Hydroperoxides and potassium channels: a possible mechanism for vasodilation in septic shock.

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Abstract

In septic shock (SS), hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) are released by inflammatory cells and have been implicated in tissue damage and inflammation. Recently, H_2O_2 has been established as an important signaling molecule and an important component of SS. The pathways involved in this process are not completely understood, but the formation of hydroperoxides (HPs), arachidonic acid (AA) metabolites and potassium (K^+) channels have been implicated. In this study, we used a canine carotid ring preparation as a bioassay to determine the role of peroxyacetic acid (POX), a hydroperoxide (HP), in causing vasodilation and elucidate the subsequent pathways involved. We removed internal carotid artery segments from dogs and placed them in an organ bath. The segments were preconstricted after which we added POX to the preparation. We found that POX produced an endothelium and nitric oxide independent vasodilation in the carotid artery ring preparation. This decrease in tension could be prevented by high concentrations of K^+ in the bath. This suggested that K^+ channels were involved in POX's action. Further investigation showed that the particular K^+ channels implicated were the combination of small (SK_{Ca}) and intermediate conductance calcium activated K^+ channels (IK_{Ca}). In addition we found that the prostaglandin H synthase (PGHS) inhibitor, indomethacin, could block POX's mechanism of action. This finding indicates that PGHS takes part in the vasodilation caused by POX. Our results suggest that HPs that are released from inflammatory cells in sepsis could stimulate the PGHS pathway leading to prostaglandin synthesis and subsequently activating SK_{Ca} and IK_{Ca} to produce vasodilation. Inhibition of this pathway may be important component in the treatment of SS.

Key words: sepsis; vasodilation; hydroperoxides; prostaglandins, potassium channels

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Abbreviations

17-ODYA	17-Octadecynoic Acid
1-EBIO	1-Ethyl-2-benzimidazolinone
4-AP	4-Aminopyridine
AA	Arachidonic acid
AC	Adenylate cyclase
APA	Apamin
ATK	Arachidonyl trifluoromethyl ketone
BK_{Ca}	Large conductance calcium activated potassium channels
BP	Blood pressure
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
ChTX	Charybdotoxin
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
DAMPs	Damage-associated molecular patterns
EPO	Epoxygenase
EPOP	Epoprostenol
ETI	Eicosatrienoic acid
GC	Guanylate cyclase
H_2O_2	Hydrogen peroxide

HAVSMC	Human aortic vascular smooth muscle cell
HP/HPs	Hydroperoxide/Hydroperoxides
IK_{Ca}	Intermediate conductance calcium activated potassium channels
K^+	Potassium
K_{ATP}	Adenosine triphosphate sensitive potassium channel
K_{Ca}	Calcium activated potassium channel
KCl	Potassium chloride
K_{IR}	Inward rectifier potassium channel
K_V	Voltage activated potassium channel
$_{L}$-NMMA	N^G-monomethyl-$_L$-arginine
LO	Lipooxygenase
Lzm-S	Lysozyme-S
NO	Nitric oxide
NOSs	Nitric oxide synthase
ODQ	1H-[1, 2, 4]oxadiazolo[4, 3-a]quinoxaline-1-one
PAMPs	Pathogen-associated molecular patterns
PG/PGs	Prostaglandin/Prostaglandins
PGHS	Prostaglandin H synthase
PGI_2	Prostaglandin I_2 (Prostacyclin)
PLA_2	Phospholipase A_2
POX	Peroxyacetic acid
PRRs	Pattern recognition receptors

ROS	Reactive oxygen species
sGC	Soluble guanylate cyclase
SKA-31	Naphtha [1, 2-d] thiazol-2-ylamine
SK_{Ca}	Small conductance calcium activated potassium channel
SQ-22,536	9-(Tetrahydro-2-furanyl)-9H-purin-6--amine
SS	Septic shock
TRAM-34	1-[(2-Chlorophenyl)diphenylmethyl]--1H-pyrazole
VSMC/VSMCs	Vascular smooth muscle cell/Vascular smooth muscle cells
VSML	Vascular smooth muscle layer

Introduction

Sepsis derives from the Greek word *"sepo"* which means decomposition or putrefaction and the word has persisted like that for many years[1]. Current knowledge indicates that sepsis is the abnormal systemic response to an infection process produced by pathogenic microorganisms[1,2]. Clinically, sepsis is defined by the presence of infection plus the appearance of systemic inflammatory manifestations such as fever or hypothermia, tachycardia, tachypnea and leukocytosis or leukopenia[1]. If the infectious process persists, a complex interaction between microorganism and host appears, leading to a complicated and not well known cascade of events that may produce conditions that reside in the end of the septic spectrum such as severe sepsis and septic shock (SS)[2]. As mentioned before, the pathophysiologic events that culminate in SS are intricate and not completely understood. In previous years it was thought that sepsis and SS occurred due to an exaggerated inflammatory response of the host against an infection mainly characterized by the presence of inflammatory response mediators which activated multiple deleterious signals[2]. However, clinical trials that have used different types of anti-inflammatory drugs have been unsuccessful, raising the suspicion that inflammation is not the only culprit in the occurrence of sepsis[3]. Recently, due to extensive basic and clinical research, the paradigm of sepsis and SS has moved towards a concept of abnormal interaction or "mismatch" between the microorganism and the host. This pathologic interaction produces not only a systemic inflammatory response, but also an immunologic dysfunction and hematological abnormalities that contribute to the pathogenesis of the disease[2,3]. Furthermore, the new paradigm takes into account the role of the microorganism and other factors such as the genetic predisposition of the host in the development of the disease[3]. At the end of the

septic spectra, SS presents itself as a frequent, deadly and complicated condition that needs more research to help unravel the mechanisms responsible of the disease.

1.1 Septic shock definition and epidemiology

SS is the result of a generalized infection and a complication of the microorganism-host systemic mismatch. In the clinical setting, SS is defined as the appearance of systemic hypotension that persists despite adequate fluid resuscitation in the presence of sepsis[2] (See Table 1 for the clinical definition of sepsis and SS).

Systemic inflammatory response syndrome (SIRS): Presence of an abnormal inflammatory response demonstrated by two or more of the following features: • Body temperature >38.5°C (hyperthermia) or <35°C (hypothermia). • Heart rate >90 beats/min (tachycardia). • Respiratory rate >20 breaths/min (tachypnea) or arterial tension of carbon dioxide (PCO_2) <32 mmHg. • Leukocyte count >12000 mm^3 (leukocytosis) or <4000 mm^3 (leukopenia) or >10% of band forms.
Sepsis: SIRS in the context of a proven infection (documented by bacteriologic methods).
Severe sepsis: Sepsis and the existence of at least one feature of organ hypoperfusion or dysfunction such as. Clinical: • Areas of mottled skin. • Capillary refill time >3 seconds. • Urine output <0.5 ml/hr or need for renal replacement therapy. • Sudden changes in level of consciousness or mental status. • Acute respiratory distress syndrome. Laboratory and radiological: • Serum lactate >2 mmol/L • Platelet counts <100,000 mm^3 or evidence of disseminated intravascular coagulation. • Evidence of cardiac dysfunction assessed by echocardiography.
Septic shock: Severe sepsis plus the presence of systemic hypotension (<60 mmHg of mean blood pressure; <80 mmHg in hypertensive patients) despite adequate fluid resuscitation (use of 40 to 60 ml/kg of cristaloids or 20 to 30 ml/kg of colloids) or the need for vasopressor use (norepinephrine or dopamine).
Refractory septic shock: Septic shock that requires high doses of vasopressor to maintain systemic blood pressure above 60 mmHg or 80 mmHg in hypertensive patients).

Table 1. Clinical definitions used in sepsis.

SS is one of the most challenging conditions found in current clinical practice and it is one of the most common causes of death in critical care patients carrying also a high burden on health care resources[4]. In Canada, SS has a high mortality rate that approaches the range of 30 to 40% of all the critical care unit admissions. Furthermore, Winters et al., analyzed a series of studies and found that an event of SS has been clearly associated with an increase in morbidity and mortality after hospital discharge with a mortality of around 60% after 2 to 3 years. The same authors also reported that survivors of the disease have a long term decrease in quality of life scores, findings that are consistent across a broad range of patient populations[5].

1.2 Causes of SS

Infection occurs after the introduction of a pathogenic microorganism to a previously sterile tissue[1]. If the infection becomes systemic, the abnormal interaction between pathogen and host may lead to SS. The most frequent sites of primary infections that produce SS are in the respiratory system, the genitourinary system and the abdomen. These sites are involved in around 80% of the SS cases, being the urinary system being the least associated with mortality, whereas the highest mortality is found when there is no evident site of infection[1,2].

Gram-positive and Gram-negative bacteria are the most common microorganisms involved in the pathogenesis of SS (30-50% and 25-30% respectively); however, fungal, parasitical and viral infections are also capable of producing SS[1,46]. The main microorganisms that have been associated with SS can be viewed in Table 2.

Gram-positive bacteria ▪ Meticillin-susceptible *Staphylococcus aureus* ▪ Meticillin-resistant *Staphylococcus aureus* ▪ *Staphylococcus epidermidis* ▪ *Streptococcus pneumoniae* ▪ Mixed anaerobes ▪ *Enterococcus faecalis* **Gram-negative bacteria** ▪ *Pseudomona aeruginosa* ▪ *Escherichia coli* ▪ *Proteus mirabilis* ▪ *Klebsiella pneumoniae* ▪ *Enterobacter* species
Fungus ▪ *Candida albicans* ▪ *Candida glabratra* and other *Candida* species ▪ *Aspergillus* species ▪ Other fungus
Parasites
Viruses

Table 2. Microorganisms capable of producing sepsis and SS.

1.3 Pathophysiologic events in SS

The response of the body to infection starts with the recognition of pathogenic microbes by the immune system which can have different outcomes: a) the containment of the infectious process with permanent cure and minimal tissue damage; b) the containment of the infectious process but the presence of chronic and unresolved infection or c) the appearance of an imbalanced and exaggerated host response to the pathogen. The latter outcome is the one that leads to the appearance of SS[1,2].

1.3.1. The importance of the pathogen in sepsis and SS

Some bacterial and non-bacterial microorganisms have multiple mechanisms that allow them to attach and proliferate in tissues. Microbial components such as adhesins (bacterial proteins), flagella, fimbriae (mobility structures) and other membrane glycoproteins are important for the adherence to tissues and for the activation of the innate immune response of the host[2]. Furthermore, pathogens have factors (proteins, lipidrafts and others) that promote the invasion to the tissues as well as systems which allow them to communicate between them and establish a critical bacterial density in the tissue to avoid or resist the host immune response; this is what has been referred to as quorum-sensing systems[2]. These intricate systems are controlled by the genetic material of the pathogen as well as other virulence factors. Microorganisms are capable of changing their genetic sequences in order to expand or maintain the population and increase the expression of virulence molecules[1]. So, pathogens are well equipped to produce an infection and in recent years more recognition has been given to the important role of the pathogen in the development of severe sepsis and SS.

1.3.2. Host response to the pathogen in sepsis and SS

After the establishment of an infectious process, the host response is determined by multiple factors such as the virulence of the pathogen, age of the host, pre-morbid conditions and the genetic capability to adapt and respond to the microbial challenge[2, 3]. In addition, the immune system recruits other systems (coagulation, autonomic nervous system) to enhance the response against microorganisms. These complex system-to-system interactions further improve the effectiveness of the host to eliminate the pathogen[1]. On the other hand, in SS there is a dysfunction in virtually all components of the immune response coupled with an

exaggerated activation of the inflammatory and coagulation systems that may lead to tissue damage and ultimately, organ failure[2,3].

