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The Cardio-Protective Effects of Substance P in Both Ischemia/Reperfusion and and Short-Term Hypoxia Rat Models

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THE CARDIO-PROTECTIVE EFFECTS OF SUBSTANCE P IN BOTH
ISCHEMIA/REPERFUSION AND SHORT-TERM HYPOXIA RAT MODELS

by

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School of Medicine

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DEDICATION

I dedicate this thesis to my angel and idol, my mother. She has always been an example of sacrifice and perseverance in facing the life since my childhood. Her persistence, optimism, and wisdom have inspired me to go through all the hardship during my study.

To the memory of my father, Khaleel Jubair. I would like to thank him for advising me to explore the world and expand my knowledge. I really miss his smile.

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ABSTRACT

Substance P, a member of the tachykinin family, is found primarily in sensory nerves. In the heart, substance P-containing nerve fibers surround coronary vessels, making them ideally positioned to sense changes in coronary pressure and/or flow. Recent studies have identified substance P as being protective acutely following ischemia-reperfusion due to its ability to induce coronary blood vessel vasodilation. In addition, studies conducted on non-cardiac tissue have reported substance P to be protective against cell death through a mechanism involving activation of anti-apoptotic AKT pathway. However, the possibility of substance P being similarly cardioprotective has not been reported. Accordingly, the purpose of this study was to test the hypothesis that substance P attenuates cardiomyocyte cell death following ischemia/reperfusion. A rat isolated heart preparation was used to study the effect of substance P following global ischemia/reperfusion, while a rat left ventricular tissue slice culture preparation was used to study the effect of substance P in ischemia without reperfusion. Coronary flow was significantly increased during reperfusion and LDH release was less in substance P pretreated ischemia/reperfusion hearts compared with no-treatment ischemia/reperfusion hearts. In the cultured slice preparation, substance P was shown to be effective in decreasing hypoxic-induced LDH release, apoptosis (TUNEL), and necrosis (PAS), as well as increasing AKT activation (phosphorylation) in a dose dependent manner. Inhibition of the substance P receptor (NK1) or p-AKT resulted in an increased release of

LDH, apoptosis, and necrosis in hypoxic slices incubated with substance P, thus abolishing the protective effect of substance P. These findings indicate that, in addition to its coronary vasodilatory effect, substance P is cardioprotective via a cardiomyocyte anti-apoptotic mechanism.

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LIST OF ABBREVIATIONS

ACE.....	Angiotensin converting enzyme
ANP	Atrial natriuretic peptide
APS	Ammonium Persulfate
BCA	Bicinchoninic acid
CGRP	Calcitonin gene related peptide
dP/dt	Change of pressure with respect to time (contractility assessment)
DPIV	Dipeptidyl aminopeptide IV
E/A ratio.....	Early (E) to late (A) ventricular filling velocities
EDTA.....	Ethylenediaminetetraacetic acid
EMCV	Encephalomyocarditis virus
ERK.....	Extracellular-signal-regulated kinases
FBS	Fetal bovine serum
Foxo1	Forkhead box protein O1
GAPDH.....	Glyceraldehyde 3-phosphate dehydrogenase

H.....	Hypoxia
H100.....	Hypoxia with 100nM Substance P
H100+AKT INH.....	Hypoxia with 100nM substance P and AKT inhibitor
H100+NK1 INH.....	Hypoxia with 100nM substance P and NK1 receptor inhibitor
H30.....	Hypoxia with 30nM substance P
H300.....	Hypoxia with 300nM substance P
I/R.....	Ischemia/reperfusion
ICAM-1.....	Intercellular adhesion molecules-1
IFN.....	Interferon
IHD.....	Ischemic heart disease
IL.....	Interleukin
LDH.....	Lactate dehydrogenase
LV.....	Left ventricle
NK.....	Neurokinin
LVEDP.....	Left ventricular end diastolic pressure
MAPK.....	Mitogen-activated protein kinase
MMP.....	Matrix metalloproteinases
N.....	Normoxia

NEP.....Neutral endopeptidase

NF- κ BNuclear factor kappa-light-chain-enhancer of activated B cells

NKA Neurokinin A

NKBNeurokinin B

NPKNeuropeptide K

NTS.....Nucleus of tractus solitarius

p-AKT..... Phosphorylated AKT

PAGE Polyacrylamide gel electrophoresis

PAS Periodic acid-Schiff

PEP..... Postproline endopeptidase

PGE2..... Prostaglandin E2

PI3K Phosphatidylinositide 3-kinase

PKC..... Protein kinase C

PLCPhospholipase C

SDSSodium dodecyl sulfate

SHR..... Spontaneous hypertension

sICAM-1 soluble Intracellular adhesion molecule-1

SP Substance P

SP I/R Substance P treated ischemia/reperfusion

SP-DE Substance P-degrading enzyme

TBS Tris buffered saline

TBS-T Tris buffered saline-Tween

TEMED Tetramethylethylenediamine

TIMP-2 Tissue inhibitors of metalloproteinase-2

TNF- α Tumor necrosis factor alpha

TRPV1 Transient receptor potential cation channel subfamily V member 1

TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling

CHAPTER 1

INTRODUCTION

Ischemic heart disease (IHD) is a major health problem in the industrialized countries. IHD usually evolves as a result of a pathological imbalance between coronary blood supply and myocardial oxygen demand (Shiny KS et al. 2005). The intrinsic compensatory reaction to acute myocardial infarction includes neural and neuro-humoral mechanisms. For example, the massive acute increase in catecholamines (Schömig A, 1990) and specific neuropeptides such as substance P (SP) occur following acute myocardial ischemia and infarction suggesting the activation of the sympathetic nervous system and cardiac sensory afferent nerves (Franco-Cereceda A 1988, 1989; Milner P et al. 1989; Hua F et al. 2004a, 2004b; Zhang and Guo 2006). Specifically, the increase of specific sensory neuropeptides, including calcitonin gene related peptide (CGRP) and SP (Franco-Cereceda A et al. 1987; Kallner G et al. 1998; Szallasi and Blumberg 1999) within the myocardium indicates the involvement of afferent nerves and their neuropeptides during acute myocardial ischemia and infarction (Skofitsch and Jacobowitz 1985; Sigrist S et al. 1986; Dipette DJ et al. 1987; Franco-Cereceda A et al. 1987; Lappe RW et al. 1987; Maggi and Meli 1988; Gardiner SM et al. 1988, 1989).

SP is a prominent member of the tachykinin family of neuropeptides and is widely distributed in sensory afferent neurons that have both afferent and efferent

functions (Bayliss WM 1901; Franco-Cereceda A 1988; Otsuka and Yoshioka 1993; Regoli D et al. 1994). The cardiac effect of SP is mediated by the NK1 receptor (Franco-Cereceda A et al. 1989; Milner P et al. 1989), a subtype of neurokinin receptors possessing a high affinity for SP (Maggi CA et al. 1993), which are present in the heart (Schoborg RV et al. 2000).

Substance P is known to have negative inotropic and chronotropic effects on the normal heart (Hoover DB 1990, 2000). The negative effect is due in part to chronic TAC1 up-regulation with subsequent myocardial MMP activation, inflammation, apoptosis, hypertrophy, fibrosis and cardiac dysfunction (Weglicki WB et al. 1994; D'Souza M et al. 2007; Robinson P et al. 2009; Mak IT et al. 2011; Melendez GC et al. 2011). However, several studies have reported SP to have a protective vasodilatory effect following short-term ischemia-reperfusion (Ustinova EE et al. 1995; Wang and Wang 2005; Zhong and Wang 2007; Ren JY et al. 2011) In addition, an anti-apoptotic effect of SP on tenocytes following ischemia via AKT pathway activation has been reported (Backman LJ and Danielson 2013). This later observation suggests the possibility that SP might also be cardioprotective via a cardiomyocyte mechanism apart from its coronary vasodilatory effect. However, this remains to be determined.

Accordingly, the purpose of this study was to test the hypothesis that SP attenuates cardiomyocyte cell death following ischemia/reperfusion. To this end, the effect of SP following ischemia/reperfusion was tested in an isolated heart preparation and in short term hypoxia without reperfusion experiments using a left ventricular tissue slice culture preparation. Accordingly, this experimental design enabled the comparison

of the protective effect of SP with and without reperfusion in order to distinguish between the vasodilatory and antimyocyte damage capabilities of SP.

CHAPTER 2

LITERATURE REVIEW

2.1 Substance P overview:

Substance P is a member of the tachykinin family of sensory nerve neuropeptides, which also includes neurokinin A (NKA), neurokinin B (NKB), neuropeptide K (NPK), and neuropeptide gamma. Substance P and NKA are found in the central nervous system and peripheral afferent sensory neurons and encoded by the TAC1 gene, while NKB is restricted to the central nervous system and encoded by TAC3 gene (Brain SD et al. 2006). The expression of pre-mRNA of TAC1 gene can produce four mRNA isoforms (α , β , γ , and δ). Substance P can be encoded by all four isoforms, while only the β and γ isoforms give rise to NKA. Accordingly, SP can be expressed without NKA. However, both SP and NKA are synthesized, stored, and released together due to the fact that the β and γ isoforms are the most abundant (Brain SD and Cox 2006).

2.2 Substance P Discovery and Localization:

Substance P was originally discovered in 1931 by Ulf Von Euler and John H. Gaddum. It was initially isolated as an extract from equine brain and gut, and found to cause smooth muscle contraction in vitro (Euler and Gaddum 1931). It was named substance P where P referred to the powder obtained after the extraction procedure by

Gaddum and Schild (1934). Decades later, Lembeck detected high concentrations of SP in the dorsal root of the spinal cord and proposed that SP was a neuronal sensory transmitter associated with pain transmission (Lembeck F 1953). Later on, Otsuka and Konishi found upon electrical stimulation that SP immunoreactivity increased in the perfusate taken from the isolated spinal cord of newborn rats (Otsuka and Konishi 1976). Today, it is fully documented that SP is released from both the central and peripheral endings of primary afferent neurons and acts as a neurotransmitter (Otsuka and Yoshioka 1993).

The origin of SP is very diverse. Dalsgaard noticed a marked reduction in SP in the right atria of the guinea pig heart upon bilateral removal of the stellate ganglia, and upon vagus nerve depletion with capsaicin; while neither intervention affected SP in the left ventricle. This suggests separate origins of ventricular SP containing nerves that lie outside the stellate ganglia (Dalsgaard CJ et al. 1986). On the other hand, an increase in SP in the T4 region of the spinal cord was detected upon left anterior descending coronary artery occlusion in rats using microprobes coated with an antibody to SP (Hua F et al. 2004). Likewise, studies conducted on spontaneously hypertensive rats (SHR) (Aline BP et al. 2005), and dog dorsal root ganglia (Hoover DB et al. 2008) indicate that SP-containing neurons from the ventricles probably connect with the thoracic region of the spinal cord. Cardiac afferent neurons were traced to the nodose ganglion of the vagal nerve and nucleus tractus solitarius (NTS) where 17% of nodose ganglia cells were SP positive and only a small subpopulation of cardiac afferent axons in the NTS were SP positive (Corbett EK et al. 2005). These studies highlight the diverse origins of SP-

containing nerves from the heart and the intricacy of the neural networks associated with the heart.

While C-fiber sensory neurons are the predominant source of SP, it is not the only source since selective degeneration of C-fiber with capsaicin resulted in an incomplete loss of SP (60%) in the rat heart (Holzer P et al. 1982) thus suggesting other sources. It was found in the rat heart that only a small population (~5–10%) of coronary artery endothelial cells contains SP and is surrounded by non-SP-containing endothelial cells (Milner P et al. 1989). These cells respond rapidly to hypoxic conditions by releasing SP. In addition to the above-mentioned sources, a few other non-neuronal cells such as eosinophils, endothelial cells, and macrophages have also been reported to produce SP (Weinstock JV et al. 1988; Linnik and Moskowitz 1989; Pascual and Bost 1990).

The distribution of SP differs with species. In rats, for example, initial studies were unable to detect SP containing fibers in the heart (Hougland and Hoover 1983). However, subsequent studies by Papka and Urban revealed SP containing fibers in both the atrial and ventricular epicardium and myocardium of the rat heart (Papka and Urban 1987). In the rat the number of SP producing C-fiber sensory neurons were relatively few compared to the guinea pig heart (0.33 pmol/g of SP in the rat heart versus 2.7 - 4.2 pmol/g in the guinea pig left ventricle) (Wharton J et al. 1981; Holzer P et al. 1982; Dalsgaard CJ et al. 1986).

In mice, SP-containing nerves were found to be most prevalent in the epicardium and in ganglia adjacent to the heart hilum, and they were also located in the intrinsic nerve bundles and interganglionic nerves (Rysevaite et al. 2011). These nerves were

located close to blood vessels and were mainly thin and mixed with choline acetyltransferase and tyrosine hydroxylase positive neurons. It was reported that SP content in protein extract from mice with myocarditis is far greater than that of uninfected mice (2807 pg of SP/mg of protein in myocarditis mice versus 71 pg/mg in uninfected mice) (D'Souza M et al. 2007; Robinson P et al. 2009).

In guinea pig hearts, the entire coronary arterial system was reported to be innervated by SP containing nerves in 1980 (Reinecke M et al. 1980). Later on, it was found that plentiful numbers of SP-containing nerve fibers were also present in the atria and ventricles (endo-, epi-, and myocardium) as well as the mitral and tricuspid valves (Hougland and Hoover 1983). Substance P-containing nerves were reported to be more prevalent in the endocardial regions, chiefly around the trabeculae and papillary muscles of the ventricles compared to the epicardial regions (Wharton J et al. 1981). More fibers were noticed to be at the base of the heart than the apex with no significant difference between right and left ventricles (Wharton J et al. 1981; Hougland and Hoover 1983). These fibers were found to be associated with branches of the bundle of His in the ventricular septum (Wharton J et al. 1981), and also associated with the ascending aorta and pulmonary trunk (Hougland and Hoover 1983). Consistent with Hougland's study, Papka and Urban observed SP containing neurons in the epicardium and myocardium of the atria, the mitral and tricuspid valves, and in pericellular baskets around intrinsic cardiac ganglia as well as the parietal portion of the pericardium (Papka and Urban 1987). It was reported that SP-containing fibers in the atria were four times as abundant as those in the ventricles (Dalsgaard CJ et al. 1986).

