Peroxynitrite Effects on Smooth Muscle Contractility

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by

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A Thesis Submitted to the School of Graduate Studies
In Partial Fulfillment of the Requirements For the Degree
Master of Science

McMaster University

August 2002

Master of Science (2002) McMaster University
Department of Biology

Title: Peroxynitrite Effects on Smooth Muscle Contractility.

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Number of Pages: 75

ABSTRACT

Peroxynitrite is formed in blood vessels upon reaction of superoxide anion with nitric oxide (NO). It can oxidize proteins and thiols and nitrosylate free or protein bound thiols and tyrosine residues, thereby producing vascular dysfunction. Peroxynitrite therefore, may contribute to hypertension and cardiovascular diseases. We investigated the *in vitro* effects of commercially available peroxynitrite. De-endothelialized rings from the left descending coronary artery of pig were treated with peroxynitrite for 30 min, washed and then contracted with cyclopiazonic acid (CPA) or by membrane depolarization with KCl. Tissues pre-treated with peroxynitrite showed inhibition of the CPA-induced contraction with an IC_{50} of ≈100 uM but there was no effect on KCl-induced contraction. Peroxynitrite is stable only at alkaline pH and it may decompose to form superoxide and NO. However, including superoxide dismutase + catalase along with peroxynitrite did not change its effect.

Next, we used the same protocol to compare the effects of peroxynitrite and NO generating agents: 3-morpholino sydnonimine (SIN-1), s-nitroso-N-acetylpenicilliamine (SNAP), sodium nitroprusside (SNP) and spermine nonoate. The effectiveness of these agents to inhibit the CPA-induced contraction was SNAP > spermine nonoate ≥ SIN-1 > SNP. SNAP was the most effective in inhibiting the KCl-induced contraction with spermine nononoate being less effective and SIN-1 and SNP not producing any significant inhibition. We further investigated the effect of SNAP. Catalase, superoxide dismutase or CPTIO (a NO scavenger) did not prevent the effects of SNAP on the KCl- or the CPA-induced contractions. The guanylate cyclase inhibitor ODQ, partially reversed the effects of only low concentrations of SNAP. Thus, pretreatment with NO

generating agents such as SNAP and spermine NONOate appear to be more effective in inhibiting the contraction of the pig coronary artery than with peroxynitrite or the peroxynitrite generating agent SIN-1. Since SIN-1, SNAP, SNP and NONOates produce different amounts of peroxynitrite, nitric oxide and S-nitrosylation products, their effects may be used to delineate the molecular basis of the actions of peroxynitrite and NO on the arterial function.

Acknowledgements

This thesis represents the culmination of my wonderful time at the McMaster University. I have to sincerely thank Dr Grover for the support, encouragement and guidance throughout my undergrad and my graduate school. I wouldn't be where I am today but for him. He has helped me be professional and taught me many valuable lessons in life that I will always hold close to me. Thank you, Dr. Grover for making me a better person on the whole. This thesis work was in part supported by Heart & Stroke of Ontario.

Dr Werstuik, a very modest person has helped me through academic difficulties. Thank you for being personal and appreciate your kind words.

I thank Dr Kwan for being there during those critical moments! And Sue, you were a great help in untangling those messy technical problems. Thanks for the invaluable technical support.

I have to indulge in a huge note of gracious gratitude to all my friends (Vicky, Meeta, Moni, Sagina, Ayesha, Uma, Winty & Gurjit) for being there for me when I needed them, for checking up on me and overall for making me hold on to memorable times in Hamilton.

Tej, thanks for everything and being a wonderful friend.

For the last 2 years I have been coming home to a very pleasant and homely housemate, Melanie. Thank you for making my time at 106 Carlingview a sweet nostalgia. You listened to my problems, were a wonderful roommate, fellow student and above all, a great friend.

Jyoti, thank you for the constant encouragement, patience and words of wisdom.

Reem, the best nurse, the tastiest cook and an ocean of kindness. Thank you for all the concern, kindness and culinary treats I was lucky to experience!

If "Laughter is the best medicine", then Prameet and Victor would definitely be my doctors as they made me smile and smiled with me unconditionally even when my jokes weren't the best in the world! Thank you guys for the hilarious yet valuable memories.

I thank my mom (for the fantastic food), dad and my sisters for the vital support, emotionally and in every other aspect that I can think of. Their presence just a 30 minute drive away was a pillar of indescribable strength.

"Dear Father, whatever conditions confront me, I know that they represent the next step in my unfoldment. I welcome all tests because I know that within me is the intelligence to understand and the power to overcome"

-Paramahansa Yogananda

My grandfather believed the above & taught me this since I was a kid. I wish he was here to see this achievement of his granddaughter but from where he is I know he is always with me in spirit and soul, looking after me. Thank you, grandpa for believing in me and imbibing in me the core values and morals that makes me who I am today. I miss you a lot and want to say that you are best grandfather in this whole world. I dedicate my thesis to you. May God rest your soul in peace.

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List of Abbreviations

Ach	Acetylcholine
CAT	Catalase
CPA	Cyclopiazonic acid
Ca^{2+}	Calcium
$[Ca^{2+}]_I$	Intracellular Calcium
cGMP	cyclic guanosine monophosphate
CPTIO	(2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DAG	Diacylglycerol
EDRF	Endothelium dependent relaxing factor
EDTA	Ethyl-di-tetraacetic acid
EGTA	Ethylene Glycol bis N, N, N', N' tetraacetic acid
eNOS	Endothelial-nitric oxide synthase
FAD	Flavin adenine diphosphate
FMN	Flavin mono nucleotide
GTP	Guanosine triphosphate
HDL	High density lipoprotein
IP_3	Inositol triphosphate
I/R	Ischemia reperfusion
iNOS	Inducible-nitric oxide synthase
kD	kilodalton
LAD	Left anterior descending
LDL	Low dendity lipoprotein
MLCK	Myosin light chain kinase
NOS	Nitric oxide synthase
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
$ONOO^-$	Peroxynitrite
PMCA	Plasma membrane Ca^{2+}-Mg^{2+} ATPase
PSS	Physiological saline solution
PIP_2	Phosphatidylinositol biphosphate
ROS	Reactive oxygen species
SIN-1	3-morpholinosydnonimine, hydrochloride
SNAP	S-nitroso-N-acetylpenicillamine
SNP	Sodium Nitroprusside
SP-NONOate	Spermine NONOate
SOCCs	Store operated Ca^{2+} channels
SERCA	Sarcoplasmic reticulum Ca^{2+}-Mg^{2+} ATPase
SOD	Superoxide dismutase
sGC	soulble Guanylate cyclase
VOCCs	Voltage operated Ca^{2+} channels

CHAPTER 1: INTRODUCTION

Myocardial ischemic-reperfusion injury produces excess free radicals such as peroxynitrite, hydrogen peroxide, superoxide and hydroxyl radicals[30,32,40,41,52]. The main objective of this study is to determine the effect of peroxynitrite and various nitric oxide donors on smooth muscle contractility in porcine coronary artery. The introduction focuses on calcium homeostasis, the structure and function of coronary arteries, the effects of reactive oxygen-nitrogen species during ischemia-reperfusion on the arteries, the antioxidative mechanisms to overcome the oxidative and nitrosative stress, the formation and action of peroxynitrite, and the nature of nitric oxide donors.

1.1 CALCIUM

1.1.1 Calcium Homeostasis

Mammalian cells maintain very low concentrations of free Ca^{2+} in the cytosol ($[Ca^{2+}]_I$) (~10^{-7} M) as compared to much higher extracellular Ca^{2+} concentrations (~10^{-3} M)[4,33,46]. $[Ca^{2+}]_I$ is also lower relative to that in the sarcoplasmic reticulum (SR). $[Ca^{2+}]_I$ increases rapidly even with a small influx from the extracellular space. $[Ca^{2+}]_I$ is a primary mechanism in regulating coronary vascular tone, although changes in cAMP, cGMP and other metabolites may also modulate tone[46]. Therefore, changes in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) plays an important role in transmitting information from the extracellular space to the intracellular sites. The exact mechanisms of the contraction will be discussed later in Section 1.3.1.

There are four main pathways by which extracellular Ca^{2+} can enter the cytosol of vascular smooth muscle cells[4,33,46]. These include: 1) voltage-operated Ca^{2+} channels (VOCCs) which are activated by depolarization, 2) Na^{+}-Ca^{2+} exchange, 3) passive Ca^{2+}

leak, and 4) store-operated calcium channels (SOCCs) (Figure 1). The Ca^{2+} gradient is maintained by Ca^{2+} pumps such as the plasma membrane Ca^{2+}-Mg^{2+} ATPase (PMCA) which actively transports Ca^{2+} out of the cell and the sarcoplasmic reticulum Ca^{2+}-Mg^{2+} ATPase (SERCA) which serves to sequester Ca^{2+} into an intracellular store, and possibly by a Na^{+}-Ca^{2+} exchanger[4,15,18,46]. The properties of both pumps will be discussed later in detail.

Opening of the voltage and receptor operated calcium channels in the plasma membrane (PM) causes an increase in the $[Ca^{2+}]_i$[46]. Calcium is released from the sarcoplasmic reticulum (SR) into the cytosol by the production of second messengers through the binding of specific ligand, such as acetylcholine (Ach) binding to the G-protein-linked receptors[46]. This binding activates phospholipase C, which in turn hydrolyzes phosphatidylinositol biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). Calcium release from the SR into the cytosol increases when IP_3 binds to the IP_3-gated Ca^{2+} release channels and thus increasing the $[Ca^{2+}]_i$[4,46,52]. A Ca^{2+}-induced Ca^{2+} release mechanism exists in skeletal and cardiac muscle but its role in coronary artery smooth muscle is not known. In any event, calcium increase in the cytosol is transient because smooth muscle SR Ca^{2+} ATPase pump (SERCA2b), plasma membrane Ca^{2+} ATPase pump (PMCA1b) and Na^{+}-Ca^{2+} exchanger rapidly pump Ca^{2+} back into the SR[15,18].

Thus mobilization of Ca^{2+} from extracellular fluid and the SR is responsible for smooth muscle contraction and relaxation (Figure 1). The exact mechanism of the contraction-relaxation cycle is reviewed in detail in a later section.

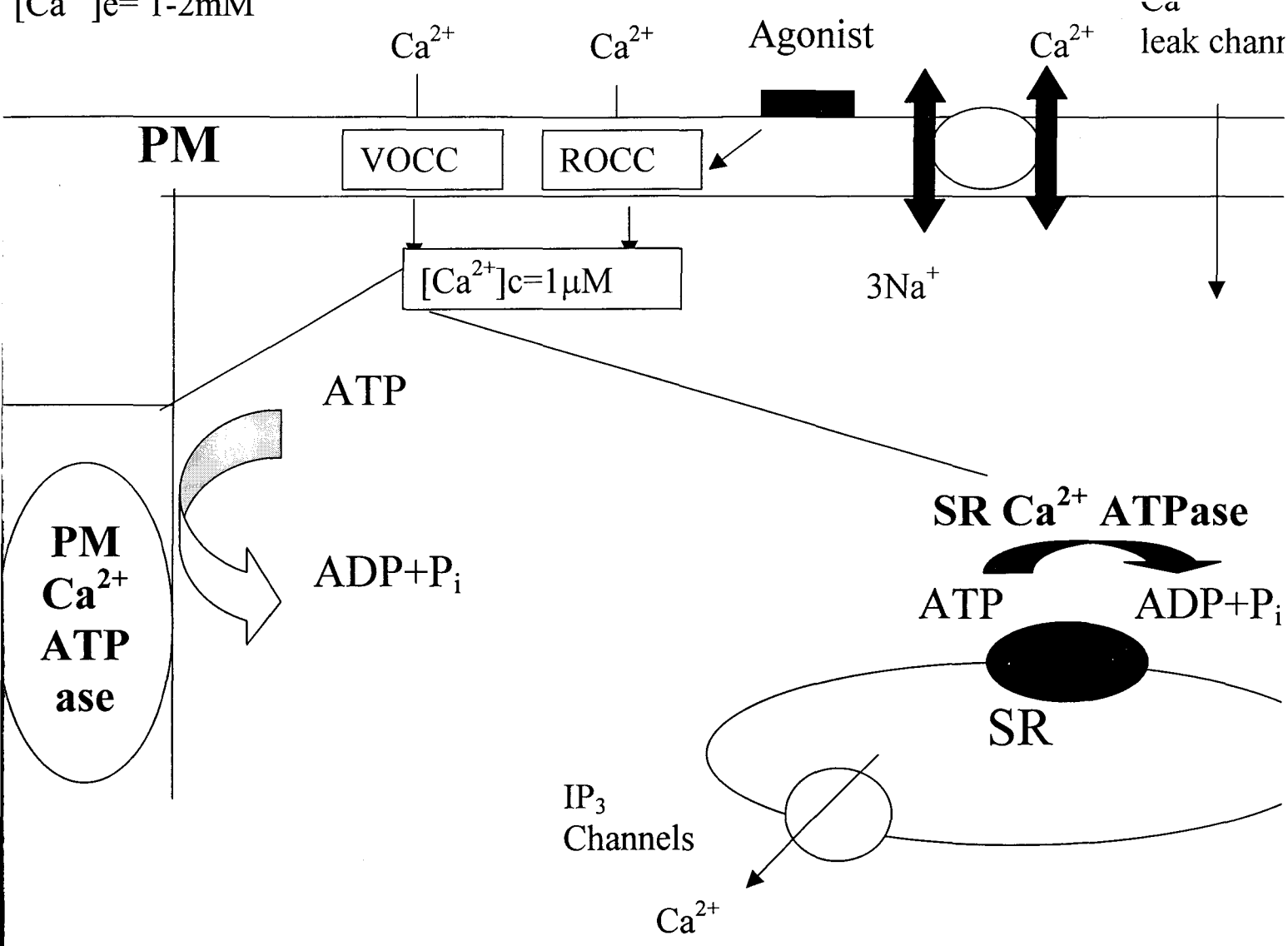


Figure 1: Main Mechanisms involved in the maintenance of the cytosolic free Ca^{2+} concentration in a vascular smooth muscle cell. Calcium homeostasis in the cytosol is maintained by Na^+/Ca^{2+} exchanger, plasma membrane (PM) and sarcoplasmic reticulum (SR) Ca^{2+} ATPases. Calcium can enter the cell via VOCCs, Na^+ - Ca^{2+} exchanger, and Ca^{2+} leak channels. P_i, inorganic phosphate; $[Ca^{2+}]_e$, extracellular free Ca^{2+} concentration; R, receptor. This figure was taken from reference 46.

1.1.2 Calcium pumps

Calcium pumps play a vital role in relaxing the contracted smooth muscle[7,15,18,57]. As mentioned earlier, PMCA pumps calcium from the cytoplasm against its concentration gradient to the extracellular space and SERCA sequesters Ca^{2+} from the cytoplasm and stores in the SR[7,15].

The activity and amount of SERCA pumps and PMCA pumps varies between the arteries and veins. Furthermore, the contribution of the pumps in Ca^{2+} homeostasis varies among different tissues and species[18]. Both PM and SR Ca^{2+} pumps derive energy from ATP hydrolysis to move calcium from the cytosol against its electrochemical gradient. But these two types of pumps are not only structurally different but are also regulated by different compounds[15,18]. Various SR Ca^{2+} pump isoforms have a molecular weight of 100-115 kD and the PM Ca^{2+} pump isoforms are 130-140 kD[15,18]. During the contraction-relaxation cycle, both pumps are regulated by phosphorylation but PMCA and SERCA are activated by calmodulin whereas SERCA is inhibited by phospholamban[15,51,53]. Ca^{2+} is necessary for the activation of calmodulin for PMCA activity. However, for SERCA activity, Ca^{2+} or phosphorylation of phospholamban by cAMP-dependent protein kinase is required for the dissociation of phospholamban from the calcium pump[15,18,53]. Therefore Ca^{2+} ATPase pumps in SR and PM both effectively lower the $[Ca^{2+}]_i$, and cause the contracted smooth muscle to relax.

There are 4 genes that encode PMCA - PMCA1, PMCA2, PMCA3 and PMCA4[18,51]. PMCA1 is ubiquitous and is found in abundance in the coronary artery. There are 3 genes that encode SERCA pump isoforms - SERCA1, SERCA2 and SERCA3[12,18]. SERCA1 gene is expressed mostly in the fast-twitch muscle and SERCA2

gene in the cardiac and slow twitch skeletal muscle. SERCA3 isoforms are most abundant in the endothelial cells, platelets, pancreas and kidney[34,51]. SERCA2 gene is alternatively spliced to produce SERCA2a and SERCA2b[12,51]. SERCA2a is most abundant in the skeletal muscle and SERCA2b in the vascular smooth muscle cells, cerebellum and cerebrum[51]. All isoforms of the SERCA pump share a common characteristic that they can be inhibited by cyclopiazonic acid (CPA) and thapsigargin[15,51]. In contrast, there are no known specific inhibitors for PMCA pump activity.