The host's first line of defense is the innate immune system. This system utilizes mechanical barriers, enzyme secretion, activation of fast-response white blood cells and other mechanisms to attenuate the infectious process[3]. In one of the first key steps in the host response to infection, cells of the innate immune system recognize the presence of a microorganism by the activation of pattern recognition receptors (PRRs) located on the membrane of immune cells[2]. PRRs are activated by specific motifs of the microorganisms termed pathogen-associated molecular patterns (PAMPs); these structures together with molecules that derive from local tissue damage are usually referred to as damage-associated molecular patterns (DAMPs)[1]. The activation of PRRs produces a complex cascade of signaling cellular effects that range from pro-inflammatory to anti-inflammatory[1,2,3]. One of the most the prominent and well known effects of PRR activation is the increased expression of inflammatory cytokines (low molecular weight glycoproteins), which in turn induce the synthesis of pro-inflammatory mediators and activation of specific immune cells targeted at the infected site[2]. On the other hand, the activation of PRRs in immune cells that have an anti-inflammatory profile (i.e. type 2 helper T cells) suppress the expression of cytokines and reduce the migration and recruitment of inflammatory cells to the infected area mitigating the inflammation but favoring the infection process[1]. Current theories postulate that in SS there is an increase in the expression and activation of PRRs[2], leading to an abnormal mismatch between the pro-inflammatory and anti-inflammatory profiles which generates a state of immune dysfunction that further contributes to the severity of the clinical

entity[2,3]. In addition, genetic factors modulate the type of predominant response in a particular individual and may be the reason for the clinical variability observed with this condition[3]. As mentioned before, a myriad of factors come into play in the host response to an infection that occurs in SS, however more knowledge is needed in key areas of the pathogenesis which would certainly improve the management of the disease.

1.3.3. Inflammatory mediators in SS

The over-expression and production of cytokines and other inflammatory mediators that occurs in sepsis is one of the most studied aspects of this condition which has lead to the development of multiple treatment strategies, unfortunately all of them have been so far unsuccessful[3]. Nonetheless, the inflammatory mediators are vital in the generation and propagation of the immunological response in SS.

As we can see in Table 3, sepsis and SS induces the synthesis and release of cytokines and non-cytokine mediators that contribute to the host response to infection and are important in the development of abnormalities within multiple systems and organs[1,2].

Inflammatory Cytokines	**Source**	**Effects in sepsis and SS**
Tumor necrosis factor α (TNFα)	Macrophages, lymphocytes.	Activation of T cells, myocardial dysfunction, cachexia and fever.
Interleukin-1 (IL-1)	Macrophages, lymphocytes.	Activates neutrophils, induces metalloproteinases and prostaglandins synthesis fever, hypotension.
Interleukin-6 (IL-6)	Lymphocytes, endothelial cells, fibroblasts, monocytes.	Proliferation of T and B lymphocytes.
Interleukin 8 (IL-8)	Monocytes	Promotes neutrophil chemotaxis.

Interleukin-12 (IL-12)	Macrophages	Increase interferon-γ production.
Interleukin 17 (IL-17)	Activated T cells	Induces synthesis of other cytokines (IL-6, TGF-β).
Interferon-γ (IFN-γ)	T cells	Activates T cells, macrophages and natural killer cells, induces expression of adhesion molecules.
High mobility group box 1 (HMGB1)	Macrophages	Induces expression of adhesion molecules and inflammatory cytokines.
Anti-inflammatory cytokines		
Interleukin-10 (IL-10)	Monocytes, activated T cells, natural killer cells.	Decreases the production of TNF-α, IL-1 and IL-6.
Interleukin 1Ra (IL-1Ra)	Monocytes	Blocks the activity of IL-1 by competitive binding to IL-1 receptors.
Transforming growth factor-β (TGF-β)	Lymphocytes, macrophages	Interferes with macrophages phagocytosis.
Non-cytokine mediators		
Chemokines (CXC and CC)	Neutrophils, eosinophils and lymphocytes	Increase pro-inflammatory mediator's secretion.
Adhesion molecules (Selectins, ICAM and VCAM-1)	Endothelial cells and leukocytes	Promote the initial step in the migration of the leukocytes (adhesion).
Arachidonic acid metabolites	Multiple cells	Increase local inflammation, vascular permeability and other effects.
Lysozyme-S (Lzm-S)	Leukocytes of the spleen and other organs	Systemic vasodilation and myocardial dysfunction.
Gaseous mediators (reactive oxygen species, NO and others)	Multiple immune cells, endothelial cells	Alterations in vasomotor tone. Myocardial depression.

Table 3. Mediators that contribute to the host response in sepsis and SS.

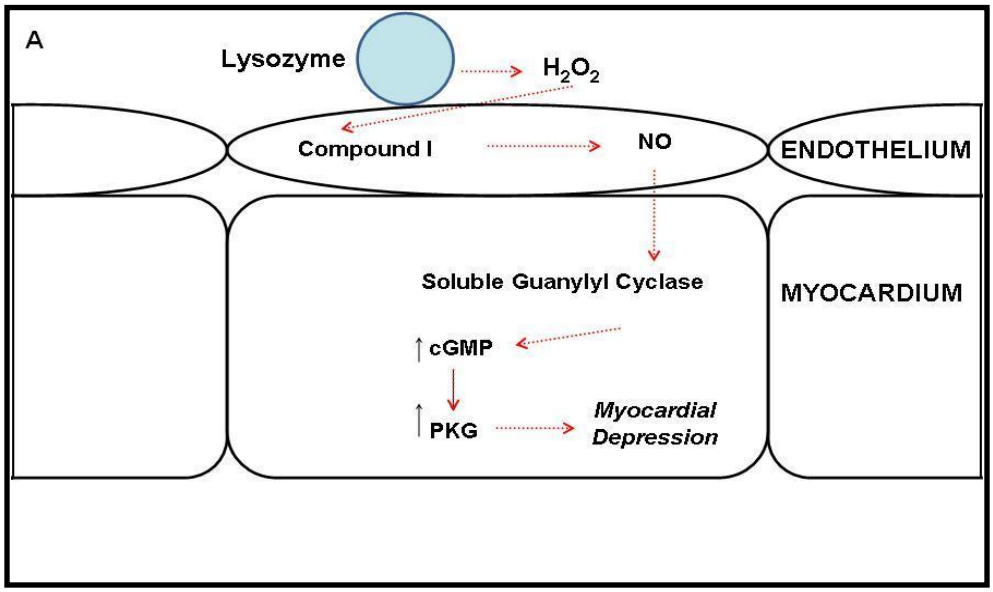
1.3.4. Lysozyme's contribution in SS

Lysozyme (Lzm-S) is an enzyme that has bactericidal properties – cleaves to the bacterial wall and induces the hydrolysis of glycoprotein linkages of the bacterial wall- and is primarily located

in granulocytes, monocytes and tissues such as bone marrow, lungs and kidneys[6]. Recently, Lzm-S has been found to be increased in an animal model of SS and also contributing to the cardiovascular dysfunction that occurs in that disease[7]. Evidence obtained from these studies show that Lzm-S can produce myocardial depression[7] and arterial vasodilation[8], both prominent features of the disease and important factors that generate systemic hypotension in the disease.

The mechanism of action by which Lzm-S produces myocardial dysfunction and arterial vasodilation involves the generation of H_2O_2, well known reactive oxygen species (ROS), which contributes to cardiovascular cell signaling and vascular tone modulation under various pathophysiologic conditions[9]. Previous studies[7, 8] have found that Lzm-S is able to intrinsically produce H_2O_2 by the mechanism described by Wentworth et al.[10,11]. These group of authors proved that proteins (antibodies and others) can generate H_2O_2 from singlet oxygen ($^1O_2$*) and water by a dismutase reaction and that these chemical events are relevant in immunological responses. Mink et al.[7], showed that Lzm-S is also capable of forming H_2O_2 in a manner similar to the one described by Wentworth. Following its generation, H_2O_2 activates dependent pathways that ultimately lead to cardiovascular dysfunction in SS[7,8]. The pathway in Lzm-S action, involves the generation of a catalase derivative called compound I (see Figure 1) obtained through the metabolism of H_2O_2 by endogenous catalase[7,8,12,13]. Compound I is a molecule with high oxidizing potential that is the intermediate metabolite of the natural reaction between H_2O_2 and native catalase[14]. Compound I contains an oxoferryl group and a π-cation radical on a porphyrinic ring, structure that allows a two electron oxidation on the ferryl

and porphyrinic group which is relevant in its metabolism and confers the molecule its oxidizing capabilities[15]. The formation of this metabolite depends entirely in the H_2O_2 concentration[14]. Furthermore, when there is a stable flow of H_2O_2, compound I suffers a two electron reduction, thus returning the molecule to native catalase, a process that has been called "the catalytic cycle"[14]. Under physiological conditions, this reaction occurs naturally in the peroxisomes and in the cytosol[8] facilitating the interaction of compound I with other signaling molecules such as soluble guanylyl cyclase (sGC)[7,8]. Other investigators have shown the importance of compound I in the H_2O_2 metabolism and vascular tone modulation. Wolin et al., showed that compound I could activate sGC, leading to vasodilation in a preconstricted pulmonary arterial preparation, and that this effect could be inhibited by the peroxide-metabolizing agents *Aspergillus niger* catalase, ethanol and methanol[12, 13] demonstrating a decrease in the production of compound I by the latter agents, thus, blocking the vasorelaxation.



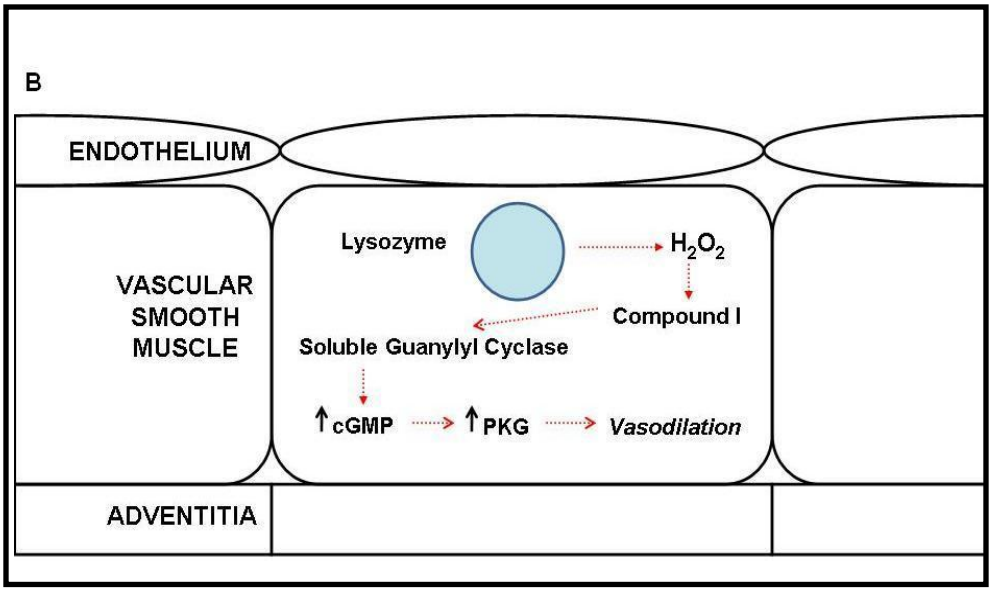


Figure 1. Lzm-S mechanism of action in the myocardium and in the vascular smooth muscle. A) In the myocardium Lzm-S intrinsically produces H_2O_2 which reacts with catalase to produce Compound I which then activates GC to induce myocardial depression. B) Inside the VSMC, Lzm-S generates H_2O_2 and Compound I to induce vasodilation by the cGMP pathway.

Recently, the role of Lzm-S in SS has been further clarified[16]. It has been shown that Lzm-S can produce H_2O_2 in plasma which then diffuses to the vascular smooth muscle layer (VSML) of arteries to promote vasodilation. Furthermore, Lzm-S dilates smooth muscle cells in close proximity to them. Unpublished data suggests that Lzm-S accumulates in the VSML of arteries and other organs in an animal model of SS, which would support its importance in the pathophysiology of SS. From the Lzm-S studies it can be concluded that ROS and particularly H_2O_2 are molecules that interact with multiple intracellular pathways in the systemic arteries and the myocardium contributing to the cardiovascular collapse that it's seen in SS.

1.3.5. The importance of hydroperoxides in SS

Multiple ROS are generated during sepsis and SS[12,18]. In these diseases the generation of ROS is increased due to several mechanisms[17]: a) enhancement of the mitochondrial respiratory chain reaction; b) augmentation of the release of AA by phospholipases (inflammation); c) increase of

the xantine-oxidase system; and d) over stimulation of the granulocytes (in particular neutrophils) to activate lysozomal enzymes and other mechanisms.

Hydroxyl radicals (OH), $^{1}O_2$*, O_2^- and H_2O_2 are among the intermediates generated after reduction of oxygen. These compounds are highly reactive and are capable of producing cellular damage due to membrane peroxidation, DNA damage and protein denaturation[12]. Moreover, ROS are now recognized as important signaling molecules that could promote different effects in multiple diseases such as SS[19].