Substance P was found to be present in the descending coronary and circumflex coronary arteries of the dog heart (Brum JM et al. 1986). In cats, SP-containing nerves were found in the atrial and ventricular myocardium as well as the endocardium (Zhu and Dey 1992). In non-human primates, SP positive areas have been identified in both varicose and non-varicose nerve fibers, in the interatrial septum, in the cardiac ganglia and musculature and in perivascular networks around blood vessels (Tay and Wong 1992).

In the normal heart of humans, few SP-containing neurons mainly around neural cell bodies in intrinsic ganglia and in nerve trunks have been reported (Wharton J et al. 1990). However, in patients with congestive or hypertrophic cardiomyopathy, endomyocardial biopsies revealed that SP-containing nerve fibers were located close to arterioles, capillaries and veins (Weihe E et al. 1981). Substance P-containing nerves were also identified between cardiomyocytes and around blood vessels in atrial biopsies taken from patients undergoing open-heart surgery (Rechartd L et al. 1986). They have also been found surrounding the adventitia of coronary vessels in atherosclerotic regions of human coronary arteries (Laine P et al. 2000). Finally, it has been reported that SP was present in nerve fibers in the right atrial ganglionated plexus in patients undergoing coronary artery bypass grafting (Hoover DB et al. 2009).

In summary, the SP-containing nerves in the heart differ among species. Generally, SP-containing nerve fibers seem to be located around coronary vessels and in the intrinsic ganglia of the heart. In addition to a small number of coronary endothelial cells, limited numbers of fibers in normal ventricles appear to be SP positive. However, SP levels do increase significantly in the diseased heart.

2.3 Substance P Biochemistry:

Chemically, partly purified SP was found to be soluble in water and alcohol and insoluble in ether and chloroform. It was found to tolerate boiling at pH 1 to 7 but was rapidly destroyed in an alkaline environment (Euler and Gaddum 1931; Euler USv 1936a, 1942). Pernow found during electrophoresis, SP traveled to the cathode at pH < 10 and its isoelectric point was near 9 (Pernow B 1955). Trypsin and pepsin appeared to destroy the biological activity of SP (Euler USv 1936b), while carboxypeptidases had no effect (Franz J et al. 1961). Substance P prepared from brain and intestine seemed to show no physicochemical or biological differences (Eliasson R et al. 1956).

In 1971, Chang et al. identified the structure of SP from bovine hypothalamus as being an undecapeptide with a sequence: H-Arg1-Pro2-Lys3-Pro4-Gln5-Gln6-Phe7-Phe8-Gly9-Leu10-Met11-NH₂ (M. Wt. 1347.6 g/mole) (Chang MM et al. 1971), which was subsequently introduced as part of the tachykinin family (Erspamer V 1983). Substance P is metabolized by several enzymes including: neutral endopeptidase (NEP: metalloendopeptidase EC.- 3.4.24.11., (Matsas R et al. 1984)); substance-P-degrading enzyme (SP-DE: EC.3.4.24., (Probert and Hanley 1987)); angiotensin-converting enzyme (ACE: EC.3.4.15.1, (Skidgel and Erdos 1987)) dipeptidyl aminopeptidase IV (DPIV: EC.3.4.14.5., (Heymann and Mentlein 1978)); postproline endopeptidase (PEP: EC.3.4.21.26., (Blumberg S et al. 1980)); cathepsin-D (EC.3.4.3.23., (Azaryan and Galoyan 1988)) and cathepsin-E (EC.3.4.23.34., (Kageyama T 1993)). Despite the fact that all of these enzymes cleave SP due to their specific cellular localization in in vitro models, it appears that NEP and/or ACE are mainly involved in the cleavage of SP within the periphery (Nadel JA 1991). It has been shown that NEP is involved in the metabolism

of SP in the brain (Hooper and Turner 1987), spinal cord (Sakurada T et al. 1990) and in peripheral tissues (Di Maria GU et al. 1998), while SP is degraded by ACE in plasma (Wang LH et al. 1991), cerebrospinal fluid, and substantia nigra. ACE participates in degrading SP fragments released from NEP. Together, NEP and ACE catalyze the hydrolysis of Phe⁸-Gly⁹ or Gly⁹-Leu¹⁰ bonds of SP that leave the peptide missing the carboxyl terminal regions needed to bind to the tachykinin receptors (Skidgel and Erdos 1987).

2.4 Neurokinin (NK) Receptors:

The biological actions of SP are mediated by tachykinin (neurokinin: NK) receptors. NK receptors belong to rhodopsin-like membrane structure, consisting of seven hydrophobic transmembrane domains, connected by extra and intracellular loops and coupled to G-proteins (Nakanishi S 1991; Gerard NP et al. 1993; Maggi and Schwartz 1997). Three types of tachykinin receptors have been described. NK1 receptor exhibits preference for SP, while NK2 and NK3 receptors exhibit preferences for neurokinin A and neurokinin B, respectively (Regoli D et al. 1994). Still, cross reactivity between endogenous tachykinins and NK receptors occur based on receptor availability and/or peptides' concentrations. For this reason SP can activate not only NK1 receptors, but also NK2 and NK3 receptors in different tissues (Regoli D et al. 1994). Also a new designated receptor NK4 has been proposed (Donaldson LF et al. 1996). The NK1 receptor is found in both the central and peripheral nervous system. It is also present in vascular endothelial cells, muscle, gastrointestinal tracts, genitourinary tract, pulmonary tissue, thyroid gland and different types of immune cells (Saria A 1999; Almeida T et al. 2004; Datar P et al. 2004; Satake and Kawada 2006).

Regarding NK receptors in the heart, a pharmacological approach has been taken to determine the presence of neurokinin receptor subtypes in intrinsic cardiac neurons in canines (Thompson GW et al. 1998). The response has been shown to be diverse among species. A selective NK-1 receptor agonist, for example, reduced activity of right atrial neurons in some animals, while it increased activity in others. It was shown that right atrial neuron activity was reduced by selective NK-2 receptor agonist, while selective stimulation of NK-3 receptors increased its activity. Hoover and Hancock were able to identify SP binding sites in the parasympathetic ganglia located within the epicardial connective tissue adjacent to the pulmonary trunk, ascending aorta and right atrium, as well as in coronary arteries in the guinea pig heart, but were unable to detect binding sites in the atria, ventricles, ascending aorta and pulmonary trunk (Hoover and Hancock 1988). Later on, radiographic studies conducted in Wistar rats found SP binding sites on clusters of connective tissue cells within the skeleton of the heart, the cusps of the cardiac valves and within the adventitia of the great vessels and coronary arteries. While they did not find any evidence of SP binding sites on cardiomyocytes (Walsh RJ et al. 1996), genes for the NK-1 and NK-3 receptors (but not NK-2) were shown to be expressed in isolated neonatal rat cardiomyocytes (Church DJ et al. 1996). Levick's laboratory was able to detect the NK-1 receptor on isolated adult cardiac fibroblasts (Dehlin and Levick 2014). NK1 receptors showed high degree of sequence homology among species including man, mouse, rat, guinea pig upon using cloned NK1 receptors (Gerard NP et al. 1993).

2.5 Substance P Actions:

As a member of tachykinin family, SP is involved in many processes including smooth muscle contraction, vasodilation, nociception, and modulation of

inflammatory/immune cell functions (Rameshwar P et al. 1993; Kavelaars A et al. 1994; Vergnolle N et al. 2001; Azzolina A et al. 2003; Massaad CA et al. 2004). Substance P induces vasodilatation via activation of NK1 receptors in large arterial vessels and the subsequent release of nitric oxide (NO) from the endothelium (Fiscus RR et al. 1992). However, electrical stimulation of sensory nerve endings in rat isolated vessels showed the vasodilatory response was induced by the release of CGRP rather than SP (Kawasaki H et al. 1988). The CGRP vasodilatory effect being dominant over that of SP has also been observed in the mammalian heart.

2.5.1 Substance P Modulatory Actions on Inflammatory/Immune Cell Function:

The substance P modulatory activity has been demonstrated presynaptically via evoking both inhibitory and excitatory effects on the ganglia upon exogenous SP administration in different animal species including: cat spinal cord (Randic M et al. 1982), chick sympathetic and ciliary ganglion (Dryer and Chiappinelli 1985). It has also been demonstrated that peripheral inflammation (Smith GD et al. 1992; Levine JD et al. 1993) and noxious stimulation could cause an increase in the expression of SP in the spinal cord (Oku R et al. 1987; Duggan AW et al. 1988; Schaible HG et al. 1990) and an increase in the expression of SP-related genes, preprotachykinin (PPT) mRNA in sensory neurons in the dorsal root ganglia (Noguchi K et al. 1988). Moreover, SP can be released from the somata of primary afferent neurons (Neubert JK et al. 2000) with cross-excitation possible among adjacent neurons (Devor and Wall 1990). It has been reported that SP can induce its own release with subsequent release of calcium ion from internal stores induced by inositol-1,4,5-trisphosphate (Malcangio and Bowery 1999). This

autoreceptor role may have been implicated in the pathophysiology of nerve injury, noxious stimuli, and inflammation.

It was shown that primary and secondary lymphoid organs have a peptidergic innervation with nerve endings in close vicinity to immune cells, which support the notion that neuropeptides such as SP act as a link between the nervous and the immune system (Felten DL et al. 1985; Stead RH et al. 1987; Fink and Weihe 1988; Weihe E et al. 1989, 1991; Bellinger DL et al. 1990; Kurkowski R et al. 1990; Zentel HJ et al. 1991). Binding studies have shown that distinct subpopulations of T and B-lymphocytes, as well as macrophages possess SP receptors (Payan DG et al. 1984a, 1984b; Hartung HP et al. 1986; Payan DG et al. 1986; Stanisz AM et al. 1987; Bost KL 1988); and shown that SP has been implicated in the modulation of immune responses such as T cell proliferation (Payan DG et al. 1983; Payan and Goetzl 1985), immunoglobulin synthesis (Stanisz AM et al. 1986; Laurenzi MA et al. 1989; Eglezos A et al. 1990; Pascual DW et al. 1991; Bost and Pascual 1992), lymphocyte traffic (Moore TC et al. 1989), macrophage activation (Bar-Shavit Z et al. 1980; Bost KL 1988; Pascual and Bost 1990), mast cell degranulation, and release of histamine, in addition to mast cell-dependent granulocyte infiltration (Fewtrell CM et al. 1982; Lowman MA et al. 1988; Iwamoto I et al. 1992).

It has been shown that tachykinin release upon stimulation of the peripheral endings of primary sensory neurons induces a major proinflammatory response represented by neurogenic plasma extravasation (Piedimonte G et al. 1993). This response occurs due to SP activation of NK1 receptors located on endothelial cells of post-capillary venules (Bowden JJ et al. 1994), and subsequent opening of the gaps between endothelial cells and the flux of plasma proteins from the vascular lumen to the

interstitial space. Substance P induced plasma extravasation is reported in different cases such as: cigarette smoking (Lundberg JM et al. 1983), ovalbumin challenge in sensitized animals (Bertrand C et al. 1993a, 1993b), or baseline plasma extravasation in NEP knockout animals (Lu B et al. 1997). In human, wheel formation was observed in the human skin upon intradermal SP injection due to microvascular leakage in postcapillary venules (Fuller RW et al. 1987). Furthermore, nasal albumin secretion in man is increased after intranasal SP administration (Braunstein G et al. 1991).

Regarding SP regulation of inflammatory cells in the heart, extensive studies have demonstrated that SP is a mediator of neurogenic inflammation and up-regulator of pro-inflammatory cytokines. In mouse viral myocarditis model, deletion of the TAC1 gene prevented the infiltration of inflammatory cells into the heart (Robinson P et al. 2009). However, the direct effects of SP on inflammatory cells in the heart have not been extensively investigated. In a guinea pig ischemia reperfusion model, SP and renin were demonstrated to be released together with evidence of a SP role in stimulating renin release from mast cells as determined by the prevention of renin release with NK-1 receptor antagonist or mast cell stabilizer (Morrey C et al. 2010). Furthermore, it was found that SP (100 μ M) caused production of angiotensin II by a mixed population of isolated rat cardiac inflammatory cells (T cell, mast cells and macrophages) (Levick SP et al. 2010), and it also can induce TNF- α production by this mixed population of cardiac inflammatory cells (Melendez GC et al. 2011). Studies conducted using a spontaneous hypertension (SHR) rat model (Dehlin HM et al. 2013) and an atherosclerosis mouse model (Bot I et al. 2010) revealed that SP also activates cardiac mast cells in vivo. To be more specific, Bot et al. demonstrated that SP increased mast cell activation in the

perivascular region of coronary arteries of mice and the subsequent promotion of intraplaque hemorrhages. Considering the necessity of mast cell activation for mast cell density to increase (Li J et al. 2012), Melendez found that mast cell density did not increase in rats treated with an NK-1 receptor blocker (Melendez GC et al. 2011) suggesting a role for SP in mast cell activation.