1.2 CORONARY ARTERIES

1.2.1 Function of Coronary Arteries

Coronary arteries supply cardiac muscle with oxygen and nutrients[41]. Any obstruction in coronary blood flow can lead to the development of cardiovascular diseases. The most important factor in regulating coronary blood flow is vascular tone. There are also other factors that participate in the regulation of coronary vascular resistance such as local metabolites and endothelium-derived constricting and relaxing substances[41,69].

1.2.2 Anatomy of Coronary Arteries

The heart is composed of coronary arteries, veins and the lymphatic system. The cardiac vessel consists of the left and right coronary arteries[41]. The left main coronary artery passes between the left atrium and the pulmonary trunk to reach the atrioventricular groove, where it divides into the left anterior descending (LAD) coronary artery and the circumflex artery[41]. The LAD and its branches supply blood to the apical wall of the left ventricle, right and left bundle branches and the papillary muscle of the

left ventricle. The right main coronary artery gives rise to the posterior descending artery and nourishes the apical half of the ventricular septum[41]. The coronary arteries run on the surface of the heart and are embedded in sub-epicardial fat and ventricular muscle.

A coronary artery consists of three layers of tissue: adventitia, intima-media and endothelium. The adventitia of the artery is a thin layer of connective tissue made up of elastic and collagen fibers[41]. The middle layer consists of elastic fibers and smooth muscle cells arranged circularly around the lumen[41]. It is due to the elastic fibers in the smooth muscle cells that the walls of the arteries can easily stretch or expand without tearing in response to small increases in pressure. The lumen or the innermost layer of the artery is a smooth mono-layer of endothelial cells[41]. In order to regulate blood pressure and blood flow, both vascular endothelial cells and smooth muscle cells play a vital role in altering vascular tone.

1.3 SMOOTH MUSCLE

The contractile apparatus of smooth muscle cells in the medial layer of a coronary artery consists of thick, thin and intermediate filaments[31,41,57]. The thick filaments contain myosin and the thin filaments contain actin. It is the overlap between myosin and actin that generates the tension in smooth muscle that ultimately causes the contraction or dilation of the coronary artery.

1.3.1 Contraction Mechanism of the Artery

The contraction of smooth muscle in the coronary artery is initiated by an increase in $[Ca^{2+}]_i$[31,41,57]. Calcium ions flow into smooth muscle cytosol from both the extracellular fluid and the sarcoplasmic reticulum. The contraction of smooth muscle involves the binding of a regulatory protein called calmodulin to calcium in the cytosol.

Ca^{2+}-Calmodulin activates myosin light chain kinase (MLCK) enzyme[31,53,57]. This enzyme uses ATP to phosphorylate a portion of the myosin head. The phosphorylated myosin head binds to actin, and contraction can occur. Smooth muscle cells contract or relax in response to stretching, hormones, neurotransmitters or local factors such as changes in pH, oxygen and carbon dioxide levels, temperature, and ion concentrations[53,57].

1.3.2 Relaxation mechanism of the Artery

The phosphorylated myosin is dephosphorylated by an enzyme phosphatase that relaxes a contracted smooth muscle[53]. During contraction and otherwise, the phosphatase is present in its active form, thus, the rate of myosin phosphorylation by MLCK must exceed the rate of dephosphorylation during contraction[22,53]. On the other hand, in order for the muscle to relax, the rate of dephosphorylation must exceed the rate of phosphorylation[7,53]. Thus relaxation is accomplished by decreasing the amount of phosphorylated myosin, which is ultimately controlled by lowering the cytosolic calcium in order to inactivate the MLCK.

1.4 ENDOTHELIUM

The endothelium is a single layer of cells that line the entire inner surface of the cardiovascular system[41]. It covers approximately $700m^2$ and weighs 1.5 kg. Endothelial cells are generally oriented in the direction of blood flow parallel to the main axis of the artery. The orientation of endothelial cells is different among veins and arteries. There are numerous microvilli present on the luminal surface of endothelial cells. It is suggested that these microvili prevent the available contact surface area between the endothelial cells and the superfusing blood[41]. Endothelial cells also contain various

receptors for many substances circulating in the blood. Endothelial cells are held together by gap junctions which are involved in cell-to-cell communication[41]. The exact mechanism of cell communication is not known. However, the abundance of the gap junctions in the endothelial cells probably co-ordinate the release of contraction and relaxation factors that are produced by one activated endothelial cell due to a receptor-mediated mechanism[41].

1.4.1 Function of Endothelium

Endothelial cells play a wide variety of critical roles in the control of vascular function. They form a barrier that contains circulating blood within the lumen of the vessel[28]. The endothelium prevents thrombosis by the expression of antithrombotic surface molecules such as heparin sulfate, thrombomodulin and plasminogen activators. It also resists clot formation through the release of platelet inhibitors such as prostacyclin and endothelium-derived relaxing factor[39,41]. The endothelial cells secrete vasoactive substances such as the endothelins that directly modulate contraction of smooth muscle cells in the underlying medial layer. They also produce vasodilator substances such as nitric oxide and prostacyclin and contribute to relaxation of the smooth muscle, and thus increase the diameter of the vessel[41]. Therefore, the vascular tone is dependent on the balance between vasoconstricting and vasorelaxing agents produced by the endothelium in the normal artery. The endothelial cells also inhibit smooth muscle cell migration and proliferation via secretion of heparin sulfate and endothelium derived relaxing factor (EDRF-NO), which may also serve an important anti-atherosclerotic role[39,41].

In diseased states, many of the normal endothelial functions are perturbed and major risk factors for the development of endothelial dysfunction include dyslipidemia

(high LDL or low HDL), hypertension, atherosclerosis, smoking, and diabetes[39,41,44]. The injured endothelium promotes vasoconstriction, smooth muscle cell migration and has reduced anti-thrombotic properties because of decreased secretion of prostacyclin and EDRF-NO[44].

1.4.2 Endothelium dependent relaxation pathways

Nitric oxide (NO) acts as a signaling molecule in mammalian cells[43,28]. It is lipophilic and hence readily crosses membranes. Normal arterial endothelial cells synthesize vasoactive substances such as prostacyclin (an arachidonic metabolite) and endothelium-derived relaxing factors (EDRF) that contribute to the modulation of vascular tone[28] (Figure 2).

NO is the major EDRF. It is a highly reactive free radical that lasts less than 10 seconds before it combines with oxygen and water to form inactive nitrates and nitrites[28]. Nitric oxide is produced in mammalian cells by three isoforms of nitric oxide synthases (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS)[2,28,49]. All three isoforms of NOS share a similar catalytic scheme that involves the five-electron oxidation of the terminal guanido nitrogen of the amino acid L-arginine to form NO and L-citrulline[2,28]. The reaction also involves molecular oxygen, NADPH as co-substrates and reduced thiols, FAD, FMN and tetrahydrobiopterin as cofactors[2,43].

NO synthesis from all three NOS isoforms depends on the ability of the enzyme to bind to calmodulin (calcium regulatory protein)[2]. For eNOS and nNOS to become fully activated, increases in the resting $[Ca^{2+}]_I$ is essential for the binding to calmodulin[2,49]. For example, intracellular $[Ca^{2+}]$ increases when acetylcholine is released by autonomic nerves in the walls of the blood vessel and binds to endothelial cells which

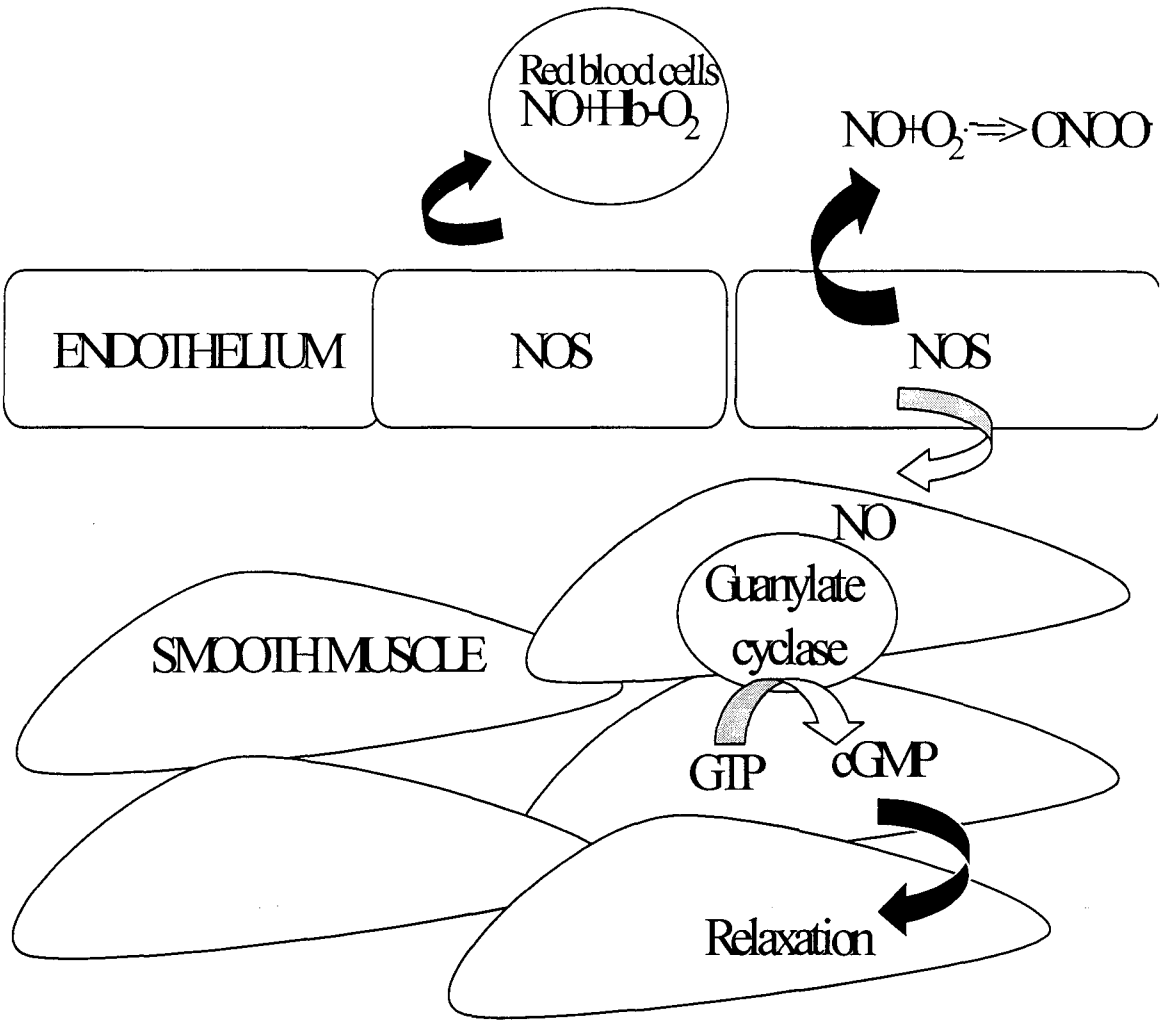


Figure 2: Endothelium Dependent Relaxation. Nitric Oxide is produced by NOS in the endothelium. NO diffuses rapidly into the underlying smooth muscle layer and binds to the heme moiety of guanylate cyclase (sGC). The activation of sGC converts GTP to cGMP and causes relaxation. Nitric oxide can be dissociated by hemoglobin in the circulating blood vessels or it can combine with superoxide anion ($O_2^{\cdot-}$) to produce peroxynitrite. This Figure is an adaptation from Reference 37.

activates eNOS and NO is synthesized[2,28]. NO then rapidly diffuses from the endothelium to the adjacent smooth muscle and activates guanylyl cyclase (G-cyclase) by binding to its heme moiety[28,47]. G-cyclase in turn forms cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP)[28,47] (Figure 2). The increase in intracellular cGMP activates cGMP-dependent protein kinases that phosphorylate SERCA-regulating protein phospholamban as well as Ca^{2+} transporters and channel proteins[28,43]. This results in smooth muscle cell relaxation by a reduction in cytosolic calcium and thus inactivating the MLCK as described earlier.

1.5 Reactive Oxygen Species (ROS)

ROS are loosely referred to as oxygen free radicals. Free radicals contain one or more unpaired electrons. Free radicals can be anionic, cationic and neutral. ROS is a chemical compound that has an unpaired electron in an outer orbit of the oxygen atom[6]. The unpaired electron of the ROS can be donated to cellular lipids and proteins to form peroxyl derivatives. This oxidation reaction can lead to changes in the nature of the lipids and proteins that ultimately results in several biological dysfunctions[6]. However, specified amounts of ROS are formed in all aerobic organisms by enzymatic and nonenzymatic reactions. The dioxygen molecule physiologically undergoes successive reductions yielding the superoxide anion, hydrogen peroxide (H_2O_2), and the hydroxyl radical[6].

1.5.1 ROS Generation during Ischemia followed by Reperfusion

ROS formation increases during ischemia followed by reperfusion[6,40,45,74]. Under physiological conditions ROS can be generated by the mitochondria, xanthine oxidase,

neutrophils and arachidonic acid metabolism[6,40]. Alternatively, in diseased states, ROS production is enhanced by each of these systems.

Mitochondria are the main source of ROS in healthy cells where oxidation-reduction reactions occur and the metal containing cytochromes are present[6]. The reduction of O_2 and subsequent catalysis with superoxide dismutase forms superoxide anion and H_2O_2 by the donation of the electrons from either NADH dehydrogenase or ubiquinone cytochrome b regions of the electron transport chain to molecular oxygen[6].

Several ROS such as superoxide anion, hydroxyl radical, and hydrogen peroxide can also be produced by stimulated neutrophils[6]. NADPH oxidase is an enzyme that is located within the plasma membrane of the endothelial cell and is a highly efficient source of superoxide anion[6]. This enzyme is activated by pathogens and cytokines that oxidizes NADPH on the cytoplasmic side and reduces O_2 to superoxide on the exterior surface of the membrane[6].

The generation of ROS in the endothelium by xanthine oxidase is the major source during ischemia or reperfusion injury[6,38,44]. Calcium dependent proteases which convert xanthine dehydrogenase to xanthine oxidase are activated by an increase in cytosolic calcium during ischemic injury. Xanthine oxidase in turn converts hypoxanthine to xanthine, uric acid, superoxide anion and H_2O_2[6].

In arachidonic acid metabolism, cyclooxygenase catalyzes the oxidation of arachidonic acid to prostaglandin endoperoxide[6]. ROS is also generated as a by-product when prostaglandin endoperoxide converts to other bioactive products.

1.5.2 Cellular damage by ROS

The reactive oxygen molecules can damage membrane lipids, proteins and nucleic acids[6]. They can do so by interacting with several cells and cause lipid peroxidation, oxidation of protein sulfhydryl groups and DNA damage[6].

Lipid peroxidation is the most important target of free radical reactions and the resultant oxidized lipids can travel throughout the circulatory system[6]. This can increase plasma membrane permeability, the breakdown of transmembrane ion gradients, the loss of secretory functions, and inhibit certain metabolic processes through a variety of reactions including amino acid oxidation and protein-protein cross-linking[6].

DNA damage caused by reactive oxygen species produced during normal cellular metabolism or under physiologic stress contributes to mutation of critical target genes[6]. Oxidative damage is thought to be mediated by excessive exposure of cells to reactive oxygen species (ROS), which can be generated following cell lysis, oxidative bursts (as part of the immune response) or by the presence of an excess of free transition metals[6]. ROS cause both base damage and strand breaks in DNA[6]. The alterations in the genome due to DNA damage can cause errors in transcription, mutations, recombinations and rearrangements[6].

Additionally, it has been suggested that accumulation of mutations and deletions in mitochondrial DNA with their associated defects in oxidative phosphorylation have been implicated in diabetes, ischemic heart disease, Parkinson's disease, demyelinating polyneuropathy, cancer and aging[6,40].

1.5.3 Cardiovascular damage by ROS

Reactive oxygen species produced in excess in the heart cause an imbalance in the Ca^{2+} homeostasis mechanism[6]. ROS have been shown to reduce the ability of the SERCA and PMCA to pump Ca^{2+} out of the cell by depleting high-energy phosphates resulting in Ca^{2+} overlaod[6,40]. Therefore, Ca^{2+} overload is responsible for the contractile abnormalities in myocardial ischemia-reperfusion (I/R) injury. Numerous studies have shown that excess ROS are formed during myocardial I/R[6,40,45,74]. There are several cell types that are involved in myocardial I/R injury such as coronary endothelial cells, circulating blood cells (leukocytes, neutrophils, platelets) and cardiac myocytes[6,30]. All of these cell types are able to produce ROS and in the diseased heart, ROS production is increased tremendously. These ROS have the ability to injure vascular cells and cardiac myocytes directly[30,40,44].