Among ROS, organic peroxides (hydroperoxides, HPs) are generated after the interaction of ROS with components of the inflamed tissue[20,21]. Lipids[21], proteins, RNA and DNA[22] are all susceptible of peroxidation by ROS (See Figure 2 for a description of the mechanism of peroxidation). From the previous molecules, and due to their abundance in cells, proteins are a major target for peroxidation[22]. Multiple studies have shown that amino acids and peptides are able to react with ROS (H_2O_2 and $^{1}O_2$*) to generate organic peroxides[21,22]. The consequences of macromolecule peroxidation and HP formation in cells include: a) disruption of the normal cell membrane architecture; b) enzymatic inhibition in the cell cytoplasm by changing the native enzyme structure; c) accumulation of toxic metabolites such as aldehydes; and d) increase in apoptosis[21,22]. All of the former consequences have been shown to occur in the inflammatory response of SS and are relevant in cell damage and organ dysfunction[2].

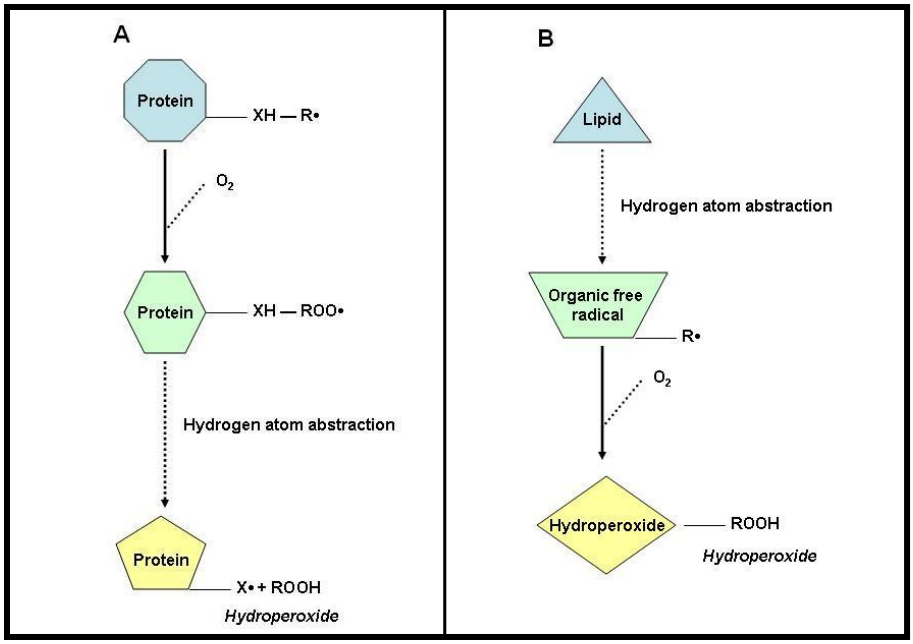


Figure 2. Diagram showing protein and lipid peroxidation in tissues. A) ROS oxygenate proteins and then by hydrogen atom abstraction there is a generation of a peroxide radical attached to the protein. B) Lipids are subject to a hydrogen atom abstraction to generate an organic free radical which after oxygenation forms a HP radical.

One of the most important features of SS is the presence of inflammation produced in part by the increase in number and function of immune cells[1,2]. Granulocytes are key cells in the initial immune response and in the generation of the microorganism-host "mismatch" seen in SS. These cells have a very high oxidative capacity due to the presence of multiple key enzymes in the cytosol[17]. Besides the usual ROS, leukocytes are capable of generating HPs. Okazaki et al.[20] showed that as part of the inflammatory response, leukocytes are able to form HPs which may induce cell membrane peroxidation and damage. Moreover, Claster et al. found that activated neutrophils have the capacity of producing peroxidation of erythrocytes and induce hemolysis[23]. However, granulocytes are not the only cells involved in the generation of HPs,

other organs such as liver and spleen, have been shown to make HPs after the stimulation with endotoxins[21].

The increase in oxidative stress seen in SS has been associated with an elevation in the measured levels of HPs in animal models of sepsis and some human studies. In an animal model of neonatal sepsis[24], the level of total HPs content was increased fourfold as compared to control animals after sepsis induced with cecal ligation and perforation. In addition, Takeda et al., found that HPs from lipid peroxidation doubled in plasma, liver and lung after sepsis was induced in rats[25]. Similar results have been obtained in human studies, where HPs were measured in critically ill patients and found to be augmented, particularly in patients with SS and multiple organ failure[26]. Confirming the latter study, Keen et al. showed that HPs levels were high in septic patients suggesting that the HPs level could potentially be a marker of infection severity and tissue damage in patients with SS[27].

In summary, these observations suggest that HPs are a type of ROS produced and released by inflammatory cells such as granulocytes in sepsis and SS. After their release, HPs may induce cell and tissue damage, however, more research is needed to clarify whether HP's have cell signaling properties in normal conditions and its role in disease states such as SS.

1.4 Cardiovascular collapse in SS

Systemic vasodilation and reversible myocardial depression are main features of the systemic hypotension responsible for the cardiovascular collapse that occurs in SS[28]. Both of them are associated with an increase in the mortality of patients with SS[29]. International efforts have been undertaken in order to ensure that patients diagnosed with SS receive the adequate

treatment to restore BP by the pertinent use of fluid resuscitation and vasopressor therapy[29]. Despite these efforts, mortality of SS is still unacceptably high, pinpointing the need for more treatment strategies. The knowledge of the underlying mechanisms that produce systemic hypotension in SS is vital to find better treatment options.

1.4.1. Systemic vasodilation in SS: contribution of inflammatory mediators

Physiologically, vascular tone is regulated by several mechanisms that determine the basal tone of the blood vessel[30]. Some of these mechanisms are generated in the vessel while others are originated in the surrounding structures or in distant tissues, but all of them alter in a way the basal tone of the vasculature[31]. One of the most important mechanisms in vascular tone modulation is the balance between vasoconstrictors and vasodilators acting on the blood vessel[30, 31]. In addition to the effect of vasoactive substances in vascular tone, changes in the resting membrane potential of the vascular smooth muscle cell (VSMC) can have profound modifications in vascular tone[31]. In general, vasoconstriction occurs as a result of an increase in intracellular calcium to the VSMC. Intracellular calcium binds to calmodulin to activate myosin light chain kinase (MLCK) which in turn phosphorylates the myosin light chain to induce the interaction of myosin and actin to generate the contractile response; on the contrary, vasodilation occurs when the concentration of intracellular calcium decreases and there is an increased activity of myosin light chain phosphatase (MLC) which dephosphosrylates the phosphorylated myosin, thus, relaxing the contractile apparatus of the cell (See Figure 3)[31]. Multiple diseases alter in distinct manners the physiological regulation in vascular tone; thus, modifying the systemic balance between vasoconstriction and vasodilation. One of these conditions is SS where a marked imbalance towards vasodilation exists.

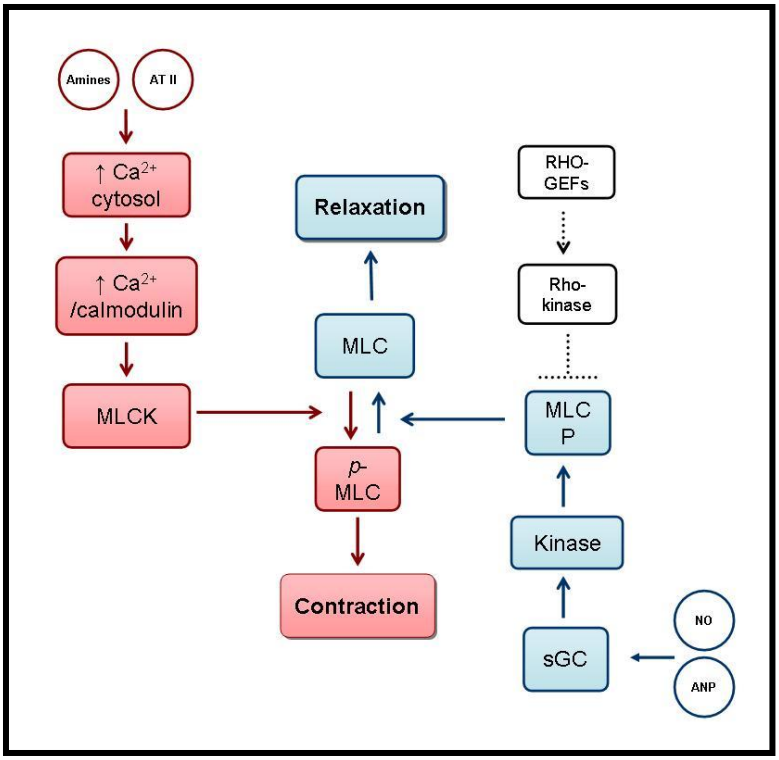


Figure 3. General scheme of vasodilation and vasoconstriction in VSMCs. Vasoconstriction occurs as a result of an increase in intracellular Ca^{2+} to the VSMC. Intracellular calcium activates myosin light chain kinase (MLCK) to induce the interaction of myosin and actin. On the contrary, vasodilation occurs when the concentration of intracellular Ca^{2+} decreases and the activity of myosin light chain phosphatase (MLC) increases, thus, relaxing the myocyte.

In SS, there is a profound systemic vasodilation which reduces peripheral resistance, thus, decreasing BP leading to tissue hypoperfusion and possibly organ failure[28]. The mechanisms that produce this vasodilation are not completely understood, however, there seems to be an inability of the VSMC to constrict in response to multiple factors[31]. Endothelial injury, microcirculatory alterations, vascular hyporesponsiveness, alterations of ionic channels and inflammatory mediators are all participants in a complex vascular response to severe infection that ultimately contribute in the generation of the vasodilatory shock seen in this disease[30].

Inflammatory mediators have been mentioned as one of the most important factors in decreasing vascular tone in sepsis and SS. These substances are produced and released as part of the extensive inflammatory process that occurs in SS[1,2]. These mediators come in close contact with the vascular endothelium due to the recruitment of leukocytes to this area inducing the endothelial release of cytokines and vasodilators such as nitric oxide (NO), and prostaglandins (PGs)[8, 31]. NO, PGs and other AA metabolites levels are increased in experimental and clinical models of SS[32], which appear to contribute in the exaggerated inflammatory process and in the cardiovascular dysfunction of SS. Nonetheless, NO synthesis blockers and cyclooxygenase inhibitors have been tried in multiple clinical trials of SS and have been shown to be ineffective in the treatment of the human condition[32] which underlines the importance of determining the exact mechanisms by which these mediators contribute to the pathogenesis of the disease.

1.4.2. ROS as vasoactive substances in SS

As part of the inflammatory response, ROS are generated by inflammatory cells and blood vessels in SS[33], and been implicated as vasoactive factors with the potential of promoting vasodilation or vasoconstriction[34, 35, 36, 39] as well as cyto-toxic effects[38].

Among ROS, H_2O_2 has been found to be an important mediator that contributes to cardiovascular cell signaling and vascular tone under various pathophysiological conditions such as SS[40]. Previous studies have shown that the administration of exogenous H_2O_2 elicits a dichotomous response in vascular tone, either dilation[41-43] or contraction[44,45] under different vascular beds and study models. Cseko and colleagues[46] found that the vascular response in

skeletal muscle arterioles to the administration of H_2O_2 it's dependent on its concentration and occurs by the activation of diverse endothelial or smooth muscle cell pathways. There is also increasing evidence suggesting that H_2O_2 mediates changes in the ionic conductance of the cellular membrane, modifying the currents in and out of the membrane[47-49], particularly, related to its hyperpolarizing potential[18,34,36] and consequently vascular relaxation. Notwithstanding, the exact molecular mechanism by which H_2O_2 induces vasodilation is not fully clarified.

As mentioned before, HPs are a type of ROS that are also elevated in SS[27]. Recent evidence by Mink et al.[7, 8] suggests that some HPs can produce myocardial depression in experimental conditions. These authors showed that the HP, POX, caused myocardial depression in a right ventricular trabecular preparation by the endothelial release of NO and activation of guanylate cyclase (GC). The explanation for these observations is that POX's structure is similar to the structure of H_2O_2 (See Figure 4). Furthermore, the authors showed that POX is a pseudosubstrate for catalase in the formation of compound I, a catalase derivative, which is formed after the interaction of catalase with H_2O_2[7]. So the effect of POX may be related to its chemical structure as well as its ability to produce compound I. These novel observations open another mechanism in the cardiovascular dysfunction of SS and warrants further research in the matter.

