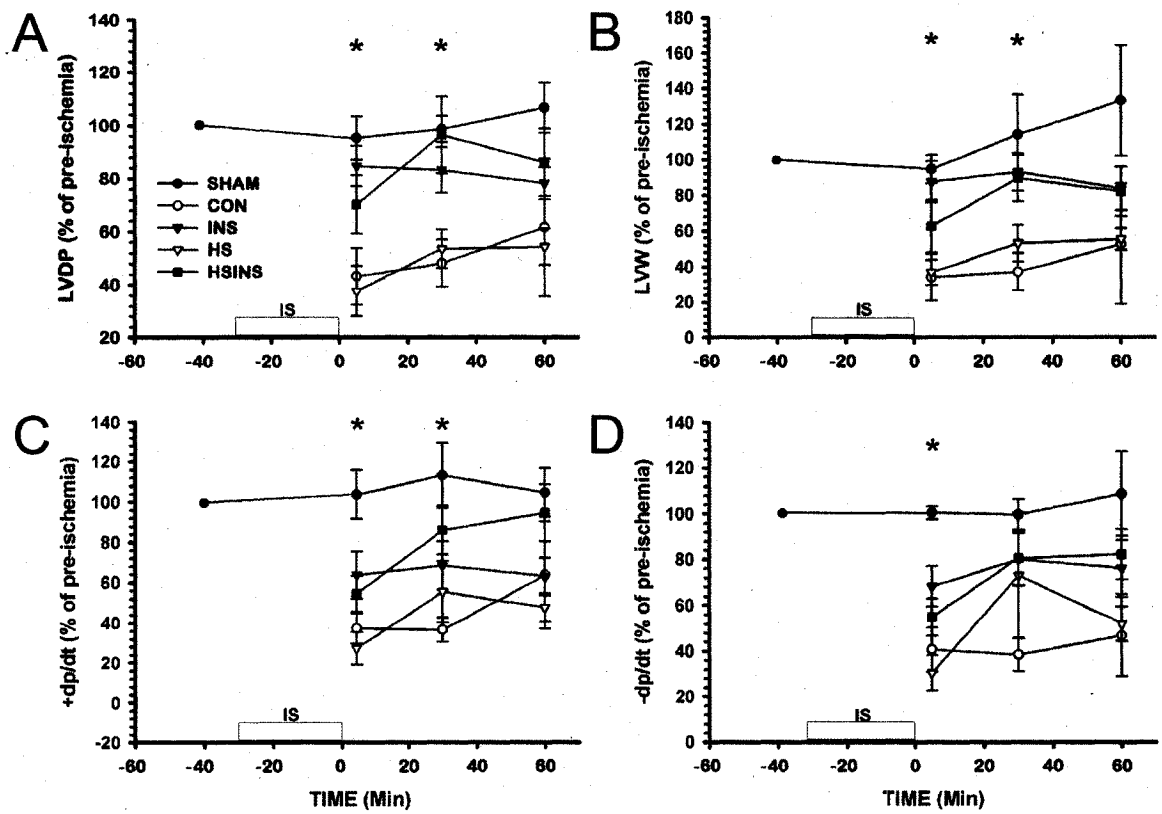
A
B
C
D
LVDP (% of pre-ischemia)
LVW (% of pre-ischemia)
+dp/dt (% of pre-ischemia)
-dp/dt (% of pre-ischemia)
SHAM
CON
INS
HS
HSINS
IS
TIME (Min)

Figure 3.1

Figure 3.2. Insulin reversed the effect of HS on coronary vascular resistance (CR). At 5 min of reperfusion HS hearts had greater CR than SHAM, CON and HSINS hearts ($p = 0.009$, $p < 0.001$ and $p = 0.043$, respectively) and at 30 and 60 min of reperfusion, HS hearts had greater CR than SHAM, CON, INS, HSINS ($p<0.001$ to 0.024, all p values are presented in the results).

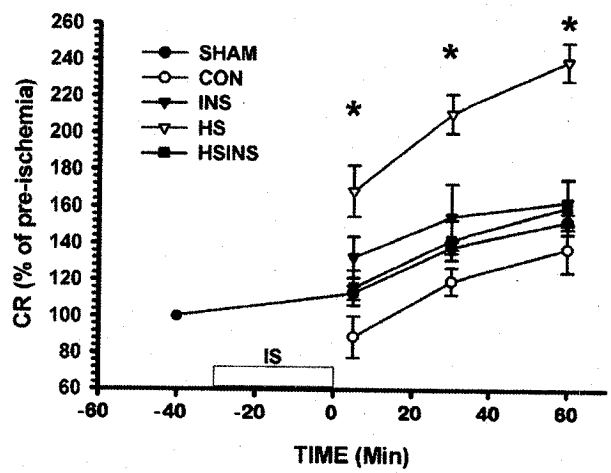
260
240
220
200
180
160
140
120
100
80
60
CR (% of pre-ischemia)
SHAM
CON
INS
HS
HSINS
*
*
*
IS
-60
-40
-20
0
20
40
60
TIME (Min)

Figure 3.2

Figure 3.3. Insulin and heat shock activate DNA binding activity of HSF1 as shown by electrophoretic mobility shift analysis. A. Specific binding activity is shown by U937 (heat shocked U937 cell extract + biotin-labeled HSE oligonucleotide, as positive control), and U937 + cold probe (competition with 66 X unlabeled HSE oligonucleotide). In addition, specific binding activity is shown by HS 0.5 (0.5 hour post-heat shock rat heart extract + biotin-labeled HSE oligonucleotide, as a positive control), and HS 0.5 + cold probe (competition with 66 X unlabeled HSE oligonucleotide). Experimental samples are 6 hours after treatment. HSF1-HSE binding is strongly activated 6 hours after insulin (INS) and heat shock and insulin (HSINS) treatments compared to control (CON) or sham (SHAM) animals. Heat shock (HS) showed weak HSF1-HSE binding at 6 hours after treatment. HSF1, inducible HSF1 binding complexes; CB, constitutive HSF1 binding complexes; NS, non-specific; Free, free probe. B. Specific binding activity is shown by HS 0.5 (0.5 hour post-heat shock rat heart extract + biotin-labeled HSE oligonucleotide, as a positive control), and HS 0.5 + cold probe (competition with 66 X unlabeled HSE oligonucleotide). In addition, supershift analysis, INS + anti-HSF1 (INS 6 hour extract + anti-HSF1) and HSINS + anti-HSF1 (HSINS 6 hour extract + anti-HSF1) showed that the HSF1-HSE binding activity was compromised with the addition of anti-HSF1. Experimental samples are 6 hours after treatment and are independent of those in A. C. Histogram shows semi-quantification of binding activities of HSF1- HSE of 6 hour experimental samples normalized to CON (mean ± SEM, n = 3 for each group, ANOVA, $p = 0.109$).

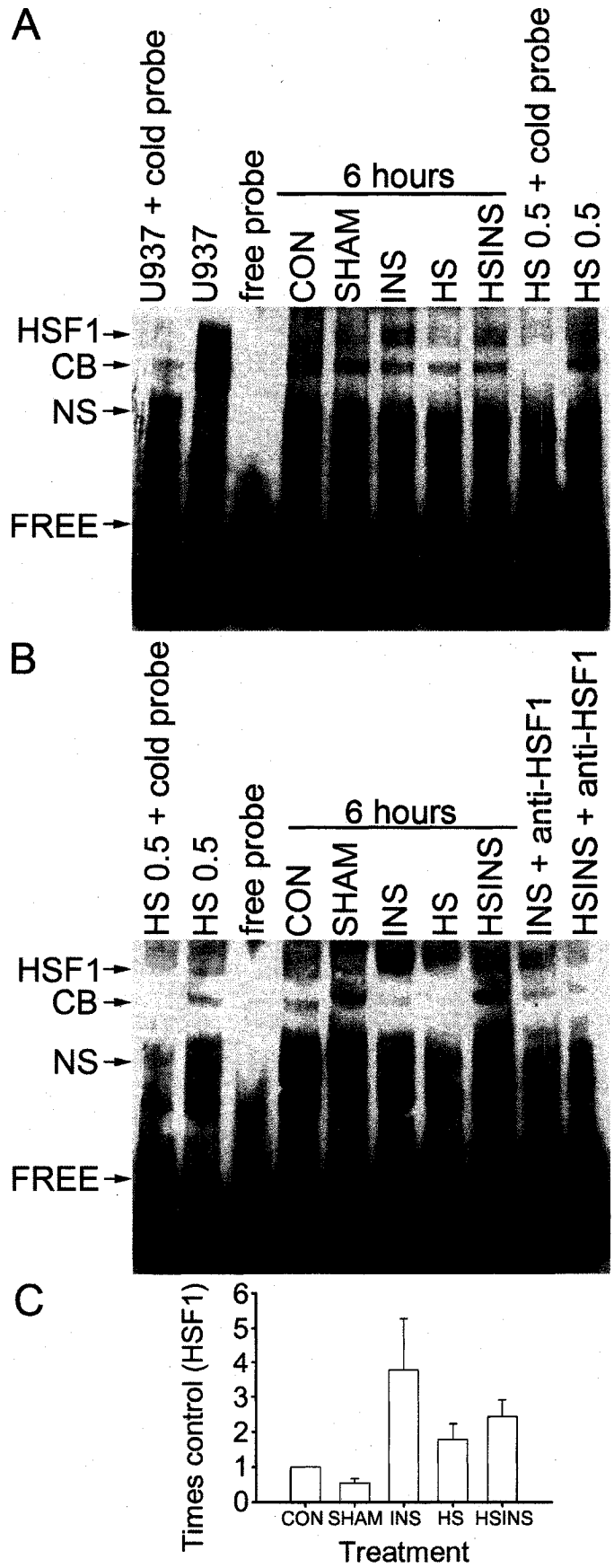
A
U937 + cold probe
U937
free probe
6 hours
CON
SHAM
INS
HS
HSINS
HS 0.5 + cold probe
HS 0.5
HSF1
CB
NS
FREE
B
HS 0.5 + cold probe
HS 0.5
free probe
6 hours
CON
SHAM
INS
HS
HSINS
INS + anti-HSF1
HSINS + anti-HSF1
HSF1
CB
NS
FREE
C
Times control (HSF1)
0
1
2
3
4
5
6
CON SHAM INS HS HSINS
Treatment

Figure 3.3

Figure 3.4. Insulin and heat shock had minimal effect on Hsc70 in heart homogenate, cytosol, membrane and pellet fractions (n = 3, each group) as shown by Western blot and densitometric analysis. Actin was used as a loading control for Western analysis. Hsc70 was detectable in each fraction and levels did not appear to change with experimental treatment. Histogram results are relative to the control group (times mean control values and standard error of mean) for each fraction. There was no statistical difference among the groups ($p > 0.05$). CON, naïve control; SHAM, sham control; INS, insulin treated; HS, heat shock treated; HSINS, heat shock and insulin treated.

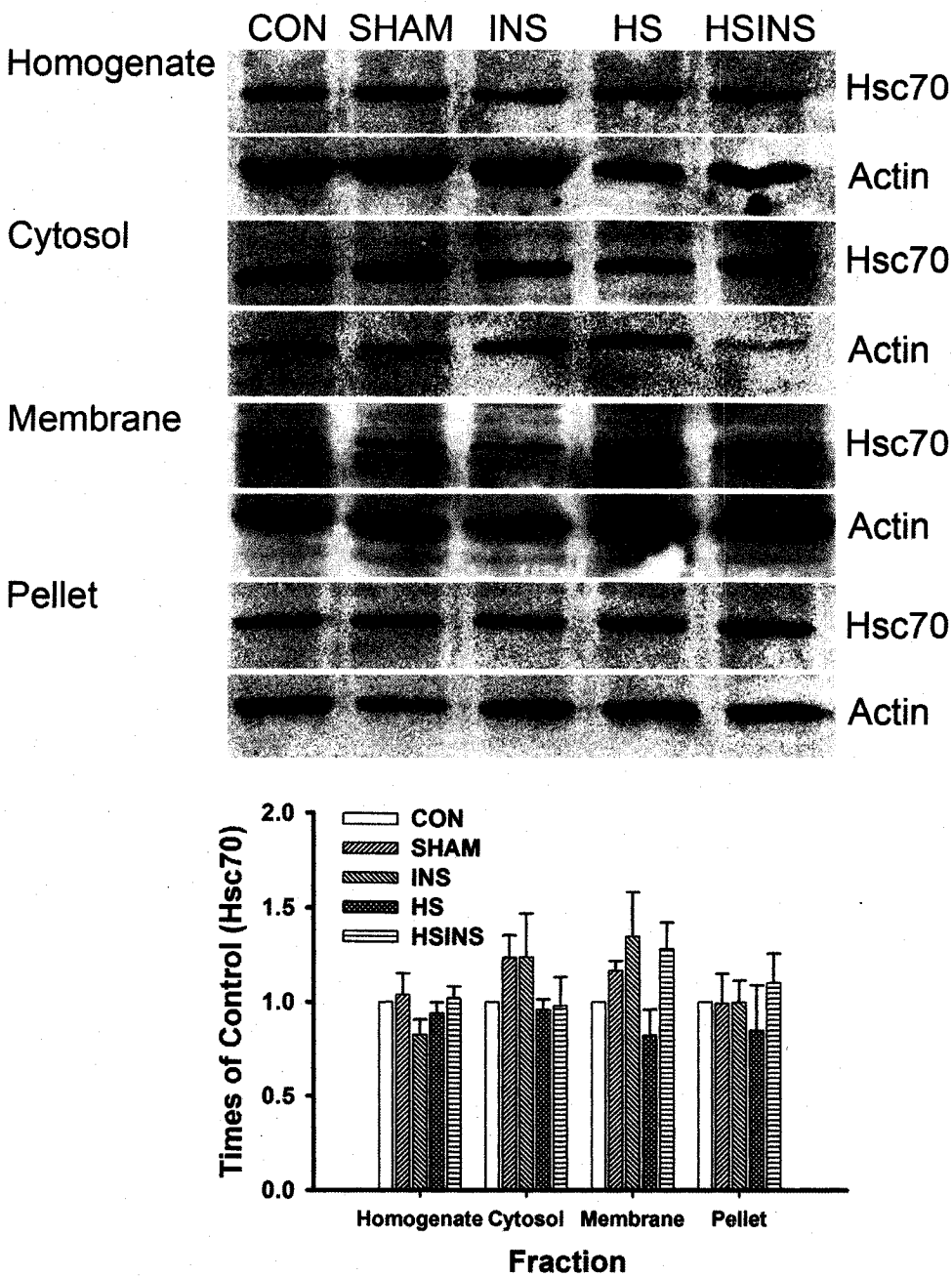
CON SHAM INS HS HSINS
Homogenate
Hsc70
Actin
Cytosol
Hsc70
Actin
Membrane
Hsc70
Actin
Pellet
Hsc70
Actin
Times of Control (Hsc70)
2.0
1.5
1.0
0.5
0.0
CON
SHAM
INS
HS
HSINS
Homogenate
Cytosol
Membrane
Pellet
Fraction

Figure 3.4

Figure 3.5. Insulin and heat shock had minimal effect on Hsc70 and dystrophin distribution in ventricular sections examined by confocal microscopy. Sections were labeled with primary anti-Hsc70 and secondary antibody AlexaFluor 546 conjugated anti-rabbit IgG (red), and primary anti-dystrophin and secondary antibody AlexaFluor 488 conjugated anti-mouse IgG (green). Dystrophin is localized mainly to the plasma membranes. A-C, Naïve control. D-F, Sham control; there is constitutive existence of Hsc70 in unstressed cardiomyocytes of rat. G-I, insulin treated; there is no apparent change in Hsc70 expression. J-L, heat shock treated. M-O, heat shock and insulin treated; there is no apparent change in Hsc70 expression. Images are representative of 3 animals in each treatment group. Bar = 20 μm.

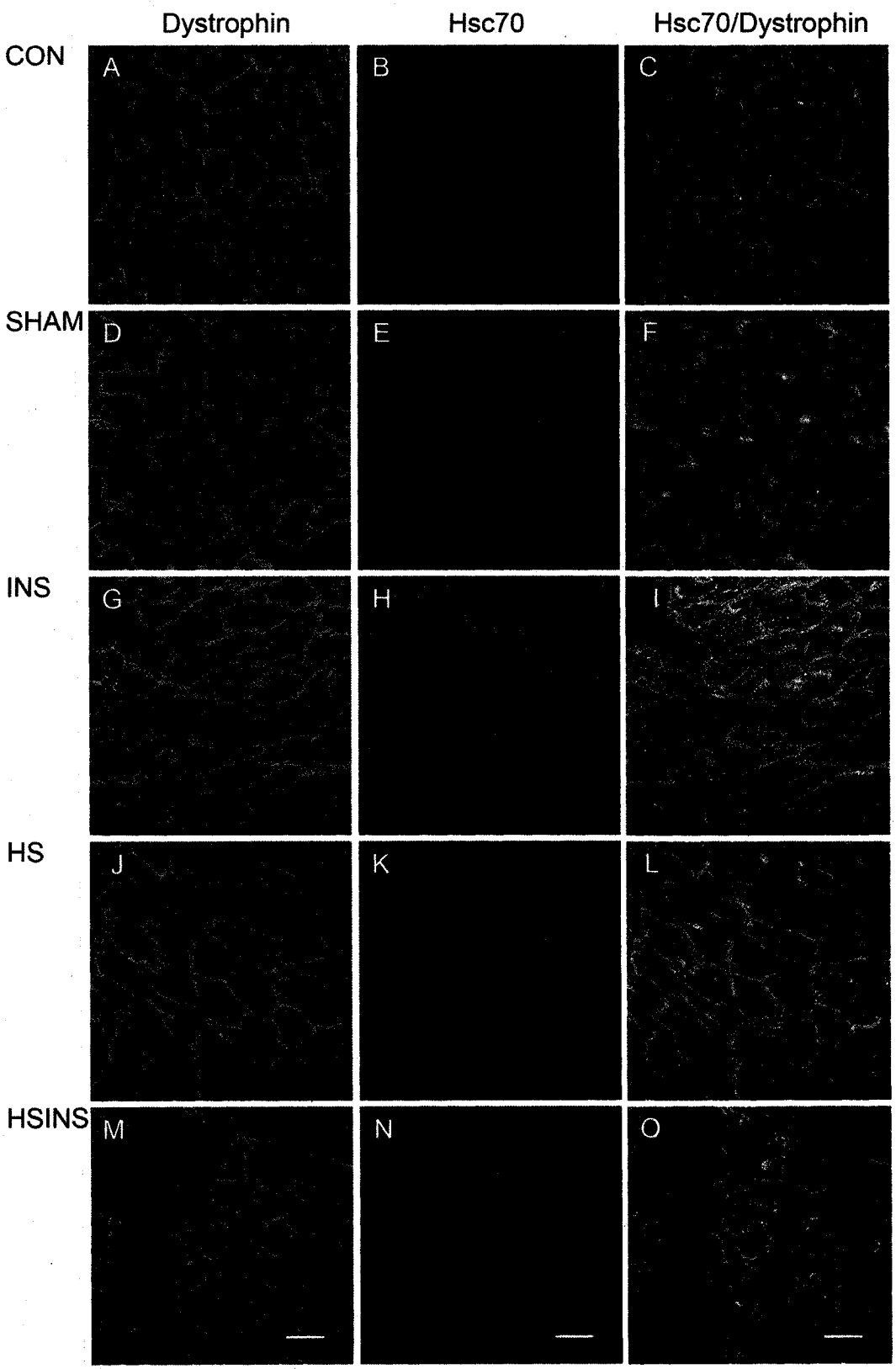
Dystrophin
Hsc70
Hsc70/Dystrophin
CON
SHAM
INS
HS
HSINS
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 3.5

Figure 3.6. Insulin and heat shock had minimal effect on Hsc70 and α-tubulin distribution in ventricular sections examined by confocal microscopy. Sections were labeled with primary anti-Hsc70 and secondary antibody AlexaFluor 546 conjugated anti-rabbit IgG (red), and primary anti-α-tubulin and secondary antibody AlexaFluor 488 conjugated anti-mouse IgG (green). α-tubulin is localized mainly to the microtubular network. A-C, Naïve control. D-F, Sham control. G-I, insulin treated. J-L, heat shock treated. M-O, heat shock and insulin treated. Images are representative of 3 animals in each treatment group. Bar = 20 μm.