Physiologically, approximately 5% of the oxygen consumed by the tissue is converted into ROS[6]. There are endogenous enzymatic free radical scavengers present in the normal tissue such as catalase, superoxide dismutase and glutathione peroxidase that can detoxify basal levels of ROS[6,23,42]. However, in myocardial I/R injury, an excess production of ROS can exceed the ability of endogenous antioxidant mechanisms to detoxify ROS[23,42]. Studies have shown that endothelial cells are responsible for the production of excess ROS and superoxide anion is the predominant ROS formed after reperfusion[32,36]. Superoxide formed from endothelial cells can rapidly interact with NO and leads to its toxicity. This reaction is very fast and will be discussed in the preceding section in detail.

1.6 Peroxynitrite

1.6.1 Formation

Peroxynitrite ($ONOO^-$) is formed when superoxide anion and NO react which occurs at a diffusion-limited reaction rate and physiologically NO competes with superoxide dismutase (SOD) for its substrate[1,5,21]. $ONOO^-$ is formed at a rate of $6.7x10^9 Msec^{-1}$ and this rate is three times faster than the interaction of superoxide anion with its endogenous scavanger, superoxide dismutase[5,43]. $ONOO^-$ is a very powerful but selective oxidant that reacts slowly with most biological membranes[5].

In vivo, superoxide anion is generated by several enzymes. These include membrane located NADPH-oxidase in the endothelium and xanthine/xanthine oxidase system[6]. Mitochondria can also enhance the production of superoxide anion by a specific one-electron-reduction of dioxygen by the ubiquinone system of the respiratory system[6]. Therefore, all the above-mentioned superoxide generating systems alone or in concert with each other can enhance the production of superoxide anion. Since, NO is formed in excess in many events such as shear stress, enhanced $[Ca^{2+}]_I$ as a result of ischemia. The simultaneous production of NO and superoxide anion leads to a fast reaction between them and causes vasoconstriction[5,43,58].

1.6.2 Action

Peroxynitrite is a powerful oxidant that has both physiologic and cytotoxic effects[5]. Physiologically, in low amounts, it induces vasodilation, inhibits platelet aggregation and leukocyte adhesion to the endothelium similarly to NO[72]. It can also dilate cerebral arteries in a cGMP-independent way[5].

Peroxynitrite also exerts many deleterious effects by oxidizing lipids, thiols, proteins and nucliec acids[5,55]. $ONOO^-$ causes lipid peroxidation and forms lipid hydroperoxyradicals that propagate the free radical reaction[26,62]. It can also inhibit DNA repair enzymes and can oxidize protein-bound thiols[5]. It is also suggested that it can modulate the activity of SERCA and thus alter the contraction-relaxation cycle in the skeletal muscle[5]. It can also inhibit MnSOD in the mitochondria by nitration of tyrosine residues. It also inhibits complex I, complex II and complex V by binding to the {Fe-S} clusters of the enzymes. Inhibition of mitochondrial respiration by $ONOO^-$ is also implicated in myocardial I/R injury and endotoxic shock[5]. It can oxidize mitochondrial proteins and membrane lipids that lead to the opening of the permeability transition pore, resulting in Ca^{2+} efflux[55]. It can damage DNA by oxidation and nitration of DNA bases or by induction of nicks and breaks in the DNA strand[5,6,43]. It can nitrate the phenolic ring of tyrosine forming 3-nitrotyrosine (3-NT) which is considered a specific biomarker for $ONOO^-$[5,24,25]. Nitrated tyrosine affects both protein structure and function. For example, tyrosine kinases have shown to inhibit their phosphorylation and thus tyrosine kinase-dependent downstream signaling is altered[5]. $ONOO^-$ is a very unstable molecule and is dissociated rapidly into hydrogen peroxide and superoxide anion[5]. Thus, $ONOO^-$ produces both nitrosative and oxidative stress[5]. However, it is not known if all the cytotoxic actions mentioned above are caused directly by $ONOO^-$ or by its dissociated products.

1.6.3 Effect of ROS on calcium pump

Several studies show that SERCA is extremely susceptible to free radicals as compared to PMCA[14,16,17,19]. It is now known that SERCA damage by reactive oxygen

species leads to decreases in Ca^{2+} extrusion from the cytoplasm and results in an increase in cytosolic Ca^{2+} concentration. The decrease in SERCA activity is due to the oxidation of sulfhydryl groups in the SR membrane[16,19]. In addition, these modified sulfhydryl groups also lead to the depression of Na^{+}-K^{+} ATPase, Na^{+}-Ca^{2+} exchange and voltage-operated Ca^{2+} channels because sulfhydryl groups modulate the activity of these membrane-bound-ion-transporting systems[32,40].

ROS such as hydrogen peroxide, superoxide, peroxynitrite and hydroxyl anions generation increases rapidly in myocardial I/R injury[32,40]. Studies show that hydrogen peroxide and superoxide anion damage the SERCA pump activity by oxidizing sulfhydryl groups in the SR membrane[16,17,32]. It should be noted that peroxynitrite can also nitrate tyrosine and thiol residues[5,68]. Several studies have been conducted on the cardiac muscle SR Ca^{2+}-ATPase (SERCA2a) demonstrating that the depression in SERCA2a pump activity is due to its nitration by $ONOO^{-}$[67,70]. The exposure of SR membranes to $ONOO^{-}$ in *in vitro* experiments selectively nitrates SERCA2a in the presence of excess SERCA1, and suggests that $ONOO^{-}$ may be one possible source as the nitrating agent *in vivo*[70,71]. No such studies have been done on coronary artery smooth muscle (SERCA2b) or endothelial (SERCA3) isoforms.

1.7 NITRIC OXIDE DONORS

1.7.1 Nitric Oxide

Nitric Oxide (NO) mediates various physiological and pathophysiological processes in the heart as discussed in Section 1.4.2. NO plays a key role in myocardial contractility by at least two biochemical mechanisms. First, NO binds to the heme site of soluble guanylyl cyclase (sGC) leading to the production of cGMP as discussed in detail in

Section 1.4.2. Second, NO can also react with sulfhydryl moieties on proteins. The transfer of a nitric oxide group to cysteine sulfhydryls and tyrosine residues is known as S-nitrosylation and O-nitrosylation respectively[8,66]. It has been shown *in vitro* that protein nitrosylation activates the L-type calcium channel and the ryanodine receptor that are involved in the regulation of myocardial contractility[73]. S-nitrosylation can influence many protein functions and thereby perturb enzymes, G-proteins, transcription factors, and ion-channels[8]. It has now been recognized that nitrosylation occurs *in vivo* for various proteins such as S-nitrosoalbumin and S-nitrosoglutathione[8,47].

Endothelial dysfunction in atherosclerosis, hypertension, heart failure, coronary artery disease and stroke leads to insufficient amounts of NO[44]. Insufficiency of NO could be due to deficit of NO synthesis, impaired availability of bioactive NO, or enhanced NO inactivation[43]. Thus, the replacement of endogenous NO by exogenously administered NO donors with a prolonged half-life are very useful tools for evaluating the important role of NO in cardiovascular physiology and pathology. There are two types of nitric oxide donors: those that spontaneously release NO in a solution and others that require enzymatic biotransformation *in vivo* to liberate NO. Several studies have concentrated on their synthesis, properties and reactions that lead to the formation of NO[48,56,59,60,61,65]. However, it has been impossible to measure the exact levels of NO in an aqueous solution. There are some assays available that measure the levels of nitrite and nitrate but they do not permit unambiguous resolution of whether the damage is due to NO or its other forms (nitrite and nitrate). There are various Nitric Oxide donors available commercially and will be discussed in detail in the following sections.

1.7.2 Spermine NONOate (Diazeniumdiolates)

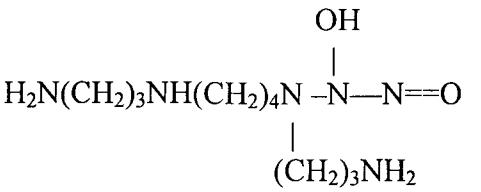


Figure 3: Structural formula of SP-NONOate

Diazeniumdiolates (NONOates) are a group of compounds that contain complexes of NO with nucleophilic adducts[29,48]. There are several NONOates available commercially in a solid form with varying rates of decomposition in aqueous solution ranging from releasing NO within a few seconds to generating NO over a period of hours. NONOates are capable of spontaneously releasing 2 NO radicals per molecule in solution and stimulating sGC in various biological systems[48]. A number of NONOates available commercially with different half-lives in aqueous solution are listed in Table 1. The choice of NONOate for a specific use is based on the required rate of release of NO and duration of its action[48].

Table 1: Range of NONOates with an approximate half-life in an aqueous solution.[48]

NONOates	Half-life in aqueous solution (pH 7)
DEA NONOate	2 minutes
SPER NONOate	39 minutes
DPTA NONOate	3 hours
DETA NONOate	20 hours

1.7.3 SNAP (S-Nitrosothiols)

S-Nitrosothiols are compounds that are green or red solids synthesized with the nitrosylation of reduced thiols and have a characteristic absorption peaks at 340 and 520nm[29,48]. The half-life of S-nitrosothiols varies from milliseconds to hours. S-nitrosothiols like S-nitroso-N-acetylypenicillamine (SNAP), S-nitroso-N-acetylcysteine (SNAC), and S-nitrosocysteine (SNOCys) decompose spontaneously in solution to their disulfide form and generate NO[29,48]. SNAP is one of the most common S-nitrosothiols used in the research experimental settings as shown in Figure 4. It has been suggested that SNAP mediates vasodilation and platelet aggregation by activation of sGC. However, the exact mechanism of NO release from SNAP is unknown.

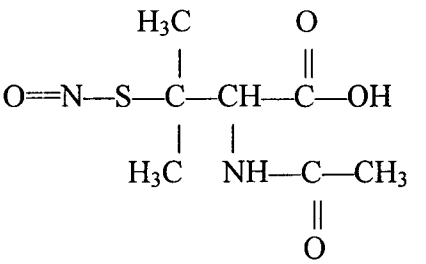


Figure 4: Structural formula of S-nitroso-N-acetylypenicillamine (SNAP)

1.7.4 Sodium Nitroprusside (SNP)

Sodium Nitroprusside (Figure 5) decomposes spontaneously to NO when exposed to light. It is therefore very important to protect SNP from light prior to administration in order to avoid release of NO[63,48]. SNP has vasodilator effects on smooth muscle but the mechanism of its action is not yet clear. However it is suggested that vasodilation exerted by SNP is mediated by generation of NO through cGMP-dependent and cGMP-independent mechanisms[63,48]. SNP is used as a common NO donor drug in many clinical and basic research studies despite the above-mentioned limitations.

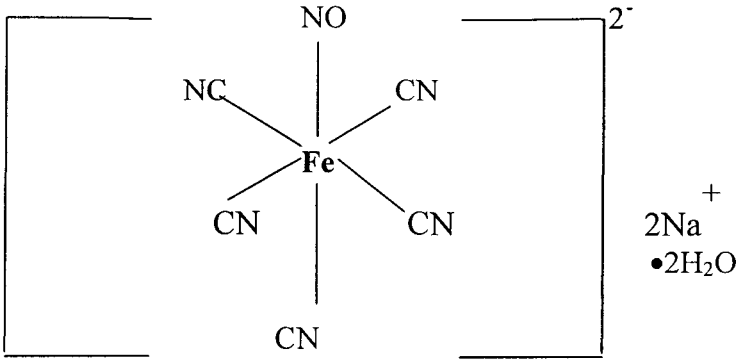


Figure 5: Structural Formula of Sodium Nitroprusside.

1.7.5 3-morpholinosydnonimine, hydrochloride (SIN-1)

SIN-1 is a zwitterionic compound derived from molsidomine by combining morpholine and a sydnonimine (Figure 6)[27,48,54,65]. SIN-1 is commonly used in the laboratory as a peroxynitrite donor because it decomposes spontaneously at physiological pH to NO and superoxide anion[27,48]. SIN-1 causes vascular relaxation and inhibits platelet activation but the exact mechanism is not clear. SIN-1 has also been shown in experimental models to protect against thrombotic occlusion in blood vessels after balloon angioplasty. It also inhibits oxidation of low-density lipoprotein and smooth muscle proliferation[48]. It is believed that SIN-1 increases cGMP levels but the extent of the elevation of cGMP levels cannot be measured. It is also not possible to measure the exact levels of NO or superoxide anion or peroxynitrite generated from SIN-1. There are some assays available such as Griess Reactions that can measure the levels of nitrite and nitrate by spectrometry. Nitrite and nitrate are the by-products of both NO and $ONOO^-$. However, it is difficult to quantitate the levels of NO, and $ONOO^-$. Nitrotyrosine is a bio-marker for the formation of peroxynitrite *in vitro* and *in vivo*[25]. There are some nitrotyrosine antibodies available commercially. But these antibodies are also limited for use depending on the tissue and protein available for nitrosation.

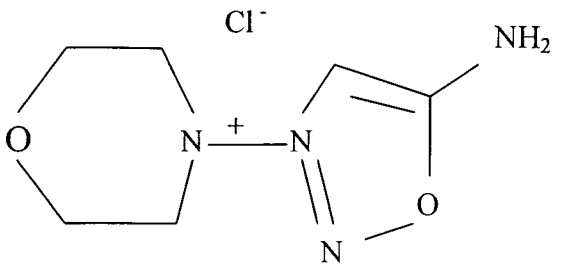


Figure 6: Structural Formula of 3-morpholinosydnonimine, hydrochloride (SIN-1)

1.7.5 Nitric Oxide Antagonist

Nitric Oxide antagonists are molecules that can be used *in vitro* and *in vivo* to inhibit the physiological effects mediated by NO[48]. Carboxy-PTIO (CPTIO) is a water-soluble blue solid that reacts with nitric oxide to form carboxy-PTI derivatives[48]. It is therefore used in the laboratory to scavange NO in various biological systems. Another molecule called 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) is a potent and selective inhibitor of soluble guanylate cyclase (sGC)[64]. As mentioned in Section 1.4.2, NO mediates vasodilation by binding to the heme group of sGC and thus catalyzing the formation of cGMP from GTP. ODQ reduces sGC activity by irreversibly oxidizing the ferrous form of the enzyme to its ferric form[64]. The ferric form of sGC enzyme is suggested to exhibit poor NO sensitivity[64]. Therefore, ODQ is a valuable tool to study the mechanism of action of NO-mediated signal transduction in various experimental models.

1.8 OBJECTIVES OF THE STUDY

NO production increases rapidly during acute reperfusion and also the production of superoxide anion is greatly enhanced in the endothelial cells[6,40]. It is likely that $ONOO^-$ is produced in large amounts in the endothelium during myocardial I/R injury. It was also shown that the generation of $ONOO^-$ was prevented by inhibition of eNOS or by removal of superoxide anion by superoxide dismutase[6]. However, the effect of $ONOO^-$ and NO on smooth muscle contractility has not been studied and detailed knowledge of the mechanisms involved in damage to vascular smooth muscle cells is still lacking. **In order to better understand the mechanism involved in perturbing Ca^{2+} homeostasis by peroxynitrite and nitric oxide, this proposed research will examine the effects of $ONOO^-$ and nitric oxide donors on smooth muscle contractility and also determine the possible mechanisms of damage to smooth muscle contractility.** In this study, organ bath studies were conducted using pig coronary arteries in order to determine:

1) peroxynitrite effects on smooth muscle contraction.
2) whether catalase and superoxide dismutase prevent the damage by $ONOO^-$
3) the effect of nitric oxide donors such as SNAP, SNP and SP-NONOate and the peroxynitrite donor (SIN-1) on smooth muscle contraction.

It was expected that increasing concentrations of peroxynitrite would lead to inhibition of CPA-induced contractions and this inhibition will not be due to its by-products (hydrogen peroxide, superoxide anion and NO). Also it was hypothesized that nitric oxide donors (SNAP, SNP and SP-NONOate) will be less damaging to smooth muscle contractility as compared to peroxynitrite donor (SIN-1).

CHAPTER 2: METHODS

Pig hearts were obtained from a Burlington slaughterhouse in the morning on each day of the experiment. The hearts were immediately placed in an ice-cold physiological saline solution consisted of 138mM NaCl, 2mM $CaCl_2$, 10mM glucose, 10mM HEPES, 5mM KCl, 1mM $MgCl_2$ at pH 6.4. The left descending arteries were dissected from two pig hearts. Coronary arteries were dissected in Kreb's solution by removing the connective tissue and fat. The Kreb's solution consisted of 115mM NaCl, 5mM KCl, 22mM $NaHCO_3$, 1.1mM $MgCl_2$, 1.7mM $CaCl_2$, 1.1mM KH_2PO_4, 0.03mM EDTA and 7.7mM glucose. The endothelium was removed from all the arteries and then four rings of approximately 3mm in diameter were taken from the middle section of each artery. The artery rings were hung in an organ bath to measure contractility.