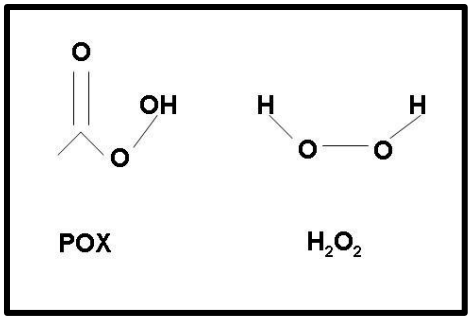


Figure 4. POX's chemical structure is similar to H_2O_2. POX structure presents a hydroxyl radical as compared to the simple oxygen and hydrogen bonds found in the H_2O_2 molecule.

1.4.3. The role of K^+ channels in SS

In recent years there has been a greater recognition for the role of the VSMC membrane potential in the cardiovascular dysfunction that occurs in SS[31]. The normal resting membrane potential of the VSMC membrane is around -30 to -60 mV[30]; a small alteration in this potential produces a substantial change in vascular tone. A less negative membrane potential (depolarization) and a more negative potential (hyperpolarization) generate modifications in the intracellular concentrations of calcium; thus, producing vasoconstriction and vasodilation respectively (see Figure 5)[31, 50, 51].

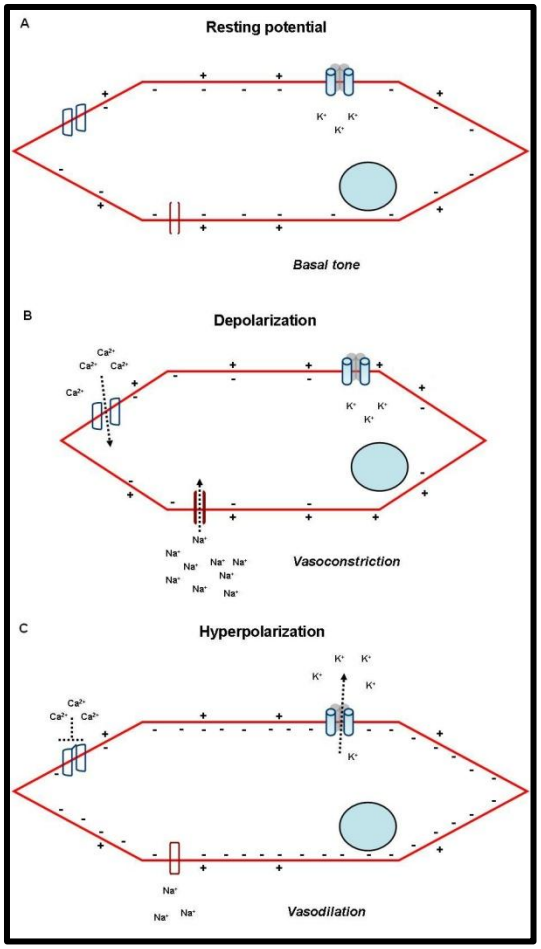


Figure 5. The opening of K+ channels produces VSMC hyperpolarization and consequently vasodilation. A) Normal vascular resting potential (-30 to -60 mV) is determined by the ion balance across the membrane. B) This potential is modified by the sudden opening of Na^+ channels to increase the intracellular potential (depolarization) which opens voltage-dependent Ca^{2+} channels and induces vasoconstriction. C) When the cell depolarizes up to a point, K+ channels are activated which produces an efflux of K+, decreasing the membrane potential.

Numerous ionic channels contribute to the establishment of resting membrane potential in the VSMC[51]. From these channels, K^+ channels are key contributors to the basal membrane potential of VSMC in physiologic conditions[50] and have been implicated in the pathophysiology of SS[31,51]. Four different types of K^+ channels have been identified in VSMC: a) Ca^{2+} activated K^+ channels (K_{Ca}), b) voltage activated K^+ channels (K_V), c) ATP-sensitive K^+ channels (K_{ATP}) and

inward rectifier K^+ channels (K_{IR})[34,50,51]. Most of the evidence in SS seems to link the K_{ATP} channel in experimental and clinical studies of the condition[52,53]. It was initially hypothesized that excessive activation of K^+ channels (in particular the K_{ATP} channel) occurred in the arterioles of patients with SS and that this would be the cause of the profound systemic vasodilation observed in this disease[31,50,51]. Indeed, multiple basic and clinical studies were done to test this hypothesis. In animal models of SS using rats, sheep and pigs, the administration of glibenclamide (a sulfonylurea that blocks competitively the K_{ATP} channel) reversed the hypotension that developed after the production of sepsis and also, increased the systemic vascular resistance in some of the animal groups[53]. However, in the clinical setting, glibenclamide was not effective in improving the drop in BP or reducing the amount of vasopressor needed in a cohort of patients with severe sepsis and SS[54]. The disappointing results with glibenclamide can be explained by multiple reasons. Firstly, K_{ATP} channels are not the main contributors for physiologic vasodilation[34,50], on the contrary, it appears that the K_{Ca} channels are the most relevant in the vasodilation process since they are extensively expressed in all VSMC[34, 50, 51]. Secondly, glibenclamide blocks the sulfonylurea receptor subunit of the channel and not the other portions of the K_{ATP} channel which may reduce its effectiveness[54]; and finally the clinical studies had several methodological difficulties which limit the value of the results[52]. Given these results, multiple investigators have explored the involvement of the other K^+ channels in SS. Cauwels, et al[55]; found that inhibitors of the small-conductance calcium activated channel (SK_{Ca}) and large-conductance calcium activated channels (BK_{Ca}) - subtypes of the K_{Ca} channel family – protected mice from death due to injected endotoxins. Furthermore, the vascular hyperpolarization observed in animal models of SS could be reversed with BK_{Ca}

blockers which suggest the role of these channels in SS induced vasodilation[56]. Despite the vast amount of evidence involving different types of K^+ channels in SS, further research is needed to determine the exact combination of K^+ channels that are involved in the systemic vasorelaxation that is seen in SS.

The mechanism of K^+ channel activation in SS is still a matter of debate; although it seems to be multifactorial. Inflammatory mediators, vascular shear stress and endothelial substances have been implicated as K^+ channel activators in SS[31, 32]. From the inflammatory mediators, PGs and other AA metabolites directly or indirectly activate K^+ channels[57]. There is evidence showing that AA, can affect K^+ channels by producing transient K^+ currents across the VSMC membrane[57, 58]. The same has been suggested for PGs and particularly, prostacyclin (PGI_2). However, there is limited evidence regarding the mechanism by which this activation occurs and further research in this area is needed.

Besides the usual inflammatory mediators, ROS are also generated in excess in SS[17] and they are important signaling molecules that may activate ionic channels[9]. From all the ROS, multiple studies have proven that H_2O_2 is capable of modulating vascular tone through several mechanisms[9]. One potential mechanism that explains the changes in vascular tone that occur with H_2O_2 is the activation of K^+ channels[47, 48]. H_2O_2 activates BK_{Ca} and SK_{Ca} channels in VSMC[47,49], increasing the efflux of K^+, thus, inducing membrane hyperpolarization and consequently vascular relaxation[33, 34, 36]. Notwithstanding, the exact molecular mechanism by which H_2O_2 activates K^+ channels is not completely understood as well as the type of K^+ channels involved.

The activation of K^+ channels has been suggested as an important mechanism for the vasodilation that occurs in SS; however the clinical studies done with some K^+ channel blockers have been so far unsuccessful, which warrants more investigation on the type of mediators that activate the channels and the specific type of K^+ channels involved in the systemic vasorelaxation of SS.

1.5. Study Rationale

In SS there is an increase in the production and release of cytokines and non-cytokine mediators that contribute to the host-pathogen mismatch responsible of the cardiovascular dysfunction seen in SS. Recently, Lzm-S has been discovered as an important mediator in SS that could contribute to the hypotension and the myocardial depression seen in this condition. Lzm-S intrinsically generates H_2O_2 which activates dependent pathways to produce the dysfunction. Similar to what happens with Lzm-S, HPs are a type of ROS that are generated by granulocytes and other cells and are also augmented in SS. HPs can produce various forms of cellular damage and may contribute to the cardiovascular collapse of the disease. The HP, POX, has a very similar structure to H_2O_2 except an additional hydroxyl moiety attached to the molecule and one of its advantages is that it is capable of forming compound I after its reaction with catalase as demonstrated by Jones and Midlemiss[31]. In preliminary studies POX was capable of producing myocardial depression in a right ventricular trabecular preparation[7, 8] which was related to its ability to generate the catalase derivative compound I. Due to these special characteristics we considered that POX was a suitable drug to investigate the effects of HPs in the arterial vasculature and determine whether POX could produce arterial vasodilation in a manner similar to one observed with Lzm-S as well as establish the pathway of this effect. The knowledge of this mechanism may prove to be important in the treatment of SS.

Our main hypothesis was that the HP, POX, can produce a decrease in tension in the carotid artery rings.

1.6. Study objectives

In this study we used an arterial ring preparation as a bioassay to test the main hypothesis. We removed arterial segments from dogs and pretreated them with several inhibitors and treated it with POX. We also used other techniques to clarify further our results.

Our primary objective was to investigate whether POX was able to produce vasodilation in an arterial organ bath preparation and the pathway involved in this effect.

After the initial results, we also examined whether this effects could be replicated in a cell culture preparation.

Materials and Methods

Drugs and reagents used

POX, bovine catalase, L- monomethylarginine (L-NNMA), SQ-22, ODQ, indomethacin, arachidonyl trifluoromethyl ketone (ATK), quinacrine, 17-ODYA, miconazole, barium chloride, glyburide, 4-AP, TRAM-34, iberiotoxin APA, ChTX, were purchased from Sigma Aldrich, St. Louis, MO, USA.

SKA-31 and 1-EBIO were purchased from Tocris Bioscience, Bristol, UK.

Animal tissue work

All of the experiments were approved by the University Animal Care Committee and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (NIH Publication No 85-23, 1996)[59].

2.1. Carotid artery ring preparation

We used a carotid artery ring preparation as a bioassay to determine the role of POX in causing vasodilation of the carotid rings and the subsequent pathway involved. We used the method described by Mink et al[8]. As a first step, we removed internal carotid artery segments (4 cm length) from previously anesthetized (pentobarbital 45 mg/kg) mongrel dogs (15 to 25 kg). The tissue was immediately stored in cold HEPES buffer until the start of the experiment (less than 5 minutes). Then, we dissected the arteries free from the surrounding tissues and cut the artery to obtain rings ≈ 4mm in length and 4 mm outer diameters, which were then, suspended using 2 stainless steel triangles to stretch the rings to optimal length (≈ 4 g). We placed the rings into a 10 ml organ bath filled with HEPES-buffered physiological solution (in mmol/L: 118 NaCl, 2.5

$CaCl_2$, 1.2 $MgCl_2$, 1.4 KH_2PO_4, 4.9 KCl, 25 HEPES and 11 glucose), bubbled the solution with 100 % O_2 into the bath, and set the temperature at 37^o C with a pH of 7.35. Tension was measured using a force transducer (Grass Instrument, West Warick, RI). This carotid artery preparation allowed us to obtain a greater number than from other systemic vessels. In a subgroup of experiments we determined whether POX caused the same effect in a superior mesenteric artery ring preparation.

In this protocol, we initially preconstricted the carotid rings with phenylephrine (10^{-5} mol/L)[7] to obtain a stable tension and when we observed that the tension reached a plateau, we made the appropriate measurements (approximately 20 minutes post- phenylephrine instillation). In the present study, we used POX to produce a vasorelaxation of the arterial rings. We prepared stock solutions of POX ($5x10^{-3}$) by diluting it with distilled water as described by Jones and Middlemiss[60]. Then, 30 minutes before its utilization, we added bovine catalase ($2\ x10^{-9}$) to the POX solution to promote the formation of compound I and to prevent artifacts arising from the presence of H_2O_2. We kept the resulting solution under refrigeration to ensure the formation of the derivative. On the basis of our previous experiments[7,8], we tested two different concentrations of POX in the bioassay, $1.25\ x10^4$ and $2.5\ x10^{-4}$ mol/L in order to determine which one of these resulted in a more consistent arterial vasodilation.

After the phenylephrine induced contraction, we waited for a stable plateau, and then added POX to the preparation in which we made measurements at 5, 15 and 30 minutes post-POX. A graphic display of the arterial preparation protocol can be seen in Figure 6.