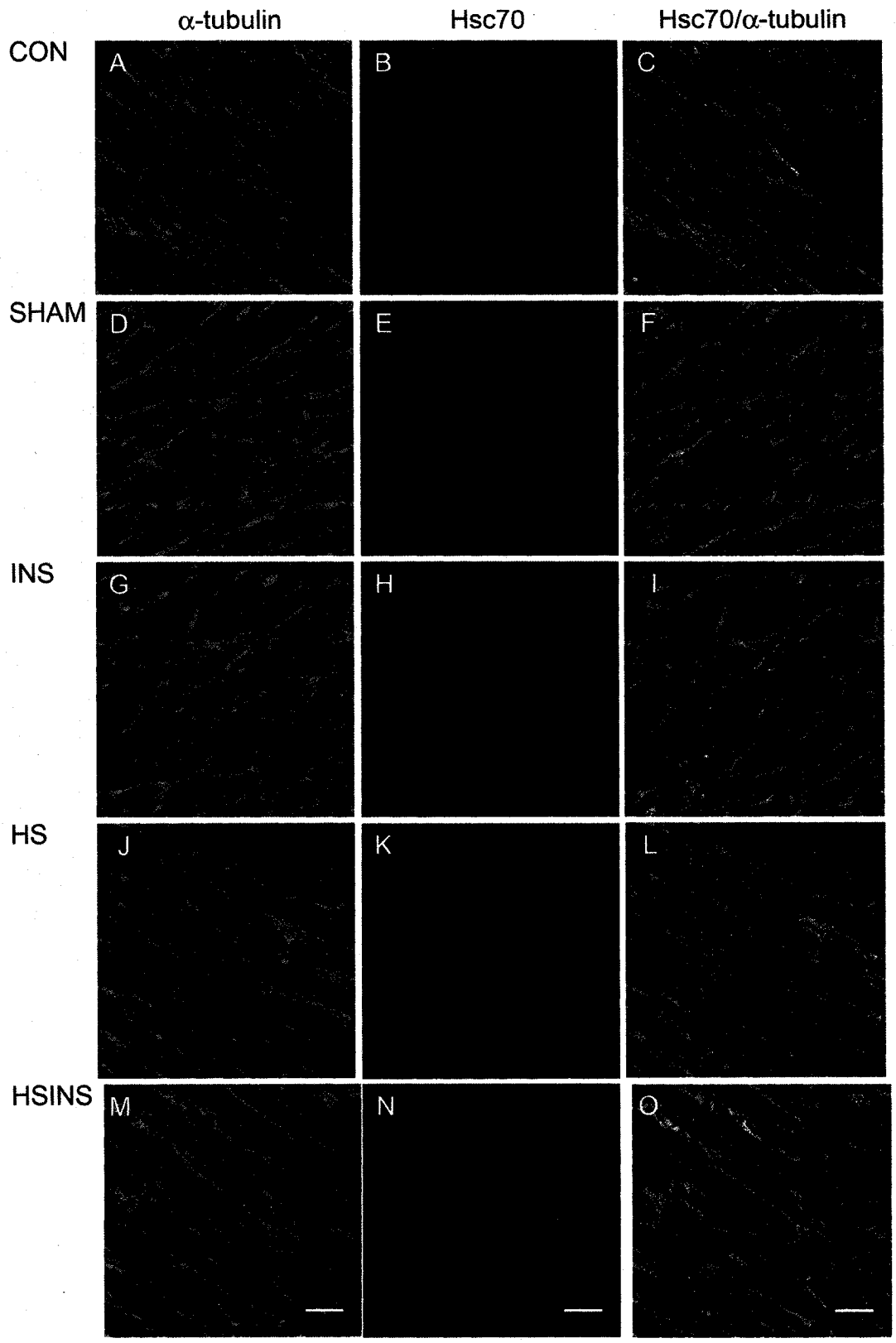
α-tubulin
Hsc70
Hsc70/α-tubulin
CON
SHAM
INS
HS
HSINS
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 3.6

Figure 3.7. Insulin elevated Hsp70 in the membrane fraction of hearts. Hsp70 was analyzed by Western blot and densitometric analysis in heart homogenate, cytosol, membrane and pellet fractions (n = 6, each group). Actin was used as a loading control for Western analysis. In the homogenate, Hsp70 appears to be elevated in the INS, HS and HSINS hearts. While there is a modest increase in abundance of Hsp70 after INS treatment as seen in the homogenate compared to HS or HSINS treatment, this increase is most evident in the membrane fraction. Histogram results are relative to the control group (times mean control values and standard error of mean) for each fraction. One-way analysis of variance with Bonfferoni multi-comparison test, confirmed with a non-parametric test, revealed differences in the homogenate, CON vs HS and HSINS, $p = 0.04$; cytosolic fraction, CON vs HS and HSINS, $p = 0.02$; membrane fraction, CON vs INS and HSINS, $p = 0.03$; pellet fraction, CON vs HSINS, $p = 0.03$.

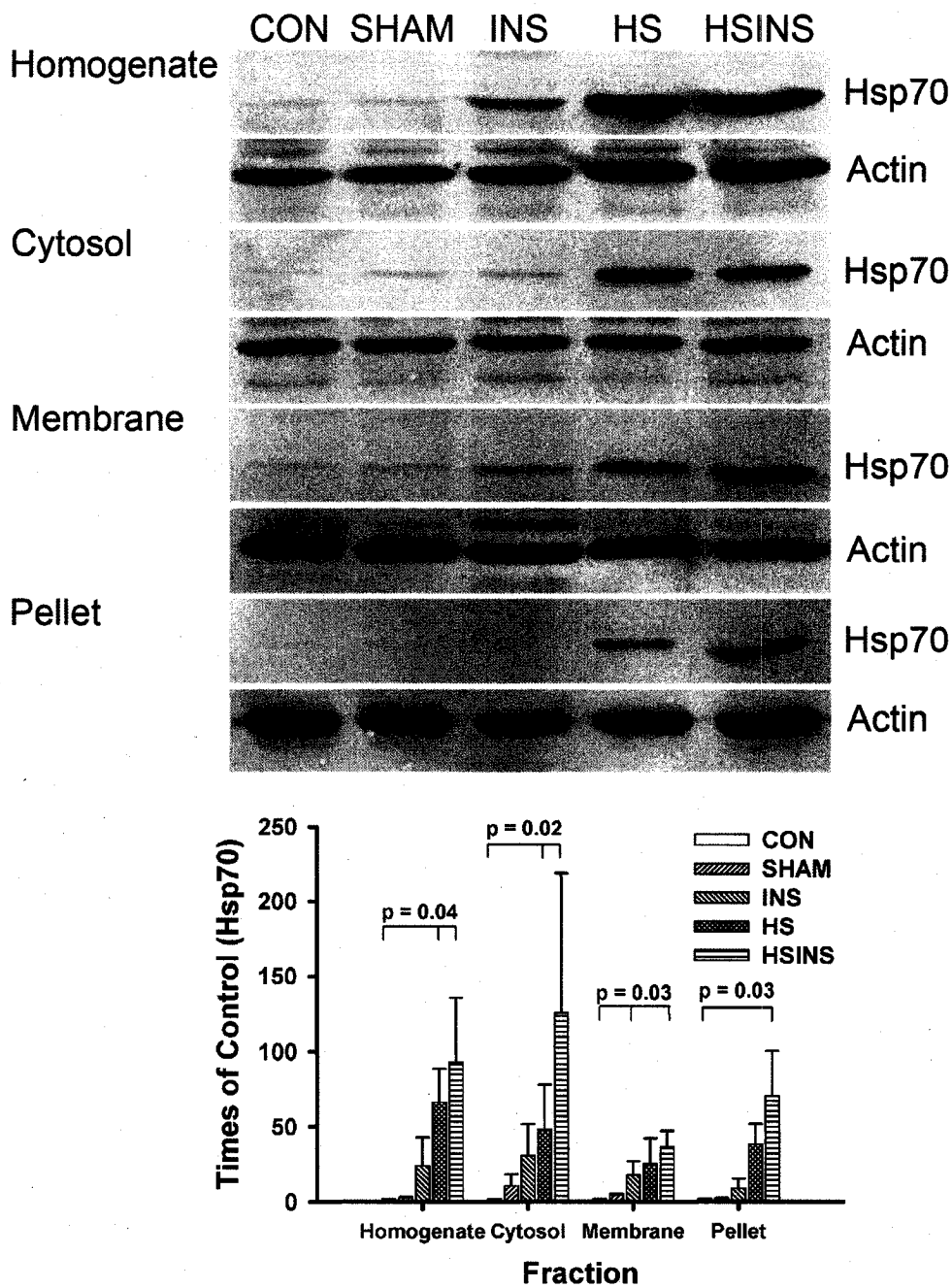
CON SHAM INS HS HSINS
Homogenate
Hsp70
Actin
Cytosol
Hsp70
Actin
Membrane
Hsp70
Actin
Pellet
Hsp70
Actin
Times of Control (Hsp70)
250
200
150
100
50
0
p = 0.04
p = 0.02
p = 0.03
p = 0.03
CON
SHAM
INS
HS
HSINS
Homogenate
Cytosol
Membrane
Pellet
Fraction

Figure 3.7

Figure 3.8. Insulin and heat shock have specific distributions of Hsp70 compared to dystrophin distribution in ventricular sections examined by confocal microscopy. Sections were labeled with primary anti-Hsp70 and secondary antibody AlexaFluor 546 conjugated anti-rabbit IgG (red), and primary anti-dystrophin and secondary antibody AlexaFluor 488 conjugated anti-mouse IgG (green). Dystrophin is localized mainly to the plasma membranes. A-C, Naïve control. D-F, Sham control; there is little or no expression of Hsp70 in unstressed rat hearts. G-I, insulin treated; Hsp70 immunofluorescence is detectable (H) and appears to be mostly co-localized with dystrophin immunofluorescence (I). J-L, heat shock treated; Hsp70 immunofluorescence is abundant and mostly expressed between cardiomyocytes in peri-vascular elements (K). M-O, heat shock and insulin treated; Hsp70 immunofluorescence is mostly expressed in peri-vascular elements (N), and some co-localized with dystrophin immunofluorescence is evident (O). Images are representative of 3 animals in each treatment group. Bar = 20 μm.

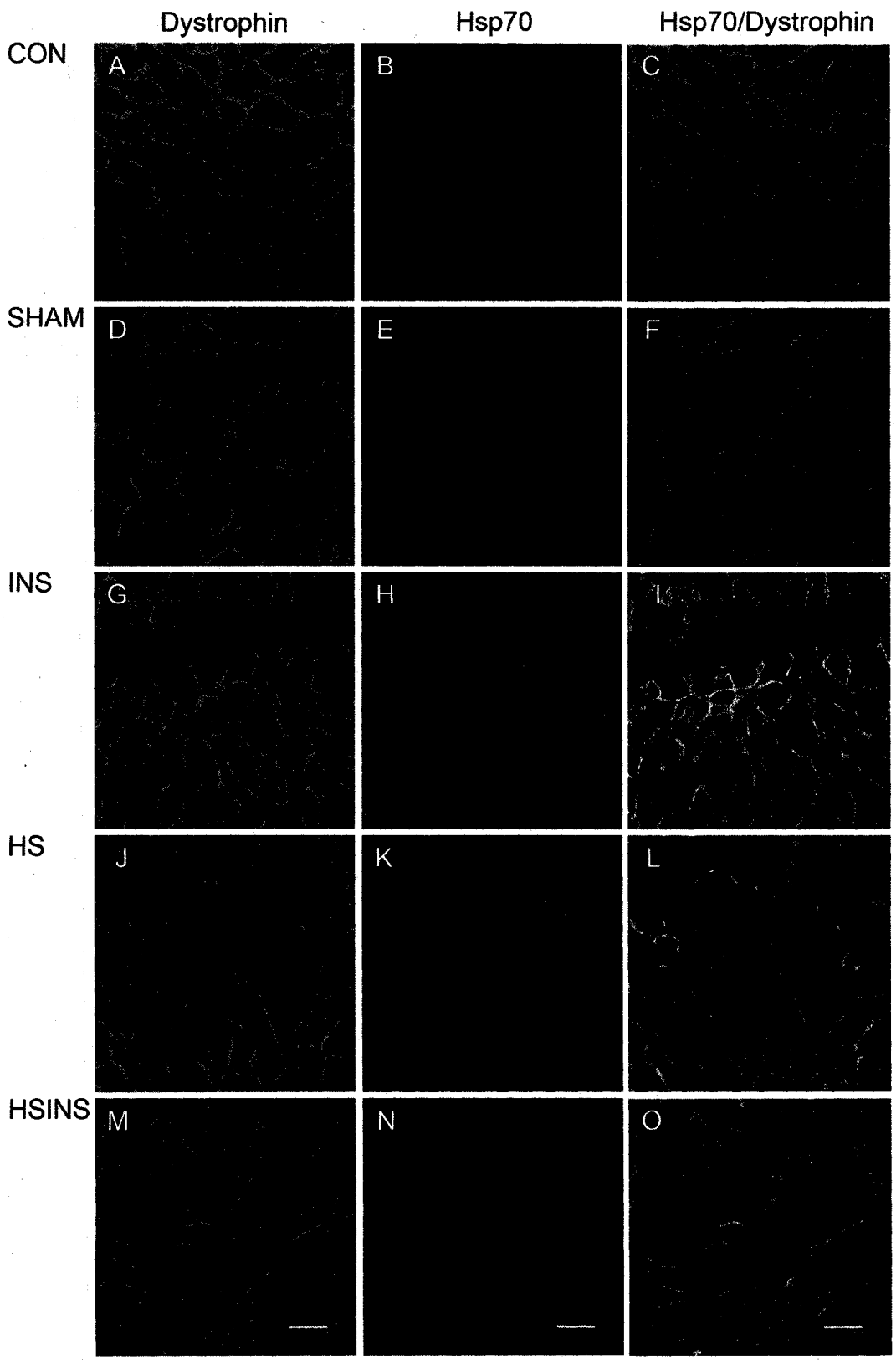
Dystrophin
Hsp70
Hsp70/Dystrophin
CON
SHAM
INS
HS
HSINS
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 3.8

Figure 3.9. Insulin and heat shock have specific distributions of Hsp70 compared to α-tubulin distribution in ventricular sections examined by confocal microscopy. Sections were labeled with primary anti-Hsp70 and secondary antibody AlexaFluor 546 conjugated anti-rabbit IgG (red), and primary anti-α-tubulin and secondary antibody AlexaFluor 488 conjugated anti-mouse IgG (green). α-tubulin is localized mainly to the microtubular network. A-C, Naïve control. D-F, Sham control; there is little or no expression of Hsp70 in unstressed rat hearts. G-I, insulin treated; Hsp70 immunofluorescence is detectable (H) and appears to be mostly localized with contractile elements and cell membranes (I). J-L, heat shock treated; Hsp70 immunofluorescence is abundant and mostly expressed between cardiomyocytes in peri-vascular elements (K), and some co-localization with α-tubulin immunofluorescence is evident (L). M-O, heat shock and insulin treated; Hsp70 immunofluorescence is mostly expressed in peri-vascular elements (N), and some co-localized with α-tubulin immunofluorescence is evident (O). Images are representative of 3 animals in each treatment group. Bar = 20 μm.

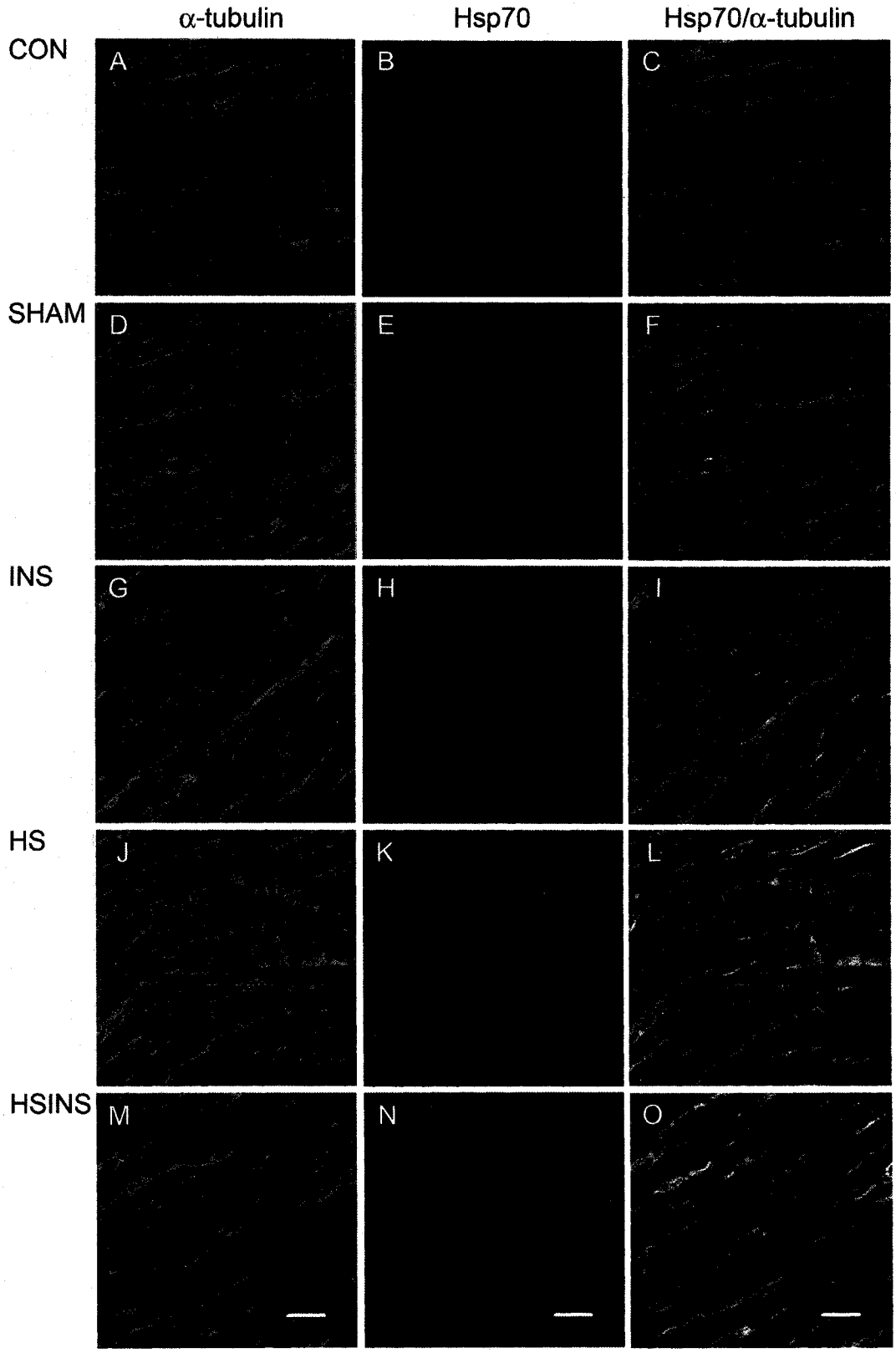
α-tubulin
Hsp70
Hsp70/α-tubulin
CON
SHAM
INS
HS
HSINS
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 3.9

Discussion

This study reports that insulin treatment improves post-ischemic myocardial contractility, activates HSF1, induces a small amount of Hsp70 increase in rat heart, and interestingly, this insulin induced expression of Hsp70 appears to be localized to cell membranes. In contrast, heat shock treatment 6 hrs before isolation of hearts did not improve post-ischemic myocardial contractility, but induced strong Hsp70 expression mostly in capillaries and peri-vascular compartments. Heat shock and insulin treatment caused further apparent increase in Hsp70 expression in the rat heart.