2.1 Materials

Peroxynitrite was obtained from Calbiochem dissoleve in 0.1N NaOH. It was aliquoted in small volumes under compressed nitrogen gas and stored in -80^0C freezer. One aliquot of peroxynitrite was thawed on ice just before its use in an experiment. Peroxynitrite was assayed using spectrometer at absorbance 302 mm with concentrated HCl. 0.1N NaOH was used as a blank. SIN-1, SNAP, SNP, SP-NONOate, ODQ, CPA, catalase, superoxide dismutase and CPTIO were obtained from Sigma Chemical Company (St. Louis, MO). SNP and SP-NONOate were diluted in 0.1N NaOH just before the experiment so that dissociation of NO would take place in the organ bath. NaCl, $CaCl_2$, glucose, HEPES, KCl, $MgCl_2$, $NaHCO_3$, KH_2PO_4 and EDTA were obtained from McMaster Scientific Stores. 95% O_2 – 5% CO_2 tanks were obtained from Vital Air

(Mississauga, ON). Surgical thread was obtained from McMaster Scientific Stores that was black braided (4-0 silk), non-sterile and non absorbable.

2.2 Contractility Measurements

The organ baths were attached to a polygraph machine and all the pen deflections caused by the change in the tension of the force transducer were recorded in the chart. At the beginning of each experiment, the machine was balanced and then calibrated with a 0.5 g weight.

The artery ring with approximately 3mm size was placed between two steel hooks that were suspended vertically in the organ bath. The hooks were then placed in a 5ml organ bath containing 4ml Kreb's solution at 37^0C during the course of the experiment by a circulating water bath. The organ baths were also bubbled with $95\%O_2 - 5\%CO_2$ at all times during the experiment. The upper hook was attached to the force transducer and the lower hook was held by a tissue holder clamped in place. A tension of 3g was applied to each tissue and was then allowed to rest for 30 minutes. The tension was corrected to 3g and the tissue was allowed to rest for 30 minutes. This amount of total tension has been determined previously.[20] The tissues were depolarized with 3M KCl in order to give 60mM final concentration for 20 minutes. This concentration has been known to give maximal response[20]. The tissues were then washed with normal Krebs' five times for 10 minutes. The tissues were depolarized with 3M KCl in order to give 30mM final concentration for 20 minutes. The tissues were then washed with normal Krebs' five times for 10 minutes, and they were allowed to equilibrate for 15 minutes. The rings were then treated with varying concentrations of peroxynitrite, SIN-1, SNAP, SP-NONOate and SNP for 30 minutes. The tissues were then washed for four times for

45 to 60 minutes with normal Krebs' solution. The rings were then depolarized with 30mM KCl again and both peak and steady state contractions were measured. The rings were then again washed four times and were allowed to equilibrate for 30 minutes. The rings were then treated with 10μM CPA. The tissues were taken off the hooks, blotted and weighed at the end of the experiment. The average weight of the artery rings was determined to be 5mg.

2.3 Statistical Analysis

The statistical analysis for this study included calculation of means of all values of force of contraction, the standard errors in these means, and the t-test values in order to compare the mean differences among different treatment groups. The analysis of the data was done in three different ways. The analysis of only mN data was graphed in order to compare the data visually. The means and the standard errors were calculated using Lotus 1-2-3 Release 4 (Lotus Development Corp., USA). The graphs were drawn using Fig-P (The Scientific Fig. Processor version 1.2c, Fig. P Software Corp., USA). And the t-test values were obtained by using one-way ANOVA test (Minitab Software).

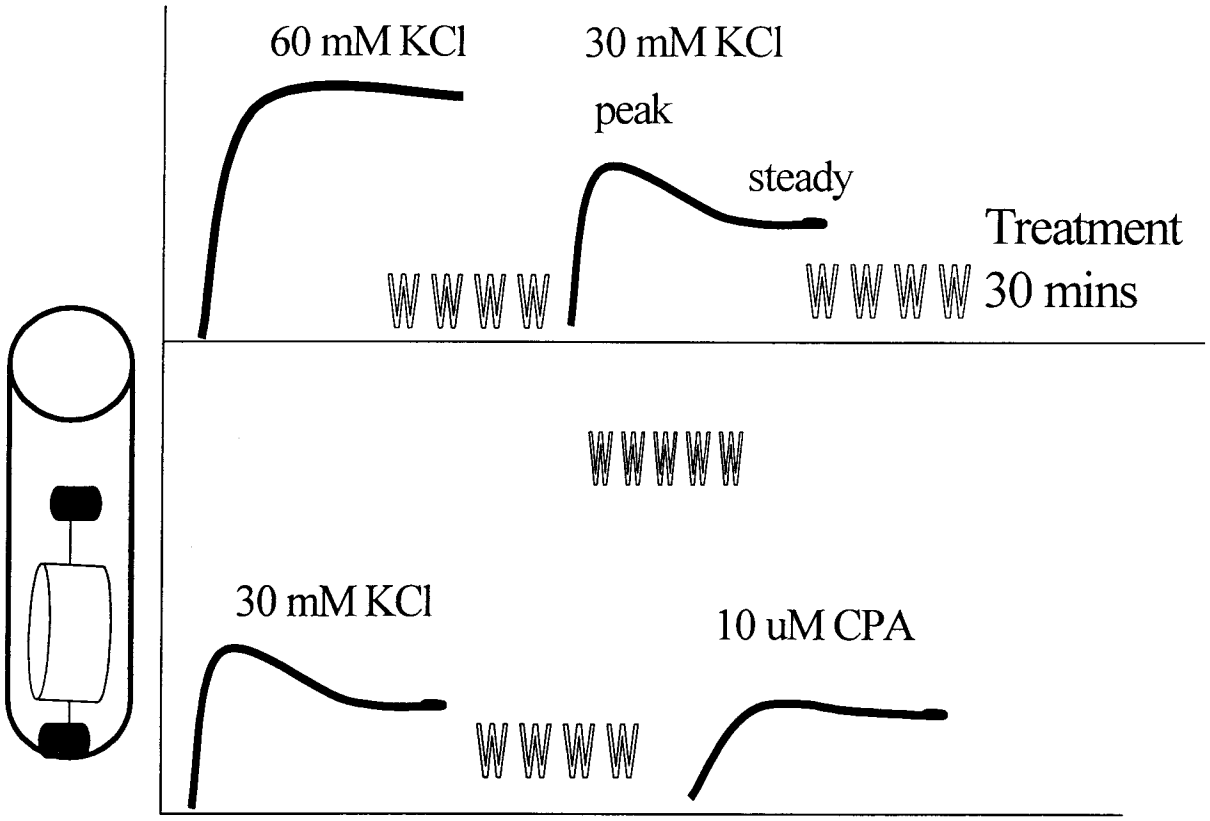


Figure 7: Timeline for a Typical Contractility Experiment. De-endothelialized 3mm artery rings were hung in an organ bath. A tension of 3g and then upto 3g was applied to each tissue. The tissues were then depolarized with 60 and 30mM KCl. Each W in the above figure represents 5 minutes wash after specified treatment. 30mM KCl produced a peak and a steady state contraction as shown in the figure.

CHAPTER 3: RESULTS

My Hypothesis is that pre-treating with increasing concentrations of peroxynitrite will decrease the ability of the coronary artery smooth muscle to contract with CPA and this decrease is not mediated by hydrogen peroxide or superoxide anion formed upon dissociation of peroxynitrite. In order to test this hypothesis, contractility experiments on de-endothelialized coronary artery rings were conducted in an organ bath. This allowed direct examination of peroxynitrite actions on smooth muscle without the intervention of endothelium. The effects of peroxynitrite, NaOH, nitric oxide donors and peroxynitrite donors on smooth muscle contraction were determined in these experiments.

3.1 Effects of Peroxynitrite.

Contractility experiments were performed in order to determine the effects of $ONOO^-$ pretreatment on smooth muscle contraction. The timeline in Chapter 2 shows a typical tracing of a contractility experiment. Since the contractions of the artery are known to vary from orifice to the tapered end,[50] each artery ring was consistently cut from the middle portion of the artery and hung in an organ bath as explained in chapter 2. SERCA and PMCA pump are important components in Ca^{2+} mobilization of a cell as explained in chapter 1. SERCA is more susceptible to damage by ROS than PMCA. However, the effects of $ONOO^-$ are not known on smooth muscle contraction. In contractility experiments, KCl and CPA contractions were determined. KCl produces a contraction by depolarizing the membrane and opening voltage operated calcium channels. The first contraction was induced by depolarizing the membrane with 60 mM KCl. It was previously found that 60 mM KCl gives a maximum contraction. Thus, the force of contraction for this concentration of KCl confirmed that the tissues are healthy

and alive. The second contraction was induced with 30 mM KCl that produced a sub-maximum contraction. The artery rings were treated with peroxynitrite or NaOH for 30 minutes and were then washed four times to wash all of peroxynitrite or NaOH. 30 mM KCl and 10 μM CPA-induced contractions were then measured. 30 mM KCl response produced peak and steady contractions and both components of the contraction were recorded. CPA inhibits SERCA pump and produces contraction. This protocol allowed for monitoring the effects of peroxynitrite-induced damage to the tissues by eliminating the possibility that peroxynitrite or NaOH could react directly with the contracting agents such as CPA. The force of contraction for KCl and CPA was measured by using two methods of analysis: mN and mN/g tissue. The force of contraction with mN/g tissue showed the same pattern as the force of contraction with mN. The force of contraction produced by CPA as percentage of the KCl contraction in the same tissue was also found to be highly variable. Therefore, all the preceding sections show the results with only the force of contraction with mN.

3.1.1 Does treatment of the coronary artery rings with $ONOO^-$ and NaOH in an organ bath affect the ability of the arteries to contract with CPA & KCl?

SERCA is more susceptible to ROS than PMCA (see chapter 1 for details). It is therefore expected that $ONOO^-$ treatment would be more damaging to CPA contractions. In fact, Figure 8A shows that CPA contraction was lowered when the artery rings were treated with varying concentrations of $ONOO^-$. The mean ± SEM value for non-treated artery rings was determined to be 5.0 ± 0.6 mN. This value of force of contraction was reduced to 3.1 ± 0.5 mN, 2.5 ± 0.8 mN, 1.4 ± 0.3 mN and 0.0 mN for 50, 100, 200 and

500 μM $ONOO^-$ respectively. Artery rings treated with 50, 100, 200 and 500μM $ONOO^-$ produced a significant ($p<0.05$) decrease in CPA contraction.

NaOH treatment was used in this experiment as a control. $ONOO^-$ was commercially available dissolved in 0.1M NaOH. $ONOO^-$ is stable only at basic pH. Therefore, NaOH treatment was used in parallel with $ONOO^-$ treatments to ensure that the inhibition was not due to NaOH. Figure 8B shows CPA-induced contraction after artery rings treated with different concentrations of NaOH. The mean ± SEM value for the untreated artery rings was determined to be 5.0 ± 0.6mN. This value of force of contraction did not change significantly ($p>0.05$) when the artery rings were treated with 250, 500, 1000 and 2500μM NaOH. Thus NaOH had no effect on CPA-induced force of contraction.

Figure 9A shows the force of contraction produced with 30mM KCl after the artery rings were bathed in an organ bath with $ONOO^-$and washed with Krebs solution. 30mM KCl response produced a peak and steady contractions as explained in Methods. The mean ± SEM value for non-treated artery rings was determined to be 26.2 ± 1.5mN for peak contraction and 24.7 ± 1.42mN for steady contraction. This value of force of contraction for peak and steady contractions for artery rings treated with $ONOO^-$did not differ significantly ($p>0.05$) from those of untreated group. Figure 9B shows that varying concentrations of NaOH had no effect on 30mM KCl-induced contraction. The mean ± SEM value for untreated artery rings was determined to be 26.2 ± 1.5mN for peak contraction and 24.7 ± 1.4mN for steady contraction. When the tissues were treated with 250, 500, 1000 and 2500μM NaOH, this value was not significantly ($p>0.05$) different.

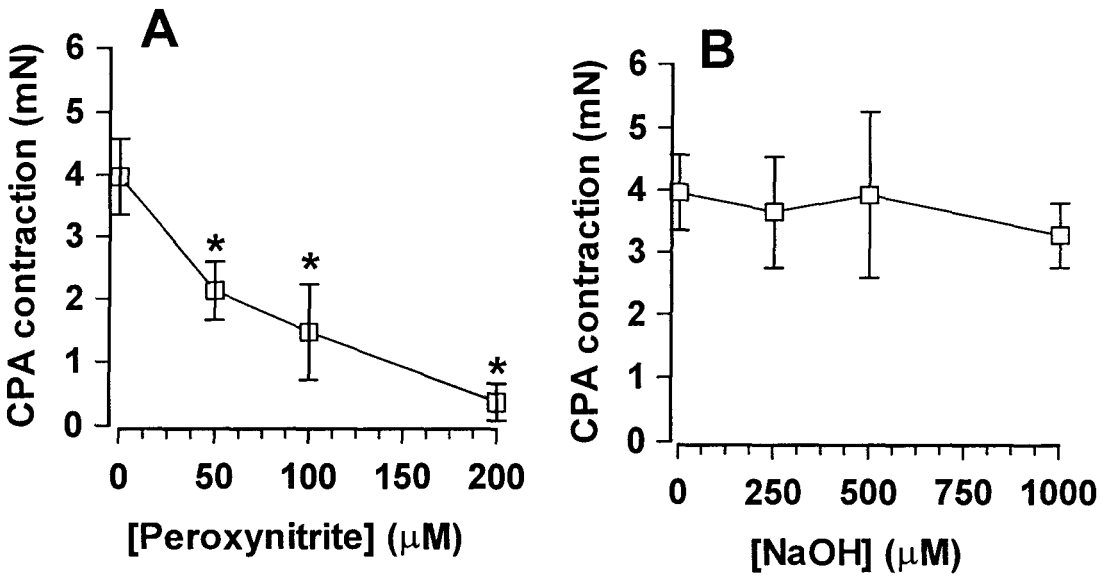


Figure 8: CPA-induced contraction of the coronary artery rings treated with different concentrations of $ONOO^-$ and NaOH.

The graph shows 10 μM CPA-induced contraction on a 3mm artery ring hung in an organ bath after treated with specified concentrations of $ONOO^-$ and NaOH. The values shown are mean ± SEM of 51, 19, 20, 18 and 5 replicates for 0, 50, 100, 200 and 500μM $ONOO^-$ and 51, 20, 17, 26 and 4 replicates for 0, 250, 500, 1000 and 2500μM NaOH respectively. In graph A, "*" indicates that this value is significantly ($p<0.05$) different from those of the untreated group. In graph B, NaOH concentrations do not differ significantly ($p<0.05$) from those of 0μM NaOH.

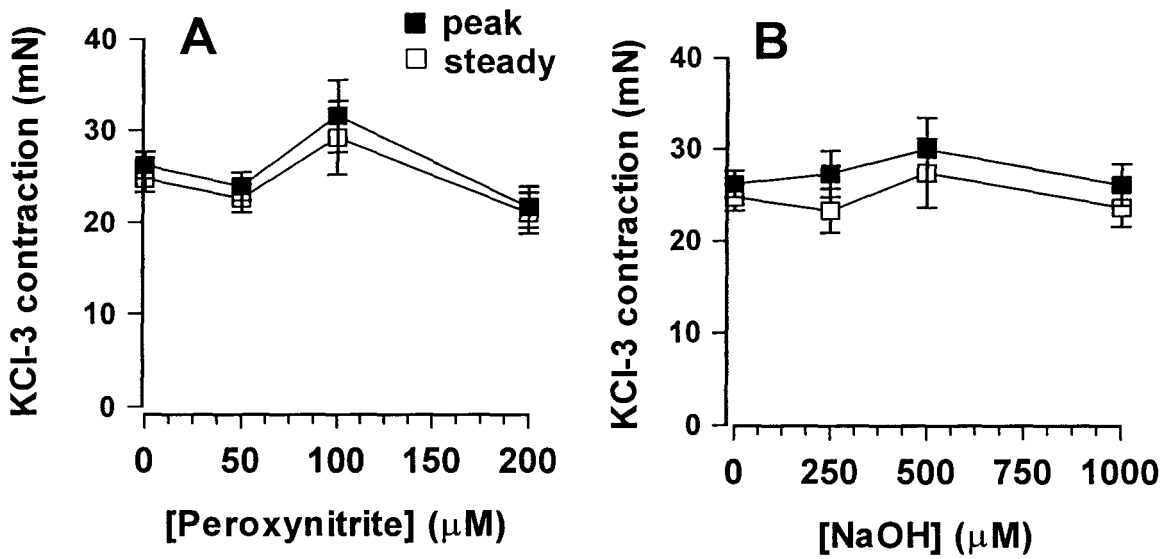


Figure 9: KCl-induced contraction of coronary artery rings treated with various concentrations of $ONOO^-$ and NaOH.