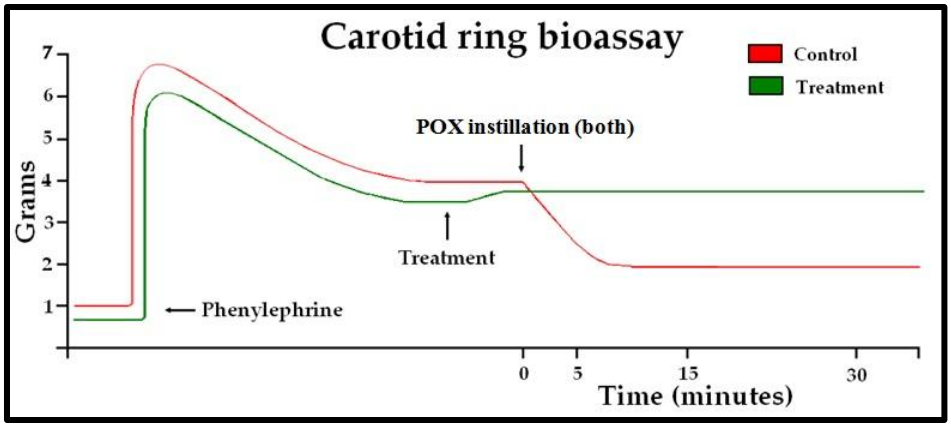


Figure 6. Graphic display of the protocol used in the carotid artery preparation. We constricted the arterial rings with phenylephrine. Then we waited until the tension stabilized to pre-treat the preparation with any given inhibitor. POX instillation was used after that and the results were measured at the treatment, at 5, 15 and 30 minutes post POX.

2.1.1. Experiments to determine if the endothelium, NO formation, sGC or the adenylate cyclase pathways are involved in POX-induced vasodilation:

To determine the role of the endothelium, we mechanically denuded the carotid artery endothelium with a cotton-tipped applicator[8,12,13] after which we determined POX's vasodilatory activity in this preparation. To confirm the denudation of the endothelium we used acetylcholine as described by Furchgott and Zawadzki who showed that the endothelium presence was necessary for the relaxation of an isolated artery in response to acetylcholine[62], an effect that is generated by the NO-cyclic guanosine monophosphate (cGMP) pathway[63]. The data analyzed for these set of experiments came from the acetylcholine confirmed denuded endothelium carotid artery rings.

In another set of experiments and with a similar protocol, we pre-exposed the carotid artery rings to the nonspecific NO synthases (NOSs) inhibitor N^G -monomethyl-$_L$-arginine ($_L$-NMMA) to establish if NO production by the endothelium was necessary for POX's induced vasodilation.

To further observe if the treatment had a dose-effect relationship, we used 2 different concentrations of $_{L}$-NMMA, 10^{-5} and 10^{-6} mol/L in which we incubated the carotid artery preparation for ~30 minutes, allowing the maximal inhibitory effect of the treatment[7, 8] after which we compared the results to a control group without $_{L}$-NMMA.

The intracellular second messengers, cGMP and cyclic adenosine monophosphate (cAMP) are produced by the activity of guanylyl cyclase (GC) and adenyl cyclase (AC) respectively. They are involved in vascular smooth muscle cell relaxation through the activation of dependent protein kinases[63]. We determined whether these pathways were relevant to POX's vasodilatory effect. In one experiment, we treated the rings with 1H-[1, 2, 4]oxadiazolo[4, 3-a]quinoxaline-1-one (ODQ) at 10^{-5} mol/L, a potent and selective inhibitor of GC, while in a second experiment, we added the selective AC inhibitor SQ-22,536 at 10^{-5} mol/L to examine the role of AC in POX's induced vasodilation.

2.1.2. Experiments to determine whether the AA pathway is involved in POX's-induced decrease in vascular tone.

AA metabolites, such as prostanoids, are produced by the PGHS pathway (i.e. COX) and are widely recognized as vascular tone modulators[34,64,66]. By using indomethacin, a slow tight-binding inhibitor of PGHS[67], we tested whether POX's action could be related to the production of prostanoids by PGHS. We pretreated the carotid artery preparation with four different concentrations of indomethacin (10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} mol/L) to examine the dose-relationship effect.

In subsequent experiments, we also explored whether AA metabolism by means of the (lipoxygenase) LO and epoxygenase (EPO) pathways were involved in POX's vasodilatory effect. We treated the carotid ring preparation with eicosatrienoic acid (ETI) a general inhibitor of the LO pathway (21), 17-octadecynoic acid (17-ODYA) a potent inhibitor of LTB4 ω-oxidase, and with miconazole a general blocker of EPO pathway in respective experiments to determine whether any of these inhibitors prevented POX's vasodilatory activity.

Phospholipase A_2 (PLA_2) is an enzyme that hydrolyzes membrane phospholipids to yield AA[64]. PLA_2 has several classes, one of which is the cytosolic PLA_2 ($cPLA_2$) class that plays a major role in AA release and has been shown to be activated by H_2O_2[65]. To determine whether PLA_2 was involved in POX's effect, we pretreated the carotid ring preparation with the $cPLA_2$ specific inhibitor arachidonyl trifluoromethyl ketone (ATK) at three concentrations: 10^{-3}, 10^{-4} and 10^{-5} mol/L and with the non-specific PLA_2 inhibitor quinacrine (10^{-4} mol/L) in respective experiments.

2.1.3. Measurement of PGI_2 production

Since we found that indomethacin could block the effect of POX, we determined whether POX could increase PGI_2 production to cause this effect. In these experiments, we obtained fluid samples from the buffer of the arterial preparation at baseline, at the moment of stabilization in tension after the phenylephrine administration and after 20 minutes of the POX instillation. We chose the 30 min time-point of the last sample due to the fact that POX's induced vasodilation is maximal at this specific period. We determined PGI_2 levels using an ELISA-assay that quantifies the major PGI_2 metabolite, 2, 3-dinor-6-keto $PGF_{1\alpha}$, since PGI_2 half life is about 2-

3 min and then is quickly converted to its principal metabolite. The measurement was done according to the manufacturer's protocol (Cayman Chemical's ACE, Burlington, ON, Canada).

2.1.4. Experiments to determine whether POX's action may be related to potassium channels.

Since AA metabolites may activate K^+ channels, we determined whether this may be the case with POX. To assess this possibility, in a subgroup of experiments, instead of contracting the carotid artery ring with phenylephrine, we used a high potassium chloride (KCl) solution (25 mmol/L) in the preparation to generate vasoconstriction and tested whether POX could decrease the tension in these groups of rings. After the KCl induced increase in vascular tone and a stable plateau was attained, we determined the effect of POX in this preparation.

2.1.5. Experiments to determine if K^+ channels are involved in POX's induced vasodilation.

The results obtained with POX and the KCl-induced contractions suggested that K^+ channel were involved in its action, so, we analyzed separately the four known types of K^+ channels in the vascular smooth muscle. At first, we added to the arterial preparation the selective inhibitor 4-aminopyridine (4-AP) in a 10^{-4} mol/L concentration to determine if Kv channels were involved[51]. Then we investigated whether glyburide (10^{-4} mol/L), a sulfonylurea (specific inhibitor of K_{ATP} channels), could block POX's induced vasodilation. We also used (Ba^{2+}) at two concentrations in the arterial bath, 10^{-3} and 10^{-4} mol/L, to examine the role of K_{IR} channels. Furthermore, since K_{Ca} are abundantly expressed in VSMCs, we used selective blockers for each K_{Ca} subtype, such as iberiotoxin, for BK_{Ca}, TRAM-34 and charybdotoxin (ChTX) for IK_{Ca} and apamin (APA) for SK_{Ca} and a combination of selective and non-selective blockers, i.e. APA and ChTX.

Since the results suggested that there was a relationship between prostaglandins and K^+ channels in the mechanism of POX- induced vasodilation, we added epoprostenol (EPOP), a prostacyclin analogue, at two different concentrations (2 $x10^{-5}$, 5 $x10^{-5}$) instead of POX to produce vasodilation in the arterial preparation. We utilized APA and ChTX to determine if they could block the relaxation caused by EPOP. We performed measurements at 5, 15 and 30 minutes after the EPOP addition to the bath.

2.1.6. Experiments to determine whether the SK_{Ca} and IK_{Ca} channels are activated in the canine VSML.

Since we found that POX's action could be blocked by the combination of SK_{Ca} and IK_{Ca} channel inhibitors, the results suggested their role in POX's induced vasodilation. However, we were unsure of the site of K^+ channel activation, whether it was in the endothelial layer or in the VSML. Since we previously found that POX's action was independent of endothelial integrity, we hypothesized that the K^+ channel activation occurred in the VSMC[1,72]. To determine whether SK_{Ca} and IK_{Ca} were activated in the VSML we denuded the endothelium (as previously described) in a pair of carotid artery rings, while the endothelium was left intact in another two rings as a control. Then we constricted all of the rings with phenylephrine (10^{-5} mol/L). After the tension was stable, we added acetylcholine (10^{-5} mol/L) in all of the rings to confirm the successful removal of the endothelial layer in the denuded endothelium arterial segments (demonstrated by the absence in vasodilation); meanwhile, we confirmed the presence of endothelium in the intact rings (shown as a profound vasodilation). Subsequently, we washed the rings with HEPES buffer until the measured tension became stable at the baseline level. Once the tension in the rings had stabilized at the baseline level we constricted the rings again

with phenylephrine after which we added two K^+ channel activators in all the rings. SKA-31 (naphtha [1, 2-d] thiazol-2-ylamine) which is a specific activator of the SK_{Ca} channel was the first activator tested. The second activator we used was 1-EBIO (1-Ethyl-2-benzimidazolinone) which activates the IK_{Ca} channel. We compared the results between the treated denuded endothelium carotid artery rings against the non treated denuded arterial segments for every activator.

2.2 Spectroscopy experiments

Because the mechanism by which POX caused vasodilation seemed to be related to the production of PGs; we hypothesized that PGHS was also involved in this reaction. As previously mentioned, PGHS is an essential enzyme in prostaglandin synthesis that has two distinct activities: a COX and a peroxidase reaction[66,67,69]. The peroxidase reaction requires a HP to initiate the COX reaction[70]. Since POX is a HP, we tested by spectrophotometric analysis (Pharmacia Biotech, Ultraspec 3000) whether POX could act as a substrate for the PGHS peroxidase reaction. In the spectrophotometer cuvette (1 ml), we added ovine PGHS-1 (supplied in 80 mM Tris-HCl buffer, pH 8.0 with 0.1% Tween 20 and 300 µM of diethyldithiocarbamate as a preservative from Cayman Chemical, Burlington, ON, Canada) and incubated with haematin (1µM) for 1 min at 37° C and the change in absorbance of the native enzyme was recorded. Then, we added into the cuvette POX 10^{-6} mol/L solution after which we registered the difference in absorption of the PGHS-1 enzyme at 5, 10, and 15 min post POX instillation.

2.3 To determine the effect of POX in a Human aortic vascular smooth muscle cell (HAVSMC) preparation

In these experiments, to support the findings observed in the carotid artery preparation, we determined whether POX could directly lengthen phenylephrine constricted human aortic vascular smooth muscle cells (HAVSMC). We obtained HAVSMC from ATCC (Manassas,VA). The cells were cultured at $37^{o}C$ in a humidified 5% CO_2 and incubated in vascular cell basal medium (ATCC PCS 100-030) supplemented with 5% fetal bovine serum, rh FGF-basic 5 ng/ml, rh insulin 5 ug/ml, ascorbic acid, 50 ug/ml, L-glutamine 10 mm, rh EGF 5ng/ml, gentamicin 10 ug/ml, amphotericin B 0.25 ug/ml, penicillin 10 units/ml, streptomycin 10 ug/ml. The cells were grown to about 50% confluence. We then exchanged the medium to serum free F12 medium (GIBCO 21700-075) that also contained 1% insulin-transferrin-selenium (CAT No 51500-056, GIBCO). The cells were incubated for another 48 hrs, after which the medium was changed to fresh F12 (1 ml) before initiation of the experiment. We measured cell length with a microscope (Olympus IX 71) attached to a computer with image –proanalyzer software (In vivo and Image – Pro-Analyzer, version 6.2, Media Cybernetics, Silver Springs, MD). We measured baseline lengths of 10 cells, then, we added phenylephrine to the medium to a final concentration of 10^{-5} mol/L to cause the cells to shorten. The lengths of the same cells were again measured after 30 minutes of phenylephrine incubation. We examined several experiments in this HASVSM preparation in which 10 cells were counted for each group within each experiment. In this experiment, we determined whether POX could lengthen cells constricted with phenylephrine. After the measurement of basal lengths and addition of phenylephrine to the preparation, we

measured the cells and then immediately instilled POX (10-6 mol/L) and measured the resulting cell lengths.