In this study insulin, but not HS had a beneficial effect of the post-ischemic recovery of myocardial function (Figure 3.1). Improved myocardial function after ischemia is seen following ischemic preconditioning (Kuzuya et al., 1993; Marber et al., 1993; Murry et al., 1986). Interestingly, two windows of protection are evident, the first being rapid and persisting for only about 2 hrs, and the second being present at about 20 to 48-72 hrs (Morimoto et al., 1992; Cornelussen et al., 1998, 2003; Rao et al., 2000). Improved myocardial function after ischemia is also seen 24 to 48 hrs following heat shock treatment (Currie, 1987; Kamazyn et al., 1990; Hutter et al., 1994; Fath-Ordoubadi et al., 1997). As shown in this study, myocardial protection is not evident at 6 hrs after HS (Cornelussen et al., 1998, 2003; Yamashita et al., 1998) even though considerable Hsp70 is present in the heart. However, as show here, the heart displays myocardial protection at 6 hr following insulin treatment. Whether there are multiple windows of protection or a continuous protection, each window or segment of the protection appears to be stimulus dependent. It is also interesting that insulin treatment appears to rescue heart functional recovery after heat shock treatment (Figure 3.1, compare HS vs HSINS).

The absence of enhanced myocardial recovery in hearts 6 hrs after heat shock treatment when Hsp70 levels are elevated has been observed repeatedly (Cornelussen et al., 1998, 2003). Newly synthesized Hsp70 may not be fully mature or folded into its functional configuration. Alternatively, the protective function of Hsp70 may be related to its differential localization after HS or insulin treatment (Figure 3.7, 3.8, 3.9). Another intriguing possibility is the elevation of coronary resistance (Figure 3.2) seen during reperfusion in hearts 6 hrs after HS; the HS hearts had the highest level of CR. The elevated CR may be due to increased plasma catecholamine concentration and myocardial noradrenaline turnover after heat shock (Kregel et al., 1991, 1993). This may cause an elevated responsiveness of coronary vessels to reperfusion, and gradual vasoconstriction at short recovery times following heat shock. The elevated CR in HS hearts (6 hrs after heat shock treatment) is in contrast to 24 to 96 hrs post-heat shock hearts having significantly lower perfusion pressures compared to control (non heat shock) hearts during reperfusion following 30 min of ischemia (Karmazyn et al., 1990). Co-incidentally, transgenic mouse hearts containing high levels of human Hsp70 also had significantly lower perfusion pressures compared to litter-mate control non-transgenic hearts during reperfusion following 30 min of ischemia (Plumier et al., 1995). Part of the protective effect of insulin appears to be controlling the elevation of CR as seen in the HSINS hearts compared to the HS hearts. The elevated CR at 6 hours after HS may negate other protective mechanisms.

In this study Hsc70 expression and distribution was not affected by insulin or by heat shock treatment. Hsc70 is a constitutive member of the 70 kDa family of heat shock proteins. In the rat, Hsc70 has an apparent molecular mass of 73 kDa and is normally expressed in all cells and tissues and its expression changes minimally with injury

compared to the inducible member of this family of Hsps. Hsc70 acts as a molecular chaperone to regulate normal protein folding and helps repair damaged proteins.

In contrast, others and I have shown that Hsp70 expression was elevated following insulin treatment (Ting et al., 1989; Li et al., 2004). Hsp70 is the highly inducible member of the 70 kDa family of Hsps, and in rat has an apparent molecular mass of 71 kDa. Hsp70 is normally at low or undetectable levels in most tissues in rat, but with heat shock or injury, its expression is transiently increased many times (Currie and White, 1983; Currie and Tanguay, 1991; Armstrong et al., 1996). Hsp70 maintains the heat shock transcription factor in an inactivated form in the cell cytoplasm (Morimoto, 1998; Shi et al., 1998). Similar to Hsc70, Hsp70 is thought to act as a molecular chaperone and as a regulator of apoptotic signaling pathways (Mosser et al., 2000).

Insulin induced Hsp70 appeared to have a different localization compared to HS induced Hsp70. This localization may be related to the intensity of stimulation used to induce the expression of Hsp70. While I generally consider that Hsp70 is expressed in response to noxious stimuli, it is doubtful that insulin is such a stimulus. Insulin administration of 200 μU/g body weight does not affect blood glucose levels significantly (Li et al., 2004). Insulin is an existing circulating hormone in the body that does not normally stress or injure cells. Insulin induces low level expression of Hsp70, in contrast to the high level expression of Hsp70 after the noxious heat shock treatment. Thus the membrane localization of Hsp70 after insulin treatment may be related to the physiological induction of its expression by insulin and not a pathological induction. In fact, similar distinct stress-dependant cellular localization of Hsp70 has been reported in the brain (Krueger et al., 1999).

Localization of Hsp70 to cardiomyocyte membranes after insulin treatment compared to microvasculature elements after heat shock likely has implications for the function of Hsp70. Elevated levels of Hsp70 in microvessels, 24 hours after heat shock treatment, may be refolding damaged proteins caused by reactive oxygen species during ischemia/reperfusion to maintain overall cardiac function (Plumier et al., 1995; Leger et al., 2000). Localization of Hsp70 to cardiomyocyte membranes following insulin treatment may stabilize the cell membranes, or regulate membrane receptors or even cell signaling pathways. Interestingly, Hsp70 has been identified on plasma membranes of several carcinoma cell lines (Asea et al., 2002; Brouquet et al., 2003), particularly after heat shock (Multhoff et al., 1995), or treatment with anti-inflammatory drugs (4) or γ-irradiation (Gehrmann et al., 2004). However, membrane-bound Hsp70 is not found in normal (non cancerous) cell lines after heat shock treatment (Multhoff et al., 1995). In carcinoma cells, the role of membrane-bound Hsp70 appears to be related to immunological recognition. Tumor cells with high levels of membrane-bound Hsp70 have high rates of cell growth and metastasis (Bausero et al., 2004) and are more effectively killed by natural killer cells (Gehrmann et al., 2005; Hantschel et al., 2000). Similarly, the hydroxylamine derivative bimoclomol, induced membrane-bound Hsp70 that stabilized membrane fluidity at elevated temperatures (Torok et al., 2003). Interestingly, membrane-bound Hsp70 may be interacting with the insulin receptor (Zachayus et al., 1996). Since Hsp70 mediates apoptotic signaling pathways (Gabai et al., 2002, Mosser et al., 1988), it may be that Hsp70 at the cell membrane mediates other cell signaling events. Indeed, Hsp70 on the cell membrane also interacts with inflammatory signaling pathways to up-regulate pro-inflammatory cytokines (Asea et al., 2002). Similarly, Hsp70 in lipid rafts after HS may be

maintaining the stability of lipid raft-associated signal transduction complexes following stress (Chen et al., 2005). In contrast, our finding of Hsp70 localized to the plasma membrane after insulin treatment was in normal non-stressed and non-cancerous hearts. After insulin treatment, Hsp70 localized to plasma membranes may stabilize cells membranes or regulate pro-survival signaling pathways that are responsible for enhanced recovery.

My current study has also shown that a physiological dose of insulin activates the DNA binding activity of HSF1. Activated HSF1 is represented by two complexes, the formation of which can be abolished by excess unlabeled cold probe (Figure 3.3). The upper and lower HSF bands are considered to represent inducible and constitutive binding activity (Locke et al., 1995, 1996). Heat shock genes are regulated by HSF1. HSF1 is maintained in an inactive form by binding with Hsp70 (Morimoto, 1998) or Hsp90 (Wang et al., 2006). Under stressful conditions that denature protein, Hsp70 is recruited to the denatured protein, freeing the transcription factor. HSF1 is activated by phosphorylation, trimerizes, translocates to the nucleus, binds to the heat shock element, and initiates transcription of various Hsps (Morimoto et al., 1992; Morimoto, 1993; Sarge et al., 1993; Kiang and Tsocos, 1998). Insulin is a general regulator of protein synthesis (Proud and Denton, 1997). As a general regulator of overall protein synthesis, insulin regulates phosphorylation of S6 ribosomal protein, increases the activity of eukaryotic protein synthesis elongation factor-2 (eEF-2) and regulates the eukaryotic protein synthesis initiation factor-4E (eIF-4E) (Moule and Denton, 1997). Thus, insulin may stimulate and regulate translation through eIF-4E to increase overall protein synthesis and increase Hsp70 content in cells. Alternatively, insulin inhibits glycogen synthase kinase 3 (GSK3)

activities by activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/Akt) (Moule and Denton, 1997). GSK3 is a negative control regulator of HSF1. After inhibition of GSK3β, activity of HSF1 in heat-shocked cells increased, while overexpression of GSK3β results in significant reduction in heat induced HSF1 activity (Xavier et al., 2000). In this study, it is possible that insulin induced inhibition of GSK3 led to activation of HSF1 and elevation of Hsp70 expression.

Conclusion

Six hrs after insulin treatment, hearts have improved functional recovery from 30 min of ischemia while heat shocked hearts do not. Insulin increases the DNA binding activity of HSF1 and induces expression of Hsp70 in cardiomyocytes that appears to be localized to cellular membranes. This is in contrast to the expression of Hsp70 after heat shock that is mainly localized to vascular and peri-vascular elements between the cardiomyocytes. This differential localization of Hsp70 in response to insulin and heat shock suggests the interesting possibility of functionally distinct roles for Hsp70 in hearts that are stimulus-specific. Insulin induced Hsp70 being localized on cell membranes, may be regulating pro-survival cell signaling pathways that are contributing to the functional recovery of the hearts after ischemic injury.

CHAPTER 4:

INSULIN INDUCED MYOCARDIAL PROTECTION IN ISOLATED ISCHEMIC RAT HEART REQUIRES P38 MAPK PHOSPHORYLATION OF HSP27

This chapter is submitted to *Am J Physiol Heart Circ Physiol*

Introduction

Insulin is more than a hormone regulating glucose metabolism in cells. Insulin binds to receptors and activates several intracellular signaling transduction pathways such as phosphatidylinositol 3-kinase (PI3K) - protein kinase B (PKB/Akt) pathway, ERK1/2 pathway, and p38 MAPK pathway. Activation and phosphorylation of intermediates within these pathways regulates transcription, translation and the various aspects of glucose metabolism. In addition, insulin is effective at reducing myocardial ischemia/reperfusion injury (Jonassen et al., 2001, Li et al., 2006). The cardioprotective effect of insulin may be mediated via PI3K, PKB/Akt, and p70S6 kinase cell signaling (Sack and Yellon, 2003), or by increasing nitric oxide bioavailability (Gao et al., 2002; Kahn et al., 2000). Recently insulin has been shown to improve the recovery of the contractile function of cardiomyocytes after simulated ischemia/reperfusion through an Akt-dependent and sarcoplasmic reticulum ATPase (SERCA2a) mediated pathway (Yu et al., 2006).

Insulin also stimulates expression of the highly inducible 70 kDa heat shock protein, Hsp70, in hep3B/T2 cells (Ting et al., 1989). We recently reported that Hsp70 is indeed induced by insulin at a relatively low level in rat heart at six hours after insulin treatment. Insulin increased the expression of Hsp70 in rat heart via the activation of heat shock factor 1 (HSF1) and appeared to modulate the localization of Hsp70 to cardiomyocyte membrane in rat heart (Li et al., 2004, 2006).

Hsp70 is the highly inducible member of the 70 kDa family of heat shock proteins. While many Hsps are constitutive and have functions in normal cell homeostasis, Hsp70 is at very low levels in most cells and tissues under normal conditions. Many non-lethal but noxious stimuli induce high level expression of Hsp70 and other heat shock proteins (Hsps).

Subsequent to the induction and expression of various Hsps, cells and tissues have a remarkable resistance to further metabolic injury (Jolly and Morimoto, 2000; Beere and Green, 2001; Garrido et al., 2001; Delagu et al., 2002). For example, 24 hrs of heat shock treatment, hearts have high levels of Hsp70 and enhanced recovery of myocardial contractility after ischemic injury (Hutter et al., 1994; Currie et al., 1988). Transgenic overexpression of rat and human Hsp70 provided strong evidence for a direct role in protection of the mouse myocardium from ischemic injury (Marber et al., 1995; Plumier et al., 1995).

In general, Hsps, acting as molecular chaperons, regulate folding of nascent proteins, participate in refolding or renaturation of misfolded, damaged, or denatured proteins, stabilize structural proteins, and facilitate translocation of proteins across membranes among cellular compartments (Jakob et al., 1993, MacRae, 2000). Several Hsps prevent aggregation of protein, and target unstable or damaged protein for degradation. Hsp70 also suppresses intracellular apoptotic signaling pathways (Mosser et al., 2000).

Hsp27 is constitutive in muscle, brain stem and spinal cord, but not in cerebral cortex where it is highly inducible after stroke like injury (Currie et al., 2000). Hsp27 is constitutive in the myocardium and is associated with contractile elements, and after heat shock treatment, its abundance is minimally changed (Leger et al., 2000). However, engineered overexpression of Hsp27 in cardiomyocytes provides protection against simulated ischemia (Martin et al., 1997; Vander Heide, 2002). Similarly, overexpression of Hsp27 in transgenic mice (Efthymiou et al., 2004) protects the myocardium by reducing infarct size after ischemic injury. Hsp27 has more than one function. Specifically, Hsp27

acts as a molecular chaperone (Diaz-Latoud et al., 2005), inhibits actin and intermediate filament polymerization (Wieske et al., 2001), reduces oxidative stress related to tumor necrosis factor α (TNFα)-mediated cell death (Mehlen et al., 1995a, 1995b) and suppresses signaling events leading to apoptosis (Charette et al., 2000; Pandey et al., 2000; Concannon et al., 2001; Arrigo, 2005; Beere, 2005). In mitochondrial-mediated apoptosis Hsp27 blocks cytochrome C release and activation of caspase-3 and -9 (Bruey et al., 2000; Pandey et al., 2000; Beere, 2001) by sequestering cytochrome C and procaspase-3 (Pandey et al., 2000; Concannon et al., 2001; Paul et al., 2002). The function of Hsp27 appears to be regulated by its phosphorylation state and oligomeric size up to 800 kDa (Rogalla et al., 1996; Welsh and Gaestel, 1998). Mitogen-activated protein kinase (MAPK) activated protein kinase-2 (MAPKAPK2) phosphorylates human Hsp27 on serine (Ser)15, Ser78 and Ser82. Interestingly, ischemia/reperfusion injury in rabbit heart causes two phosphorylation patterns of Hsp27; specifically Ser15, Ser78 and Ser82, or Ser15 and Ser82 (White et al., 2006). In rat, Hsp27 has been shown to phosphorylatable at Ser13, Ser15, Ser27 and Ser86 (Hoffert et al., 2006) where rat Hsp27 Ser15 and Ser86 appear to correspond to human Hsp27 Ser15 and Ser 82. For human Hsp27, Ser82 appears to be the major site of phosphorylation (Landry et al., 1992).

Inducible cytoprotection is regulated by various kinases in several cell signaling pathways. These survival kinases, and particularly PI3K-Akt and the MEK1/2-Erk1/2 pathways are activated in ischemic preconditioning and ischemic postconditioning (Hausenloy and Yellon, 2006) and they regulate phosphorylation of Hsp27 (Dorion and Landry, 2002). Interestingly, insulin activates the survival kinases involving mitogen-activated protein kinase (MAPK) activation (Moule et al., 1995; Lessmann et al., 2006),

and MAPK activation leads to Hsp27 phosphorylation (Rouse et al., 1994; Armstrong et al., 1999). However it is unclear whether insulin induces phosphorylation of Hsp27 in rat heart.

In our previous study at 6 hrs after insulin treatment, hearts had increased expression of Hsp70 and were resistant to ischemia-reperfusion injury (Li et al., 2006). We hypothesized that at one hour after insulin treatment, insufficient time would have limited any increase in the abundance of newly synthesized heat shock proteins, and if myocardial protection where evident, other mechanisms such as MAPK activation and Hsp27 phosphorylation would be involved.

In this study we show that when hearts are isolated 1 hr after a single physiological dose of insulin, they have significantly increased levels of phosphorylated Hsp27 during ischemia reperfusion. Co-incidental with the phosphorylation of Hsp27 is a significant improvement in functional myocardial recovery from ischemic injury. Inhibition of p38 MAPK blocked Hsp27 phosphorylation and insulin induced myocardial protection.