There is a discrepancy regarding the mechanism by which SP activates mast cells. While Li et al. proposed that SP initiates mast cell activation via the NK-1 receptor in dermal tissue (Li WW et al. 2012), Lorenz et al. suggested that SP uptake in mast cells is rapid and independent of the NK-1 receptor, resulting in exocytosis of inflammatory compounds (Lorenz D et al. 1998). This may be due to the difference in the animal model used, and the mode of SP treatment as Li et al. looked at mast cell activation in vivo while Lorenz et al. treated cultured mast cells with SP in vitro. Obviously, substance P can initiate calcium signaling in mast cells via the PLC pathway, but the downstream events resulting in mast cell activation are still unclear in the heart. In macrophages, for example, SP causes NF- κ B transactivation and release of chemokines via activation of the ERK1/2, P38 MAPK, and PI3K-AKT pathways downstream of PKC (Sun J et al. 2008, 2009). It was also shown that SP causes NF- κ B transactivation and subsequent up-regulation of cytokines via NK-1 receptors in astrocytoma cells (Lieb K et al. 1997). A study conducted on hearts from magnesium-deficient rats showed that SP activation of the NK-1 receptor regulated the oxidative stress response and neutrophil production of neutral endopeptidase (NEP) (Mak IT et al. 2011). Specifically, It was found that NEP was decreased in neutrophils from magnesium-deficient hearts. Upon NK-1 receptor blockade, superoxide production by neutrophils was dramatically reduced, while NEP

activity was partially restored. Considering the role of NEP in degrading SP, substance P provides a mechanism of regulating its own level by down-regulating NEP. Studies not related to the heart have shown that murine peritoneal macrophages have the NK-1 receptor, and both IL-4 and IFN- γ causes an increase of mRNA and protein for NK-1 receptor (Marriott and Bost 2000). Recently it was reported that both IL-4 and IFN- γ are increased in the hearts of SHR (Levick SP et al. 2009), which suggests that these cytokines, when increased, would provide an environment for NK-1 receptor density to increase.

The role of SP in neurogenic inflammation is well documented in animal models. However, its role in humans is unclear. Several clinical trials have shown that using NK-1 receptor antagonism in inflammation type pathologies is not effective. In patients with asthma, for example, dual NK-1/NK-2 receptor antagonist was unable to prevent allergen-induced airway responses (Boot JD et al. 2007). NK-1 receptor blockade also failed to lower the incidence of post-endoscopic retrograde cholangiopancreatography pancreatitis (Shah TU et al. 2012).

2.5.2 Direct Effects on Cardiomyocytes:

Substance P containing DRG can interact with cardiomyocytes. Liu Z et al. found that in co-cultures of isolated rat DRG and neonatal rat cardiomyocytes, there were increasing connections between DRG projections and cardiomyocytes, and more SP (and CGRP)-containing neurons were present than in cultures without cardiomyocytes (Liu Z et al. 2008). Intriguingly, capsaicin caused more SP (and CGRP) release when cardiomyocytes were present in co-culture than in their absence. Regarding a direct effect

on cardiomyocytes, only one study has explored the direct effects of SP on cardiomyocytes. Church et al. demonstrated that neonatal rat cardiomyocytes express genes for both the NK-1 and -3 receptors, but not the NK-2 receptor (Church DJ et al. 1996). Along with other pharmacologic studies, it was shown that atrial natriuretic peptide (ANP) synthesis was induced by both SP (NK-1 receptor) and neurokinin B (NK-3 receptor). It was shown that SP activates PKC with subsequent prostaglandins release, which is necessary for ANP release. However, these experiments were conducted on neonatal cardiomyocytes, which may not completely reflect adult cardiomyocytes characteristics. Nevertheless, combined with the findings in adult mice in vivo, these results suggest that SP can induce hypertrophy via direct actions on cardiomyocytes.

2.5.3 Direct Effects on Cardiac Fibroblasts:

It is well recognized that SP mediates inflammation. Fibroblasts are capable of producing chemokines and adhesion molecules, which are associated with inflammation. It was found that SP was capable of inducing soluble ICAM-1 (sICAM-1) production via cleaving ICAM-1 from adult rat cardiac fibroblasts, through a p42/44 MAPK and PKC mechanism (Sapna and Shivakumar 2007). This implies anti-inflammatory properties of SP since cleavage of ICAM-1 may affect the amount of membrane bound ICAM-1 available for interaction with ligands (Tsakadze NL et al. 2004). Furthermore, ICAM-1 shedding blockers were found to increase monocytic cell adhesion to stimulated endothelial cells (Tsakadze NL et al. 2004). These findings seem to contradict the well-recognized pro-inflammatory role of SP. However, sICAM-1 can induce macrophage inflammatory protein-2 synthesis by astrocytes (Otto VI et al. 2002) and alveolar

macrophages (Schmal H et al. 1998), as well as alveolar macrophage TNF- α synthesis (Schmal H et al. 1998).

Substance P can also induce cardiac fibroblasts to produce PGE2 (Sapna and Shivakumar 2007). PGE2 induces rat neonatal fibroblast proliferation (Harding and LaPointe 2011). PGE2 was found to be elevated following myocardial infarction (LaPointe MC et al. 2004). Although adult rat cardiac fibroblasts proliferate in a process involving generation of superoxide in response to SP, these cells reportedly do not induce collagen synthesis in response to SP (Kumaran and Shivakumar 2002). The effects of SP on MMP production by cardiac fibroblasts have not been reported. However, in cultured human lung fibroblasts, SP has been shown to cause reduced collagen synthesis, increased collagen degradation and increased levels of MMP-1 (Ramos C et al. 2007). It has also been shown that SP increases mRNA and protein levels for MMP-1, -2, -3, 7 and -11 as well as TIMP-2 in gingival fibroblasts from healthy humans (Cury PR et al. 2008).

2.6 The Detrimental Effects of Substance P:

The role of SP in the heart appears to be dependent on disease etiology. Detrimental effects of SP have been seen in long-term non-ischemic myocardial remodeling and heart failure (figure 2.1.A). Substance P and the NK-1 receptor are increased in a dilated cardiomyopathy mouse model of myocarditis induced by *Taenia crassiceps* parasite injection (D'Souza M et al. 2007). Substance P deficient mice were protected from adverse remodeling following infection. In the SP-deficient mice, cardiomyocytes were protected against hypertrophy, while the wild type cells showed a

27% increase in cross-sectional area. Apoptosis was more evident in the wild type cardiomyocytes.

Similarly to parasite-infected hearts, encephalomyocarditis virus (EMCV) infected mice showed dramatic increases in SP, which were correlated with cardiac hypertrophy, increased cardiomyocyte cross-sectional area and apoptosis (Robinson P et al. 2009). Substance P-deficient mice hearts were protected against all of these adverse events. Inflammation and necrosis were evident in EMCV infected hearts with over a 50% mortality rate after 14 days of infection, while no death occurred after 14 days of infection in SP-deficient mice.

In a model of heart failure secondary to a sustained cardiac volume overload, Melendez GC et al. found that deletion of TAC1 protected mice from developing left ventricular hypertrophy and ventricular dilatation (Melendez GC et al. 2011). In contrast to the wild type, there was no increase in right ventricular mass or lung weight in the TAC1^{-/-} mice indicating protection from heart failure. In this model of heart failure, mast cell activation is responsible for collagen degradation via MMP activation (Brower G et al. 2002; Levick SP et al. 2008). Thus, this cardioprotection was explained by the prevention of SP-induced mast cell degranulation, which prevented an increase in TNF- α levels, matrix metalloproteinase (MMP) activation and subsequent collagen degradation. Cell death was not a significant factor in this model (Melendez GC et al. 2011).

Weglicki and Phillips found in rats with magnesium deficiency that circulating SP levels were elevated (Weglicki and Phillips 1992). Furthermore, they reported that SP was increased in the cardiac lesions of magnesium-deficient mice, and upon blockade of

the NK-1 receptor, TNF- α and IL-1 levels, but not IL-6 were significantly reduced within the lesions (Weglicki WB et al. 1994). Other studies have shown that blocking NK-1 receptors in hypomagnesemia rats improved systolic and diastolic function as determined by fractional shortening and the E/A ratio, respectively (Mak IT et al. 2011).

In ischemia reperfusion studies, magnesium deficient hearts seem to respond differently from the wild type hearts. Upon blocking NK-1 receptors following global ischemia and reperfusion there was an improvement in the developed pressure and cardiac work (Kramer JH et al. 1997). Lactate dehydrogenase and lipid hydrogen peroxide levels were decreased as well.

In case of hypertension/pressure overload, little is known regarding the role of SP in myocardial remodeling due to hypertension or pressure overload. Dehlin HM et al. showed that TAC1 is up-regulated in the SHR heart as blood pressure increases. It was also shown that SP induced expression of the fetal genes that are related to pathological hypertrophy in the hypertensive heart via NK-1 receptors. Activation of NK-1 receptors was shown to be critical for developing cardiac fibrosis. It was suggested that SP mediates fibrosis via up-regulation of endothelin-1 since there were functional changes in isolated cardiac fibroblasts incubated with SP (Dehlin HM et al. 2013).

2.7 The Beneficial Effects of Substance P:

In a rat model where neuropeptide was depleted by capsaicin, there were reductions in heart rate recovery, coronary flow and left ventricular developed pressure following ischemia reperfusion compared to non-capsaicin pretreated hearts. It is important to mention that capsaicin causes the depletion of other sensory nerve

neuropeptides including calcitonin gene-related peptide (CGRP). Substance P replacement (1 nM–1 μ M) caused restoration of contractile function and coronary flow; while inhibition of NK-1 receptors prevented these beneficial actions (Ustinova et al. 1995). Contrariwise, guinea pig hearts pretreated with NK-1 receptor antagonists or capsaicin and subjected to global ischemia followed by reperfusion were found to have significantly improved left ventricular developed pressure and left ventricular end diastolic pressure (LVEDP) following reperfusion (Chiao and Caldwell 1996). These contradictory findings might be related to the fact that the studies were conducted using different species with different ischemia/reperfusion durations (rat model with 20 min ischemia and 30 min reperfusion versus guinea model with 15 min ischemia and 60 min reperfusion).

However, in ischemia-reperfusion mice models, SP is consistently shown to be an important factor in functional recovery of the myocardium during acute reperfusion via stimulation of the transient receptor potential vanilloid type 1 (TRPV1) present on sensory nerves. Specifically, 40 min of global ischemia followed by 30 min of reperfusion in isolated mouse heart preparation using TRPV1 gene deficient mouse hearts produced less SP in comparison to the wild type (Wang and Wang 2005). Coronary flow and developed pressure were reduced while LVEDP was increased in TRPV1^{-/-} hearts. Upon SP (1 μ M) treatment prior to ischemia in TRPV1-deficient hearts, the adverse responses in LVEDP, developed pressure, and coronary flow were all attenuated. The addition of NK-1 receptor antagonist to wild type mice was shown to produce converse effects by worsening these functional parameters. Preconditioning of TRPV^{-/-} mice through subjecting them to three cycles of 5 min of ischemia followed by 5 min of

reperfusion, before 30 min of global ischemia and 40 min of reperfusion was ineffective as LVEDP increased associated with reduction in coronary flow, +dP/dt, and developed pressure (Zhong and Wang 2007).

In diabetic rats, the role of SP in ischemia-reperfusion was investigated using an isolated heart apparatus and a post-conditioning protocol by which, hearts were exposed to 30 min of global ischemia, followed by 5 cycles of 10 s of reperfusion and 10 s of global ischemia, before a final 40 minute period of reperfusion. It was found in non-diabetic rats that LVEDP, +dP/dt and -dP/dt, coronary flow, and developed pressure were improved following the post-conditioning protocol while these effects were lost in diabetic rats. Since SP levels increased following post-conditioning in the normal heart, cycles of SP infusion (1 μ M) to mimic the post-conditioning protocol in normal heart were used in diabetic hearts and were found to be effective in improving cardiac functional parameters along with decreases in creatine kinase and cardiac troponin I, which indicates a possible reduction in cell death (Ren JY et al. 2011). This would suggest that the loss of post-conditioning benefits was because of the decrease in SP in the diabetic heart. The SP protective effects in ischemia-reperfusion injury were suggested to be due to its potent vasodilatory actions allowing improved reperfusion (figure 2.1.B).

Accordingly, one would expect a reduction in the infarct size in situations where the left anterior descending coronary artery is ligated for a short duration and then released to allow reperfusion. Still, studies on the effect of SP in ischemia/reperfusion model utilizing left anterior descending coronary artery ligation are lacking. Furthermore, it is unknown whether reperfusion is required for SP to show its protective effects. If so,

SP would be of limited benefit in case of myocardial infarction where there is no reperfusion. It is not known what happens to SP levels following permanent myocardial infarction. It was reported that following permanent ligation of the left anterior descending coronary artery in the rat heart, SP levels progressively increase over the first hour (Wang LL et al. 2011). It was also shown in the rat hearts that SP was elevated at 3 h post-occlusion, and had returned to normal by 6 h (Zhang RL et al. 2012). The use of NK-1 inhibitor did not affect the infarct size suggesting that reperfusion is required for SP to be protective. However, this study did not measure any functional parameters and was conducted after only 3 hrs. of infarction, which leaves many unanswered questions regarding the acute effects of SP on cardiac function and the long-term effects on both structure and function.

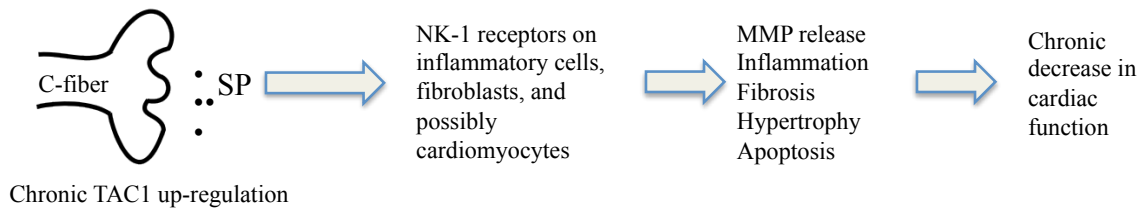
On the other hand, it was found that SP levels were elevated 24 h after ligation of the left anterior descending coronary artery (Amadesi S et al. 2012). This discrepancy of SP levels may be due to differences between species or may suggest the existence of a biphasic response where SP increases immediately upon alteration of coronary blood flow and there is a latent increase of SP upon sustained up-regulation of the TAC1 gene (Dehlin and Levick 2014).