2.4 Statistical Analyses

We compared the results obtained for a given inhibitor concentration in the organ bath preparation by a two –way ANOVA (between-within) analysis in which there are different groups (ie. one level of comparison) and different time periods (second level of comparison) (GB-Stat V8, Dynamic Microsystems, Inc., Silver Springs, MD). From the results of the ANOVA table, we performed a Student Newman Keuls' comparison test to determine where differences were observed among groups at a specific time period. The results are reported as mean $\pm$ 1SD. We considered a p value of < 0.05 to be statistically significant. We did the analysis of the spectroscopy experiments using a one –way ANOVA in which a value of $p < 0.05$ was considered as statistically significant.

Results

3.1 Carotid artery ring preparation

After a phenylephrine induced contraction, POX produced a consistent fall in the measured force of the arterial preparation that occurred a few minutes after its instillation. These results are shown in Figure 7. From this figure, we can observe that there was a dose –response effect in which the higher concentration of 2.25 x 10^{-4} mol/L POX caused an even greater decrease in tension than the lower concentration (e.g. 1.25 x10^{-4} mol/L POX) at 15 and 30 minutes post instillation. Based on these results we used the higher POX concentration (2.25 x10^{-4} mol/L) in the remaining experiments.

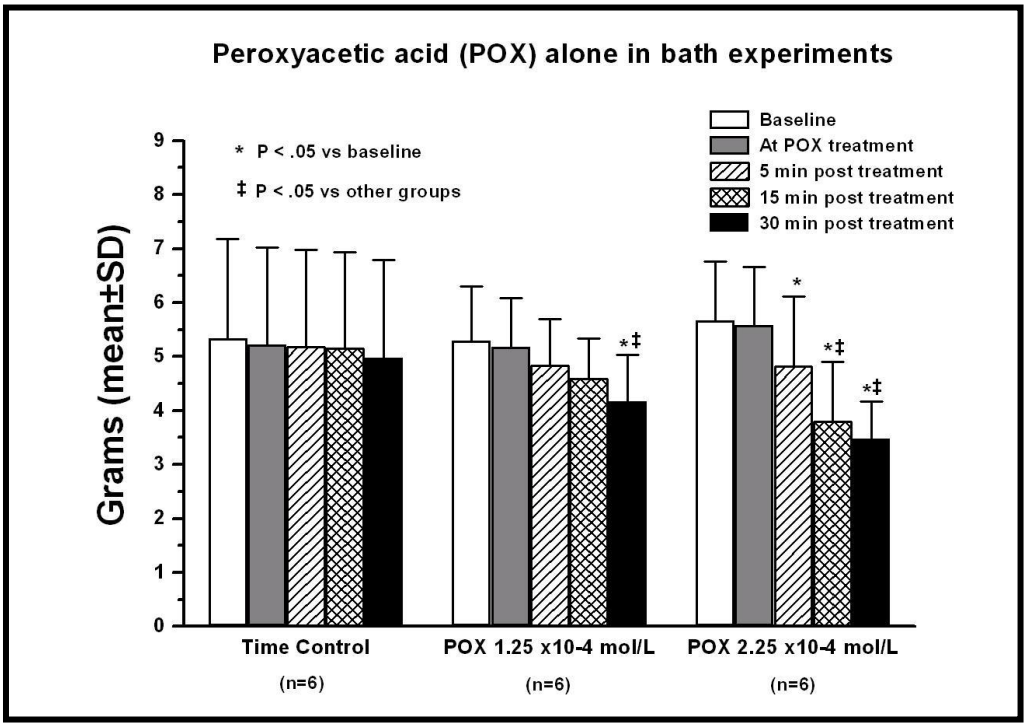


Figure 7. POX's induced vasodilation experiments. POX produced a consistent decrease in tension of the arterial rings, particularly with the highest POX concentration tested.

3.1.1. Experiments to determine if the endothelium, NO formation, sGC or adenylate cyclase pathways are involved in POX's vasodilation.

In one set of experiments, we determined whether the endothelium was required for POX's vasodilatory response. We found that the vasodilatory response for the endothelium-denuded group at 5, 15 and 30 minutes post POX did not differ from the one observed in the intact endothelium group (77 ± 10% from baseline tension *vs* 74 ± 11% at 30 minutes post POX). The only statistical significant difference encountered throughout this experiment was the absolute value of lower contractile force by the arteries in the removed endothelium group as compared to the intact endothelium group (4.9 ± 0.8 grams of tension *vs* 5.43 ± 0.9). The mean results are shown in Figure 8A.

We also pretreated the arterial preparation with 2 concentrations of the NOSs inhibitor $_{L}$-NMMA and found that this treatment did not prevent the vasodilatory effect of POX (see Figure 8B). To test whether cGMP was relevant as a second messenger for vasodilation in our preparation, we pretreated the arterial rings with ODQ, a potent and selective inhibitor of GC. As shown on Figure 8C, at the start of the experiment, ODQ augmented the tension in the preparation, but, there was no statistical significant difference ($p < 0.05$) between the ODQ treated and the control groups at 30 minutes post POX (4.57 ± 0.5 *vs* 4.06 ± 1.6 grams). There was also no difference observed with the AC inhibitor SQ 22,536 treated group versus the control group at the end of the experiment (4.93 ± 0.5 *vs* 4.87 ± 0.3 grams). See Figure 8D.

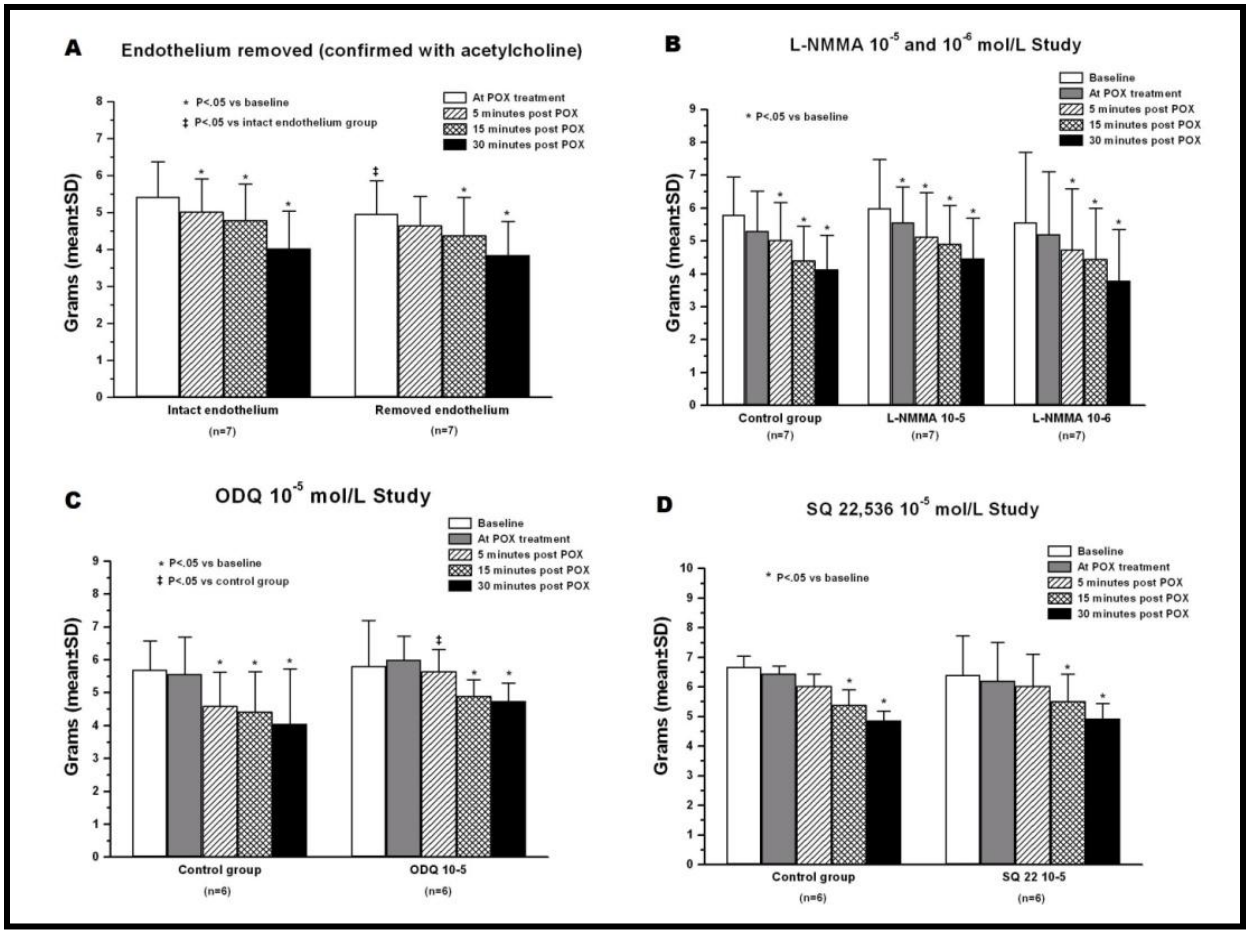


Figure 8. POX induced vasodilation is not related to the effects of: a) endothelium integrity; b) NO synthesis; c) GC pathway and d) AC pathway. A) POX effect is independent of the endothelium integrity; B) POX does not appear to increase the synthesis of NO in the preparation; C) The GC specific blocker, ODQ failed to inhibit the decrease in tension produced by POX; D) SQ, the AC inhibitor did not block POX's induced vasodilation.

3.1.2. Experiments to examine the role of the PGHS enzyme in POX-induced vasodilation.

In another set of experiments, we determined whether the AA pathway is involved in POX's decrease of vascular tone. To accomplish this, we pretreated the arterial preparation with different concentrations (10^{-5}, 10^{-6} and 10^{-7} mol/L) of the PGHS non-specific inhibitor indomethacin. We observed that POX's vasodilatory response was blocked by all the tested concentrations of indomethacin. Furthermore, at the highest concentration used (10^{-5} mol/L)

we observed a significant and consistent increase in vascular tone (at 5, 15, and g at 30 minutes post POX, $p < 0.05$). For the other indomethacin concentrations (10^{-6} and 10^{-7} mol/L), there was an inhibition of vasodilation compared to the control group, particularly at the 30 minute interval post instillation. See Figure 9.

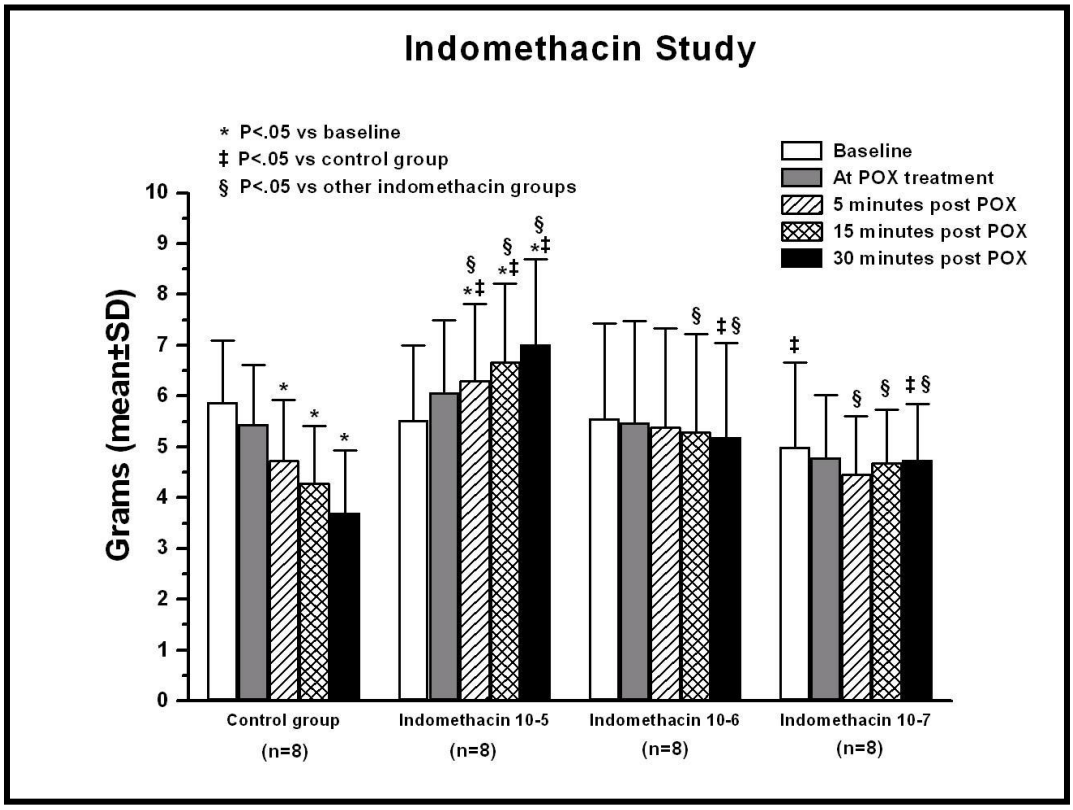


Figure 9. Indomethacin inhibited the decrease in arterial tension produced by POX. The COX inhibitor, indomethacin, significantly blocked POX's induced vasodilation. The highest concentration of indomethacin increased the tension in the arterial rings.