Materials and Methods

Animals

Male Sprague-Dawley rats (250–300 g, Charles River, St. Constant, QC, Canada) were used in these experiments. All animal care, handling, and experimental procedures on animals were in accordance with the *Guide to Care and Use of Experimental Animals* of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

Experimental Protocols and Groups

Two sets of experiment were done. In the initial experiments to determine the effects of a single injection of insulin, rats were randomized into five groups, 1) naïve control, CON; 2) sham control, SHAM; 3) insulin treated, INS; 4) heat shock treated, HS; 5) heat shock and insulin treated, HSINS. To identify the effects of Hsp27 phosphorylation in myocardial protection induced by insulin, rats were randomized into five groups, 1) naïve control, CON; 2) sham control, SHAM; 3) insulin treated, INS; 4) SB203580 and insulin treated, SBINS; 5) PD098059 and insulin treated, PDINS. For all groups, rats were anesthetized with sodium pentobarbital (50 mg/kg) i.p. Naïve control rats received an injection of sodium pentobarbital, but were not heated or injected with insulin. In addition, sham control rats received an injection of normal saline intramuscularly, similar to the insulin treatment group. Insulin treated rats were injected intramuscularly in the thigh with 200 μU/gm body weight. Heat shock treated rats were placed on a temperature-controlled heating pad (48-50 °C) and monitored with a rectal thermometer until core body temperature reached 42 °C. Core body temperature was maintained between 42 °C to 42.5 °C for 15 min. Heat shock and insulin treated rats were subjected to the heat shock

treatment first and at 10 minutes after the heat shock treatment, were injected with insulin (200 μU/gm body weight) intramuscularly in the thigh. SB203580 and insulin treated rats were injected with the selective, p38 mitogen activated protein kinase (p38 MAPK) blocker, SB203580 (1.0 mg/kg, intraperitoneally) and 30 minutes later, were injected with insulin (200 μU/gm body weight) intramuscularly in the thigh. PD098059 and insulin treated rats were injected with the selective extracellular signal regulated kinase (ERK1/2) blocker PD098059 (0.3 mg/kg, intraperitoneally) (Fryer et al., 2001) and 30 minutes later, were injected with insulin (200 μU/gm body weight) intramuscularly in the thigh. Both blockers were purchased from LC Laboratories (Catalogue number S-3400 and P-4313, Woburn, MA, USA). After treatment all rats were returned to their cages.

At one hour after the various treatments, rats were injected with sodium pentobarbital (50 mg/kg) and decapitated. For Langendorff perfusion, isolated hearts were immediately immersed in ice chilled normal saline, and quickly mounted on a cannula for perfusion. For Western analysis, hearts were perfused briefly and washed with normal saline at 4 °C to remove blood, and then immediately freeze-clamped. The cardiac tissue was also collected from Langendorff perfused hearts after ischemia/reperfusion. For comparison, hearts were also collected from rats at 3 hours after the various treatments. The frozen tissues were stored at -70 °C before protein concentration determination (Lowry et al., 1951) and Western analysis. For confocal microscopy, fresh heart samples were immersed in 2% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4, 4 °C) overnight.

Langendorff Perfusion and Cardiac Function

Isolated hearts were perfused in the non-working Langendorff mode as recently described in detail (Li et al., 2006). Modified Krebs-Henseleit bicarbonate buffer gassed with 95% O_2 - 5% CO_2 at 37 °C was perfused into hearts at a constant rate of 10 ml/min with a static roller pump. Hearts were equilibrated to the perfusion system for 30 min. Heart rate (HR) and perfusion pressure were continuously recorded with a Grass model 7 polygraph (Grass Instruments Inc, Quincy, MA, USA). A balloon connected to a pressure transducer (P-23D, Gould Inc., USA), was inserted into the left ventricle through the mitral valve to measure left ventricular end-systolic pressure (LVESP) and left ventricular end-diastolic pressure (LVEDP) as well as the first derivative of the left ventricular pressure (±dp/dt). The maximal left ventricular developed pressure (LVDP) was calculated. After 30 min of equilibration, all hearts except for SHAM were subjected to 30 min no-flow global ischemia. SHAM hearts were continually perfused at 10 ml/min for 180 min. Following ischemia, hearts were reperfused at 10 ml/min for 120 min. Physiological measurements were taken at 10 min before initiation of ischemia (time point = -40 min in the figure) during pre-ischemic perfusion and at 5, 30, 60, 90 and 120 min of reperfusion. Analysis of left ventricular function and calculation of pressure-volume relationship were done according to Burkhoff and Sagawa (1986) and as recently described by Li et al. (2006).

Change of Hsp27/Phosphorylated Hsp27 in the Myocardium

Collected cardiac tissues were homogenized in 1 ml of homogenization buffer with a sonicator, as described previously (Li et al., 2004, 2006). The homogenized samples were immediately frozen in liquid nitrogen, and then kept at -70 °C until analyzed.

Western Analysis

Heart tissues samples containing 20 μg of protein were solubilized in sodium dodecyl sulfate (SDS) sample buffer (33), heated at 95 °C for 10 min and loaded onto a mini-SDS-polyacrylamide gel (2.5% stacking gel, 7.5 -12% running gel), according to recently described methods (Li et al., 2004; 2006). For dephosphorylation of proteins, samples were incubated with 10 U of alkaline phosphotase (catalogue number M0290S, New England Biolabs, Ipswich, MA, USA) for 15 min at room temperature before adding sample buffer. Proteins were separated by electrophoresis at 75 volts for 20 min and 125 volts for ~60 minutes and then electrotransferred onto PVDF membrane. Membranes were incubated in 5% skim milk in 1X TBST (pH 7.6, Tris-buffered saline with Tween 20, 0.1%) as recently described (Li et al., 2004; 2006) for one hour to block non-specific binding of primary antibody. Membranes were incubated at 4 °C overnight with the following primary antibodies: rabbit polyclonal anti-hsp25 antibody (1:5,000, catalogue number SPA-801, StressGen, Victoria, Canada), rabbit polyclonal anti-phospho-Hsp27(serine 15) (pHsp27Ser15; 1:1,000, catalogue number SPA-525, StressGen, Ann Arbor, USA), rabbit polyclonal anti-phospho-Hsp27(serine 82) (pHsp27Ser82; 1:1,000, catalogue number SPA-524PU, StressGen, Ann Arbor, USA), mouse monoclonal anti-Hsp70 (1:2,000, catalogue number SPA-810, StressGen, Ann Arbor, USA) or rabbit polyclonal anti-actin antibody (1:2,500, catalogue number A-2066, Sigma, St Louis, USA) in 5% milk in 1 X TBST. Next day, membranes were incubated in secondary horse anti-mouse horseradish peroxidase conjugated antibody (1:10,000, catalogue number PI-2000, Vector Laboratories, Burlingame, USA) or goat anti-rabbit horseradish peroxidase conjugated antibody (1:5,000, catalogue number SAB-300, StressGen, Ann Arbor, USA). After washes, membranes

were incubated in ECL Plus™ solution for horseradish peroxidase labeled antibody (catalogue number RPN2132, Amersham Biosciences, UK) for five minutes, and then washed in distill water. Chemiluminescence was directly detected on a STORM 840 scanner with a fluorescence setting at excitation of 430 nm, emission of 503 nm, Photo Manager Two (PMT) 600 Volts and Pixel size 100 micron (STORM 840, Molecular Dynamics, UK). Densitometric analyses for 1D gels were done with imaging software (ImageQuant TL V.2003, Amersham Biosciences, UK).

Confocal Immunofluorescence Microscopy

Tissue Preparation

Following overnight fixation in 2% paraformaldehyde in 0.1 M PBS (pH 7.4, 4 °C), tissues were cryoprotected in 30% sucrose in 0.1 M PBS (pH 7.4). Tissue sections were then cut at 20 μm with a freezing slicing microtome and sections were kept in Millonig's solution at 4 °C.

Immunofluorescence

To characterize the localization of Hsp70, Hsp27 and pHsp27Ser82 in cardiac tissue and their relationship with components of the cell, sections were double labeled for either dystrophin, a cell membrane protein or α-tubulin, a cytoskeletal protein. Sections were incubated with primary rabbit polyclonal antibody against Hsp70 (1:1,000, catalogue number SPA-812, StressGen, Victoria, Canada), primary rabbit polyclonal antibody against Hsp27 (1:1,000, catalogue number SP-801, StressGen, Victoria, Canada), or primary rabbit polyclonal antibody against pHsp27Ser82 (1:1,000, catalogue number SPA-524PU, StressGen, Ann Arbor, USA), and either mouse monoclonal anti-dystrophin, (1:100 in 0.01 M phosphate-saline buffer with 0.2% Triton x-100, pH 7.4 (PBST) and 1% BSA, catalogue

number D 8043, Sigma, St Louis, USA) (Li et al., 2006), or mouse monoclonal anti-α-tubulin (1:1000 in PBST and 1% BSA, catalogue number T 5168; Sigma, St Louis, USA) (Li et al., 2006). For immunostaining of Hsp27, pHsp27Ser82, Hsp70, tissue sections were processed as recently described (Li et al., 2006). In brief, tissue sections were blocked with 10% normal goat serum in 1 X PBST for one hr. Tissue sections were incubated in primary antibodies at the concentration mentioned above at 4 °C overnight. Next day, the tissue sections were incubated in secondary antibodies (1:400 in PBS and 1% BSA, Alexafluor 546 conjugated goat anti-rabbit IgG and Alexafluor 488 conjugated goat anti-mouse IgG, Molecular Probe Inc, USA) at room temperature for two hrs. Finally, tissue sections were mounted on gelatinized slides and dried in the dark overnight at room temperature. Sections were cover-slipped with ProLong® Gold mounting media (Molecular Probe Inc., USA) and sealed. In each batch of sections stained for confocal fluorescence microscopy, some sections were incubated without primary antibody or secondary antibody to serve as controls for non-specific staining. A Carl Zeiss Axiovert 200 laser-scanning microscope was used to Z section for confocal imaging at 1 μm. Images were captured with a CCD camera and LSM 510 META software (Version 3.2). Captured images were edited adjusting only brightness and contrast and composed with Photoshop (Version 7.0, Adobe Systems Incorporated, USA).

Statistical Analysis

Relative densitometric values for Hsp27, pHsp27 and actin were obtained from Western blots. The densitometric values of each treatment group were standardized with actin and normalized to the sham control group. Quantitative densitometric data were analyzed by analysis of variance with or without covariant and Bonfferoni multi-

comparison analysis. MANOVA with repeated measurement and Bonfferoni multi-comparison analysis was used for analysis of effects of treatment for cardiac function data. (SPSS, V13.0.1, SPSS Inc, Chicago, IL, USA). The results are expressed as mean ± SEM. Significance was set at $p \leq 0.05$.

Results

Analysis of Heart Function

Pre-ischemic values for cardiac function are presented in Table 4.1. No significant differences ($p > 0.05$) were detected in the pre-ischemic function between the experimental groups for heart rate (HR), LVDP, LVW, and ± dp/dt (Table 4.1). SHAM hearts were not subjected to ischemia and their function did not change significantly during 3 hours of perfusion (Figure 4.1). During the 30-min of no-flow global ischemia contractility decreased quickly to zero for the CON, INS, HS, and HSINS groups. During reperfusion, there were no statistical differences ($p > 0.05$) between the groups or with the pre-ischemic values for heart rate (data not shown).

Following ischemia, during reperfusion, INS hearts had the highest recovery of LVDP (Figure 4.1A), LVW (Figure 4.1B), +dp/dt (Figure 4.1C), and -dp/dt (Figure 4.1D), compared to the CON, HS and HSINS hearts. Recovery of function for INS hearts approached the function of the SHAM hearts (not subjected to ischemia). Average percent (± SEM) recovery of heart function during 2 hr of reperfusion and significant differences among the treatment groups for treatment effects are summarized in Table 4.2.

Western Blot and Densitometric Analysis for Hsp70 and Hsp27

Hearts from each treatment group were examined at 1 and 3 hr after treatment for relative levels of Hsp70 and Hsp27 (Figure 4.2). Little or no Hsp70 immunoreactivity was detected in SHAM, CON or INS hearts. Hsp70 immunoreactivity was detected at significantly elevated levels in HS ($p \leq 0.006$, $n = 3$) and HSINS ($p \leq 0.003$, $n = 3$) hearts compared to SHAM, CON, or INS hearts at 3 hr after treatment. Immunoreactivity for

Hsp27, a constitutively expressed protein, was detected in all hearts and its level was unchanged between 1 and 3 hrs after treatment or by the treatments.

Hsp70 and Dystrophin Immunofluorescence Microscopy

At 1 hr after treatment, hearts were prepared for histology. Confocal micrographs of heart sections, double labeled with primary anti-Hsp70 and secondary antibody (AlexaFluor 546 conjugated anti-rabbit IgG, red) and primary anti-dystrophin and secondary antibody (AlexaFluor 488 conjugated anti-mouse IgG, green) are presented in Figure 3. Dystrophin immunoreactivity was localized to plasma membranes in all experimental groups (Figure 4.3A, D, G, J, M) and appeared to be unchanged by experimental treatments. Little or no Hsp70 immunoreactivity was detected in SHAM or CON hearts (Figure 4.3B, E). After INS, Hsp70 immunoreactivity was occasionally detected and localized mainly to plasma membranes (Figure 4.3H, I). Following HS, Hsp70 immunoreactivity was detectable in cardiac tissue, and appeared to be localized mostly between the cardiomyocytes and associated with microvessels (Figure 4.3K, L). After HSINS, Hsp70 immunoreactivity was mostly localized between the cardiomyocytes and associated with microvessels (Figure 4.3N, O).

Hsp27 and α-Tubulin Immunofluorescence Microscopy

At 1 hr after treatment, hearts were prepared for histology. Confocal micrographs of heart sections, double labeled with primary anti-Hsp27 and secondary antibody (AlexaFluor 546 conjugated anti-rabbit IgG, red), and primary anti-α-tubulin and secondary antibody (AlexaFluor 488 conjugated anti-mouse, green) are presented in Figure 4.4. The α-tubulin immunoreactivity was localized throughout the cytoplasm with some concentration along cell membranes, and microtubular networks were apparent in

cardiomyocytes in all experimental groups (Figure 4.4A, D, G, J, M). Hsp27 immunoreactivity was detectable in SHAM (Figure 4.4B, C), CON (Figure 4.4E, F), INS (Figure 4.4H, I), HS (Figure 4.4K, L), and HSINS (Figure 4.4N, O) hearts and was localized throughout the cytoplasm with limited concentration along cell membranes. In INS treated hearts some Hsp27 immunoreactivity appears to be concentrated on 3 to 5 μm diameter structures. In HS treated hearts some Hsp27 immunoreactivity appears to be concentrated with α-tubulin on the cytoskeleton. No other obvious change in Hsp27 distribution was noted.

Western Analysis of Phosphorylated Hsp27

Hearts from each treatment group were examined at 1 and 3 hrs the treatments for phosphorylated Hsp27 (Figure 4.5). The antibodies specific for pHsp27Ser15 (Figure 4.5A) and pHsp27Ser82 (Figure 4.5B) revealed immunoreactive product at 1 and 3 hrs after treatment in various groups. Little or no pHsp27 immunoreactivity was detected in SHAM or CON hearts. While pHsp27Ser15 immunoreactivity appeared to be about 5 times elevated at 1 hr in the HS and HSINS groups (Figure 4.5C) and pHsp27Ser82 immunoreactivity appeared to be about 5 times elevated at 1 hr in the INS, HS and HSINS groups (Figure 4.5C) compared to the SHAM group, the differences were not significantly different ($p > 0.05$, $n = 3$ to 6).

Dephosphorylation of Hsp27

Hearts from each treatment group were examined at 3 hrs after treatment for phosphorylated Hsp27 and for abolition of pHsp27 immunoreactivity after treatment of samples with alkaline phosphatase (Figure 4.6). The antibodies specific for pHsp27Ser15 (Figure 4.6A) and pHsp27Ser82 (Figure 4.6B) revealed immunoreactive product in various

groups. After incubation of samples with alkaline phosphatase no pHsp27 immunoreactivity was detected.

Phosphorylated Hsp27 and Dystrophin Immunofluorescence Microscopy

At 1 hr after the various treatments, hearts were prepared for histology. Confocal micrographs of heart sections, double labeled with primary anti-pHsp27Ser82 and secondary antibody (AlexaFluor 546 conjugated anti-rabbit IgG, red), and primary anti-dystrophin and secondary antibody (AlexaFluor 488 conjugated anti-mouse, green) are presented in Figure 4.7. Dystrophin immunoreactivity was localized along cell membranes in all experimental groups (Figure 4.7A, D, G, J, M). Little or no pHsp27Ser82 immunoreactivity was detectable in SHAM (Figure 4.7B, C), or CON (Figure 7E, F) hearts. Diffuse pHsp27Ser82 immunoreactivity was detected in the cytoplasm and associated with cell membranes in the INS (Figure 4.7H, I), HS (Figure 4.7K, L), and HSINS (Figure 4.7N, O) hearts. pHsp27Ser82 immunoreactivity appeared to be less abundant and more aggregated in HS hearts compared to INS hearts.

Phosphorylated Hsp27 Levels After 3 Hours of Perfusion

At 1 hr after treatment, hearts were isolated and perfused as in Figure 4.1. All hearts were subjected to 30 min of ischemia, except the SHAM hearts which were continually perfused for 3 hrs. Hearts from each treatment group were examined for phosphorylated Hsp27, total Hsp27 and Hsp70 (Figure 4.8). The antibodies specific for pHsp27Ser15 (Figure 4.8A) and pHsp27Ser82 (Figure 4.8B) revealed immunoreactive product in various groups. Hsp27 immunoreactivity was unchanged while the HS and HSINS groups had elevated Hsp70 immunoreactivity compared to the CON, SHAM and

INS hearts. pHsp27Ser82 immunoreactivity in the INS group was significantly different ($p = 0.046$, $n = 3$) from that of the SHAM group of hearts (Figure 4.8C).

Inhibition of p38 MAPK with SB203580 Suppresses Insulin Induced Myocardial Protection

To investigate whether insulin increased phosphorylation of Hsp27 through activation of MAPK pathways, rats were treated with either SB203580 or PD098059, inhibitors of p38 MAPK and ERK1/2 pathways, respectively. Five groups of hearts, SHAM, CON, INS, SBINS and PDINS, were isolated and perfused as described for hearts in figure 1. SHAM hearts were not subjected to ischemia and their function did not change significantly during 3 hours of perfusion (Figure 4.9). During the 30-min of no-flow global ischemia contractility decreased quickly to zero for the CON, INS, SBINS, and PDINS hearts. During reperfusion, there were no statistical differences ($p > 0.05$) between the groups or with the pre-ischemic values for heart rate (data not shown).

Following ischemia, during reperfusion, recovery of LVDP (Figure 4.9A), LVW (Figure 4.9B), +dp/dt (Figure 4.9C), and -dp/dt (Figure 4.9D) was blocked for SBINS hearts compared to the INS hearts. PDINS hearts had recovery of function that approached that of the INS and SHAM hearts. Average percent (± SEM) recovery of heart function during 2 hr of reperfusion and significant differences among the treatment groups for treatment effects are summarized in Table 3.

Inhibition of p38 MAPK with SB203580 Suppresses Insulin Induced Phosphorylation of Hsp27

At the end of the 3 hr perfusion period (Figure 4.9), hearts were examined for total and phosphorylated Hsp27 (Figure 4.10). The antibodies specific for pHsp27Ser15 (Figure 4.10A) and pHsp27Ser82 (Figure 4.10B) revealed immunoreactive product in various

groups. SBINS and PDINS hearts appeared to have diminished pHsp27Ser15 immunoreactivity (Figure 4.10A) compared to INS hearts. SBINS hearts appeared to have diminished pHsp27Ser82 immunoreactivity (Figure 4.10B) compared to INS and PDINS hearts. Hsp27 immunoreactivity (Figure 4.10A and B) appeared to be unchanged among the hearts. While densitometeric analysis suggested that SBINS hearts had diminished pHsp27Ser82 immunoreactivity compared to INS hearts, significant differences ($p = 0.004$ to $p = 0.019$, $n = 3$ for each group) were evident only between PDINS hearts and SHAM, CON and SBINS hearts (Figure 4.10C).