Summary:

Substance P is a member of tachykinin family of sensory neuropeptide. It is found in the central nervous system and peripheral afferent sensory neurons and encoded by the TAC1 gene. C-fiber sensory neurons are the predominant source of SP. However, a few other non-neuronal cells such as eosinophils, endothelial cells, and macrophages have

also been reported to produce SP. In the heart, though, it is shown to be also produced by small population of coronary endothelial cells. The distribution of SP in the heart differs with different species. It is degraded by various enzymes (predominantly ACE and NEP). It induces its effects through neurokinin receptors (preferably NK-1 receptors). It acts as a neurotransmitter and is involved in pain transmission, smooth muscle contraction, vasodilation, nociception, and modulation of inflammatory/immune cell functions. Substance P has detrimental effects on the heart in long-term non-ischemic myocardial remodeling and heart failure. It also has beneficial effects in short-term ischemia-reperfusion. Most studies investigating the effect of SP following ischemia and short-term reperfusion have indicated that the protective effect of SP is due to its vasodilator properties upon reperfusion. However, other reports were not able to demonstrate this mechanism, which opens the possibility of SP having other protective mechanisms. For example, SP was shown to reduce Anti-Fas-induced apoptosis in human tenocytes in vivo. This study demonstrated that the anti-apoptotic effect of SP is mediated through NK-1 receptors and AKT-specific pathways (Backman and Danielson 2013). The possible cardiac cell protective effect of SP in short-term hypoxia without reperfusion has not been investigated which is the subject of our hypothesis.

(A) Long-Term Non-Ischemic Remodeling (SP detrimental effects)



(B) Short-Term Ischemia-Reperfusion (SP beneficial effects)

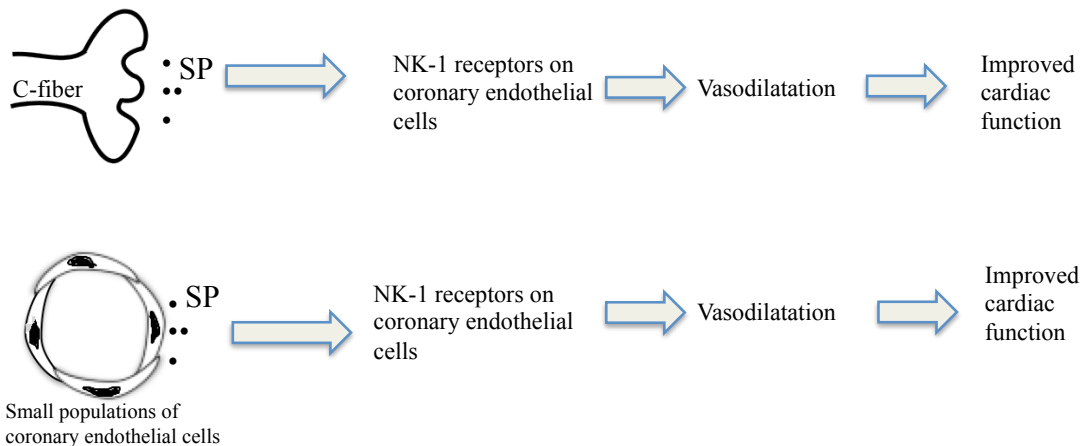


Figure 2.1. Schematic indicating the long-term and short-term substance P effects in response to altered coronary flow.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals:

The study protocol was approved by the Institution of Animal Care and Use Committee and conformed to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Seven-week old male Sprague–Dawley rats were purchased from Harlan Laboratories. The animals were housed under standard environmental conditions and were maintained on a normal rodent diet and tap water ad libitum.

3.2 Isolated Heart Experiments:

For the whole heart ischemia reperfusion experiments, a non-recirculating Langendorff Heart preparation was used. Rats were anesthetized with intraperitoneal sodium pentobarbital injection (100 mg/kg). The right femoral vein was then exposed and heparin (1000 IU) was administered intravenously. After allowing the heparin to circulate, the heart was then rapidly excised and placed in an ice-cold Krebs-Henseleit buffer where the aorta was isolated for subsequent cannulation. After cannulation, a retrograde perfusion was started at a constant perfusion pressure of 60 mmHg with Krebs-Henseleit buffer that contains: NaCl (107 g/L), KCl (5.96 g/L), NaHCO₃ (32.26 g/L), MgCl₂·6H₂O (3.25 g/L), CaCl₂·2H₂O (5.17 g/L), 10 mM HEPES. The buffer was

filtered, maintained at 37°C, and bubbled with 95% O₂ and 5% CO₂ in advance. Hearts were allowed an acclimatization period a 20 min after being un-paced and then, global ischemia was induced by turning off the flow of buffer to the heart. Following 30 min of ischemia, flow was restored at a perfusion pressure of 60 mmHg for a period of 30 min. Coronary flow was recorded throughout the experiment with coronary perfusate collected at: baseline (immediately before ischemia), immediately following the initiation of reperfusion, 10 min of reperfusion, 20 min of reperfusion, and 30 min of reperfusion. Non-ischemic control hearts were continuously perfused for 60 min. Hearts treated with SP were given a 1 mL bolus of SP (1µM) 10 min prior to the initiation of ischemia. After finishing the experiment, the hearts were taken down from the Langendorff apparatus, the atria and right ventricle were removed, and a transverse section of the left ventricle and septum were fixed in zinc formalin for histological analysis. The apex of the left ventricle was frozen at -80°C for subsequent western blot analysis.

3.3 Rat Left Ventricular Tissue Slice Culture and Hypoxia:

The rats were deeply anesthetized with an intra-peritoneal injection of pentobarbital sodium (70 mg/kg) and the hearts removed, washed in cold sterile saline, and transferred to Joklik media, Sigma-Aldrich, St. Louis, USA (Joklik+10% FBS, PS, AmphoB, gent.). The LV and septum were separated from the rest of the heart, the LV chamber filled with 2.5% agarose (Cat. No.: BP1356-100, Fisher BioReagents, CA, USA) that was heated to 40°C in order to keep it liquefied, and inserted within a metal cylinder containing agarose. Once the agarose was solidified, a Brendel/Vitron Tissue Slicer (Vitron Organ Slicing Tech., Tucson, AZ) was used to obtain 15 to 20 slices that were 250 to 300µm in thickness.

The slices were incubated in calcium-free Joklik media at room temperature for 30 min and then transferred to fresh Joklik media containing 0.2 μM of calcium chloride and incubated for 30 min in a culture incubator (37 °C, 95% O₂ and 5% CO₂). Next, additional calcium chloride was added to obtain a final concentration of 0.4 μM and the slices were further incubated for 30 min. There were two major groups with the slices in one group being incubated with three concentrations (30, 100, and 300 nM) of SP (Cat. No.: 1156, Tocris Bioscience, Minneapolis, MN, USA) and in the other group the slices were incubated with either 100nM SP, 100nM SP with 10 μM NK-1 inhibitor, L-732, 138 (Cat. No.: 0868, Tocris Bioscience, Minneapolis, MN, USA), or 100nM SP with 50 μM AKT inhibitor, LY294002 (Cat. No.: 9901, Cell Signaling Technology, Danvers, MA, USA). In both groups, the slices were divided randomly and placed into five wells (i.e., representing groups: normoxia, hypoxia, and hypoxia with different doses of SP (30, 100, and 300nM) in the first group; while normoxia, hypoxia, hypoxia with SP, and hypoxia with SP and either NK-1 inhibitor or AKT inhibitor in the second group) with at least 3 slices per well.

Tissue hypoxia was induced by placing the slices into pre-deoxygenated, serum free Waymouth medium, Sigma-Aldrich, St. Louis, USA (Waymouth+PS, AmphoB, gent.) and incubated in 1% O₂, 94% N₂ and 5% CO₂ at 37 °C, for 45 min. Tissue normoxia culture was conducted in Waymouth medium with serum, Sigma-Aldrich, St. Louis, USA (Waymouth+10%FBS, PS, AmphoB, gent.) and 95% O₂, 5% CO₂ at 37 °C for 45 min. Afterwards, media from each well was collected and analyzed for lactate dehydrogenase (LDH) and the slices weighed. Some of the slices from each well were snap frozen and stored at -80°C for subsequent biochemical analysis and the remaining

slices were fixed in 10% buffered formalin for 24 hr. at room temperature and embedded in paraffin and sectioned to a 5 µm thickness for subsequent staining.

3.4 LDH assay:

Lactate Dehydrogenase (LDH) is a cytosolic enzyme that is usually released to the surrounding media as a result of sarcolemmal membrane rupture. To determine the amount of tissue injury induced by hypoxia, the culture media was collected and LDH activity in the media was immediately assayed by using LDH cytotoxicity detection kit (Cat. No.: 630117, Clontech, Mountain View, CA). The manufacturer's instructions state that the optimal cell concentration should be between 5×10^3 - 2×10^4 cells/well. Therefore, we diluted our media 500 times. Three controls were run: 1) Background control (media without reaction mixture), 2) Low control (Normoxia), 3) High control (100% dead cells using 2% Triton X-100 with the media). Reaction Mixture was made immediately before the experiment by adding 250ul catalyst to 11.25ml dye thoroughly mixed in a dark room. Diluted media and controls were run in triplicate using 100ul media plus 100ul reaction mixture per well. The plate was covered and incubated at room temperature for 30 min. The plate was read with a micro-plate reader (VersaMax) at 490 nm wavelength and the data collected using Software-Max. The relative LDH activity in the culture media was normalized by the tissue weight.

3.5 Protein extraction and measurement:

The tissue slices that were snap frozen at -80C were put on ice to melt. The slices were added to T-PER buffer (Cat. No.: 78510, Thermo-Scientific, Rockford, IL, USA) to which protease-phosphatase inhibitor cocktail and EDTA (Cat. No.: 78440, Thermo-

Scientific, Rockford, IL, USA) were added. They were then minced in the buffer with scissors, vortexed and homogenized (Ultra-Turrax T8, IKA LABORTECHNIK, Wilmington, NC, USA). The vortexing and homogenization procedure was repeated and the homogenate centrifuged at 10,000g for 5 min at 4° C. Some of the supernatant was used for protein measurement and Western blotting with the remainder was snap-frozen in liquid nitrogen.

For protein measurement, a BCA protein assay kit (Cat. No.: 23227, Thermo-Scientific, Rockford, IL, USA) was used. Standard solutions and working reagents were prepared according to the manufacturer's instructions. The tissue slice protein had to be diluted ten times to fall within the standards range. All standards, unknowns, and blank were run in duplicate via the micro-plate method using 25ul samples per well to which 200ul of working reagent was added (working reagent was prepared by adding the kit's reagent A to reagent B in a ratio 50:1). The micro-plate was incubated for 30 min at 37°C, read using a micro-plate reader at 562 nm wavelength and the data were collected using Software-Max.

3.6 Western Blots:

The gels for Polyacrylamide Gel Electrophoresis (PAGE) were made in the lab using a discontinuous buffer system method with the gel separated into two sections (a large -pore stacking gel on top of a small-pore resolving gel). The resolving gel (12%) was made for 2 gels of 1mm thickness as follows: [4.42ml H₂O, 5.2ml 30% Acrylamide/Bis (Cat. No.: 161-0156, Bio-Rad, Hercules, CA, USA), 3.25ml 1.5M Tris-HCl buffer PH=8.8 (Cat. No.: 161-0798, Bio-Rad, Hercules, CA, USA), 0.13ml 10%

SDS (Cat. No.: 161-0416, Bio-Rad, Hercules, CA, USA), 65ul 10% APS (Cat. No.: 17874, Thermo-Scientific, Rockford, IL, USA), 6.5ul TEMED (Cat. No.: 161-0801, Bio-Rad, Hercules, CA, USA)]. The stacking gel (4%) was made for two gels of 1mm thickness as follows: (3.05ml 30% acrylamide/Bis, 1.25ml 0.5M Tris-HCl buffer PH=6.8 (Cat. No.: 161-0799, Bio-Rad, Hercules, CA, USA), 0.05ml 10% SDS, 25ul 10% APS, 5ul TEMED).

Protein samples were thawed and mixed with an equal volume of sample buffer [mix 2-Mercaptoethanol (Cat. No.: 161-0710, Bio-Rad, Hercules, CA, USA) to Laemmli buffer (Cat. No.: 161-0737, Bio-Rad, Hercules, CA, USA) in a ratio 1:19] and then heated to 95°C before loading onto the gel. The running buffer [adding 50ml 10x TGS (Cat. No.: 161-0772, Bio-Rad, Hercules, CA, USA) to 450ml of DI H₂O] was then added to the electrophoresis module. A protein marker (Precision Plus Kaleidoscope, Cat. No.: 161-0375, Bio-Rad, Hercules, CA, USA), positive control for p-AKT (Cat. No.: 9273, Cell Signaling Technology, Danvers, MA, USA) were also loaded on the gel. The gels were run at 100V for 1.5 hr. and then transferred to nitrocellulose membrane using transfer buffer [adding 85ml 10x TG (Cat. No.: 161-0771, Bio-Rad, Hercules, CA, USA) to 595ml DI H₂O to 170ml methanol] at 100V for 1 hr. Next, the membranes were washed for 5 min with TBS-T and blocked with 5% non-fat milk in TBS-T for 1 hr at room temperature. The membranes were then incubated with primary antibody (Cat. No.: 4060, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C on a shaker (p-AKT antibody 1:2000 5% BSA in TBS-T). The membranes were washed with TBS-T 3 times for 5 min with TBS-T before incubating it with anti-rabbit secondary antibody (Cat. No.: 7074, Cell Signaling Technology, Danvers, MA, USA) for 1 hr at room temperature

(1:2000 in 5% non-fat milk in TBS-T). The membranes were washed again 3 times for 5 min in TBS-T before incubation with ECL (SuperSignal West Pico Chemiluminescent Substrate, Cat. No.: 34080, Thermo-Scientific, Rockford, IL, USA) solution for 10 min. The membranes were wrapped with lab wrap and exposed to film.