Based on the results obtained with indomethacin, we decided to explore the AA pathway (i.e. mechanism of release and metabolites) in order to determine whether POX's effect was related to the release of AA by PLA_2, in particular $cPLA_2$. We exposed the carotid rings to the specific $cPLA_2$ inhibitor ATK. The results show that there were no differences between the control and

the treated groups (see Figure 10). We also found no statistical significant differences ($p < 0.05$) with the use of the non-specific PLA_2 inhibitor quinacrine (10^{-4} mol/L) as seen in Figure 11.

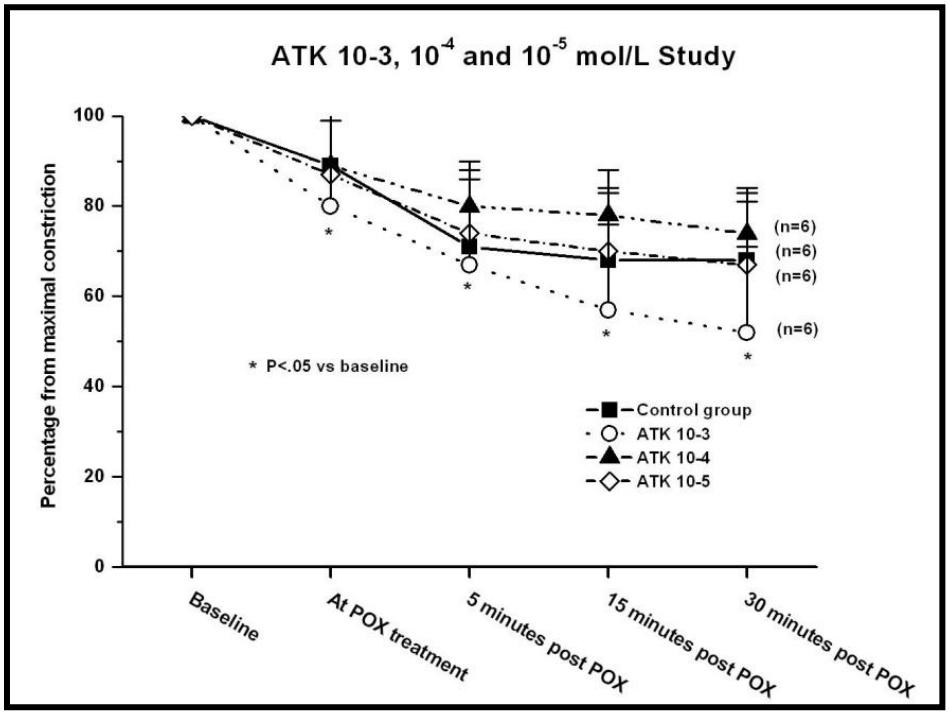


Figure 10. POX's induced-vasodilation is not associated with the release of AA by $cPLA_2$. POX's action is not related to the activation of $cPLA_2$ as shown by the failure of ATK to inhibit the decrease in arterial tension.

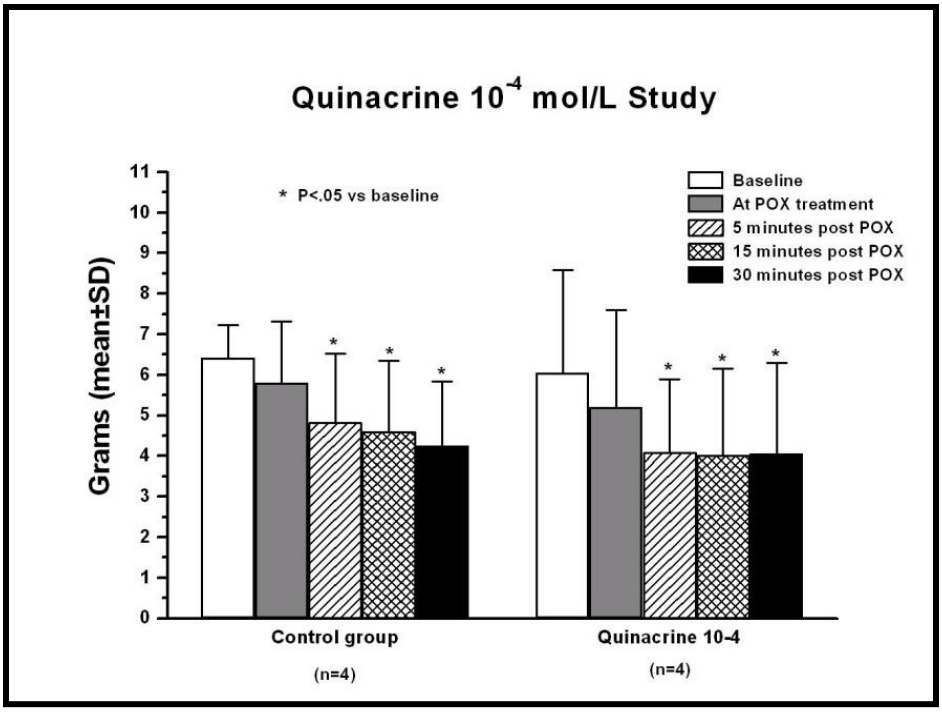


Figure 11. POX's response is not related to the action of PLA_2 on AA. Quinacrine is a non-specific blocker of PLA_2 that was incapable of attenuating POX's induced decrease in arterial tension.

In other experiments, we pretreated the carotid artery rings with LO and EPO pathways inhibitors. We used several blockers of these pathways (specific and non-specific) and we found no inhibitory effect by the agents as shown on Table 4.

Table 1. *POX action was not blocked by LO and EPO inhibitors*

Groups	n	Baseline (g)	At POX treatment (g)	5 min post POX (g)	15 min post POX (g)	30 min post POX (g)
Control group (POX alone)	3	5.7 ± 0.47	5.62 ± 0.54	4.23 ± 1.3	3.91 ± 0.52 *	3.33 ± 0.8 *
ETI 10^{-4} mol/L	3	5.29 ± 0.83	4.95 ± 0.95	4.08 ± 1.01 §	4.125 ± 0.62	3.95 ± 0.64 *
17-ODYA 10^{-4} mol/L	3	5.95 ± 1.44	5.54 ± 1.68	4.08 ± 2.37 §	3.83 ± 2.07 *	3.41 ± 1.6 *
Miconazole 10^{-4} mol/L	3	5.6 ± 0.5	4.83 ± 0.67	4.15 ± 0.45	4.08 ± 0.49 *	3.52 ± 0.94 *

Values are mean ± SD. * $P < 0.05$ vs baseline; § $P < 0.05$ vs control group .

Table 4. POX's effect is not related to the LO and EPO pathways metabolites.

3.1.3. PGI_2 measurements

We considered that the vasodilation caused by POX might be mediated by the release of PGI_2 as a result of PGHS activation. To test whether this was the case, we measured the levels of PGI_2 in the buffer solution of the arterial preparation. We found a statistically significant ($p < 0.05$) double fold increase in the production of PGI_2 at 30 min post POX addition to the bath as compared to the placebo group. See Figure 12.

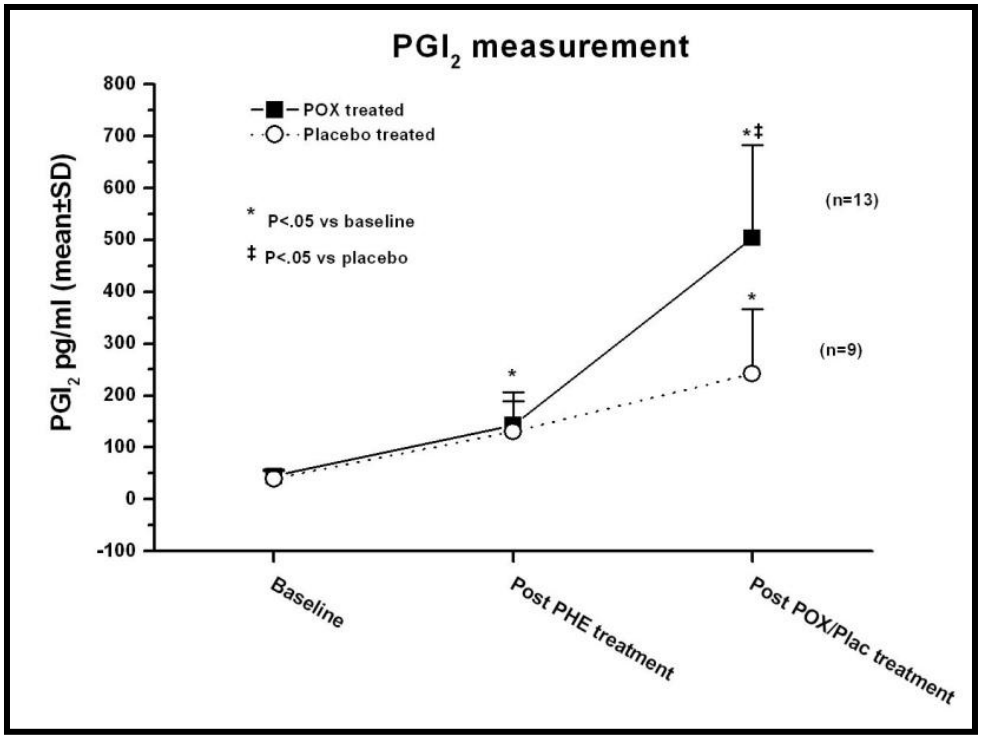


Figure 12. POX increases PGI_2 production. POX activates the PGHS enzyme to produce PGI_2 as demonstrated by the increase in the production of the PGI2 breakdown metabolite 2, 3-dinor-6-keto $PGF_{1\alpha}$.

3.1.4. POX activates K^+ channels

We performed a series of experiments to determine whether the PGHS enzyme stimulated by POX could in turn activate K^+ channels to produce vasodilation. In the first set of experiments, we determined whether high concentrations of KCl could inhibit POX induced vasodilation. We compared experiments in which we produced contraction by phenylephrine and a high concentration of KCl in the bath (25 mmol/L). Although phenylephrine generated a higher tension at the beginning of the study, we found that POX induced a profound vasorelaxation in the phenylephrine contracted group as compared with the KCl group. In Figure 13A we can see an example of the tracings obtained with these groups of experiments. The mean results are shown in Figure 13B.

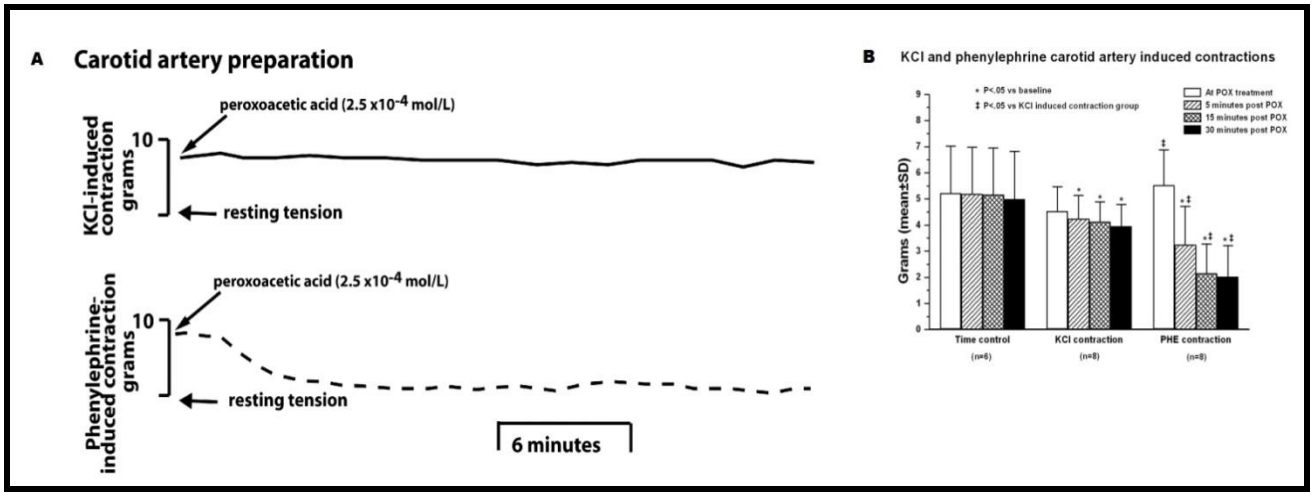


Figure 13. The vasodilation produced by POX was attenuated when the arterial rings were constricted with high concentrations of KCl. A) The vasodilation produced by POX was attenuated when the arterial rings were constricted with high concentrations of KCl. B) Absolute values show the difference between the phenylephrine constricted group vs the KCl constricted group.