Table 4.1. Pre-ischemia heart function values after 20 min of equilibration to Langendorff perfusion.

	n	HR (beat/min)	LVDP (mmHg)	LVW (mmHg*ul)	+dp/dt (mmHg/sec)	-dp/dt (mmHg/sec)	CR (mmHg/ml/min)
SHAM	6	247 ± 24	111 ± 10	10044 ± 1034	2304 ± 207	1667 ± 136	6.2 ± 0.7
CON	6	308 ± 14	111 ± 14	12493 ± 2137	3975 ± 514	2054 ± 205	9.3 ± 1.3
INS	6	319 ± 18	102 ± 8	10734 ± 858	2821 ± 409	1705 ± 106	6.6 ± 1.5
HS	6	306 ± 14	104 ± 9	10656 ± 1010	2703 ± 535	1600 ± 128	9.2 ± 1.2
HSINS	6	311 ± 12	91 ± 12	9137 ± 1310	2358 ± 480	1540 ± 201	8.7 ± 1.4

Pre-ischemia heart function values are means ± SEM. HR, heart rate; LVDP, left ventricular developed pressure; LVW, left ventricular work; +dP/d*t* and -dP/d*t*, maximal first derivative of LV pressure increase and decrease, respectively; CR, coronary vascular resistance. SHAM, sham control; CON, naïve control; INS, insulin treated; HS, heat shocked; HSINS, heat shock and insulin treated. There was no significant differences among groups ($n = 6$, $p > 0.05$).

Table 4.2. Treatment effects on cardiac function. At 1 hr after insulin treatment hearts have improved ventricular contractile recovery during post-ischemic reperfusion.

Average percent (± SEM) recovery of LVDP during 2 hr of reperfusion.

	SHAM	CON	INS	HS	HSINS
	83.9 ± 4.6	28.9 ± 3.3	68.7 ± 3.8	31.3 ± 3.3	49.5 ±3.2
		Treatment group differences			
SHAM		$p < 0.001$	$p = 0.04$	$p < 0.001$	$p < 0.001$
CON			$p < 0.001$	NS	$p < 0.001$
INS				$p < 0.001$	$p = 0.003$
HS					$p = 0.006$

Average percent (± SEM) recovery of LVW during 2 hr of reperfusion.

	SHAM	CON	INS	HS	HSINS
	87.6 ± 5.4	24.9 ± 3.5	63.3 ± 4.3	28.1 ± 3.1	40.2 ± 3.0
		Treatment group differences			
SHAM		$p < 0.001$	$p = 0.001$	$p < 0.001$	$p < 0.001$
CON			$p < 0.001$	NS	NS
INS				$p < 0.001$	$p < 0.001$
HS					NS

Average percent (± SEM) recovery of +dp/dt during 2 hr of reperfusion.

	SHAM	CON	INS	HS	HSINS
	85.1 ± 3.7	22.8 ± 2.9	66.1 ± 3.3	35.8 ± 4.8	50.0 ± 3.4
		Treatment group differences			
SHAM		$p < 0.001$	$p = 0.004$	$p < 0.001$	$p < 0.001$
CON			$p < 0.001$	NS	NS
INS				$p < 0.001$	$p = 0.024$
HS					NS

Average percent (± SEM) recovery of -dp/dt during 2 hr of reperfusion.

	SHAM	CON	INS	HS	HSINS
	80.0 ± 4.5	25.0 ± 3.1	62.0 ± 3.9	34.0 ± 4.2	47.0 ± 3.5
		Treatment group differences			
SHAM		$p < 0.001$	$p = 0.02$	$p < 0.001$	$p < 0.001$
CON			$p < 0.001$	NS	NS
INS				$p < 0.001$	NS
HS					NS

Table 4.3. Treatment effects on cardiac function. Inhibition of p38 MAPK pathway with SB203580 blocks insulin improved ventricular contractile recovery during post-ischemic reperfusion.

Average percent (± SEM) recovery of LVDP during 2 hr of reperfusion.

	SHAM	CON	INS	SBINS	PDINS
	52.7 ± 5.4	10.4 ± 2.8	55.8 ± 5.1	25.6 ± 4.9	44.7 ± 7.6
		Treatment group differences			
SHAM		$p < 0.001$	NS	$p < 0.001$	NS
CON			$p < 0.001$	NS	$p < 0.005$
INS				$p < 0.002$	NS
SBINS					NS

Average percent (± SEM) recovery of LVW during 2 hr of reperfusion.

	SHAM	CON	INS	SBINS	PDINS
	61.9 ± 7.8	11.3 ± 2.5	49.0 ± 5.1	23.8 ± 4.2	41.0 ± 7.5
		Treatment group differences			
SHAM		$p < 0.001$	NS	$p < 0.001$	NS
CON			$p < 0.001$	NS	$p = 0.012$
INS				$p = 0.014$	NS
SBINS					NS

Average percent (± SEM) recovery of +dp/dt during 2 hr of reperfusion.

	SHAM	CON	INS	SBINS	PDINS
	59.8 ± 5.0	13.4 ± 3.5	60.0 ± 5.3	28.9 ± 6.5	36.6 ± 3.9
		Treatment group differences			
SHAM		$p < 0.001$	NS	$p < 0.001$	$p = 0.02$
CON			$p < 0.001$	NS	NS
INS				$p < 0.001$	$p = 0.011$
SBINS					NS

Average percent (± SEM) recovery of -dp/dt during 2 hr of reperfusion.

	SHAM	CON	INS	SBINS	PDINS
	59.5 ± 5.2	15.8 ± 4.0	53.5 ± 4.7	25.0 ± 4.8	37.1 ± 3.8
		Treatment group differences			
SHAM		$p < 0.001$	NS	$p < 0.001$	$p = 0.007$
CON			$p < 0.001$	NS	NS
INS				$p < 0.001$	NS
SBINS					NS

Figure 4.1. At 1 hr after insulin treatment hearts have improved ventricular contractile recovery during post-ischemic reperfusion. Experimental group treatments are described in the Materials and Methods section under *Experimental protocol and groups*. Sham control (SHAM) hearts were continually perfused at 10 ml/min (not subjected to ischemia). IS indicates the 30 min ischemic interval for the control (CON), insulin treated (INS), heat shock treated (HS), and heat shock and insulin treated (HSINS) hearts. Hearts were reperfused at 10 ml/min for 120 min starting at time = 0. Data are normalized to pre-ischemia values. A. Insulin improved LVDP during reperfusion. B. Insulin improved LVW during reperfusion. C. Insulin improved recovery of +dp/dt during reperfusion. D. Insulin improved recovery of -dp/dt during reperfusion. Average percent (± SEM) recovery of heart function during 2 hr of reperfusion and significant differences between the treatment groups are summarized in Table 2 (n = 6 in each group).

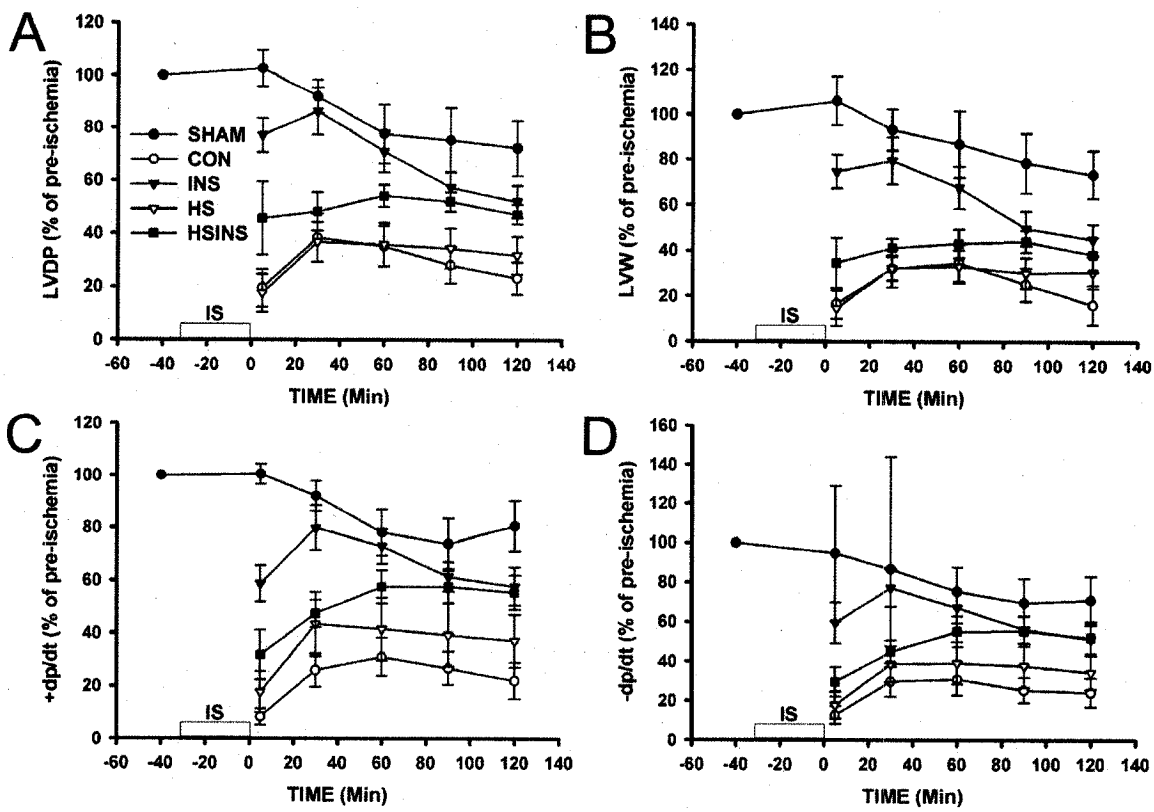
A
LVDP (% of pre-ischemia)
SHAM
CON
INS
HS
HSINS
IS
TIME (Min)
B
LVW (% of pre-ischemia)
IS
TIME (Min)
C
+dp/dt (% of pre-ischemia)
IS
TIME (Min)
D
-dp/dt (% of pre-ischemia)
IS
TIME (Min)

Figure 4.1

Figure 4.2. Western analysis of Hsp70 and Hsp27 in hearts 1 and 3 hrs after treatment. A. Hsp70 was detectable in hearts at 3 hrs after HS or HSINS treatments. Hsp27 was detectable in all hearts and minimal change was noted temporally or by treatment. Actin shows that the lanes are approximately equally loaded. B. No significant change was noted for Hsp27 levels at 1 or 3 hours after the various treatments. Hsp70 was elevated approximately 15 times the SHAM level at 3 hrs after HS or HSINS treatments. The asterisk indicates significant differences for Hsp70 in HS ($p \leq 0.006$, $n = 3$) and HSINS ($p \leq 0.003$, $n = 3$) hearts compared to SHAM, CON, and INS hearts.

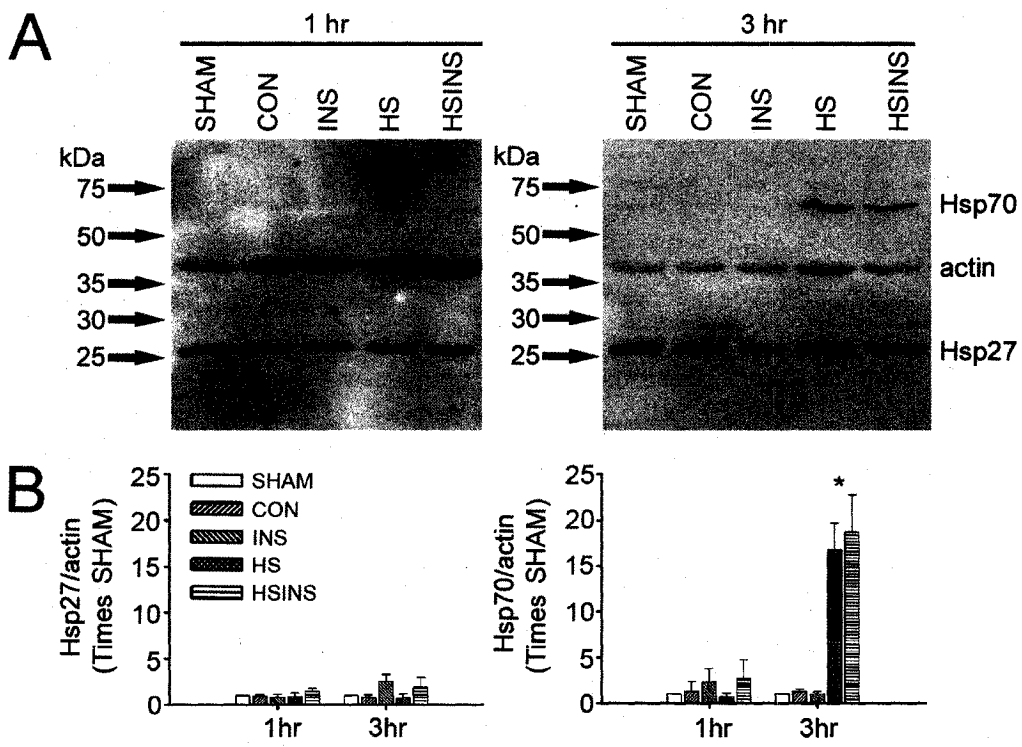
A
1 hr
3 hr
SHAM
CON
INS
HS
HSINS
kDa
75
50
35
30
25
Hsp70
actin
Hsp27
B
Hsp27/actin
(Times SHAM)
Hsp70/actin
(Times SHAM)
SHAM
CON
INS
HS
HSINS
1hr
3hr
*

Figure 4.2

Figure 4.3. Hsp70 localization compared to dystrophin 1 hr after treatment in ventricular sections examined by confocal microscopy. Sections were labeled with primary anti-Hsp70 and secondary antibody AlexaFluor 546 conjugated anti-rabbit IgG (red), and primary anti-dystrophin and secondary antibody AlexaFluor 488 conjugated anti-mouse IgG (green). Dystrophin is localized mainly to the plasma membranes. A-C, Sham control. D-F, Naïve control; there is little or no expression of Hsp70 in unstressed rat hearts. G-I, insulin treated; small amounts of Hsp70 immunofluorescence is detectable (H) and appears to be partly co-localized with dystrophin immunofluorescence (I). J-L, heat shock treated; Hsp70 immunofluorescence is detected (K) and appears to be between cardiomyocytes (L). M-O, heat shock and insulin treated; Hsp70 immunofluorescence (N) is detectable, and some co-localized with dystrophin immunofluorescence is evident (O). Images are representative of 3 animals in each treatment group. Bar = 20 μm.

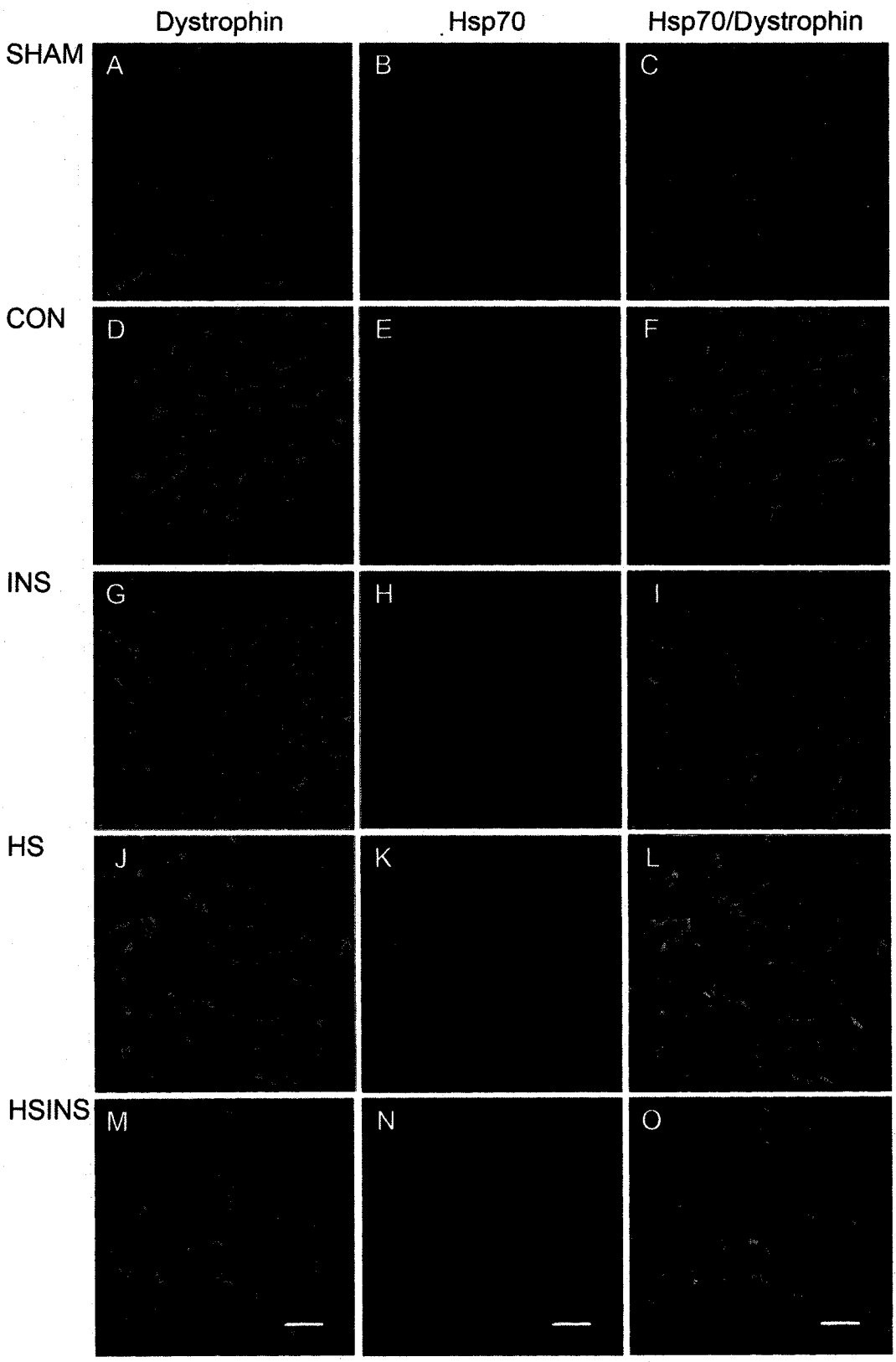
Dystrophin
Hsp70
Hsp70/Dystrophin
SHAM
CON
INS
HS
HSINS
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 4.3

Figure 4.4. Hsp27 localization compared to α-tubulin 1 hr after treatment in ventricular sections examined by confocal microscopy. Sections were labeled with primary anti-Hsp27 and secondary antibody AlexaFluor 546 conjugated anti-rabbit IgG (red), and primary anti- α-tubulin and secondary antibody AlexaFluor 488 conjugated anti-mouse IgG (green). α-tubulin is localized mainly to the cytoskeleton. A-C, Sham control; Hsp27 is constitutively expressed in rat hearts. D-F, Naïve control. G-I, insulin treated. J-L, heat shock treated. M-O, heat shock and insulin treated. Hsp27 is localized in the cytoplasm and colocalization with α-tubulin is evident in all treatment groups. Images are representative of 3 animals in each treatment group. Bar = 20 μm.