The membranes were re-probed by washing with 1x TBS for 5 min, stripped with Restore Plus Western Blot Stripping Buffer (Cat. No.: 47430, Thermo-Scientific, Rockford, IL, USA) for 5 min, and then twice washed for 5 min with 1x TBS. The membranes were blocked with 5% non-fat milk in TBS-T for 1 hr and incubated with the primary antibody for housekeeping genes e.g. GAPDH (Cat. No.: SC-20357, Santa Cruz Biotechnology, Dallas, Texas, USA) in a dilution 1:1000 in 5% non-fat milk in TBS-T overnight at 4° C. They were then washed with TBS-T for 10 min 3 times before incubation with a secondary antibody (Donkey Anti-Goat IgG, Cat. No.: SC-2020, Santa Cruz Biotechnology, Dallas, Texas, USA) in a dilution 1:2000 in 5% non-fat milk in TBS-T for 1 hr at room temperature. Finally, the membranes were washed 3 times with TBS-T allowing 10 min for each wash, incubated with ECL solution for 10 min at room temperature, wrapped with lab wrap and exposed to film.

3.7 TUNEL:

TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining was used to determine programmed cell death, i.e., apoptosis. Tissue slices from each group were deparafinized and rehydrated. TUNEL staining was accomplished by using an In Situ Cell Death Detection Kit (Cat. No.: 11684809910 Roche, Mannheim, Germany) according to the manufacture's instruction. Negative control was established by

incubating the fixed and permeabilized tissue in Label solution only (without terminal transferase) instead of TUNEL reaction mixture while a positive control was established by incubating the fixed and permeabilized tissue with DNase I recombinant for 10 min at room temperature to induce DNA strand breaks, prior to the labeling procedure. From each slice, all of the apoptotic cells in a field were counted using a fluorescent microscope (Zeiss Axiovert 200). The section area was measured using a scanner GS-800. The number of apoptotic cells was divided by the section area to obtain the number of apoptotic cells per mm².

3.8 Periodic Acid-Schiff (PAS) Staining:

Periodic acid acts upon the 1, 2 glycol linkage of carbohydrates in tissue sections to produce aldehyde, which can be highlighted with Schiff's reagent. Periodic acid-Schiff (PAS) reagent is used to depict areas of abnormal sarcolemmal permeability that occurs early during myocardial ischemia. Accordingly, the PAS positive areas are indicative of early necrotic damage. PAS staining was performed with the PAS Staining System (Cat. No.: 395B, Sigma-Aldrich, St. Louis, USA), according to the manufacturer's instructions. The tissue sections in each group were deparafinized, rehydrated, and oxidized in 0.5% Periodic acid solution for 5 min at room temperature. Then the slides were rinsed using several changes of distilled water before immersing them in Schiff reagent for 15 min at room temperature. Next the slides were washed in running tap water and counterstained in Gill 3 hematoxylin for 90 sec. They were washed with running tap water again and dehydrated, cleared, mounted and covered with cover slip. In each section, ten fields (200x magnification) were randomly selected and photographed. The PAS positive areas were quantified using the software Image-Pro Plus

4.5 and divided by the field area. The average percent PAS positive area of 10 fields was obtained for each experimental group.

3.9 Statistical Analysis:

Results are presented as mean \pm SEM. For comparison between groups, one-way analysis of variance (ANOVA) was performed, followed by the Tukey post-hoc test. Statistical significance was set at a P value < 0.05 .

CHAPTER 4

RESULTS

In the isolated heart global ischemia-reperfusion (I/R) experiments, there was a marked increase in coronary flow after reperfusion following global ischemia in the group pre-treated with SP (SP I/R) as compared to the I/R group not pre-treated with SP. Figure 4.1A shows the coronary flow to be zero during the 20 min global ischemic period for the I/R and SP I/R groups. Upon reperfusion, coronary flow was rapidly increased to a value that was 50 % greater than its baseline value in the SP I/R group (figure 4.1B) while that in the untreated I/R group was 20% below its baseline value (figure 4.1B). Furthermore, coronary flow remained above its baseline value in the SP I/R group during the subsequent 40 min reperfusion period while it significantly declined in the untreated I/R group to a value that was 50% below its baseline value (figure 4.1B).

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is released from injured cells and is used as a marker of cell death. LDH levels in the coronary efflux were measured during the time course of global ischemia and subsequent reperfusion. Results regarding the percent change in LDH activity during the ischemia-reperfusion period are provided in figure 4.2. At the end of occlusion and onset of reperfusion, LDH levels were increased in the I/R group to 240% above its basal level, while in the SP I/R group, LDH levels were increased only 97% above its basal level; the difference between the two groups was significant. Following the washout period, the LDH level in the SP I/R group

decreased to the same amount as in the Control group while it progressively increased in the I/R group indicating a continuing cell death in this latter group.

The above results for the onset of reperfusion of a significantly lower LDH in the SP I/R group indicate that, in addition to its effect on coronary flow, SP also attenuated the degree of myocardial damage that occurred during the no flow ischemic period. To further study this protective aspect of SP separate from its effect on the coronary circulation, we made use of the cultured LV tissue slice preparation. This preparation allows for the determination of the direct effect of SP on cardiac tissue without the interference of other parameters such as coronary flow, loading conditions, and neuro-hormonal influences. Accordingly, left ventricular slices were divided into five groups as follows: normoxia, hypoxia, hypoxia incubated with 30nM SP, hypoxia incubated with 100nM SP, and hypoxia incubated with 300nM SP. The tissue slice LDH assay results are shown in figure 4.3. As can be seen, LDH activity was significantly increased in the hypoxia group (H) (0.00444 ± 0.00016) compared to normoxia group (N) (0.00303 ± 0.00017). Incubation with SP significantly reduced hypoxia-induced LDH release in a dose dependent manner [(0.00321 ± 0.00019 in H30), (0.00308 ± 0.00023 in H100), or (0.00291 ± 0.00034 in H300)] indicating its cardiomyocyte protective properties.

AKT is known to promote cell survival when activated (phosphorylated) via inhibiting apoptotic pathways. Incubation with SP in a dose dependent manner increased p-AKT in the hypoxic LV tissue slices, as shown in figure 4.4A, indicating that SP enhanced cell survival by its ability to activate the downstream AKT pathway. In figure 4.4B, p-AKT values are normalized to GAPDH and the fold changes relative to hypoxia are calculated. p-AKT progressively increased with SP in a dose dependent manner

compared to the untreated hypoxia group [(1.000 ± 0.000 in H) vs. (1.351 ± 0.086 in H30), (1.903 ± 0.102 in H100), and (2.170 ± 0.472 in H300)].

The TUNEL staining results are summarized in figure 4.5. As can be seen, incubation with SP resulted in a progressive decrease in the number of TUNEL positive apoptotic cells (figure 4.5A). In figure 4.5B, the number of apoptotic cells per unit area is expressed relative to that of normoxia group. There was a significant increase in apoptotic cells in the hypoxia group compared to the N and H300 groups [(1.686 ± 0.202 in H) vs. (1.000 ± 0.000 in N), or (0.888 ± 0.124 in H300)]. These results indicate the capability of SP to directly decrease the number of apoptotic cells in a dose dependent manner.

Periodic Acid-Schiff (PAS) staining was used to determine areas of increased sarcolemmal permeability depicting necrotic cell death. In figure 4.6, the hypoxia group is seen to have a significant increase in PAS stained areas as compared to normoxia, and the SP treated groups [(0.0810 ± 0.0074 in H) vs. (0.0250 ± 0.0020 in N), (0.0394 ± 0.0034 in H100), and (0.0464 ± 0.0161 in H300)]. Accordingly, part of SP's cardioprotective properties is its ability to attenuate necrotic cell death induced by hypoxia.

From the above results, SP was shown to be effective in decreasing cardiac cell death via decreasing cell injury (LDH assay), the number of apoptotic cells (TUNEL), necrotic cells (PAS staining), and possibly by activating the cell survival pathway (p-AKT). To investigate whether SP activates AKT through the NK-1 receptor and whether SP induces cell survival through the AKT pathway, five groups of tissue slices were

studied as follows: normoxia, hypoxia, hypoxia with 100nM SP, hypoxia with 100nM SP and 50uM AKT inhibitor, or hypoxia with 100nM SP and 10uM NK1 inhibitor.

As shown in Figure 4.7, LDH was significantly less in the hypoxia group incubated with 100nM SP (H100) as compared to the hypoxia group [(0.00213 ± 0.00037 in H100) vs. (0.00432 ± 0.00051 in H)]. While LDH levels in the hypoxia groups treated with 100nM SP and 50uM AKT inhibitor or 10uM NK1 inhibitor were less than that in the hypoxia group, the reductions did not reach the level of significance [(0.00283 ± 0.00056 in H100+AKT INH) and (0.00361 ± 0.00051 in H100+NK1 INH) vs. (0.00213 ± 0.00037 in H100)]. Western blot results of myocardial phosphorylated AKT levels are presented in figure 4.8A. The p-AKT level is seen to be more prominent in H100 group compared to other hypoxia groups. In figure 4.8B, p-AKT values were normalized to GAPDH and expressed relative to the hypoxia value. The H100 group showed a 4-fold increase in AKT activation as compared to the H group (4.104 ± 1.539 in H100). Inhibiting AKT or NK1 receptors in SP pretreated hypoxia groups prevented this SP-induced increase [(0.8662 ± 0.3349 in H100+AKT INH) and (1.284 ± 0.4611 in H100+NK1 INH)]. These results indicate that the AKT pathway has been activated by SP via the NK1 receptor.

TUNEL staining results from the normoxia, hypoxia and hypoxia with 100nM SP and either 50uM AKT inhibitor or 10uM NK1 inhibitor groups are shown in figure 4.9A. The normoxia and hypoxia with 100nM SP groups have a similar number of apoptotic cells, which is much less than that in the other groups. In figure 4.9B, the number of apoptotic cells per unit area relative to that of normoxia group is shown. Hypoxia or hypoxia with 100nM SP and 50uM AKT inhibitor groups show a significant 2-fold

increase in apoptotic cell density compared to normoxia, or hypoxia with 100nM SP groups [(2.333 ± 0.498 in H), (2.187 ± 0.153 in H100+AKT INH), vs. (1.000 ± 0.000 in N), (1.044 ± 0.177 in H100)] indicating that blocking AKT which is a major survival pathway abolishes the protective effect of SP. While the difference between the hypoxia group (H) and hypoxia with 100nM SP and 10uM NK1 inhibitor group was not statistically significant [(2.333 ± 0.498 in H) vs. (1.930 ± 0.213 in H100+NK1 INH)]. It does suggest that blocking SP receptors abolishes its protective effect.

PAS staining results are shown in figure 4.10. Panel A displays representative PAS stained images in which the hypoxia, hypoxia with 100nM SP and 50uM AKT inhibitor, or hypoxia with 100nM SP and 10uM NK1 inhibitor groups show much greater PAS stained areas compared to the normoxia or hypoxia with 100nM SP groups indicating increased necrosis. The percent PAS positive areas for the groups are shown in panel B where the H, H100+AKT INH, or H100+NK1 INH groups are seen to have a significant increase in PAS positive area percentage compared to N or H100 groups [(0.0812 ± 0.0081 in H), (0.0864 ± 0.0135 in H100+AKT INH), (0.0774 ± 0.0069 in H100+NK1 INH) vs. (0.0301 ± 0.0035 in N), (0.0379 ± 0.0045 in H100)]. These results indicate that SP reduces hypoxia-induced cell necrosis via the NK1 receptor and activation of downstream AKT pathways.

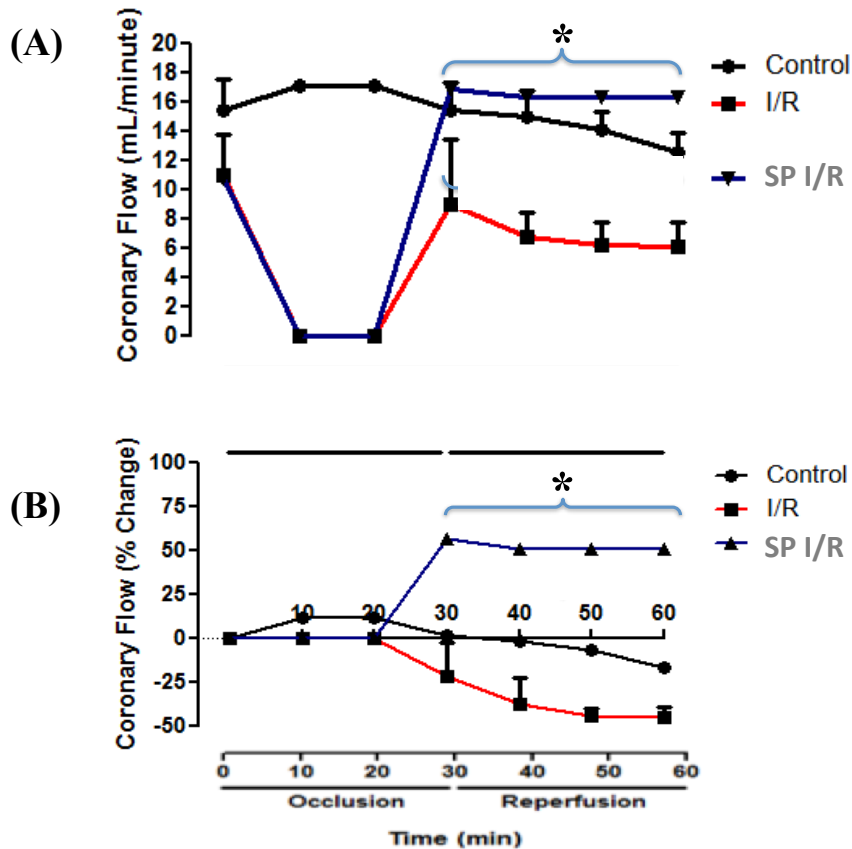


Figure 4.1. Coronary flow response to ischemia-reperfusion (I/R) in 3 groups of rat isolated hearts as follows: non-ischemic control, I/R, and SP pretreated I/R (SP I/R). Thirty min of global ischemia was followed by 30 min of reperfusion. Coronary flow was recorded every 10 min and the coronary perfusate collected at: baseline, immediately following the initiation of reperfusion, and 10, 20 and 30 min of reperfusion. Non-ischemic control hearts were continuously perfused for 60 min. Panel A - coronary flow during the time course of the experiment. Panel B - % change in coronary flow relative to baseline values. All values are mean \pm SEM. * p-value < 0.05, n=8-9.