The graph shows 30 mM KCl-induced contraction on a 3mm ring hung in an organ bath after treatment with specified concentrations of $ONOO^-$ and NaOH. The KCl response produced peak and steady state contractions. The values shown are mean $\pm$ SEM of 51, 19, 20, 18 and 5 replicates for 0, 50, 100, 200 and 500μM $ONOO^-$ and 51, 20, 17, 26 and 4 replicates for 0, 250, 500, 1000 and 2500μM NaOH. In graph A and B, the value for force of contraction for treated artery rings is not significantly ($p>0.05$) different from those of the untreated group. Therefore, $ONOO^-$ and NaOH had no effect on KCl contractions.

3.1.2 Does treatment of coronary artery rings with CAT and SOD prevent the inhibition caused by 200 μM $ONOO^-$ on CPA-induced contraction?

Peroxynitrite dissociates rapidly into $O_2^{\cdot-}$ and H_2O_2 in buffered solution. Catalase and superoxide dismutase were used in this experiment in order to test if $ONOO^-$ or its dissociated products in a solution caused the inhibition to CPA-induced contractions in Figure 8A. 200μM $ONOO^-$ showed approximately 70% inhibition of CPA contraction in Figure 8A. This concentration was chosen in this experiment so that any prevention to the inhibition of CPA contraction would be observable. The artery rings were untreated, treated with 200 μM $ONOO^-$ or with 200 units of CAT + 200 units of SOD + 200 μM $ONOO^-$ for 30 minutes as shown in the timeline in the Methods. 200 units of CAT + 200 units of SOD 200 units of CAT + 200 units of SOD were added to the Krebs solution before adding 200 μM $ONOO^-$ so that if all of peroxynitrite dissociated to $O_2^{\cdot-}$ or H_2O_2, this specified amount of CAT + SOD would be sufficient to scavenge it. The artery rings were then washed with normal Krebs solution.

The mean ± SEM value of the CPA contraction for untreated artery rings was determined to be 4.2 ± 0.7 mN and was reduced to 1.6 ± 0.3 mN for 200 μM $ONOO^-$ and 2.0 ± 0.3 mN for CAT + SOD + 200 μM $ONOO^-$as shown in Figure 10. Thus treatment with 200 μM $ONOO^-$ or CAT + SOD + 200 μM $ONOO^-$ produced a significant ($p<0.05$) decrease in force of contraction as compared to the control. But there was no significant ($p>0.05$) difference between artery rings treated with 200 μM $ONOO^-$ and CAT + SOD + 200 μM $ONOO^-$. Treatment with CAT and SOD did not prevent the inhibition to CPA-induced contraction caused by 200 μM $ONOO^-$. Therefore, peroxynitrite and not its dissociated products caused the inhibition to CPA-induced contraction.

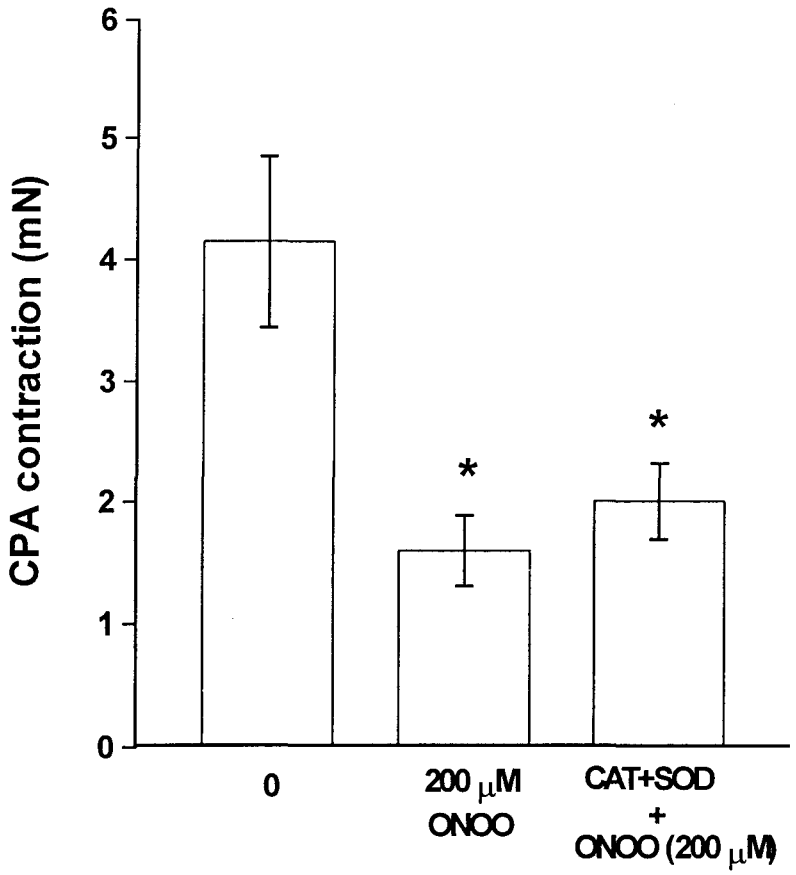


Figure 10: CPA-induced contraction of the coronary artery rings treated with $ONOO^-$ and catalase (CAT) and superoxide dismutase (SOD).

The graph shows 10μM CPA-induced contraction on a 3mm ring hung in an organ bath. The values shown are mean ± SEM of 20, 19, and 25 replicates for 0, 200μM $ONOO^-$ and 200μM $ONOO^-$ + CAT +SOD respectively. The treated groups with * indicates that they differed significantly ($p<0.05$) from those of control but there was no significant difference between 200μM $ONOO^-$ alone and CAT + SOD + 200μM $ONOO^-$.

3.2 Effects of Peroxynitrite/Nitric Oxide donors.

Nitiric oxide donors were used in this experiment so that nitric oxide and peroxynitrite could be generated continuously for 30 minutes. These donors have been reported to produce peroxynitrite and nitric oxide in different proportions. It was shown in Section 3.1 that $ONOO^-$ was added as a bolus to the organ baths that probably dissociated in less than a minute. Therefore in order to test the effects of continuously generated peroxynitrite and nitric oxide, coronary artery rings were treated with $ONOO^-$ donor (SIN-1) and different nitric oxide donors such as SNAP, SNP, and SP-NONOate for 30 minutes. In initial experiments, concentrations of each donor were kept constant at 200 μM in order to compare their effects with $ONOO^-$. The tissues were washed four times after the treatment and 30 mM KCl and 10 μM CPA contractions were then determined (see Timeline). 30 mM KCl response produced peak and steady contractions and both components of the contraction were recorded. But steady contractions followed the same pattern as peak contraction and therefore, only peak contraction is shown in the preceding sections for simplicity.

3.2.1 Does treatment of the coronary artery rings with different nitric oxide donors and $ONOO^-$ donor affect the ability of the artery rings to contract with KCl and CPA?

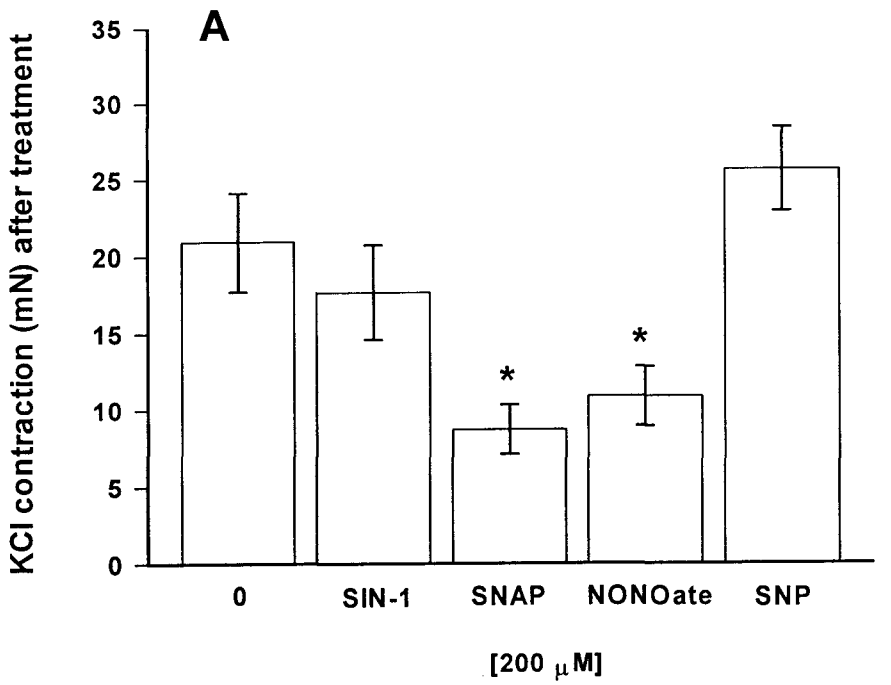
Figure 11A shows 30 mM KCl-induced contraction with 200 μM SIN-1, SNAP, SP-NONOate and SNP. The mean ± SEM value for untreated artery rings were determined to be 20.9 ± 3.2 mN. This value of force of contraction was reduced to 17.7 ± 3.0 mN for SIN-1, 8.7 ± 1.6 mN for SNAP, 10.9 ± 1.9 mN for SP-NONOate and 24.9 ± 3.6 mN for SNP. There was a significant ($p<0.05$) decrease in the force of contraction of artery rings treated with 200 μM SNAP and SP-NONOate as compared to the untreated

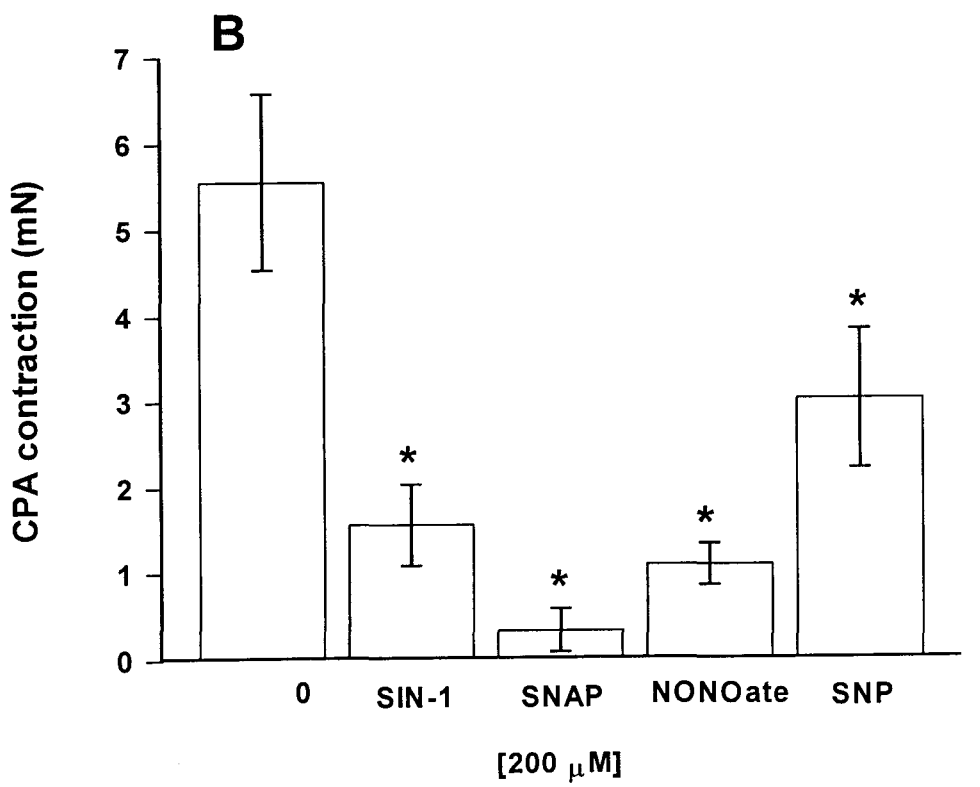
rings. But there was no significant ($p>0.05$) difference between the untreated artery rings and those treated with 200 μM SIN-1 and SNP. Therefore, SNAP and SP-NONOate are most damaging to KCl contraction.

Figure 11B shows 10 μM CPA-induced contraction on artery rings treated with 200 μM SIN-1, SNAP, SP-NONOate and SNP. The mean ± SEM value for untreated artery rings was determined to be 5.6 ± 1.0 mN. This value of force of contraction was reduced to 1.6 ± 0.5 mN for SIN-1, 0.4 ± 0.3 mN for SNAP, 1.1 ± 0.2 mN for SP-NONOate and 3.2 ± 1.2 mN for SNP. There was a significant ($p<0.05$) decrease in the force of contraction of artery rings treated with 200 μM SIN-1, SNAP, SP-NONOate and SNP as compared to the untreated artery rings. Thus, all the nitric oxide donors mentioned above inhibit CPA contraction but each nitric oxide donor has a different potency.

Figure 11: KCl & CPA-induced contraction of the coronary artery rings treated with different nitric oxide donors.

30mM KCl-induced contraction and 10μM CPA-induced contraction is shown in graph A and B respectively on a 3mm ring hung in an organ bath. The values shown are mean ± SEM of 12, 11, 11 and 12 replicates for 0, 200μM SIN-1, SNAP, NONOate and SNP respectively. In graph A, SNAP & NONOate differed significantly ($p<0.05$) denoted by * from those of untreated group and in graph B, all the treated groups with * indicates significant difference ($p<0.05$) from those of the control. The rank order of effectiveness of inhibition of different nitric oxide donors to CPA contraction was SNAP>>NONOate>SIN-1>SNP.


A
KCl contraction (mN) after treatment
35
30
25
20
15
10
5
0
*
*
0
SIN-1
SNAP
NONOate
SNP
[200 μM]


B
CPA contraction (mN)
7
6
5
4
3
2
1
0
*
*
*
*
0
SIN-1
SNAP
NONOate
SNP
[200 μM]

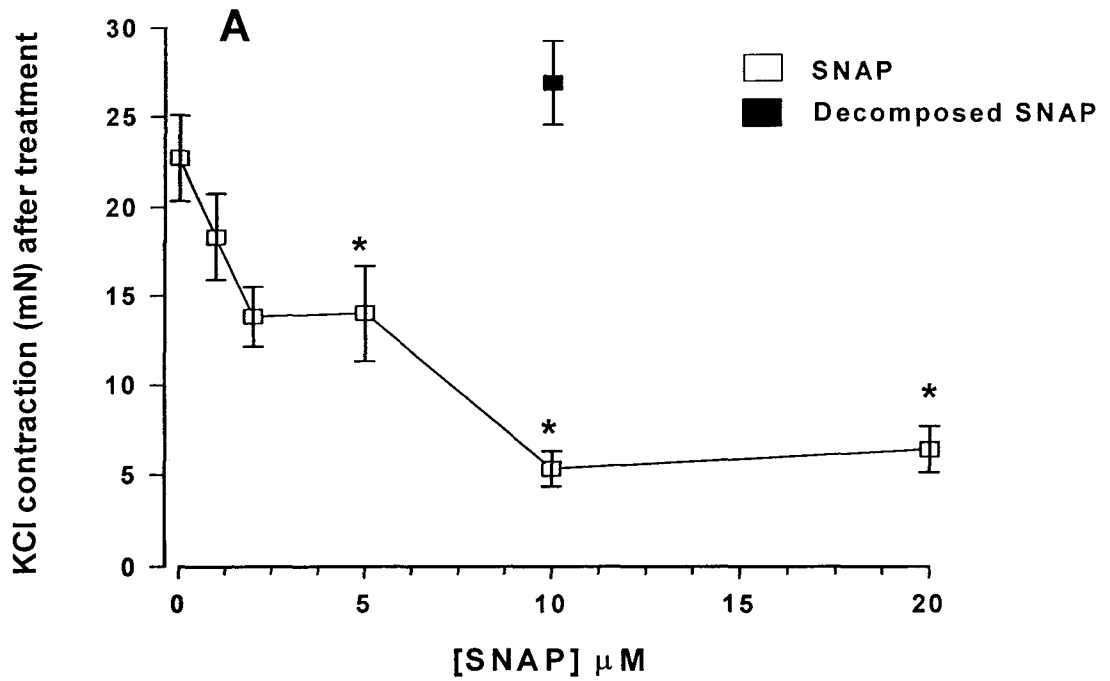
3.2.2 Concentration dependence of treatment of coronary artery rings with SNAP and SP-NONOate.