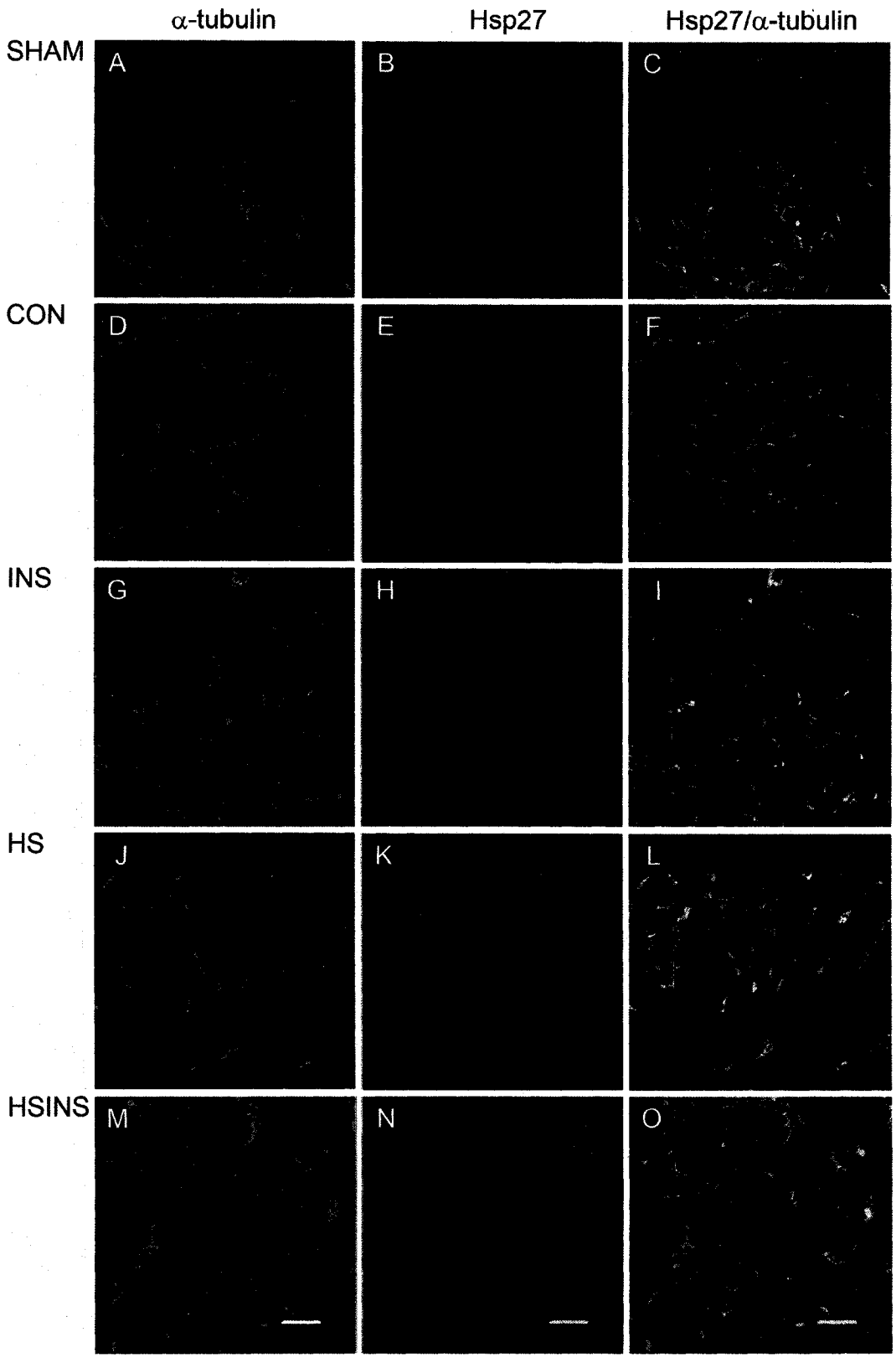
α-tubulin
Hsp27
Hsp27/α-tubulin
SHAM
CON
INS
HS
HSINS
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 4.4

Figure 4.5. Western analysis of phosphorylated Hsp27 in hearts 1 and 3 hrs after treatment. A. The antibodies specific for phosphorylated Hsp27 serine 15 (pHsp27Ser15) revealed immunoreactive product at 1 and 3 hr after treatment in various groups. B. The antibodies specific for phosphorylated Hsp27 serine 82 (pHsp27Ser82) revealed immunoreactive product at 1 and 3 hr after treatment in various groups. Actin shows that the lanes are approximately equally loaded. C. While no significant change ($p > 0.05$, $n = 3$ for pHsp27(Ser15) and $n = 6$ for pHsp27(Ser82)) was noted for phosphorylated Hsp27 levels at 1 or 3 hrs after the various treatments, pHsp27Ser15 appeared to be elevated at 1 hr after treatment in the HS and HSINS groups and pHsp27Ser82 appeared to be elevated at 1 hr after treatment in the INS and HSINS groups.

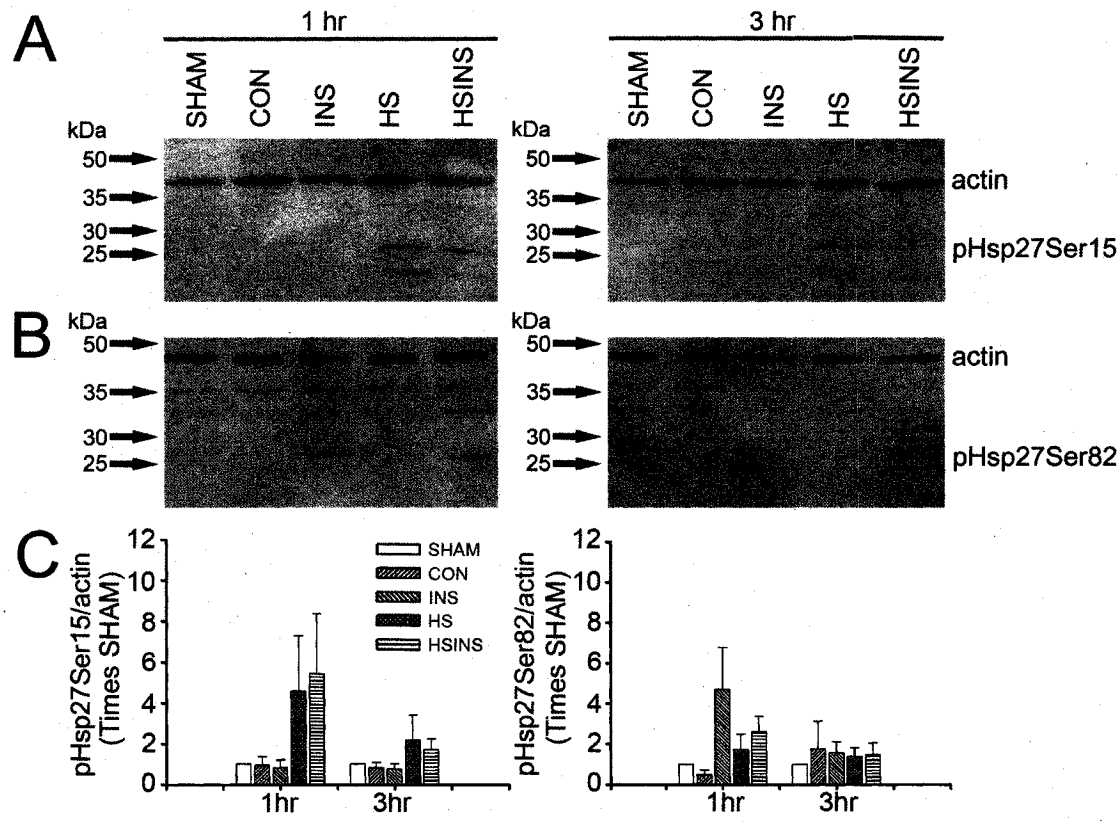
A
1 hr
3 hr
SHAM
CON
INS
HS
HSINS
kDa
50
35
30
25
actin
pHsp27Ser15
B
actin
pHsp27Ser82
C
pHsp27Ser15/actin (Times SHAM)
pHsp27Ser82/actin (Times SHAM)
12
10
8
6
4
2
0
1hr
3hr

Figure 4.5

Figure 4.6. Western analysis reveals that incubation with alkaline phosphatase abolishes immunoreactivity of phosphor specific antibodies. A. The antibodies specific for pHsp27Ser15 revealed immunoreactive product in various groups that was abolished after dephosphorylation with alkaline phosphatase. B. The antibodies specific for pHsp27Ser82 revealed immunoreactive product in various groups that was abolished after dephosphorylation with alkaline phosphatase. Actin and Hsp27 shows that the lanes are approximately equally loaded and are not changed by dephosphorylation.

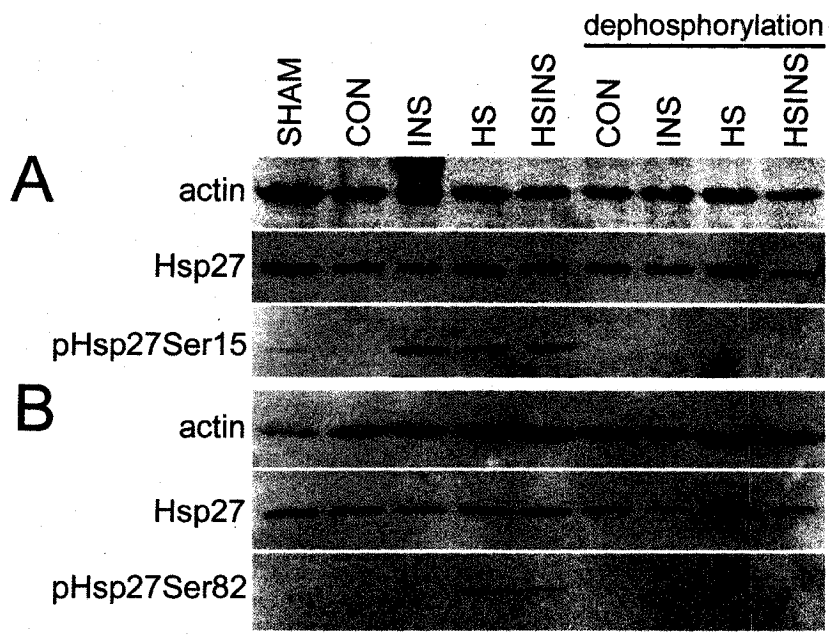
dephosphorylation
SHAM
CON
INS
HS
HSINS
CON
INS
HS
HSINS
A
actin
Hsp27
pHsp27Ser15
B
actin
Hsp27
pHsp27Ser82

Figure 4.6

Figure 4.7. Phosphorylated Hsp27 (serine 82) localization compared to dystrophin 1 hr after treatment in ventricular sections examined by confocal microscopy. Sections were labeled with primary anti-pHsp27Ser82 and secondary antibody AlexaFluor 546 conjugated anti-rabbit IgG (red), and primary anti-dystrophin and secondary antibody AlexaFluor 488 conjugated anti-mouse IgG (green). Dystrophin is localized mainly to the plasma membranes. A-C, Sham control. D-F, Naïve control; no pHsp27Ser82 was detected in the SHAM or CON hearts. G-I, insulin treated; pHsp27Ser82 was detectable colocalized with Dystrophin. J-L, heat shock treated; pHsp27Ser82 was detectable colocalized with Dystrophin. M-O, heat shock and insulin treated; pHsp27Ser82 was detectable colocalized with Dystrophin. Images are representative of 3 animals in each treatment group. Bar = 20 μm.

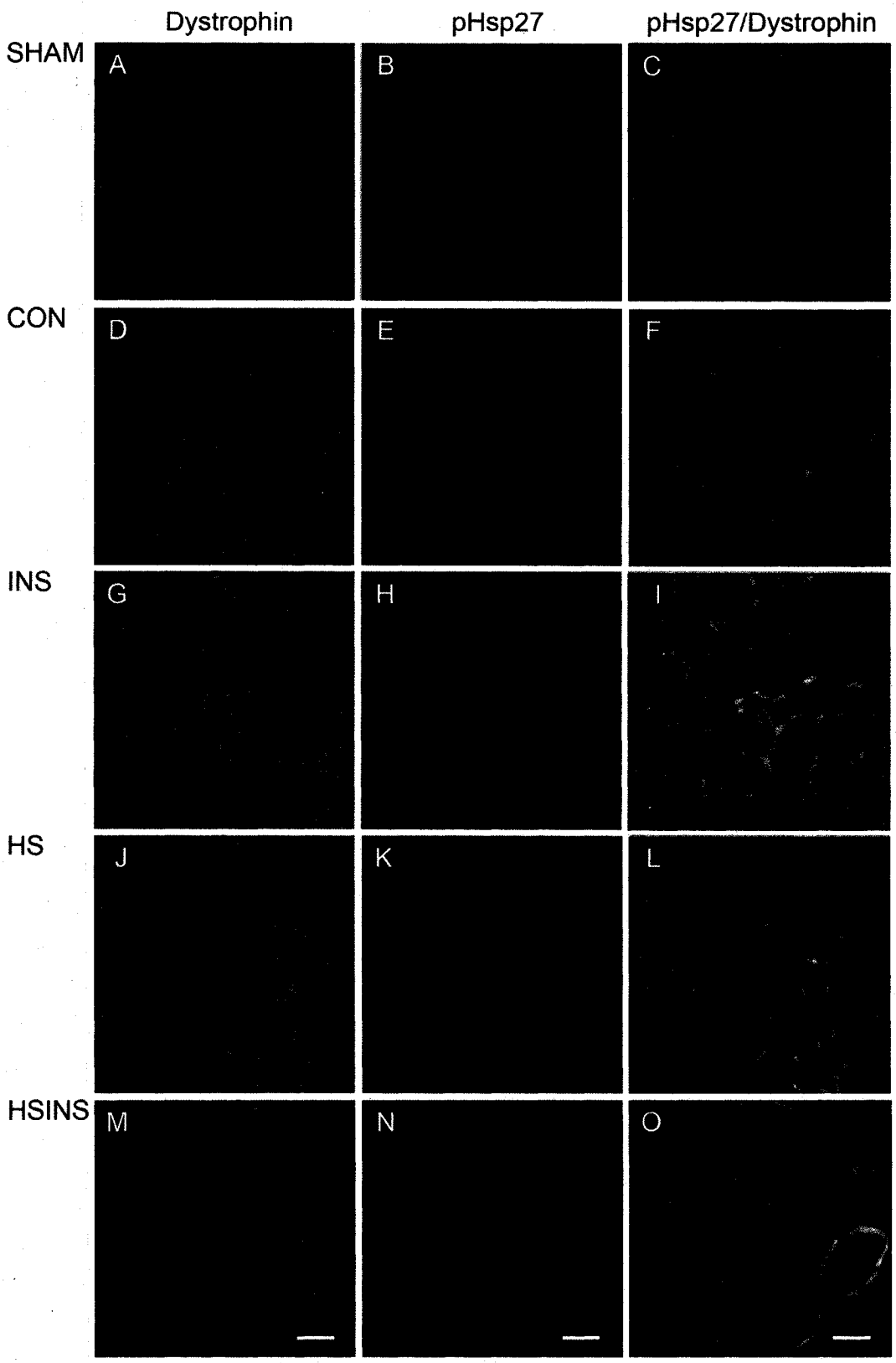
Dystrophin
pHsp27
pHsp27/Dystrophin
SHAM
CON
INS
HS
HSINS
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 4.7

Figure 4.8. Western analysis for Hsps in hearts at the end of 3 hrs of isolated perfusion. Animals in each experimental group were treated as described in the Materials and Methods section under *Experimental protocol and groups*. Animals were recovered for 1 hr before isolation and perfusion of hearts for 3 hrs as shown in Figure 1. A. The antibodies specific for pHsp27Ser15 revealed immunoreactive product mostly in INS hearts. Actin shows that the lanes are approximately equally loaded. B. The antibodies specific for pHsp27Ser82 revealed immunoreactive product mostly in INS hearts. Hsp27 was detectable in all hearts and minimal change was noted by treatment even after 3 hrs of perfusion. Hsp70 was detectable in all hearts after 3 hrs of perfusion. Actin shows that the lanes are approximately equally loaded. C. pHsp27Ser82 was significantly different (asterisk) in the INS treated hearts compared to CON hearts ($p = 0.046$, $n = 3$). While Hsp70 appeared elevated in the HS and HSINS groups compared to CON, variation was large and significance was not reached ($p = 0.059$, $n = 3$).

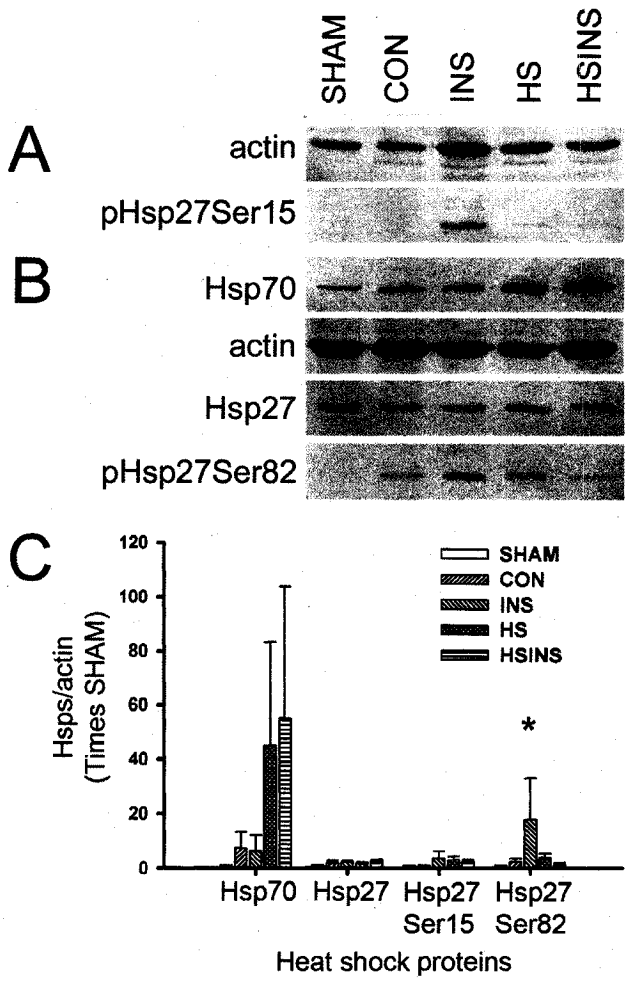
SHAM
CON
INS
HS
HSINS
A
actin
pHsp27Ser15
B
Hsp70
actin
Hsp27
pHsp27Ser82
C
Hsps/actin
(Times SHAM)
120
100
80
60
40
20
0
SHAM
CON
INS
HS
HSINS
*
Hsp70
Hsp27
Hsp27
Ser15
Hsp27
Ser82
Heat shock proteins

Figure 4.8

Figure 4.9. Inhibition of p38 MAPK pathway with SB203580 blocks insulin improved ventricular function during post-ischemic reperfusion. Experimental group treatments are described in the Materials and Methods section under *Experimental protocol and groups*. Sham control (SHAM, n = 5) hearts were continually perfused at 10 ml/min (not subjected to ischemia). IS indicates the 30 min ischemic interval for the control (CON, n = 4), insulin treated (INS, n = 6), SB203580 and insulin treated (SBINS, n = 6), and PD098059 and insulin treated (PDINS, n = 6) hearts. Hearts were reperfused at 10ml/min for 120 min starting at time = 0. Data are normalized to pre-ischemia values. A. Inhibition of p38 MAPK pathway with SB203580 blocks insulin improved LVDP during reperfusion. B. Inhibition of p38 MAPK pathway with SB203580 blocks insulin improved LVW during reperfusion. C. Inhibition of p38 MAPK pathway with SB203580 blocks insulin improved recovery of +dp/dt during reperfusion and inhibition of ERK1/2 pathway with PD098059 partially blocks insulin improved recovery of +dp/dt during reperfusion. D. Inhibition of p38 MAPK pathway with SB203580 blocks insulin improved recovery of -dp/dt during reperfusion and inhibition of ERK1/2 pathway with PD098059 partially blocks insulin improved recovery of -dp/dt during reperfusion. Average percent (± SEM) recovery of heart function during 2 hr of reperfusion and significant differences between the treatment groups are summarized in Table 3.