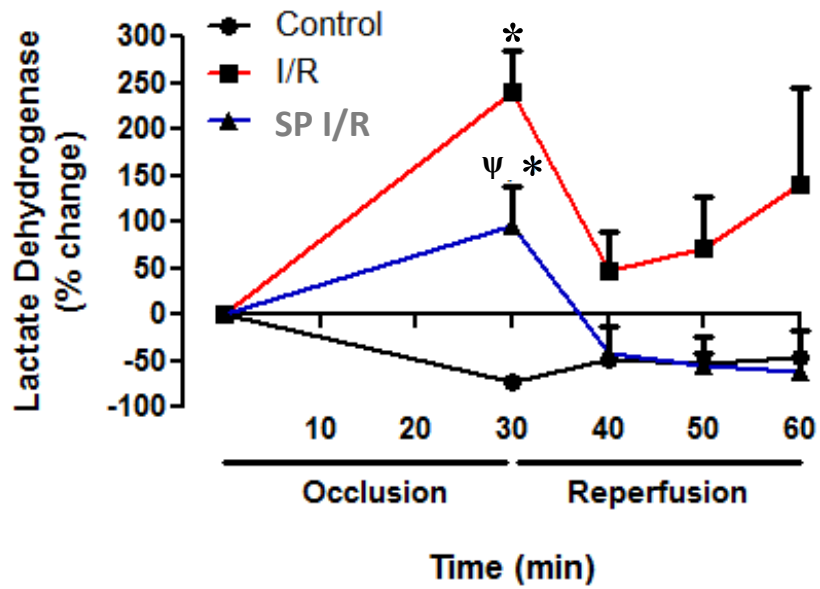


Figure 4.2. Percent change from baseline of lactate dehydrogenase activity (LDH) in the coronary perfusate during ischemia-reperfusion (I/R) in 3 groups of rat isolated hearts: non-ischemic control, I/R, and SP pretreated I/R (SP I/R). All values are mean \pm SEM, n=8-9. *p<0.05 vs. control at same time-point, ψ p<0.05 vs. I/R at same time-point.

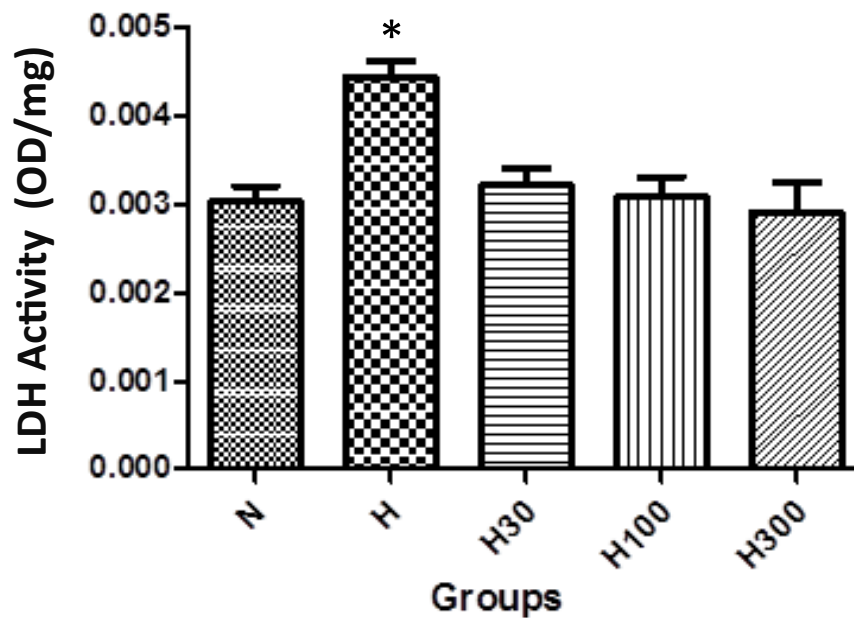


Figure 4.3. Lactate dehydrogenase activity (LDH) in the media of normoxic and hypoxic cultured left ventricular (LV) slices treated with different doses of SP. LV slices of the normoxia group (N) were incubated in Waymouth media with 10% FBS in culture incubator (37 °C, 95% O₂ and 5% CO₂) at 37 °C for 45 min., while the LV slices of all hypoxia groups: hypoxia alone (H), hypoxia with 30nM SP (H30), hypoxia with 100nM SP (H100), or hypoxia with 300nM SP (H300) were incubated in serum-free Waymouth media in a culture incubator (1% O₂, 94% N₂ and 5% CO₂) at 37 °C for 45 min. All values are mean ±SEM, N=4, *p<0.05 vs. N, H30, H100, or H300.

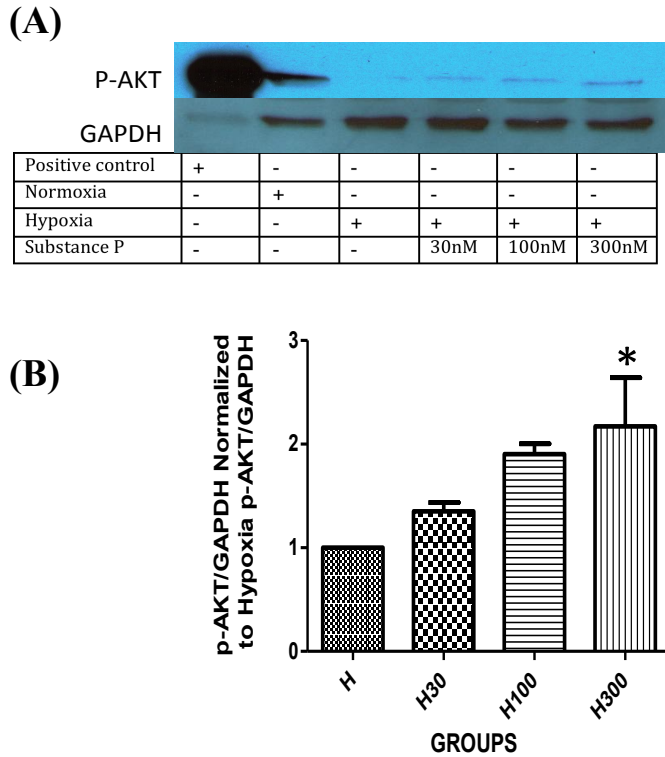
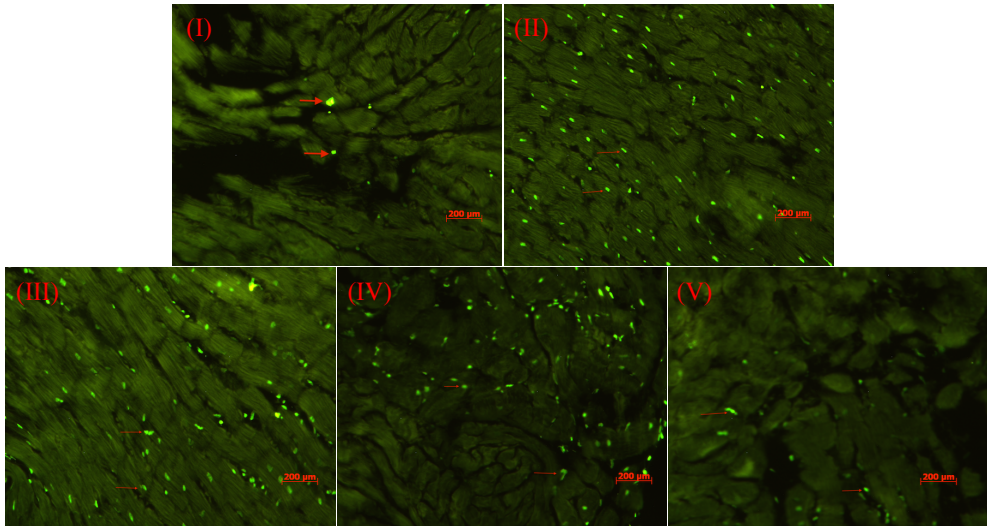


Figure 4.4. Panel A - Western blot of activated AKT (Ser473 phospho-AKT) in hypoxic LV sections incubated with and without SP in a deoxygenated culture incubator at 37 °C. (H) 45min hypoxia, (H30) 45min hypoxia with 30nM SP, (H100) 45min hypoxia with 100nM SP, (H300) 45min hypoxia with 300nM SP. Panel B - Values were normalized to GAPDH and then expressed relative to hypoxia values. All values are mean \pm SEM, n=4, *p<0.05 vs. H.

(A)



(B)

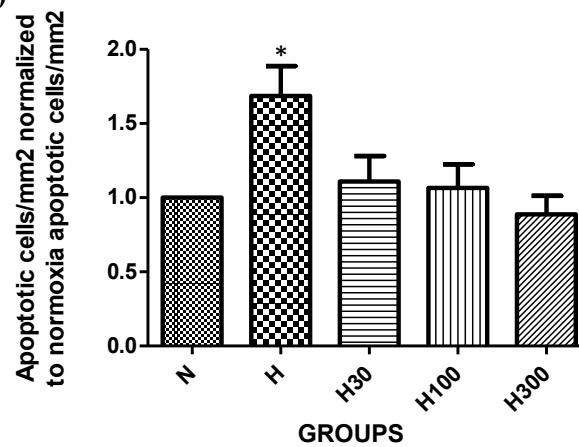
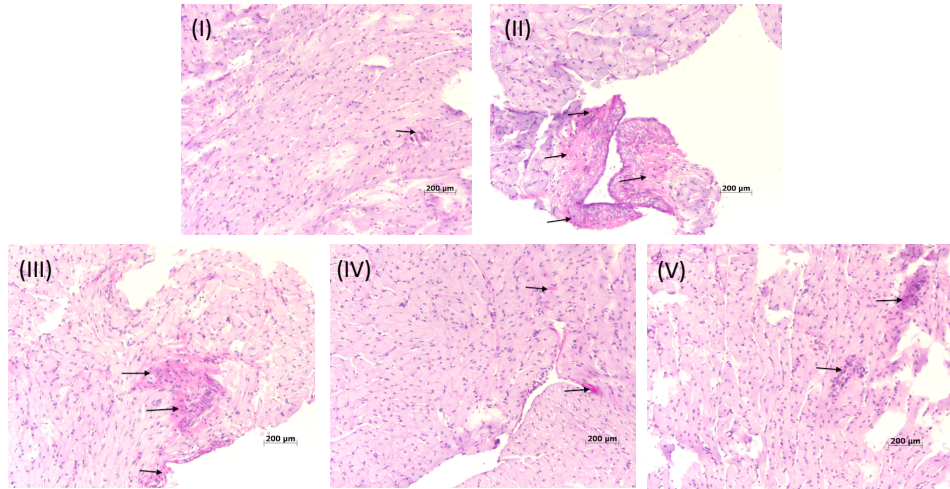


Figure 4.5. Panel A. TUNEL assay photomicrograph results of cultured left ventricular slices incubated with SP: (I) normoxia (N), (II) 45 min hypoxia (H), (III) 45min hypoxia with 30nM SP (H30), (IV) 45min hypoxia with 100nM SP (H100), (V) 45min hypoxia with 300nM SP (H300). Images were taken at 200x magnification. Panel B shows the number of apoptotic cells per unit area of LV sections normalized to the number of apoptotic cells per unit area in the normoxia group. All values are mean \pm SEM, n=5, *p<0.05 vs. N and H300.

(A)



(B)

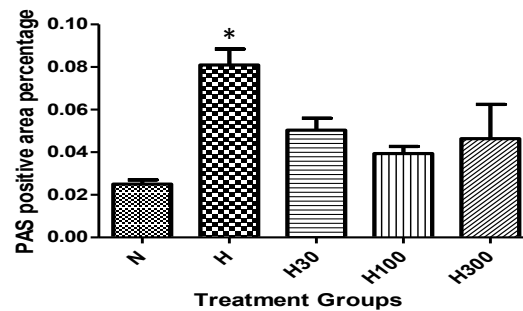


Figure 4.6. Panel A. Periodic Acid-Schiff (PAS) staining of LV sections subjected to hypoxia and incubated with different doses of SP. The pink areas (arrows) represent necrotic cell death. (I) normoxia (N), (II) 45min hypoxia (H), (III) 45min hypoxia with 30nM SP H30 (IV) 45min hypoxia with 100nM SP (H100), (V) 45min hypoxia with 300nM SP (H300). Images were taken at 200x magnification. Panel B shows the percent of PAS positive area in the LV sections. All values are mean \pm SEM, n=4, *p<0.05 vs. N, H100 and H300.