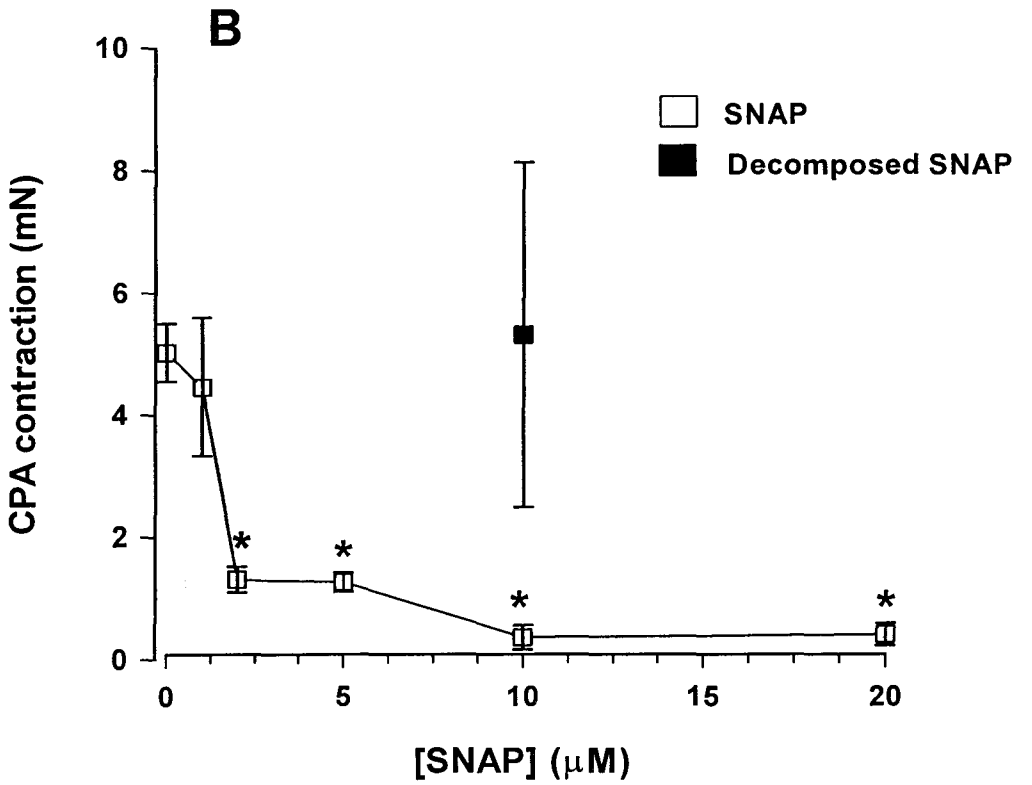
200 μM SNAP was the most potent nitric oxide donor in inhibiting KCl and CPA contractions as shown in Section 3.2.1. Figure 12A shows that when artery rings were pretreated with increasing concentrations of SNAP, the force of contraction with 30mM KCl was lowered. The mean ± SEM value for untreated artery rings was determined to be 22.7 ± 2.4mN. This value of force of contraction was reduced to 18.3 ± 2.4 mN, 13.8 ± 1.7mN, 14.0 ± 2.7mN, 5.4 ± 1.0mN, 6.4 ± 1.3mN, 7.1 ± 1.5mN and 7.9 ±1.1mN for 1, 2, 5, 10, and 20, SNAP respectively. There was a significant ($p<0.05$) difference in the force of contraction between untreated artery rings and treated artery rings with 2, 5, 10, 20, 50 and 100μM SNAP. But treatments with 1μM SNAP and decomposed SNAP did not produce a significant ($p>0.05$) decrease in the force of contraction.

Figure 12B shows 10μM CPA-induced contraction after the artery rings were treated with different concentrations of SNAP. The mean ± SEM value for the untreated artery rings was determined to be 5.0 ± 0.5mN. This value of force of contraction was reduced to 4.5 ±1.1mN, 1.3 ± 0.2mN, 1.3 ± 0.2mN, 0.4 ± 0.2mN, 0.4 ± 0.2mN, 0.5 ± 0.2mN and 0.2 ± 0.2mN for 1, 2, 5, 10, and 20, SNAP respectively. There is a significant ($p<0.05$) decrease in the contraction of the artery rings treated with 2, 5, 10, 20, 50 and 100μM SNAP as compared to the untreated artery rings. Treatment with 1μM SNAP and decomposed SNAP did not produce a significant ($p>0.05$) decrease in the force of contraction of CPA. Therefore, 10μM SNAP is as potent as 200μM SNAP.

Figure 12: Dose response curve for KCl & CPA-induced contractions of coronary artery rings treated with SNAP.

Graph A shows 30mM KCl-induced contraction and graph B shows 10μM CPA-induced contraction on a 3mm ring hung in an organ bath after treated with increasing concentrations of SNAP. The values for 2-20μM SNAP differed significantly ($p<0.05$) for both KCl and CPA contractions from those of 0μM SNAP. The values for 1μM SNAP for both graphs did not differ significantly ($p>0.05$) from those of 0μM SNAP. The values shown are mean ± SEM of 18, 13, 14, 17, 9, and 8 replicates for 0, 1, 2, 5, 10, and 20 μM SNAP.


A
KCl contraction (mN) after treatment
30
25
20
15
10
5
0
0
5
10
15
20
[SNAP] μM
SNAP
Decomposed SNAP
*
*
*

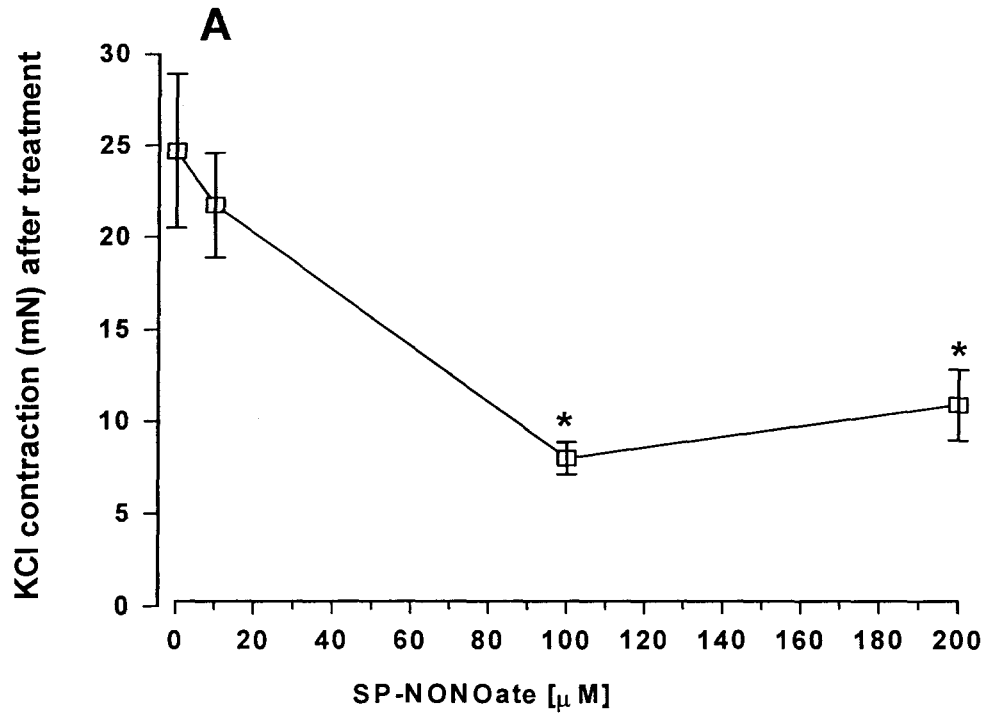

B
CPA contraction (mN)
10
8
6
4
2
0
0
5
10
15
20
[SNAP] (μM)
SNAP
Decomposed SNAP
*
*
*
*

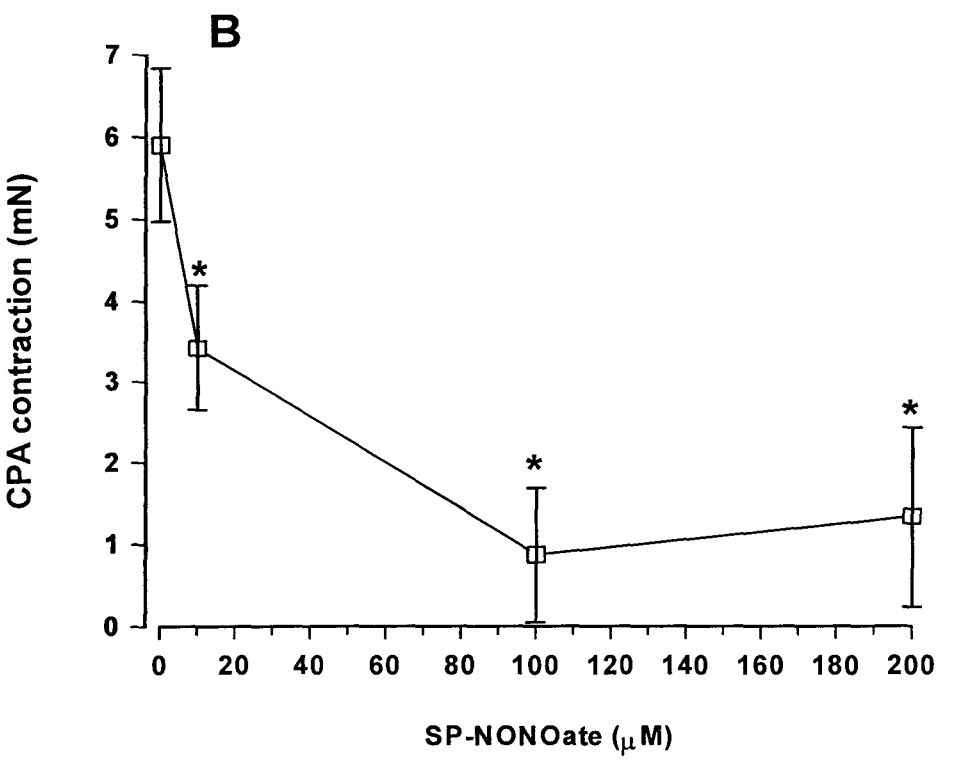
SP-NONOate was second most potent in inhibiting KCl and CPA contractions as shown in section 3.2.1. The effect of lower concentrations of SP-NONOate was measured in this experiment. Figure 13A shows that 30mM KCl-induced contraction was lowered when the artery rings in an organ bath were treated with 100 and 200μM SP-NONOate. The mean ± SEM value for untreated artery rings was determined to be 28.6 ± 3.3mN. This value of force of contraction was reduced to 21.7 ± 2.9mN, 8.0 ± 0.9mN, and 10.9 ± 1.9mN for 10, 100 and 200μM SP-NONOate respectively. There was a significant ($p<0.05$) decrease in the force of contraction of artery rings treated with 100 and 200μM SP-NONOate. But treatment of artery rings with 10 μM SP-NONOate did not produce a significant ($p>0.05$) difference as compared to the untreated artery rings.

Figure 13B shows that the coronary artery rings bathed in an organ bath with 10, 100 and 200μM SP-NONOate lowered the force of contraction produced by 10μM CPA. The mean ± SEM value for untreated artery rings was determined to be 5.3 ± 0.8mN. This value for force of contraction was reduced to 3.4 ± 0.8mN, 0.8 ± 0.2mN and 1.1 ± 0.3mN for artery rings treated with 10, 100 and 200μM SP-NONOate respectively. There was a significant ($p<0.05$) decrease in the artery rings treated with 10, 100 and 200μM SP-NONOate as compared to untreated artery rings. Therefore, 10μM SP-NONOate was not as potent as 100 or 200μM SP-NONOate.

Figure 13: KCl & CPA-induced contraction of the coronary artery rings treated with different concentrations of SP-NONOate.

The graph A and B show 30mM KCl & CPA-induced contraction respectively on a 3mm ring hung in an organ bath after treated with varying concentrations of SP-NONOate. The values shown are mean $\pm$ SEM of 9, 8, 13 and 11 replicates for 0, 10, 100, and 200μM SP-NONOate respectively. The values for 100 and 200μM SP-NONOate differed significantly ($p<0.05$) denoted by "*" from those of 0μM SP-NONOate in graph A but the values for 10μM SP-NONOate did not differ significantly ($p>0.05$). In graph B, all the treated groups differed significantly ($p<0.05$) indicated by "*" from those of the untreated group.


A
KCl contraction (mN) after treatment
30
25
20
15
10
5
0
0
20
40
60
80
100
120
140
160
180
200
SP-NONOate [μ M]
*
*
*


B
CPA contraction (mN)
7
6
5
4
3
2
1
0
0
20
40
60
80
100
120
140
160
180
200
SP-NONOate (μ M)
*
*
*

3.3 Does treatment of coronary artery rings with 10 μM ODQ in addition to peroxynitrite generating agents prevent the inhibition to KCl & CPA-induced contraction caused by SIN-1 or SNAP?

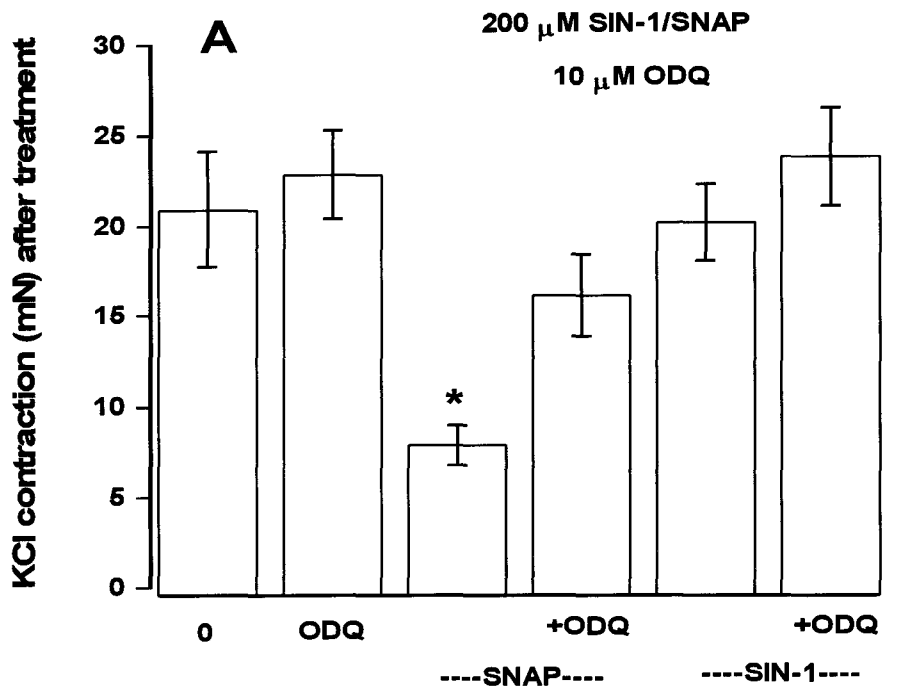
ODQ is a selective inhibitor of guanylate cyclase (see chapter 1 for details) and it can be used to determine if the action of nitric oxide is mediated by activating guanylate cyclase. ODQ was used in this experiment to determine if the inhibition to KCl & CPA-induced contractions was cGMP dependent or independent. ODQ antagonizes NO stimulation of sGC with an IC_{50} of 84 ± 15nM. Excess ODQ (10μM) was used so that all of GC in the tissue could be blocked. The effect of ODQ alone was also determined on KCl & CPA contractions. Figure 14A shows that 30mM KCl was not affected in coronary artery rings treated with 10μM ODQ alone. The mean ± SEM value for untreated artery rings was determined to be 20.9 ± 3.2mN and 22.8 ± 2.5mN for artery rings treated with 10μM ODQ. There was no significant difference ($p>0.05$) between the artery rings that were untreated and treated with 10μM ODQ. The force of contraction was reduced as compared to the control when coronary artery rings were pre-treated with 200μM SIN-1/SNAP ±10μM ODQ. The mean ± SEM for force of contraction decreased to 7.9 ± 1.1mN, 16.1 ± 2.3mN, 20.2 ± 2.1mN and 23.8 ± 2.7mN for artery rings treated with 200μM SNAP, 10μM ODQ + 200μM SNAP, 200μM SIN-1, and 10μM ODQ + 200μM SIN-1 respectively. Artery rings pre-treated with 200μM SNAP produced a significant ($p<0.05$) decrease in the force of contraction as compared to the untreated artery rings. Treatment with 10μM ODQ + 200μM SNAP, 200μM SIN-1, and 10μM ODQ + 200μM SIN-1 did not produce a significant difference in the force of contraction as compared to the untreated artery rings. There was also a significant ($p<0.05$)

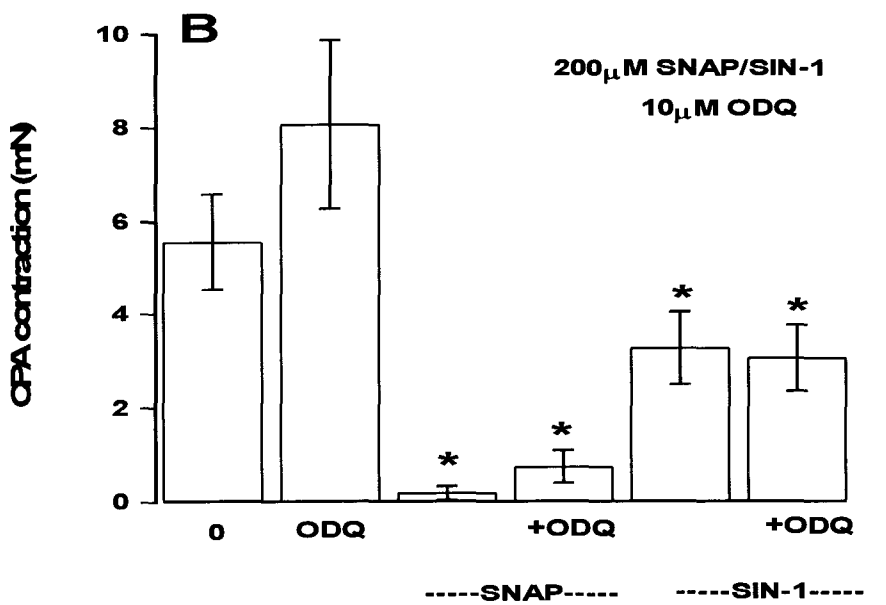
difference between artery rings treated with 200μM SNAP and 10μM ODQ + 200μM SNAP. This shows that ODQ prevented the inhibition caused by SNAP to KCl-induced contraction. Thus inhibition to KCl-induced contraction caused by SNAP is partially overcome by ODQ.