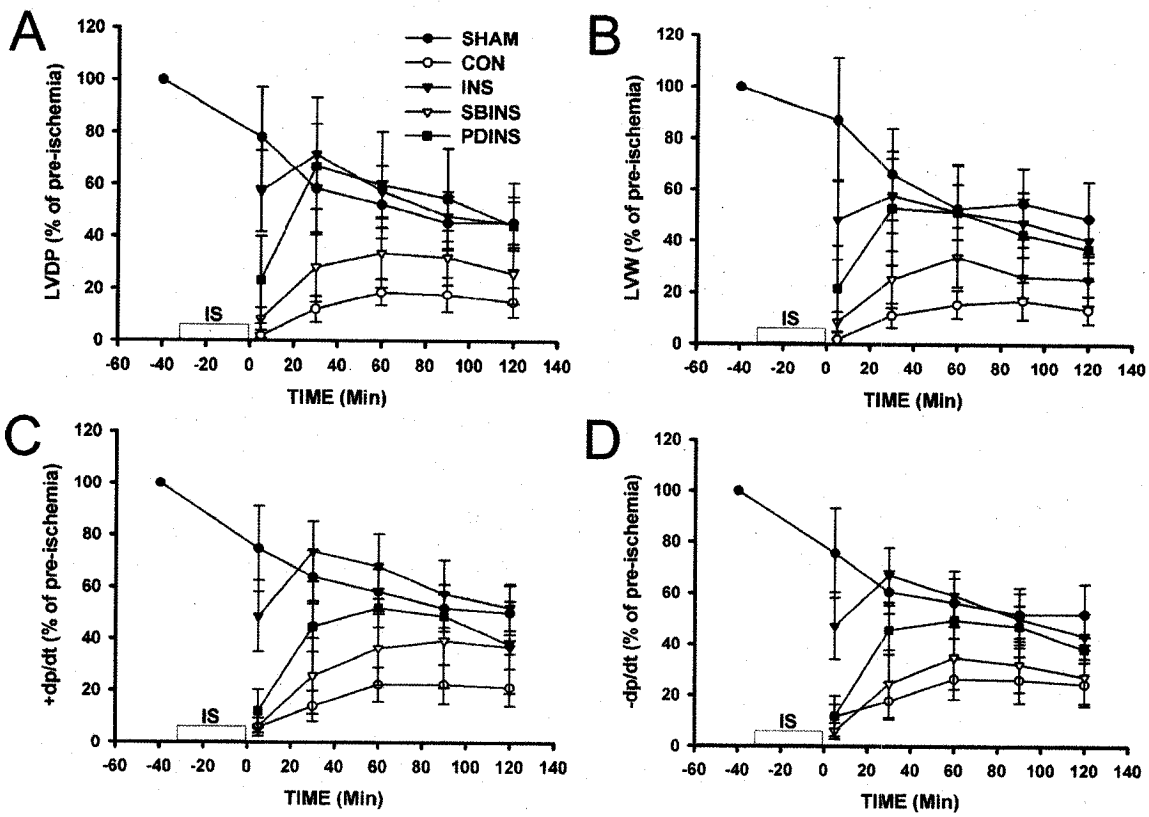
A
B
C
D
SHAM
CON
INS
SBINS
PDINS
LVDP (% of pre-ischemia)
LVW (% of pre-ischemia)
+dp/dt (% of pre-ischemia)
-dp/dt (% of pre-ischemia)
IS
TIME (Min)
0
20
40
60
80
100
120
-60
-40
-20
0
20
40
60
80
100
120
140

Figure 4.9

Figure 4.10. Western analysis for phosphorylated Hsp27 in hearts at the end of 3 hrs of isolated perfusion. SB203589 (SBINS, n=3) blocked insulin-induced phosphorylation of Hsp27, but not PD098059 (PDINS, n=3). Neither kinase blocker affected non-phosphorylated Hsp27. Animals in each experimental group were treated as described in the Materials and Methods section under *Experimental protocol and groups*. Animals were recovered for 1 hr before isolation and perfusion of hearts for 3 hrs as shown in Figure 9. A. The antibodies specific for pHsp27Ser15 revealed immunoreactive product mostly in INS hearts. Hsp27 was detectable in all hearts and minimal change was noted by treatment even after 3 hrs of perfusion. Actin shows that the lanes are approximately equally loaded. B. The antibodies specific for pHsp27Ser82 revealed immunoreactive product mostly in INS and PDINS hearts. SB203589 appears to suppress INS induced phosphorylation of Hsp27. Hsp27 was detectable in all hearts and minimal change was noted by treatment even after 3 hrs of perfusion. Actin shows that the lanes are approximately equally loaded. C. pHsp27Ser82 was significantly different (asterisk) in the SHAM, CON and SBINS treated hearts versus PDINS treated hearts, $p = 0.005$, $p = 0.004$ and $p = 0.019$, respectively.

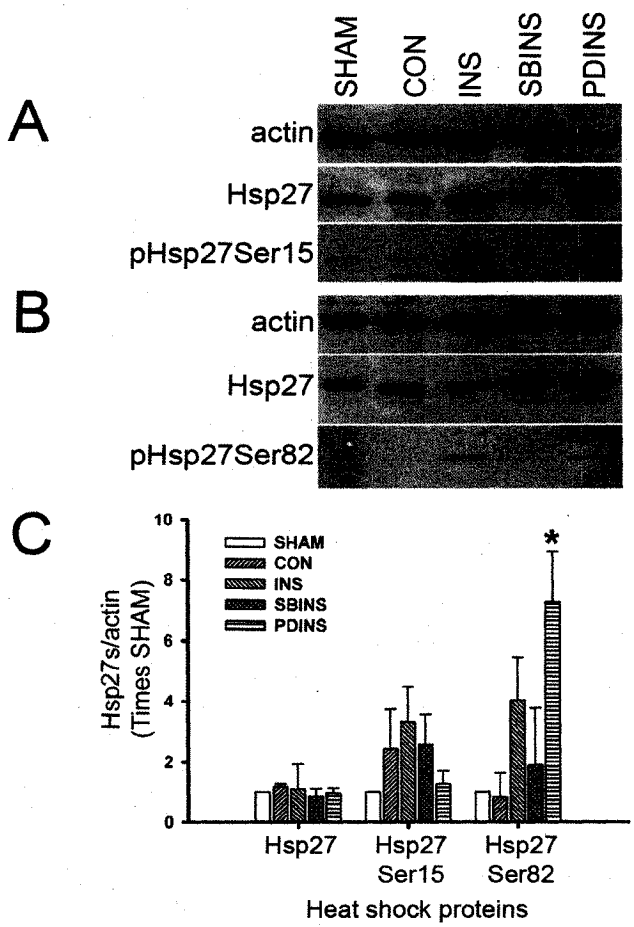
SHAM
CON
INS
SBINS
PDINS
A
actin
Hsp27
pHsp27Ser15
B
actin
Hsp27
pHsp27Ser82
C
Hsp27s/actin
(Times SHAM)
10
8
6
4
2
0
SHAM
CON
INS
SBINS
PDINS
*
Hsp27
Hsp27
Ser15
Hsp27
Ser82
Heat shock proteins

Figure 4.10

Discussion

Here we show that at 1 hr after a single 200 μU/g body weight injection of insulin, isolated hearts have a significant improved myocardial functional after 30 minutes of ischemic injury. This protection is unlikely to be due to elevated levels of Hsp70 or Hsp27. However, phosphorylation of Hsp27 appears to be elevated by insulin and heat shock treatments. Interestingly, at the end of the ischemia/reperfusion period, only insulin treated hearts contained significantly elevated levels of phosphorylated Hsp27. Most interestingly, the insulin-induced improvement of myocardial function was blocked by SB203580, a p38 MAPK pathway blocker, and was associated with an apparent decrease of phosphorylated Hsp27. PD098059, a blocker of the ERK1/2 pathway, did not inhibit phosphorylation of Hsp27 and the insulin-induced improvement of myocardial function was only partially blocked. This work suggests that insulin may be modulating Hsp27 phosphorylation. It is intriguing to consider that the phosphorylation state of Hsp27 may be associated with the improved myocardial function seen in the insulin treated hearts, and p38 MAPK pathway is responsible for the phosphorylation of Hsp27.

Insulin in a cocktail of glucose - insulin - potassium (GIK) cocktail has beneficial effects through changes in energy metabolism (Oliver and Opie, 1994). During ischemia, the components of GIK favour a shift from the anaerobic FFA metabolism with generation of toxic FA-CoA, to the less toxic anaerobic glucose-dependent metabolism, and facilitates glycolysis to produce more energy. Most clinical studies suggest GIK reduces both morbidity and mortality (van der Horst et al., 2003; Langley and Adams, 2006). However, some studies show that GIK is of minimal benefit (Ceremuzynski et al., 1999; Mehta et al., 2005). In animal studies, whether GIK or insulin infusion protect cardiac contractile

function when given before and immediately following ischemia. The benefits of insulin are thought to be through insulin intracellular mechanisms or signaling pathways (Hausenloy and Yellon, 2006).

Previously we have shown that a single physiological dose of insulin followed by 6 hrs of recovery induced the expression of Hsp70 and provided improved functional recovery of hearts against ischemia/reperfusion injury (Li et al., 2006). This dose of insulin had no effect on blood glucose levels (Li et al., 2004). While good evidence suggests that transgenic overexpression of Hsp70 is directly involved in protecting the heart from ischemia/ reperfusion injury (Marber et al., 1995; Plumier et al., 1995), at 6 hrs after insulin injection, it remains an open question whether Hsp70 is contributing to the functional recovery of the hearts after ischemic injury. In the present experiments, little or no Hsp70 was detected in the hearts at 1 or 3 hrs after insulin injection suggesting that Hsp70 is not involved in the improved functional recovery (Figure 4.1) at 1 hr after insulin treatment. While Hsp70 was detected in hearts at 3 hrs after heat shock (HS and HSINS groups, Figure 4.2) and in hearts after 1 hr after heat shock followed by 3 hrs of isolated perfusion, (HS and HSINS groups, Figure 8), recovery of function was not evident in these groups (Figure 4.1, Table 4.2).

At 6 hrs after insulin injection, Hsp70 is localized mainly along plasma membranes of cardiomyocytes (Li et al., 2006). In the present study, at 1 hr after insulin injection, little or no Hsp70 is detected in the hearts (Figure 4.3). In contrast, at 1 hr after heat shock, Hsp70 was detectable in cardiac tissue and appeared to be localized mostly in small capillaries and in perivascular cells between the cardiomyocytes (Figure 4.3) and this localization is not different at 6 or 24 hr after heat shock (Leger et al., 2000; Li et al., 2004,

2006). At 1 hr after induction, it is unlikely that sufficient mature Hsp70 has accumulated in the cells to provide protection from ischemia/reperfusion injury. The difference in detectable Hsp70 at 1 hr (Figure 4.3) and 3 hr (Figure 4.2) after INS or HS treatment may be related to activation of signaling pathways. Heat denatures protein that recruits Hsc70/Hsp70 freeing the heat shock transcription factor-1 (HSF1). Free HSF1 is phosphorylated, trimerized, and translocated to the nucleus (Morimoto, 1998; Shi et al., 1998), where it binds to a regulatory upstream promoter heat shock element (HSE) on heat shock genes (Holmgren et al., 1981; Pelham, 1982). Insulin may be signalling through GSK3 to release its inhibition of the heat shock transcription factor. The overexpression of GSK3β resulted in significant reduction in heat-induced HSF1 activities. Activation of HSF1-HSE binding under non-heat shock condition was also observed after inhibiting GSK3β with LiCl (LiCl mimics insulin inhibition of GSK3β) (Xavier et al., 2000). Comparing our contractile data for HS and HSINS hearts, we think that the insulin is rescuing the contractile function even after HS. We are convinced that any recovery of contractile function at 1 hour after insulin is not due to Hsp70 but might be due to phosphorylation of Hsp27.

Hsp27 is abundant in the heart and is associated with contractile elements. At 24 hrs after heat shock treatment, Hsp27 distribution and abundance is minimally changed (Leger et al., 2000). In the current study, the abundance of Hsp27 is not significantly changed in the heart after insulin, heat shock or heat shock and insulin treatments (Figure 2). At 1 hr after insulin injection there appears to be some localization of Hsp27 between cardiomyocytes and possibly around blood vessels that is not obvious in the other treatment groups (Figure 4.4). As with Hsp70, an increase in protective proteins in blood vessels

could be the first line of defense in protecting the heart from reactive oxygen species during reperfusion injury (Leger et al., 2000). After heat shock, Hsp27 appears to be localized with α-tubulin (Figure 4.4) and may be acting as a chaperone to restore α-tubulin and microtubule function (Liang and MacRae, 1997).

As mentioned above, at 1 hr after insulin injection, as expected there was no obvious increase in the abundance of either Hsp70 or Hsp27. However, Survival kinase pathways such as PI3K-Akt, MEK1/2-ERK1/2 and the p38 MAPK pathways are activated by ischemic preconditioning (Hausenloy and Yellon, 2006) and are also regulating heat shock proteins and phosphorylation of Hsp27 (Dorion and Landry, 2002). The MAPK pathway is activated by many stimulatory agents and phosphorylates downstream signaling molecules (Denhardt, 1996). Insulin also activates MAPK (Moule et al., 1995; Kayali et al., 2000; Han and Lee, 2005), and MAPK activation leads to Hsp27 phosphorylation (Rouse et al., 1994; Larsen et al., 1997; Armstrong et al., 1999; Han and Lee, 2005; Kayali et al., 2000). Thus, in the present study we used phospho-specific antibodies to detect phosphorylated isoforms of Hsp27. Phosphorylated Hsp27Ser15 was detected mostly in HS and HSINS hearts and phosphorylated Hsp27Ser82 was detected repeatedly in INS, HS and HSINS hearts (Figure 4.5 and 4.6). Variation was high and significant differences were not achieved. However, we are confident that we detected phosphorylated isoforms of Hsp27, because the immunoreactivity was abolished by dephosphorylation with alkaline phosphatase (Figure 4.6). The difference in distribution of pHsp27 between INS and HS hearts may be related to damage to cellular proteins caused by high temperature and pHsp27 stabilizing the cytoskeleton (Figure 4.7).

During 3 hrs of isolated perfusion, hearts treated with INS had the strongest recovery of function after ischemic injury (Figure 4.1, Table 4.2, and Figure 4.9, Table 4.3). Most interestingly, at the end of the perfusion period, the INS treated hearts had significantly elevated levels of pHsp27Ser82 compared to the SHAM hearts (Figure 4.8). Once insulin activates the insulin receptor, the PI3K - Akt and MAPK pathways are activated (Moule and Denton, 1997). Recent evidence also indicates that insulin activates ERK1/2 and p38 MAPK (Kayali et al., 2000; Bazuine et al., 2004; Han and Lee, 2005; Lessmann et al., 2006). p38 MAPK in turn activates MAPKAPK-2 that directly regulates the phosphorylation of Hsp27 (Larsen et al., 1997; Armstrong et al., 1999; Venkatakrishnan et al., 2006). Ser15 and Ser85 phosphorylation of HSP27 by MAPKAPK- 2 is the key mechanism in reduction of apoptosis and facilitation of F-actin remodeling that protects cells from Doxorubicin toxicity (Venkatakrishnan et al., 2006). Further evidence for phosphorylation of Hsp27 having a role in myocardial protection is provided by studies on activation of the p44/42 MAPK (ERK1/2) and p38 MAPK pathways by atorvastatin (Efthymiou et al., 2005) and on activation of the adenosine A1 receptor and p38 MAPK (Dana et al., 2000).

In the present experiments, the elevated level of Hsp27 phosphorylation is associated with improved cardiac function that was diminished by SB203580. The elevated level of pHsp27 appears to be contributing to the improved cardiac function, and p38 MAPK pathway is responsible for the phosphorylation of Hsp27 induced by insulin. Phosphorylation of Hsp27 is thought to be important for its protective functions. Phosphorylation of Hsp27 alters its quaternary structure and chaperone function. Hsp27 exists in cells in either monomer and dimer forms or as multimer forms up to 600 to 800

kDa. Phosphorylation facilitates the movement of Hsp27 between the various pools (Ferns et al., 2006). Larger multimers are thought to have chaperone function and to regulate anti-oxidative activity (Rogalla et al., 1999). Phosphorylation of Hsp27 downregulates multimer size and chaperone function in favour of dimer and monomer Hsp27 regulating actin cytoskeleton stabilization. In our experiments it may be that insulin treatment mobilized some pHsp27 from unphosphorylated Hsp27 pools. The pHsp27 may be regulating actin dynamics and stabilizing the cytoskeleton during the ischemia/reperfusion injury. Multimer forms of Hsp27 are likely functioning as chaperones and also reducing reactive oxygen species damage (Préville et al., 1998). In addition to insulin stimulating an increase in the amount of Hsp27 phosphorylation, it is also likely that the ischemia/reperfusion injury stimulated an increase in the amount of pHsp27 (White et al., 2006). Moreover, phosphorylation of Hsp27 may be the key mechanism in insulin-induced myocardial protection. Insulin not only protects the cardiomyocyte by regulating glucose and free fatty acid metabolism, but also by regulating the phosphorylation state, and hence the function of Hsp27 through p38 MAPK pathway.