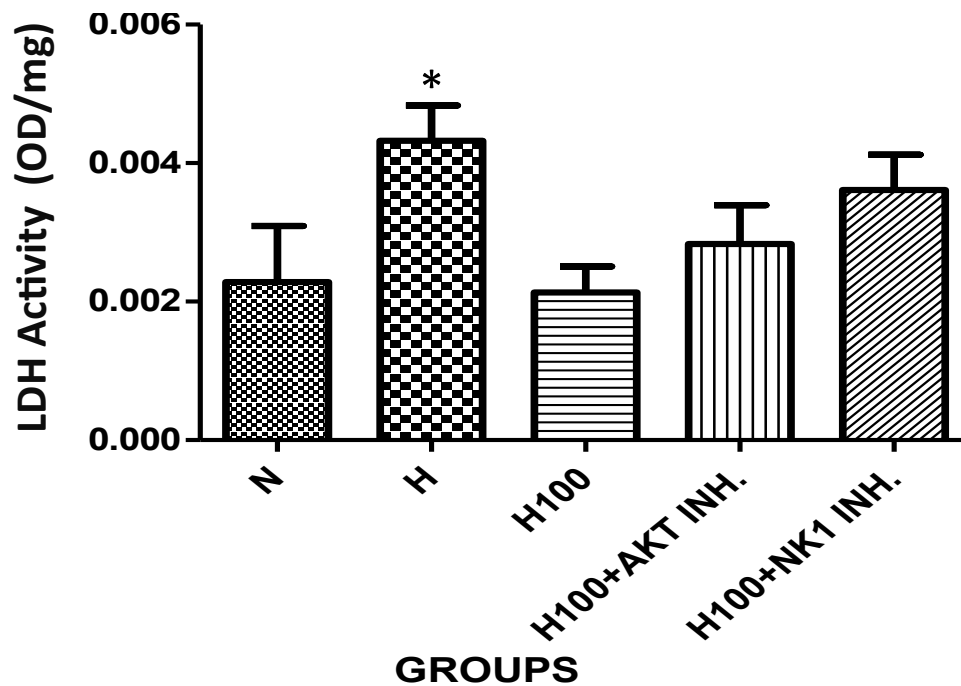
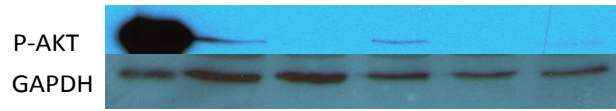


Figure 4.7. LDH activity in the media of cultured left ventricular (LV) slices incubated prior to hypoxia with SP and inhibitors of AKT and the NK1 receptor. LV slices in the normoxia group (N) were incubated in Waymouth media with 10% FBS in a culture incubator (37 °C, 95% O₂ and 5% CO₂) at 37 °C for 45 min. While the LV slices of all hypoxia groups [hypoxia alone (H), hypoxia with 100nM SP (H100), hypoxia with 100nM SP and 50uM AKT inhibitor (H100+AKT INH), and hypoxia with 100nM SP and 10uM NK1 antagonist (H100+NK1 INH)] were incubated in serum-free Waymouth media in a culture incubator (1% O₂, 94% N₂ and 5% CO₂) at 37 °C for 45 min. All values are mean ±SEM, N=4, *p<0.05 vs. H100.

(A)



Positive control	+	-	-	-	-	-
Normoxia	-	+	-	-	-	-
45min Hypoxia	-	-	+	+	+	+
100nM SP	-	-	-	+	+	+
50uM AKT inh	-	-	-	-	+	-
10uM NK1 inh	-	-	-	-	-	+

(B)

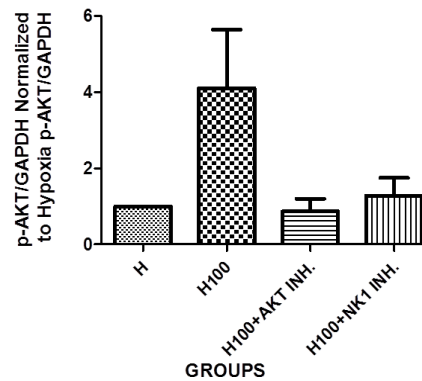


Figure 4.8. Western blot of activated AKT (Ser473 phospho-AKT) in left ventricular (LV) sections incubated with 100nM SP and inhibitors of AKT and the NK1 receptor before subjecting them to hypoxia (H) in a deoxygenated culture incubator at 37 °C for 45 min as follows: hypoxia + 100mM SP (H100), hypoxia with 100nM SP and 50uM AKT inhibitor (H100 + AKT INH), and hypoxia with 100nM SP and 10uM NK1 R antagonist (H100 + NK1 INH). Values were normalized to GAPDH and then normalized to that of hypoxia. All values are mean \pm SEM, n=4, p-value < 0.05.

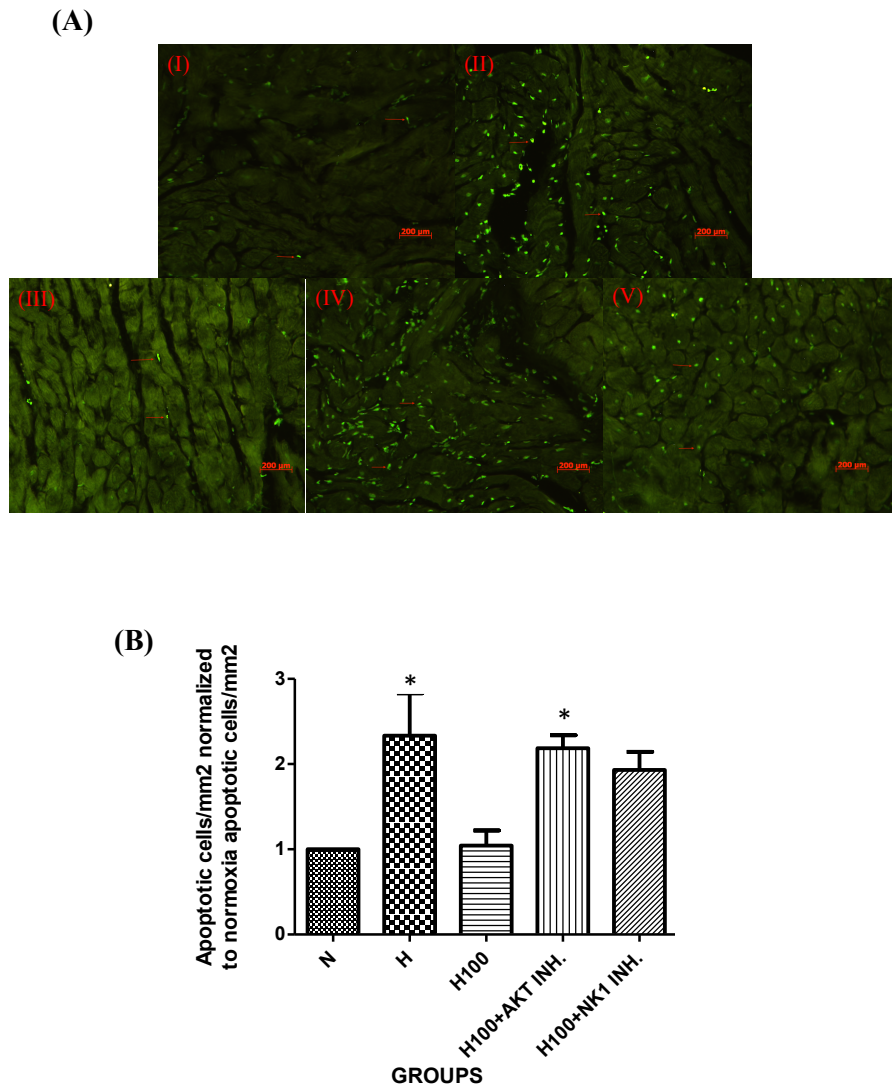


Figure 4.9. TUNEL assay of cultured left ventricular (LV) sections incubated with 100nM SP and inhibitors of AKT and the NK1 receptor before subjecting them to hypoxia (H) in a deoxygenated culture incubator at 37 °C for 45 min as follows: hypoxia + 100mM SP (H100), hypoxia with 100nM SP and 50uM AKT inhibitor (H100+ AKT INH), and hypoxia with 100nM SP and 10uM NK1 R antagonist (H100+NK1 INH). In panel A, (I) - normoxia (N), (II) - H, (III) - H100, (IV) - H100+ AKT INH and 50uM AKT inhibitor, (V) - H100+NK1 INH. Images were taken at 200x magnification. Panel B shows the number of apoptotic cells per unit area for the study groups normalized to the number of apoptotic cells per unit area in N. All values are mean \pm SEM, n=4, *p<0.05 vs. N., and H100.

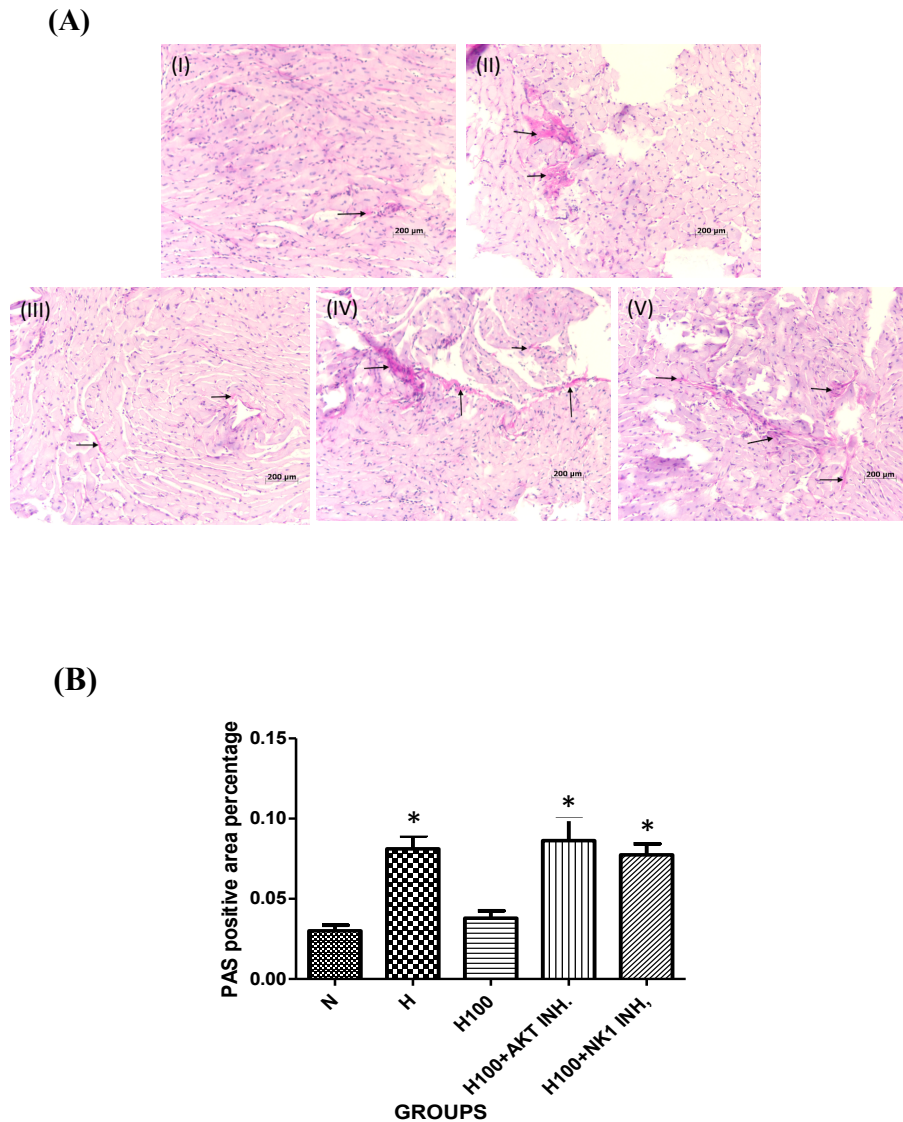


Figure 4.10. Periodic Acid-Schiff (PAS) staining of cultured rat LV sections incubated with 100nM SP and inhibitors of AKT and the NK1 receptor before subjecting them to hypoxia (H) in a deoxygenated culture incubator at 37 °C for 45 min as follows: hypoxia + 100mM SP (H100), hypoxia with 100nM SP and 50uM AKT inhibitor (H100+ AKT INH), and hypoxia with 100nM SP and 10uM NK1 R antagonist (H100+NK1 INH). The PAS pink areas (arrows) in panel A represent necrotic cell death where (I) - normoxia (N), (II) - H, (III) - H100, (IV) - H100+ AKT INH and 50uM AKT inhibitor, (V) - H100+NK1 INH. Images were taken at 200x magnification. Panel B shows the percent of PAS positive area in the LV sections of the groups. All values are mean \pm SEM, n=4, *p<0.05 vs. N., and H100.

CHAPTER 5

DISCUSSION

Current evidence has shown SP to have beneficial and adverse cardiac effects depending on the pathological setting. In general, it has been reported to be detrimental in chronic non-ischemic remodeling such as myocarditis (D'Souza M et al. 2007; Robinson P et al. 2009), magnesium deficiency (Weglicki and Phillips 1992), volume overload (Melendez GC et al. 2011), and spontaneous hypertension (Dehlin HM et al. 2013), while it has been shown to be beneficial in short-term ischemia-reperfusion (Ustinova et al. 1995). Its beneficial effect is due in part to the ability of SP to produce coronary vasodilation. A reduction in coronary blood flow induces acute SP release with subsequent vasodilation and short-term improvement of cardiac function (Dehlin HM et al. 2013). Substance P is released from sensory nerves that are distributed throughout the heart, including the ventricles, atria, valves, and connective linings (Furness JB et al. 1984). It is released from some coronary endothelial cells as well. Substance P acts via the NK-1 receptor, which is located on intrinsic cardiac ganglia, cardiac fibroblasts, and coronary endothelial cells. There is little evidence to support the existence of NK-1 receptors on cardiomyocytes and there are no reports indicating a direct effect of SP on cardiomyocytes; however, one study has indicated that genes for NK-1 receptors are expressed on isolated neonatal cardiomyocytes (Church DJ et al. 1996).