Figure 14B shows that CPA-induced contraction was not affected by pre-treatment of coronary artery rings with 10μM ODQ alone. The mean ± SEM value for force of contraction for the untreated artery rings was determined to be 5.6 ± 1.0mN and 8.1 ± 1.8mN for artery rings treated with 10μM ODQ. There was no significant ($p<0.05$) difference between untreated and treated artery rings with 10μM ODQ. Figure 14B also shows 10μM CPA-induced contraction after the coronary artery rings were treated with 200μM SNAP, 10μM ODQ + 200μM SNAP, 200μM SIN-1, and 10μM ODQ + 200μM SIN-1. The mean ± SEM value for force of contraction for untreated artery rings was determined to be 5.6 ± 1.0mN. This value for force of contraction was lowered to 0.2 ± 0.2mN, 0.8 ± 0.4mN, 3.2 ± 0.8mN and 1.9 ± 3.1mN for 200μM SNAP, 10μM ODQ + 200μM SNAP, 200μM SIN-1, and 10μM ODQ + 200μM SIN-1 respectively. There was a significant ($p<0.05$) decrease in the force of contraction with the artery rings that were untreated as compared to the treated artery rings. Therefore, pretreatment with ODQ prevented the inhibition caused by SNAP to KCl but not CPA contraction. And ODQ alone had no effect on KCl and CPA contractions. This shows that nitric oxide generated by peroxynitrite or nitric oxide generating agents does not inhibit CPA-induced contractions by activating guanylate cyclase. However, nitric oxide mediates inhibition to KCl-induced contractions by activating guanylate cyclase.

Figure 14: KCl & CPA-induced contractions of coronary artery rings treated with SNAP & SIN-1 with and without ODQ.

Graph A shows 30mM KCl-induced contraction with 10μM ODQ alone, 200μM SNAP or SIN-1 with & without 10μM ODQ. The values shown are mean ± SEM of 9 and 6 replicates for 0, and 10 μM ODQ respectively. The values for 10μM ODQ did not differ significantly ($p>0.05$) than those for 0μM ODQ. Graph B shows 10μM CPA-induced contraction after pre-treatment with 10μM ODQ alone, 200μM SNAP or SIN-1 with & without 10μM ODQ. The values for 200μM SNAP differed significantly ($p<0.05$) denoted by * from all the treated groups from those of untreated group. The values for 10μM ODQ did not differ significantly ($p>0.05$) than those for 0μM ODQ. The values for treated groups differed significantly ($p<0.05$) denoted by * from those of untreated group.

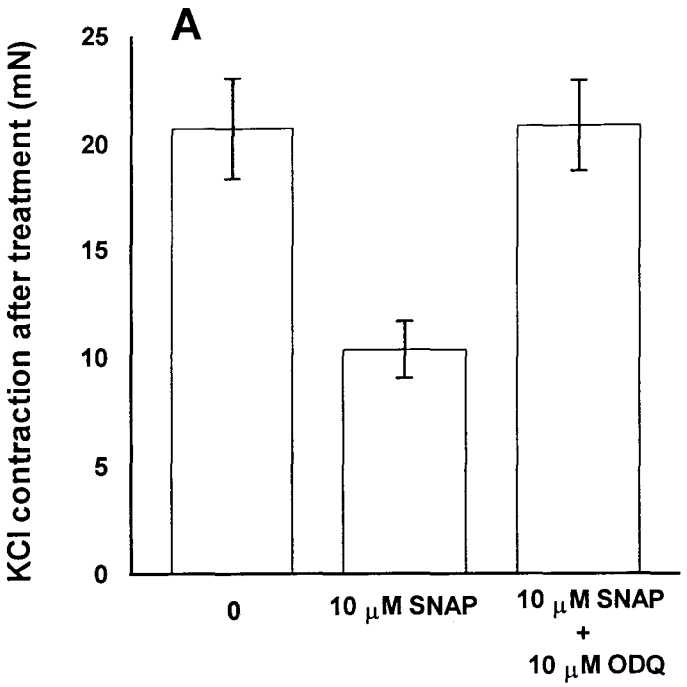

A
200 μM SIN-1/SNAP
10 μM ODQ
KCl contraction (mN) after treatment
30
25
20
15
10
5
0
*
0
ODQ
+ODQ
+ODQ
----SNAP----
----SIN-1----

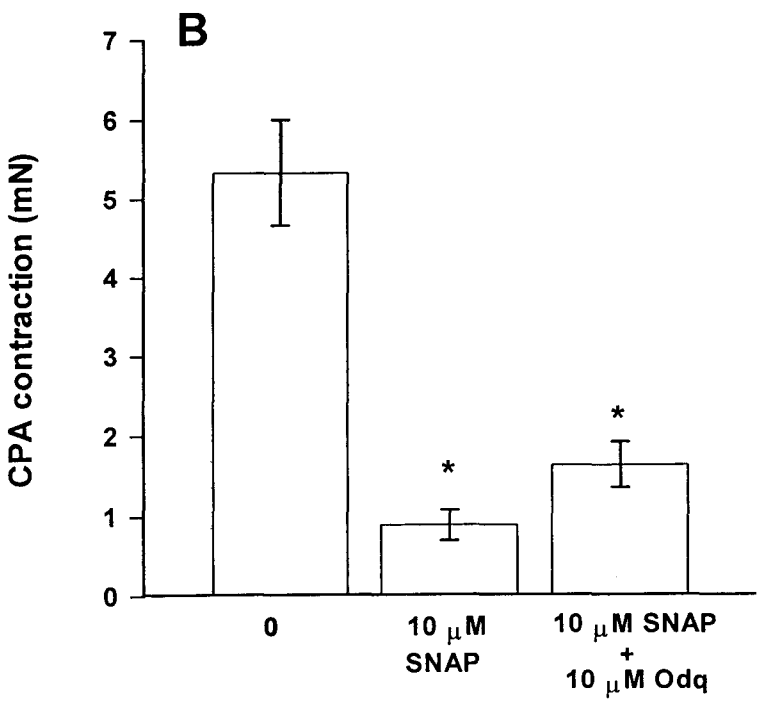

B
200μM SNAP/SIN-1
10μM ODQ
CPA contraction (mN)
10
8
6
4
2
0
*
*
*
*
0
ODQ
+ODQ
+ODQ
-----SNAP-----
-----SIN-1-----

Since QDQ prevented the inhibition to KCl contraction by 200μM SNAP. Figure 15A shows coronary artery rings in an organ bath treated with 10μM SNAP and 10μM ODQ +10μM SNAP. The mean ± SEM for 30mM KCl-induced contraction in the untreated artery rings was determined to be 20.7 ± 2.3mN. This value for force of contraction was decreased to 10.4 ± 1.3mN for 10μM SNAP. There was a significant ($p<0.05$) decrease in the force of contraction. However, the value for force of contraction for artery rings treated with 10μM ODQ +10μM SNAP did not produce a significant ($p>0.05$) difference as compared to the untreated artery rings. The value for force of contraction for 10μM ODQ +10μM SNAP was determined to be 20.8 ± 2.1mN. But there was a significant ($p<0.05$) difference between the artery rings treated with 10μM SNAP and those treated with 10μM ODQ +10μM SNAP. Figure 15B shows CPA-induced contraction after the artery rings were treated with 10μM ODQ +10μM SNAP and 10μM SNAP alone. The value for the mean ± SEM for the force of contraction for the untreated artery rings was determined to be 5.34 ± 0.7mN. This value was reduced to 0.9 ± 0.2mN and 1.7 ± 0.3mN for 10μM SNAP and 10μM ODQ +10μM SNAP respectively. There was a significant ($p<0.05$) decrease in the force of contraction from the untreated to treated arteries. And there was also a significant ($p<0.05$) difference between the artery rings treated with 10μM SNAP and those treated with 10μM ODQ +10μM SNAP. Therefore, the inhibition to KCl contraction was completely prevented and the inhibition to CPA contraction was partially prevented by 10μM SNAP.

Figure 15: KCl & CPA-induced contraction of coronary artery rings treated with SNAP with & without ODQ.

Graph A & B show KCl & CPA-induced contraction after treated with 10μM SNAP with & without 10μM ODQ. The values shown are mean ± SEM of 25, 19 and 21 replicates for 0, 10μM SNAP, and 10μM SNAP + 10μM ODQ respectively. The values for the treated groups denoted by * differed significantly ($p<0.05$) from those of untreated group in Graph B. But there was no significant difference ($p>0.05$) between the two treated groups in Graph B.


A
KCl contraction after treatment (mN)
25
20
15
10
5
0
0
10 μM SNAP
10 μM SNAP
+
10 μM ODQ


B
CPA contraction (mN)
7
6
5
4
3
2
1
0
*
*
0
10 μM
SNAP
10 μM SNAP
+
10 μM Odq

3.4 Effect of CPTIO on KCl and CPA-induced contractions prior to the addition of SNAP.

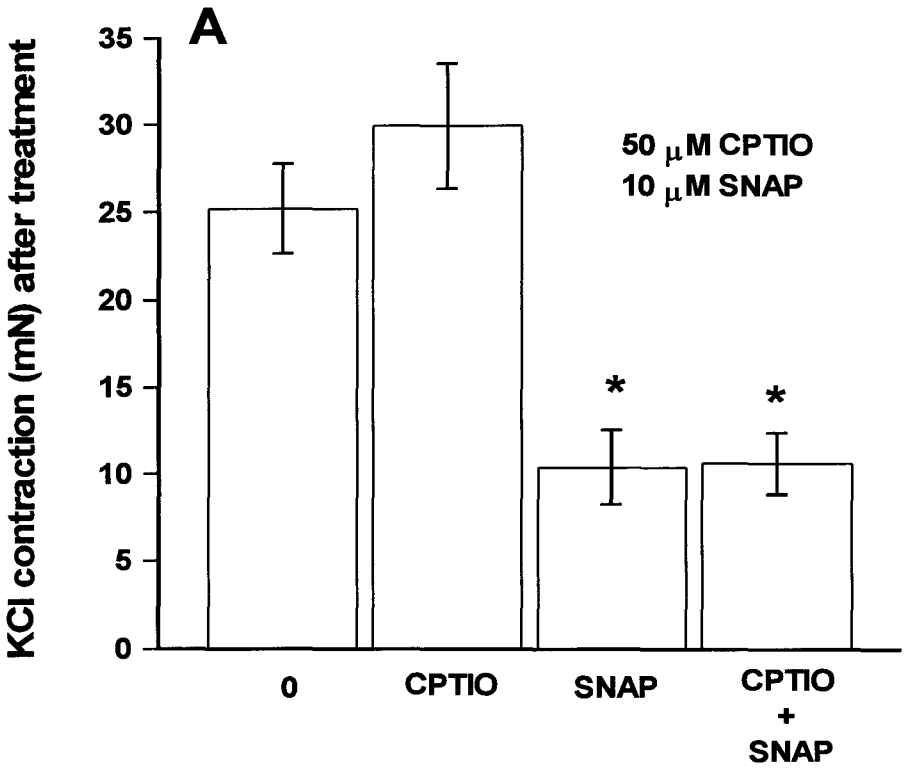
CPTIO is a NO scavenger (see chapter 1 for details). It was determined from previous sections that NO donors were more damaging to both KCl & CPA contractions than $ONOO^-$ donor. Therefore, in this experiment the effect of scavenged NO by CPTIO was determined on KCl & CPA contractions. Also the effect of CPTIO on contractile apparatus alone was measured. Figure 16A shows that treatment of 50μM CPTIO did not affect the ability of the coronary artery rings to contract with 30mM KCl. The mean ± SEM value for force of contraction for the untreated artery rings was determined to be 25.5 ± 2.8mN and 29.9 ± 3.6mN for treated rings. There was no significant ($p>0.05$) difference between the treated and untreated artery rings. Figure 16A also shows 30mM KCl-induced contraction when artery rings were treated with 50μM CPTIO +10μM SNAP or 10μM SNAP. The mean ± SEM value for the force of contraction for untreated artery rings was determined to be 25.5 ± 2.8mN. This value of force of contraction changed to 9.6 ± 2.2mN and 10.2 ± 2.7mN for the artery rings treated with 50μM CPTIO +10μM SNAP and 10μM SNAP respectively. There is a significant ($p<0.05$) decrease in the force of contraction of the treated artery rings as compared to the untreated artery rings. But there was no significant difference between the artery rings treated with 10μM SNAP or 50μM CPTIO +10μM SNAP.

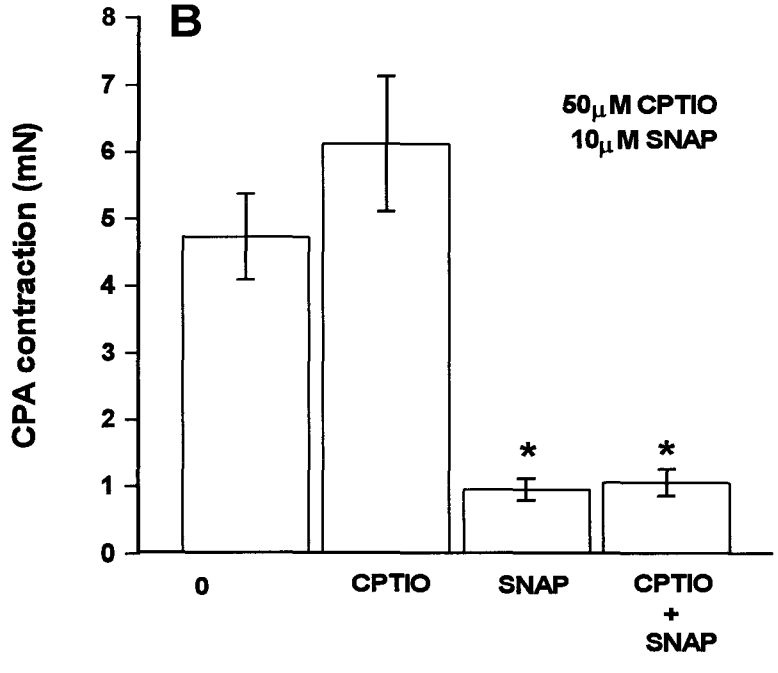
Figure 16B shows CPA-induced contraction when artery rings were treated with 50μM CPTIO. The mean ± SEM value for force of contraction was determined to be 3.8 ± 0.7mN for untreated artery rings and 6.1 ± 1.0mN for treated artery rings. There was no significant ($p>0.05$) difference between the treated and untreated artery rings. Figure

16B also shows CPA-induced contractions of the artery rings treated with 10μM SNAP or 50μM CPTIO +10μM SNAP. The mean ± SEM value for the force of contraction of the untreated artery rings was determined to be 3.8 ± 0.7mN. This force of contraction was reduced to 1.0 ± 0.2mN and 1.7 ± 0.5mN for artery rings treated with 10μM SNAP or 50μM CPTIO +10μM SNAP respectively. SNAP treatment produced a significant ($p<0.05$) but inclusion of CPTIO did not affect the inhibition ($p>0.05$) difference between the treated inhibition artery rings. Therefore, scavenging NO from SNAP by adding it to the buffered solution before treatment of SNAP did not prevent the damage to KCl & CPA contractions.

Figure 16: KCl & CPA-induced contraction of coronary artery rings treated with SNAP with & without CPTIO.