Inducible myocardial protection is clearly complex and unlikely to be due to one mechanism. Rapidly acquired protection appears to be related to activation of survival kinases (Hausenloy and Yellon, 2006), including phosphorylation of Hsp27. Delayed protection is dependant on protein synthesis and accumulation of various protective proteins, such as Hsp70. Insulin stimulation appears to regulate rapidly acquired protections as shown in this study and a longer term protection seen 6 hrs after insulin treatment that may involve expression of Hsp70 (Li et al., 2006).

Conclusion

In conclusion, insulin induces an apparent rapid phosphorylation of Hsp27 through the p38 MAPK pathway that is associated with improved functional recovery of cardiac contractile function after ischemia/reperfusion injury. After ischemia/reperfusion injury, only the insulin treated hearts had significantly elevated levels of phosphorylated Hsp27. Insulin stimulation appears to regulate the phosphorylation of Hsp27 that may be providing rapid myocardial protection.

CHAPTER 5:

DISCUSSION

General Discussion

Summary of the Work

In my thesis, the most important findings are: 1) at 1 and 6 hours after a single bolus of insulin injected i.m. hearts have protection against ischemic injury. 2) The insulin-induced myocardial protection at 1 hr after insulin treatment is associated with phosphorylation of Hsp27. 3) Insulin induced phosphorylation of Hsp27 is mainly through p38 MAPK pathway. 4) Insulin activated HSF1 binding to HSE, and increased the abundance of Hsp70 by 6 hrs after insulin injection. 5) At 6 hrs after insulin injection Hsp70 is co-localized with dystrophin to cardiomyocytes membranes. These results suggest that inducible myocardial protection may be acquired temporally through more than one mechanism.

Insulin Treatment

In this work I examined the effects of a single treatment of insulin injected intramuscularly at a dose of 200 μU/g body weight. At 1 hour after this treatment, blood glucose levels were not affected (Chapter 2). Clinically, insulin is usually administered as a component of the GIK solution and is given as an intravenous infusion continuously. In laboratory studies, continuous intravenous infusion of insulin or GIK revealed that insulin is as effective as GIK at providing myocardial protection (Jonasen et al., 2001; Gao et al., 2002; LaDisa et al., 2004; Zhang et al., 2006). These studies considered primarily the immediate beneficial effects of insulin on ischemia/reperfusion injury, based on metabolic regulation of glycolysis and FFA without considering protein metabolism.

However, stimulation of adipocytes with insulin rapidly (4 to 5 min) increases overall synthesis of proteins (Marshall, 1989). It is likely that such an increase in overall

protein synthesis is due to insulin signaling through PI3K-Akt pathway to activate eIF2B and eIF4E (Gingras et al., 1999; Figure 1.1). Interestingly, insulin also stimulates transcriptional activity and expression of Hsp70 mRNA (Ting et al., 1989). In my experiments, I focused on signaling events related to insulin and protein metabolism and not on glucose or FFA metabolism. The single low dose of insulin used in this research likely has a transient stimulatory effect. In rat hearts, the insulin receptor has an insulin unbinding constant of approximately 25 min suggesting that the direct effect of the injected insulin is transient (Eckel and Reinauer, 1982) . However, the single dose of insulin had long term changes, providing myocardial protection and triggering intracellular changes involving expression of Hsp70 and phosphorylation of Hsp27. These changes were evident long after the immediate short term effect of insulin on glucose and FFA metabolism.

Cardiac Contractile Function

Cardiac contractile function is a sensitive index of total heart metabolism. Insulin protects cardiac contractile function from ischemia/reperfusion injury (Chapters 3, 4). Contractile functional recovery after ischemia/reperfusion is dependant on the total cellular function including metabolic function, electrical conductance, membrane stability, and electrical-mechanical coupling. One of the postulated models for ischemic preconditioning is metabolic preconditioning through modulating cardiac metabolic plasticity. This metabolic plasticity includes 1) the improvement of coupling of glycolysis to glucose oxidation; 2) the role of glucose - FFA interaction, and 3) the anti-apoptotic role of insulin (Opie and Sack, 2002). In addition, heat shock proteins protect cardiac contractile function after ischemia/reperfusion injury (Marber et al., 1995; Plumier et al., 1995; Hollander et al., 2004). The chaperone function of Hsp70 and Hsp27 may directly resolve the damaged

proteins caused by ischemia/reperfusion injury. In this thesis, contrary to other studies using continuous infusion of insulin or GIK (Jonassen et al., 2001; Gao et al., 2002), I found that a single dose of insulin improved recovery of cardiac contractile function following ischemia/reperfusion injury. The improved recovery of cardiac contractile function was diminished by blocking the p38 MAPK pathway, this associated with a decrease of Hsp27 phosphorylation. On the other hand, I found only a minimal inhibition of insulin improved cardiac function by blocking the ERK1/2 pathway, without inhibition of Hsp27 phosphorylation (Chapter 4). Most interestingly, whether hearts were isolated at 1 hr or 6 hrs after insulin treatment, they had improved cardiac contractile recovery after ischemia suggesting that long term adaptive changes had occurred. Some of the long term changes in the heart include elevated expression of Hsp70 and pHsp27.

Insulin Treatment and Hsps

Evidence is strong that expression of Hsp70 and Hsp27 provide cellular protection against injury such as heat (Khoei et al.; 2004; Dokladny et al., 2006), oxidative stress (Arrigo et al., 2005; Venkatakrishnan et al., 2006), ischemic injury (Marber et al., 1995; Plumier et al., 1995; Hollander et al., 2004), inflammation (Chen et al., 2004a, 2004b, 2006) and apoptosis (Charette et al., 2000; Ravagnan et al., 2001). These studies suggest that Hsp70 and Hsp27 may play an important role in myocardial protection against ischemia/reperfusion injury through multiple intracellular mechanisms.

Time Course of Hsp70 Expression

In my studies, it is clear that heat shock treatment is a stronger stimulant than insulin for inducing elevated expression of Hsp70. However six hours after insulin treatment, Hsp70 is increased in abundance in the heart. At 1 and 3 hours after insulin

treatment an increase in the abundance of Hsp70 was not detectable. In comparison after heat shock treatment, Hsp70 is detectable at 1.5 hrs, rapidly accumulates at 3 hrs and is maximal by 6 hrs (Currie and Tanguay, 1991). Interestingly, at 6 hrs after insulin treatment, isolated and perfused hearts were resistance to ischemic/reperfusion injury (Chapter 3) while after heat shock treatment, no protection was evident at this time (Cornelussen et al., 1998; 2003; Yamashita et al., 1998), but only appears at about 24 hrs (Cornelussen et al., 1998; Currie et al., 1988, 1993; Hutter et al., 1994; Yamashita et al., 1998). While there is myocardial protection 6 hrs after insulin treatment, the contribution of the elevated level of Hsp70 to this protection is speculative for several reasons. Firstly, newly synthesized Hsp70 at 6 hrs may not be completely matured or folded into its functional configuration. However, heat shock and ischemia/reperfusion injury within 6 hours may simply overwhelm the protective chaperone activity of Hsp70. Insulin is thought not to be a noxious stimulation. Secondly, the localization of Hsp70 in the heart after insulin treatment appears to be along cardiomyocyte membranes, while after heat shock Hsp70 is mainly in small blood vessels. Localization of Hsp70 may be paramount in myocardial protection. Thirdly, at 1 hr after insulin, hearts have myocardial protection from ischemia/reperfusion injury but Hsp70 is at low or undetectable levels at this time.

Time Course of Hsp27 and pHsp27Expression

In this thesis, insulin and heat shock treatments had minimal effect on the abundance of Hsp27 in the heart (Chapter 4). Interestingly, at 1 hr after insulin treatment, myocardial protection from ischemia/reperfusion injury was evident. In the heart, Hsp27 is a constitutive protein that interacts with actin. Hsp27 protects cells against injury induced by ROS, through its ATP-independent protein chaperone activity, versus ATP-dependent

protein chaperones, Hsp70, Hsp40, Hsp90 and co-chaperones. Phosphorylation of Hsp27 is the key mechanism in reduction of apoptosis and facilitation of F-actin remodeling and it protects cells from ROS injury (Venkatakrishnan et al., 2006). Large Hsp27 oligomers are thought to be responsible for chaperone activity (Arrigo, 2007). However, a potential discrepancy is apparent between evidence for glutathione dependent protection of cells by large oligomers (Arrigo, 2007) and protection of microfilaments against cellular oxidative stress injury mediated by small oligomers of Hsp27 (Huot et al., 1995, 1996; Landry and Huot, 1995, 1999). The controversy about oligomerization state of Hsp27 and phosphorylation state of Hsp27 providing cellular protection is still on going (Welsh and Gaestel, 1998; Arrigo, 2007). In this thesis, I observed that the amount of Hsp27 was not significantly changed after insulin treatment in cardiac tissue. However, insulin induced a rapid phosphorylation of Hsp27 that was evident at the end of the ischemia/reperfusion period, and inhibition of p38 MAPK blocked the phosphorylation of Hsp27, and this was associated with a diminished myocardial protection (Chapter 4). I postulate that pHsp27 induced by insulin before ischemia plays a role during ischemia/reperfusion injury protecting actin from inappropriate interactions and denaturation.

Cellular Localization of Hsps in the Heart

In rat hearts, heat shock induced Hsp70 is localized mainly in microvessels (Amrani et al., 1998; Leger et al., 2000). In this thesis, I found that Hsp70, at 6 hrs after heat shock, was indeed localized in capillaries and/or perivascular compartments (Chapter 3). Most interestingly, I found that insulin induced Hsp70 expression was localized mainly in cardiomyocytes and along the cell membrane (Chapter 3). It is intriguing to consider

whether this distinct cardiomyocyte distribution of Hsp70 is associated with myocardial protection.

Hsp27 is abundant in the heart and is associated with contractile elements (Leger et al., 2000). The abundance of Hsp27 in the heart may obscure any increase in expression of Hsp27 induced by insulin or heat shock treatment. In my studies, no obvious change in the abundance of Hsp27 was noted after insulin or heat shock treatments (Chapter 4). However, at 1 hr after insulin treatment Hsp27 appeared to increase around or in small blood vessels.

Insulin Activates HSF1 in Rat Heart

HSFs function as specific transcription factors, and mediate the enhanced expression of heat shock protein genes when cells are stressed. HSF1 is quickly activated and bound to HSE by heat shock or hypoxia (Mosser et al., 1988; Benjamin et al., 1990). HSF1-HSE binding is also activated by salicylic acid (Cotto et al., 1997) and indomethasone (Lee et al., 1995). Interestingly, oxidative stress and heat shock have different patterns for phosphorylation of activation of HSF1 (Liu and Thiele, 1996) suggesting that various stimuli may differentially activate HSF1-dependent transcription. In this thesis, I showed that, at 6 hrs after insulin treatment, HSF1 is activated and binds with HSE, and there is increased expression of Hsp70 (Chapter 3).

Insulin Induced Myocardial Protection

Insulin Regulates Metabolism

Insulin is thought to provide myocardial protection by modulating abnormal metabolism during ischemia/reperfusion injury. Insulin increases glucose uptake and glycolytic ATP production, and inhibits mitochondria fatty acid oxidation via AMP-

activated protein kinase (Opie, 1970; Cave et al., 2000; Hue et al., 2002). Also, insulin protects survival kinases in signaling pathways. Insulin protects the myocardium even in the absence of external glucose by improving mitochondrial energy production by promotion of glycolysis and suppression of FFA metabolism (Ven Rooyen et al., 2002; Opie, 2003). Insulin protects the myocardium against ischemia/reperfusion injury by modulating multiple metabolic functions.

In this thesis, I have shown that insulin also regulates protein synthesis. While it is known that insulin regulates protein synthesis generally, here I have shown that insulin increases Hsp70 expression and Hsp27 phosphorylation. These changes in Hsp70 and Hsp27 are associated with improved recovery from ischemia/reperfusion injury. The results in this thesis also suggest that immediately (1 hr) after insulin treatment, insulin improved cardiac contractile function against ischemia/reperfusion injury requires p38 MAPK phosphorylation of Hsp27. The influence of insulin on glucose and FFA metabolism on insulin induced myocardial protection may be not enough to explain all of the effects of insulin on myocardial protection. My results suggest that insulin is regulating both protein synthesis (at least Hsp70) at the transcriptional level, and also post-translational modifications, particularly phosphorylation of Hsp27.

Pathway for Insulin-dependent Phosphorylation of Hsp27

SB203680, a p38 MAPK inhibitor, blocks insulin induced phosphorylation of Hsp27, while PD098059, an ERK1/2 inhibitor does not (Chapter 4). Insulin, acting through the insulin receptor and PI3K, activates MEK3/6 that in turn activates p38 MAPK. p38 MAPK activates MAPKAPK2 that in turn phosphorylates Hsp27 (Figure 5.1). Phosphorylated Hsp27 may bind and stabilize actin or facilitate actin remodeling during

Figure 5.1. Possible pathways involved in insulin-modulated heat shock response.

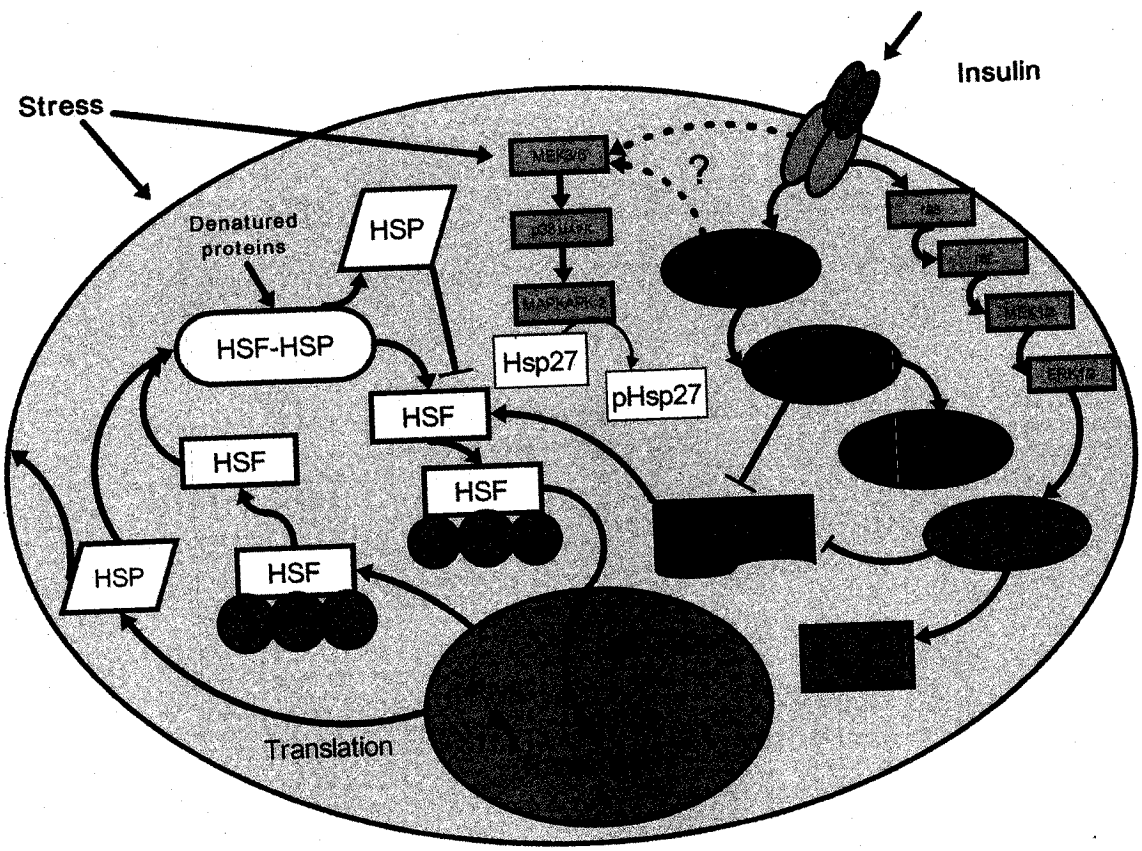
Insulin
Stress
?
Denatured proteins
HSP
HSF-HSP
Hsp27
pHsp27
HSF
HSF
HSF
HSP
HSF
Translation

Figure 5.1

ischemia/reperfusion injury, thus providing myocardium protection. This is a possible signaling pathway for the myocardial protection seen 1 hr after insulin treatment.

Pathway for Insulin-dependent Phosphorylation and Activation of HSF1

Insulin, acting through the insulin receptor and PI3K, activates Akt, that in turn suppresses the activity of GSK3 (Cohen and Frame, 2001). In addition, insulin, acting through the insulin receptor activates the ERK1/2 pathway, that also suppresses the activity of GSK3 (Chu et al., 1996; He et al., 1998; Dai et al., 2000; Xavier et al., 2000). Suppression of GSK3 releases its inhibitory effect on HSF1, freeing HSF1 to be phosphorylated (Figure 5.1). HSF1 is activated by heat shock by phosphorylation at serine 230 (Pirkkala et al., 2001; Voellmy, 2005). Once HSF1 is phosphorylated, it trimerizes, translocates to the nucleus, binds to the HSE in the promoter region of HS genes and initiates transcription. By 6 hrs after insulin treatment, elevated levels of Hsp70 are evident in the hearts and Hsp70 is strongly associated with myocardial protection. This is a possible signaling pathway for the myocardial protection seen 6 hr after insulin treatment.

Other Mechanisms for Insulin Inducing Myocardial Protection

Insulin increases nitric oxide in endothelium, and nitric oxide decreases coronary vascular resistance in a dose-dependent manner in the heart and enhances myocardial blood flow (Sundell and Knuuti, 2003). Therefore, overall myocardial protection is favoured.

Conclusion

Insulin induces myocardial protection at 1 hr and at 6 hr after injection. This thesis suggests that at 1 and 6 hr after insulin treatment the myocardial protection may be due to the stimulation of two signaling mechanisms activating two protective mechanisms. It may

be that inducible endogenous myocardial protection as seen after insulin treatment (Chapter 3 and 4) and after heat shock treatment (Currie et al., 1988; 1993) is temporally due to multiple signaling pathways and mechanisms.

Perspectives

Currently, strong evidence suggests that heat shock proteins protect the myocardium against ischemia/reperfusion injury. But heat shock as used in the laboratory is a noxious stimulation and is unlikely to be used in patients. Therefore, it seems important to find stimulants such as insulin that will gently induce Hsps and myocardial protection.

There are several questions that are evident from this thesis. Firstly, while I have shown insulin induced myocardial protection at 1 and 6 hrs after treatment, 24 hrs needs to be investigated to see if the protection matches that of HS. The time course of insulin induced myocardial protection and the contribution of other HSPs needs to be defined. Secondly, after insulin does Hsp70 bind with ion channels on the cellular membrane? It is likely that ion channels (proteins) are injured during ischemia/reperfusion. Thirdly, in clinical practice, when should insulin be given to induce myocardial protection?

The accumulated evidence suggests that ischemia preconditioning *attenuates* the injury in myocardium caused by ischemia/reperfusion, but does not facilitate full recovery. Insulin may be used therapeutically to stimulant signaling pathways to induce myocardial protection before cardiac surgery with a planned ischemic episode.

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