In the current study, we hypothesized that SP participates in cardioprotection through protective cellular mechanisms as well as through its capability to cause vasodilation. To investigate this hypothesis, a global ischemia-reperfusion rat isolated heart preparation protocol and a hypoxic rat left ventricular slices culture protocol were utilized and LDH, p-AKT, apoptosis, and ischemic areas were determined. The significant findings clearly demonstrate that SP induces its cardioprotection via coronary vasodilation and a direct effect on cardiomyocytes. Coronary flow was significantly increased in rat hearts pretreated with SP and subjected to global ischemia-reperfusion compared to rats subjected to ischemia-reperfusion without SP pretreatment. This is consistent with the already described vasodilatory effect of SP on the coronary circulation (Ustinova et al. 1995; Wang and Wang 2005; Ren JY et al. 2011).

LDH activity was significantly decreased in the Sub P I/R group compared to I/R group at the onset of reperfusion. Ren JY et al showed that diabetic hearts displayed a significant increase in other cell injury markers such as cardiac troponin I and creatine kinase due to the decrease in CGRP and SP release in diabetic hearts (Ren JY et al. 2011). However, in our study, LDH did not show a significant difference between with and without SP during the mid and late stages of reperfusion. This may be due to the fact that SP causes a vasodilation-induced increase in coronary flow, which increases the susceptibility to reactive oxygen species and consequent more release of LDH compared to I/R without SP. Also, sudden reperfusion could be a double-edged sword in that it could induce cell death *per se* (Braunwald and Kloner 1985; Piper HM et al. 1998; Yellon and Hausenloy 2007). Previous studies have showed that reperfusion of ischemic tissues is often associated with microvascular injury, mainly due to increased

permeability of capillaries and arterioles with subsequent increased diffusion and filtration of fluid across the tissues. Following reperfusion, more reactive oxygen species but less nitric oxide are produced by the activated endothelial cells, and the imbalance results in consequent inflammatory response (Carden and Granger 2000).

To avoid the influence of coronary flow on the cardiac tissue, we used a cultured LV tissue slice protocol, which has several advantages as follows: 1) myocardial cells retain their 3-D structural integrity, intercellular interactions and extracellular attachments; 2) ability to perform multiple perturbations on tissue from an individual heart thereby minimizing biological variability; 3) relatively long-term effects of a perturbation can be investigated under highly controlled conditions; and 4) the myocardial response to perturbations is solely intrinsic in that it is void of factors, such as circulating inflammatory cells, cytokines, variations in the neuro-hormonal background, and variations in preload, afterload and contractility.

In the cultured tissue slices, LDH significantly increased in the untreated hypoxia group compared to the other groups; incubation with SP significantly decreased the LDH levels in a dose dependent manner. The relative reduction in LDH release upon SP treatment is less evident in the cultured slices (H30, H100, or H300: H) compared to that of the isolated heart (SP I/R: I/R) which may indicate that SP's protective effect is more obvious when there is reperfusion due to its vasodilative properties. Nevertheless, the abovementioned slice results clearly indicate that SP has a protective effect independent of its effect on coronary flow.

AKT, also referred to as protein kinase B, is phosphorylated into its active form after stimulation with SP (Koon HW et al. 2004) and plays an important role in controlling the balance between cell survival and apoptosis (New DC et al. 2007). Activated/phosphorylated AKT (P-AKT) promotes cell survival and inhibits apoptosis by inactivating pro-apoptotic members of the Bcl-2 family (preventing cytochrome C leakage from the mitochondria), by decreasing expression of caspases and by increasing the expression of anti-apoptotic Bcl-2 family members (Yang ZZ et al. 2004; New DC et al. 2007).

Since the media of normoxia group contains 10% FBS which is known to induce AKT activation (Tari and Lopez-Berestein 2000), we omitted the normoxia group from our statistical analysis. P-AKT was progressively increased over two folds in the H300 compared to the hypoxia without SP group. This clearly indicates that with SP incubation, AKT is activated and improves cell survival by preventing apoptosis. Backman and Danielson demonstrated in their study of apoptosis in tenocytes that the anti-apoptotic effect of SP is mediated through NK-1 receptors and AKT-specific pathways (Backman and Danielson 2013). Substance P was also shown to activate AKT in human colonocytes mediating anti-apoptotic responses (Koon HW et al. 2007).

To study apoptotic cell death, the Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay was used. It detects DNA fragmentation due to the apoptotic signaling cascade. There was a significant increase in apoptotic cell density in the hypoxia group compared to normoxia and hypoxia group incubated with 300nM SP. The number of apoptotic cells increased in the hypoxia group by more than 150% than that in the normoxia group. Incubation with increasing concentrations of SP resulted in a

progressive decrease in apoptotic cells compared to hypoxia group. This clearly indicates that SP decreases apoptotic cardiac cell death. These findings are consistent with previous reports of the anti-apoptotic effect of SP in hyperoxic-induced lung injury (Huang B et al. 2014), retinal cells in diabetic rats (Yang JH et al. 2013), spinal cord injury (Jiang MH et al. 2013), human tenocytes (Backman LJ and Danielson 2013), bone marrow recovery after irradiation (An YS et al. 2011), and intestinal tissue regeneration post irradiation injury (Kang MH et al. 2009). Consistent with our findings are those reported by Melendez GC et al who used TAC1 knockout mice to study the effect of SP in mice with a sustained cardiac volume overload. While they found SP to induce adverse myocardial remodeling via mast cell activation, the number of apoptotic cells was less in wild type compared to TAC1 knockout mice (Melendez GC et al. 2011).

Periodic Acid-Schiff staining was used to delineate areas of necrotic death. The results clearly show that in the hypoxia group, the PAS stained area was significantly larger than that in the normoxia and hypoxia incubated with SP groups. Thus, SP reduces cardiac necrotic cell death during short-term hypoxia. To investigate whether it induces its effect through NK-1 receptors and whether activating AKT is the proposed mechanism beyond its cellular actions, we used NK-1 and p-AKT inhibitors. While there was a strong trend for both inhibitors to negate the beneficial effects of SP, the differences did not reach the level of statistical significance. This may be due to the doses used for both NK1 and p-AKT inhibitors not being sufficient to block all binding sites of SP and p-AKT inhibition respectively or the sample size was too small. According to our data, sample size needs to be increased to 6 rats per group to get statistically significant values with a power over 0.8.

Inhibition of AKT or NK1 receptors in SP incubated hypoxia slices resulted in a marked reduction in AKT compared to the hypoxic group incubated with SP alone. In fact, the levels of AKT activation in the slices incubated with the inhibitors were similar to that in the untreated hypoxic group. The overall p-value is significant; however, it was insignificant between head-to-head comparisons between any two groups. Again, many factors affect the significance level such as: sample size, SP dose, NK1 inhibitor dose, or p-AKT inhibitor dose. The results of Yang L et al lend additional support to the hypothesis. They demonstrated that SP promotes diabetic epithelial wound healing via AKT activation. Upon using NK-1 receptor inhibitor, all the healing effects of SP were abolished (Yang L et al. 2014).

Hypoxia, hypoxia with 100nM SP and 50uM AKT inhibitor, or hypoxia with 100nM SP and 10uM NK1 inhibitor groups showed a significant increase in apoptotic cells compared to normoxia or hypoxia with 100nM SP groups. This clearly supports our hypothesis that SP reduces cell death through activation of the AKT pathway. Consistent with our findings is a study by Koon HW et al. They found human colonocytes pretreated with AKT inhibitor followed by SP treatment showed a significant increase in apoptotic cells compared to colonocytes pretreated with SP alone [Koon HW et al., 2007]. Also the hypoxia, hypoxia with 100nM SP and 50uM AKT inhibitor, or hypoxia with 100nM SP and 10uM NK1 inhibitor groups showed significantly larger PAS stained areas compared to normoxia or hypoxia with 100nM SP treatment groups. These combined results indicate that SP treatment reduces cell necrosis induced by hypoxia while blocking the SP receptor or its downstream AKT pathway resulted in increased cell necrosis.

In summary, the findings of this study demonstrate for the first time that SP exhibits cardioprotective effects independent on its effects on coronary flow. Apoptotic and necrotic cardiac cells were significantly decreased in hypoxic LV sections upon incubation with SP in a dose dependent manner. It is also the first time that SP, via NK-1 receptors, activates the AKT pathway in cardiac tissue has been reported and that the cardioprotective effect of SP is due to its ability to induce AKT activation. While, the results of this study indicate a potential clinical application for SP administration in patients with angina pectoris or acute myocardial infarction, additional studies are required to further investigate its molecular mechanisms. For example, does SP induce activation of AKT via phosphorylating the second activation site (Threonine 308) on the AKT molecule, does SP have an effect on the other anti-apoptotic pathways such as the Ras-Raf-Erk pathway, and is Foxo1, a downstream molecule of AKT, involved?

Perspective: The results herein that SP activates AKT via NK-1 receptors to enhance cardiac cell survival are schematically summarized in Figure 5.1. These findings indicate the potential of SP to protect the heart in early stages of ischemic heart diseases and in particular during the surgical procedure to restore blood flow to an ischemic area.

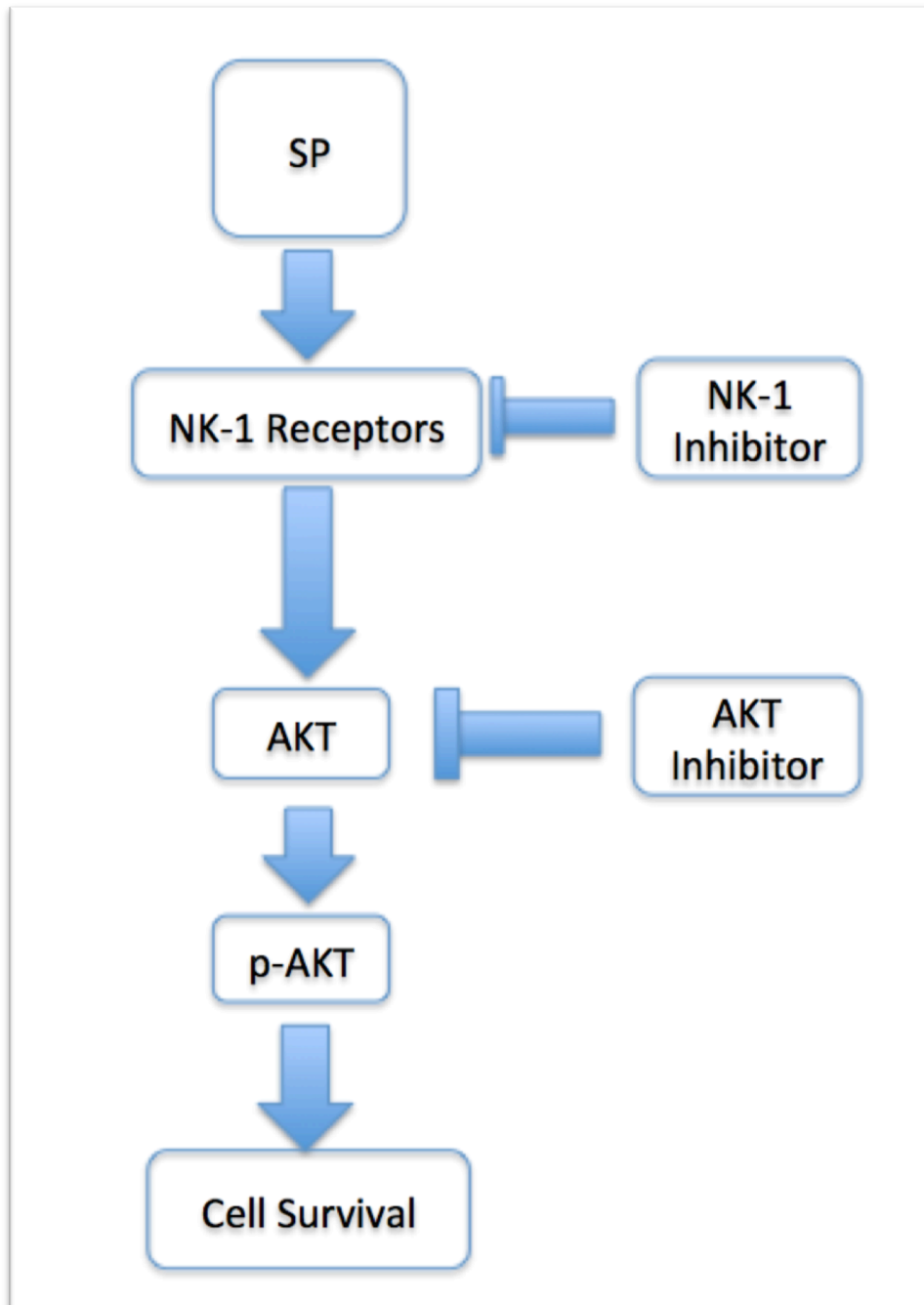


Figure 5.1. A schematic of the pathway by which substance P induces its cardio-protective effect. Substance P acts through NK-1 receptors to activate downstream AKT pathway with subsequent inhibition of cell death. The NK-1 inhibitor and AKT inhibitor acting on NK-1 receptors and AKT, respectively, prevent AKT activation resulting in reduced cell survival.

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