Graph A shows 30mM KCl-induced contraction with 50μM CPTIO alone, and with 10μM SNAP with & without 50μM CPTIO. The values for 50μM CPTIO did not differ significantly ($p>0.05$) than those for 0μM CPTIO. And there was no significant difference between the arteries pre-treated with 10μM SNAP with & without 50μM CPTIO. Graph B shows 10μM CPA-induced contraction after treated with 10μM SNAP with & without 50μM CPTIO. The values for treated groups denoted by * differed significantly ($p<0.05$) from those of untreated group. But there was no significant difference ($p>0.05$) between the treated groups. The values shown are mean $\pm$ SEM of 8, 6, 9, 11, and 18 replicates for 0, 50μM CPTIO alone, 0, SNAP & CPTIO + SNAP respectively.


A
KCl contraction (mN) after treatment
35
30
25
20
15
10
5
0
50 μM CPTIO
10 μM SNAP
*
*
0
CPTIO
SNAP
CPTIO
+
SNAP

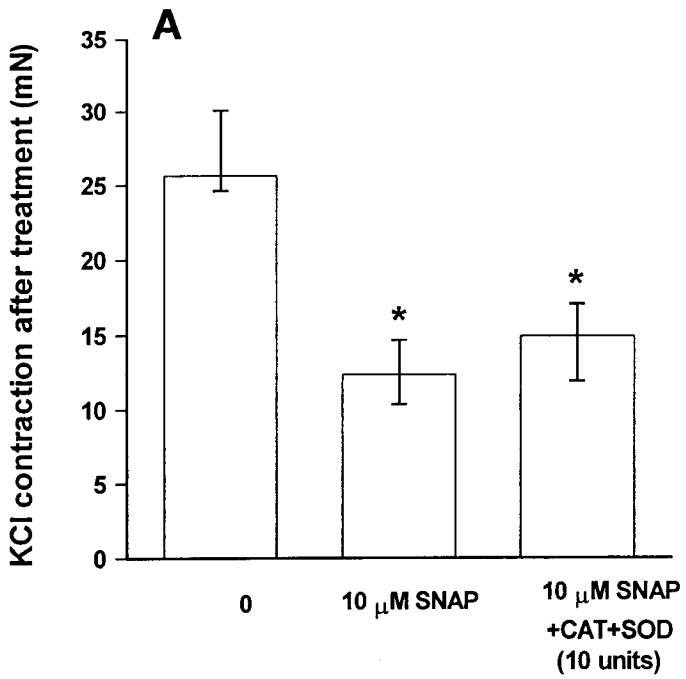

B
CPA contraction (mN)
8
7
6
5
4
3
2
1
0
50μM CPTIO
10μM SNAP
*
*
0
CPTIO
SNAP
CPTIO
+
SNAP

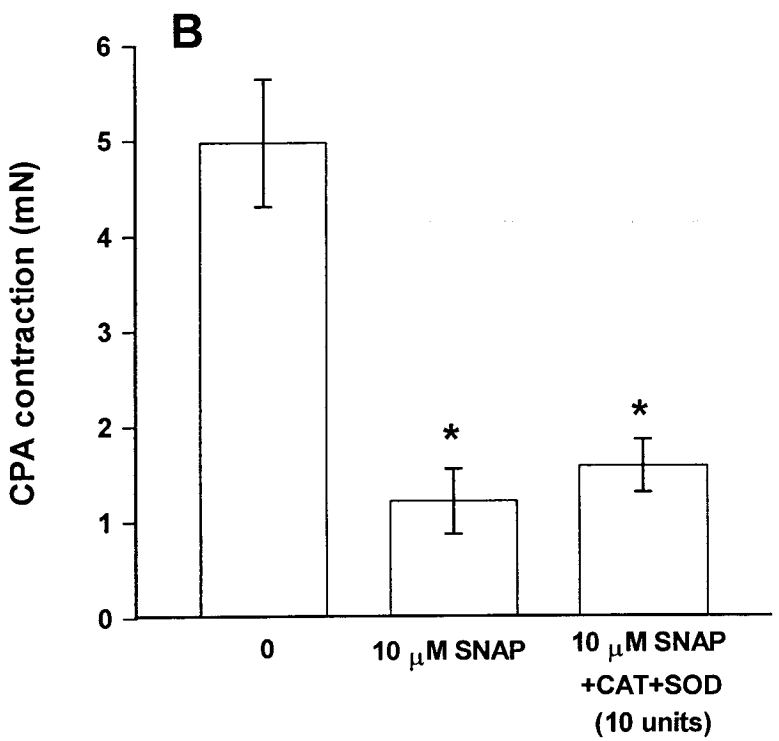
3.5 Effect of CAT and SOD prior to 10 μM SNAP treatment on KCl and CPA-induced contractions.

Scavenging NO from the tissues treated with SNAP did not prevent the damage to KCl & CPA contractions. SNAP is primarily known to produce NO but can also produce other free radicals. Therefore, CAT and SOD were added to Krebs solution prior to treatment of SNAP to rule out the inhibition to KCl and CPA contraction by $O_2^{-\cdot}$ and H_2O_2. Figure 17A shows 30mM KCl-induced contraction with artery rings treated with 10μM SNAP and 10 units of CAT + SOD + 10μM SNAP. The mean ± SEM value for the force of contraction for untreated artery rings was determined to be 25.6 ± 4.4mN and 12.4 ± 2.3mN for rings treated with 10 μM SNAP and 14.9 ± 2.1mN for rings treated with 10 units of CAT + SOD + 10μM SNAP. There was a significant ($p<0.05$) difference between the treated and untreated artery rings. But there was no significant ($p>0.05$) difference between the treated artery rings. Figure 17B shows that CPA-induced contraction was lowered when artery rings were treated with 10μM SNAP and 10 units of CAT + SOD + 10μM SNAP. The mean ± SEM value for the force of contraction for untreated artery rings was determined to be 5.0 ± 0.7mN. This value for force of contraction was reduced to 1.2 ± 0.3mN and 1.6 ± 0.3mN for artery rings treated with 10μM SNAP and 10 units of CAT + SOD + 10μM SNAP respectively. There was a significant ($p<0.05$) difference between the untreated and treated artery rings but there was no significant ($p>0.05$) difference between the treated artery rings. Therefore, SNAP alone (not its dissociated products) is involved in the inhibition of KCl & CPA contractions.

Figure 17: KCl & CPA-induced contraction of coronary artery rings treated with SNAP with & without CAT + SOD.

Graph A & B show 30mM KCl & 10μM CPA-induced contraction on a 3mm ring hung in an organ bath after treated with 10μM SNAP with & without 10 units of CAT + SOD. The values shown are mean ± SEM of 8, 13 and 19 replicates for 0, 10μM SNAP, and 10μM SNAP + 10 units of CAT +SOD respectively. The values denoted by * for the treated groups differ significantly ($p<0.05$) from those of untreated group for both graphs. But there was no significant difference ($p>0.05$) between the two treated groups in graph A and B.


A
KCl contraction after treatment (mN)
35
30
25
20
15
10
5
0
*
*
0
10 μM SNAP
10 μM SNAP
+CAT+SOD
(10 units)


B
CPA contraction (mN)
6
5
4
3
2
1
0
*
*
0
10 μM SNAP
10 μM SNAP
+CAT+SOD
(10 units)

CHAPTER 4: DISCUSSION

The present study demonstrated that in pig coronary artery, pretreatment with peroxynitrite, SIN-1 and SNP inhibited the CPA-induced contraction but not the KCl-induced contraction. In contrast, SNAP and SP-NONOate inhibited contractions induced by both the agents, with SNAP being more potent. When catalase plus superoxide dismutase was included with peroxynitrite or SNAP, it did not influence the inhibition. Including ODQ with SNAP partially relieved the inhibition while the effect of CPTIO in similar experiments was at best marginal. Instead of answering the straightforward question of whether and how peroxynitrite damages the coronary artery smooth muscle contractility, this study has raised several questions. Does the CPA-induced arterial contraction signify the damage to the SERCA pump and nothing else? Is the observed inhibition due to peroxynitrite or NO? What is the possible mechanism of this inhibition? What is the possible pathophysiological significance of damage by NO versus peroxynitrite and damage to the SERCA pump versus other Ca^{2+}-moblizing pathways? The Discussion focuses on experimental problems and addresses these questions.

4.1 Methods and Data Analysis

A protocol shown in the Experimental Methods in Chapter 2 was followed for all the contractility experiments. There were a few issues that could not be controlled in the protocol that led to the day-to-day variability. First, the pig hearts were obtained from a slaughter house and it was not known if the heart was from a male or female, from young or adult, or from fat or lean animals. It is known that the CPA induced contractions vary from the proximal (wide) to the distal (narrow) end of the artery but the KCl contractions

depend on the weight of the rings used[50]. To avoid this source of variability, the artery rings were used primarily from the middle of the artery. However, since 4 rings were cut from each artery, the position where each ring came from would still add to the variability. Further variability could come from damage to the tissues during the dissection. Half lives of various agents used was also variable. Of particular importance is peroxynitrite which had to prepared in 0.1N NaOH and was readily decomposed at the pH of the experiment. How much of the peroxynitrite would be available for the tissue action may depend on the rate of mixing, the rate of addition and where exactly in the bath this agent was added. This added further to the variability. Therefore, each experiment had to be repeated a large number of times.

One can express the CPA-induced force of contraction as mN as described, or as mN/mg tissue, or as percent of the KCl induced contraction. Since there is a decrease in size and in the KCl contraction from the proximal to the distal end of the artery but not in the CPA-induced contraction, the normalization with respect to weight or the KCl induced contraction appears to be unsuitable. Indeed, in the initial experiments (not shown) variability in the CPA contraction between tissues was observed when the data were expressed by such normalization. Therefore, the data were expressed as mN only. The KCl contraction was biphasic - more so in some experiments than in the others. In some experiments, an initially higher peak was observed followed by slightly lower more steady contractions. The possibility was considered that peroxynitrite, SIN-1 or SNAP could affect the two phases differently. First, the data for each phase was analyzed separately. No difference was observed between the effects of treatments on the two phases. Second, in each tissue the ratio of the steady to the peak value was determined.

Treatment with the various agents did not alter this ratio indicating that the steady state and the peak contractions with KCl were not affected differently. Therefore, in all the subsequent experiments, only the peak state of the contraction was monitored.

4.2 Nature of the CPA-induced arterial contraction

CPA induced contractions had been linked to the SR Ca^{2+}-pools which are also used for contraction by activation of receptors such as that bind angiotensin II and alpha adrenergic agents[19]. The premise of these experiments was that the KCl induced contractions represent Ca^{2+}-entry by the L-type of VOCC which are inhibited by dihydropyridines such as nitrendipine and the CPA induced contractions inhibit a simple change in Ca^{2+}-homeostasis due to inhibition of the SERCA pump. These assumptions are perhaps too simplistic as it does not consider other modes of Ca^{2+} mobilization. For example, the inhibition of the SERCA pump would lead to a lack of accumulation of Ca^{2+} into the SR and thus ultimate emptying of this pool due to leakage. This is likely to result in the opening of the store depletion dependent Ca^{2+}-channels which allow entry of extracellular Ca^{2+} into the cells. As these experiments were all carried out in presence of extracellular Ca^{2+}, this possibility can not be ruled out. It is noted, however, that treating the tissues with EGTA leads to a damage of the CPA-induced contractions and hence these experiments can not be carried out in the absence of Ca^{2+} thus making this issue difficult to resolve. Experiments using inhibitors of store depletion dependent Ca^{2+} entry are needed but there are no really selective agents available. Therefore, the results of this study should not be interpreted with the initial premise but also consider alternatives even if they are not proven.

4.3 Peroxynitrite or NO as the damaging species

The inhibitory effect of peroxynitrite could not be overcome using catalase plus superoxide dismutase leaving the possibility that either peroxynitrite produced the damage directly or the NO resulting from the decomposition was possible. Therefore, we used a number of peroxynitrite or NO generating agents to examine this issue. Treatment with SIN-1 which generates predominantly peroxynitrite produced an inhibition of the CPA-induced contraction suggesting that peroxynitrite may be involved. However, SNAP and SP-NONOate which are known to generate primarily NO were even more potent in producing the inhibition suggesting NO as the damaging agent. SNP which is also a NO producer, however, was less potent. It is possible that these differences relate to the rate at which these agents produce NO or the species of NO they produce. However, another major difference between the effects of these agents is that peroxynitrite at 500μM abolished the CPA response but only partially affected the KCl-induced contraction and SNAP even at 10μM affected the CPA- and KCl- induced contraction. Similarly SP-NONOate also affected both CPA- and KCl-induced contractions but SNP and SIN-1 affected only the CPA-induced contractions. Thus it is possible that the NO generating agents affected a different target than the peroxynitrite generating agent. Whether this target was at least partially in common between the two groups or the NO-producing agents affected a target such as contractile proteins which would still be needed to be explored.

SNAP was the most potent agent we examined and it is reported to produce mainly NO and not peroxynitrite. Therefore, we asked two questions. First, if CPTIO

which quenches NO would overcome the effect of SNAP. CPTIO when used at 50μM was at best partially effective in overcoming the effect of 10μM SNAP. It needs to be explored whether a higher concentration of CPTIO would be more effective. If not, it would indicate that CPTIO is not very effective in quenching the species of NO produced by SNAP. The second question concerned the mechanism by which NO that produced the damage. A possibility is that the excess NO produced guanylate cyclase activation producing sufficient cGMP that disabled the tissues from contraction even after the prolonged wash. Guanylate cyclase is inhibited by ODQ with an IC_{50} of 84 ± 15 nM[64]. ODQ (10μM) when added prior to and during incubation with 10μM SNAP produced a complete relief of inhibition of the KCl contraction but it affected the inhibition of the CPA-contraction only partially. This again suggests that there are at least two mechanism involved in the inhibition observed with the various agents. Perhaps, these can be explored by examining the effects of these agents directly on the SERCA pumps in membranes isolated from this tissue, i.e., in the *in vitro* experiments where cGMP can not be generated and hence the effects on the guanylate cyclase would be separated. Other possible mechanisms are modifications of proteins by oxidation or by nitrosylation. Peroxynitrite and NO generating agents SNAP and SPNONOate are all known to cause 3'-O-nitrosylation of tyrosines and S-nitrosylation of cysteine residues in the proteins[8,9,10,13,60,61]. For example, it has been shown that peroxynitrite can inactivate skeletal muscle SERCA1 by nitrosylation of one critical cysteine residue[71]. S-nitrosylation of the calcium release channel (ryanodine receptor) has also been postulated as another mechanism to perturb Ca^{2+} homeostasis in a cell[11,13,73].

4.4 Pathophysiological Significance

SR Ca^{2+} pools are used preferentially for contraction to agents such as angiotensin II and alpha adrenergic agents. In contrast, contraction to agents such as endothelin-1 occurs via blockage of K^{+}-channels thereby increasing extracellular entry via VOCC. ROS such as superoxide anion, hydrogen peroxide and hydroxyl radicals damage SERCA pump more severely than PMCA and VOCC. In this study we found that whereas peroxynitrite, SIN-1 and SNP primarily affected the CPA-induced contractions, SNAP and SPNONOate affected both the KCl and the CPA-induced contractions. The effects on the KCl induced contractions, however, could be prevented by the guanylate cyclase inhibitor ODQ suggesting that this could be an inactivation mechanism which was not overcome at the time scale of our experiments. As peroxynitrite and the other ROS may be produced during the inflammatory like atherosclerotic response, during ischemia-reperfusion, or stroke their actions would lead to irreversible damage of the SERCA pumps thereby a loss of regulation via the mechanisms utilizing these pathways. However, conditions such as sepsis which could produce larger amounts of NO may lead to perhaps a reversible loss of contractile mechanisms.

Conclusions

Peroxynitrite inhibited the CPA-induced contraction but the inhibition was not mediated by superoxide anion and hydrogen peroxide formed upon its dissociation. The results with the peroxynitrite and NO donors suggest that more than one type of mechanisms may be involved - possibly peroxynitrite acting on the SERCA pumps and NO acting via guanylate cyclase. This difference in the mechanisms should be

considered only a working hypothesis for future studies. In conclusion, this study demonstrates the troublesome role of NO that could be involved in both physiological and diseased states.

Future Outlook

Peroxynitrite and NO have more than one type of effects on the smooth muscle contractility. The effects of individual agents depend on the amount and the nature of the NO species produced by these different agents. In addressing the questions about effects of peroxynitrite and NO donors on smooth muscle contractility, we relied solely on contractility studies in the organ bath. This method alone does not give conclusive evidence of the Ca^{2+} mobilization pathways involved or the individual reactions which are affected. Future studies are needed to dissect out these mechanisms. The studies would involve cytosolic [Ca^{2+}] measurements, of Ca^{2+} pump activities in isolated membranes, guanylate cyclase activity in these membranes and electrophysiological studies on VOCC and possibly other channels. Also in this experimental setting, the effects of peroxynitrite and NO donors were measured in the absence of any antioxidants. The role of antioxidants and the effect of peroxynitrite and NO on total antioxidant reserve in the tissues are still to be determined. These studies will help us to further understand the role of peroxynitrite and NO in mechanisms controlling intracellular calcium in physiological and pathological states.

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