3.1.5. Experiments to determine which K^+ channels are involved in POX's induced vasodilation.

Since POX's induced vasodilation was inhibited by the use of high KCl concentrations, we examined the role of K^+ channel activation in POX's response. To study this involvement, we used several K^+ channel blockers in the arterial preparation and compared the results to their respective controls. In the first group of experiments, we found no difference in the values at the end of the experiment when the selective inhibitors for K_V and K_{ATP} channels were used in the arterial preparation. The experiments with the non-selective K_V inhibitor 4-AP and the K_{ATP} blocker glyburide are shown in Figure 14.

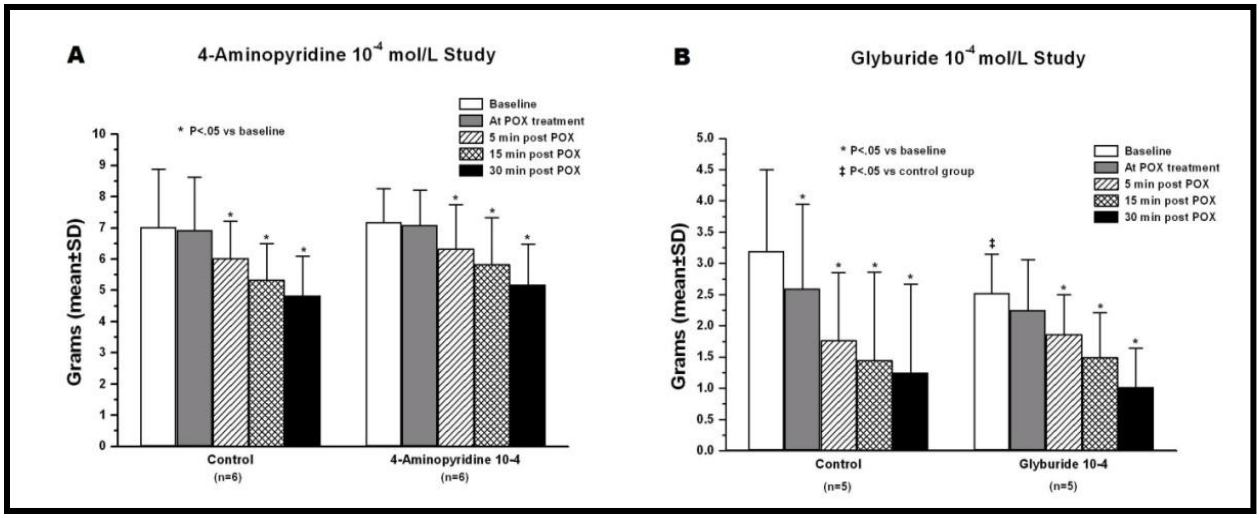


Figure 14. 4-AP and glyburide had no effect in POX's action. A) The specific inhibitor of K_v channels, 4-AP did not block POX's action. B) POX's decrease in arterial tension is not related to the activation of K_{ATP} channels.

On the other hand, when the preparation was exposed to the K_{IR} inhibitor Ba^{2+}, an initial increase in tension was seen in the two concentrations tested (3.81 ± 1.97 control *vs* 5.39 ± 2.45 at 10^{-3} mol/L and 5.6 ± 2.25 grams at 10^{-4} mol/L). However, POX's dilation was significantly ($p < 0.05$) inhibited by both concentrations for the entire experiment duration as seen on Figure 15.

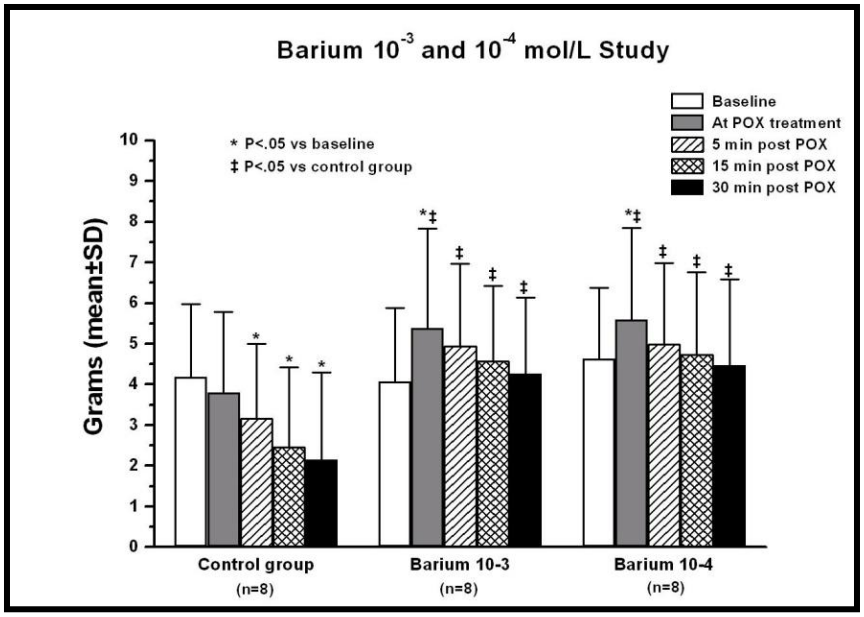


Figure 15. Ba^{2+} inhibited the vasodilation of the rings produced by POX. Ba^{2+} is a specific inhibitor of K_{IR} channels which significantly blocked the vasodilation produced by POX. Both concentrations tested were effective in blocking the effect.

We then tested multiple agents that inactivate K_{Ca} channels such as iberiotoxin (for BK_{Ca}), ChTX (for IK_{Ca}) and APA (for SK_{Ca}). In the iberiotoxin (specific BK_{Ca} inhibitor) treated group we observed a higher phenylephrine induced constriction than the control group, nevertheless, the same decrease in tension after POX was seen in both groups, with no significant differences at the end of the experiment (66 ± 14 *vs* 67 ± 13% from baseline tension in the iberiotoxin group). See Figure 16.

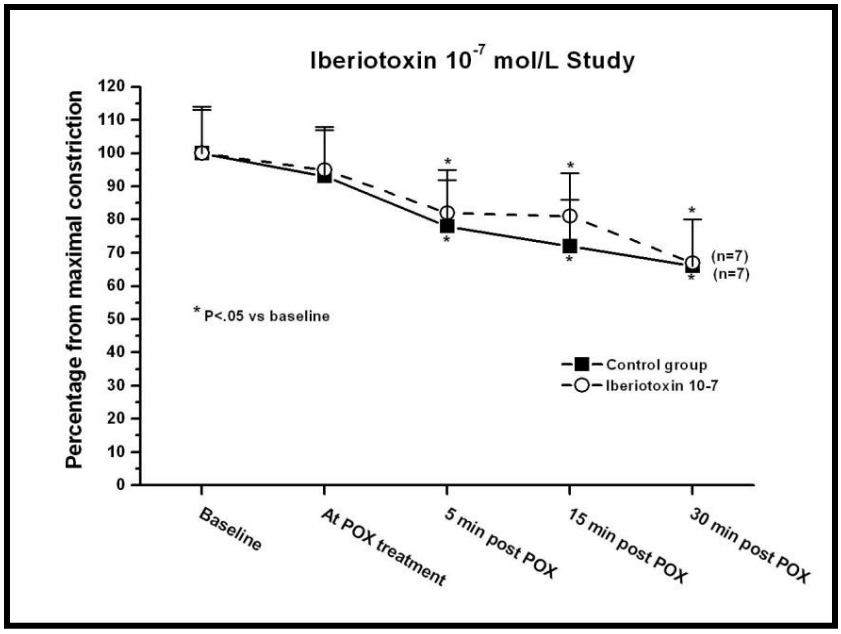


Figure 16. Iberiotoxin had no effect on POX's action. The BK_{Ca} inhibitor, iberiotoxin could not block POX's vasodilation in all the measured timepoins.

The instillation to the preparation of the SK_{Ca} blocker APA produced a modest attenuation of POX's response at the end of the experiment; meanwhile, there was no inhibitory effect when the arterial rings were treated with ChTX. However, when we used a combination of both agents, we observed a marked inhibition of POX's induced vasodilation. Figure 17 shows that two distinct concentrations of the combination of APA and ChTX blocked POX's effect in vascular muscle tone at every time point of the experiment reaching statistical significance ($p < 0.05$) at 15 and 30 minutes for both concentrations tested.

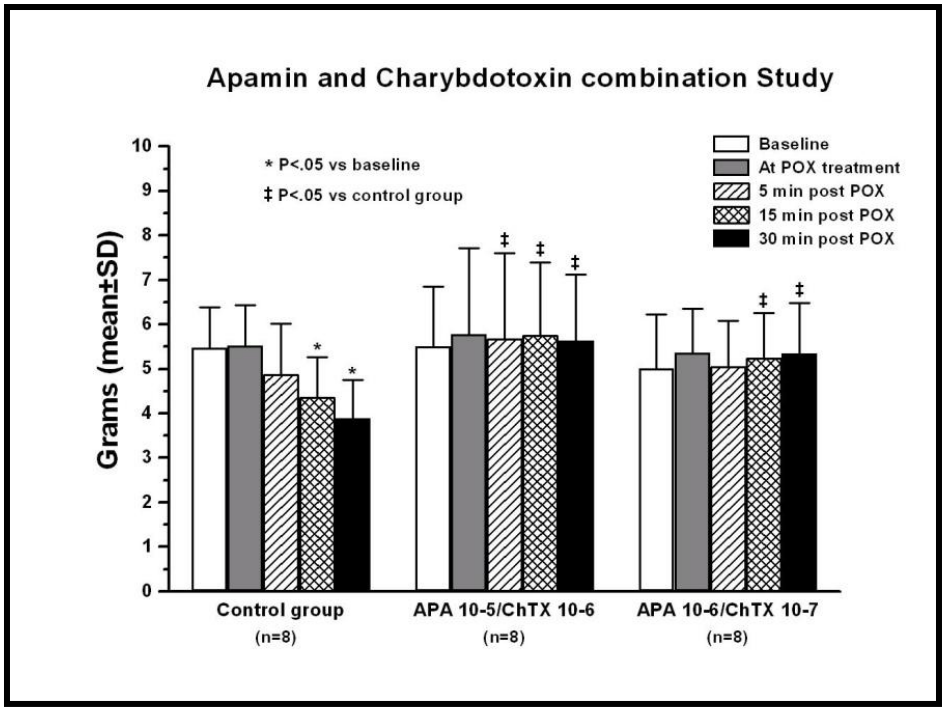


Figure 17. The combination of APA-ChTX blocked POX's generated vasodilation. SK_{Ca} and IK_{Ca} channels seem to be relevant in POX's action as proven by the ability of their respective inhibitors to inhibit the decrease in tension promoted by POX.

Since the previous results suggested that POX's action was related to the increase of PGI_2 and K_{Ca} channel activation, we wanted to examine if there was a link between these two mechanisms. In order to do that, we utilized the PGI_2 analogue EPO as a vasodilatory agent instead of POX. We observed that the dilation was quicker than the one produced by POX (see Figure 18). At 5 min post EPO treatment, the decrease in tone was abrupt and significant, while at 30 min post EPO, the tension returns almost to baseline levels. Interestingly, in the APA-ChTX combination pretreated arterial rings, the fall in vascular tone at 5 min was significantly attenuated (2.86 ± 1.34 grams). The previous result furthers confirms that PGI2 likely causes K_{Ca} channel activation.

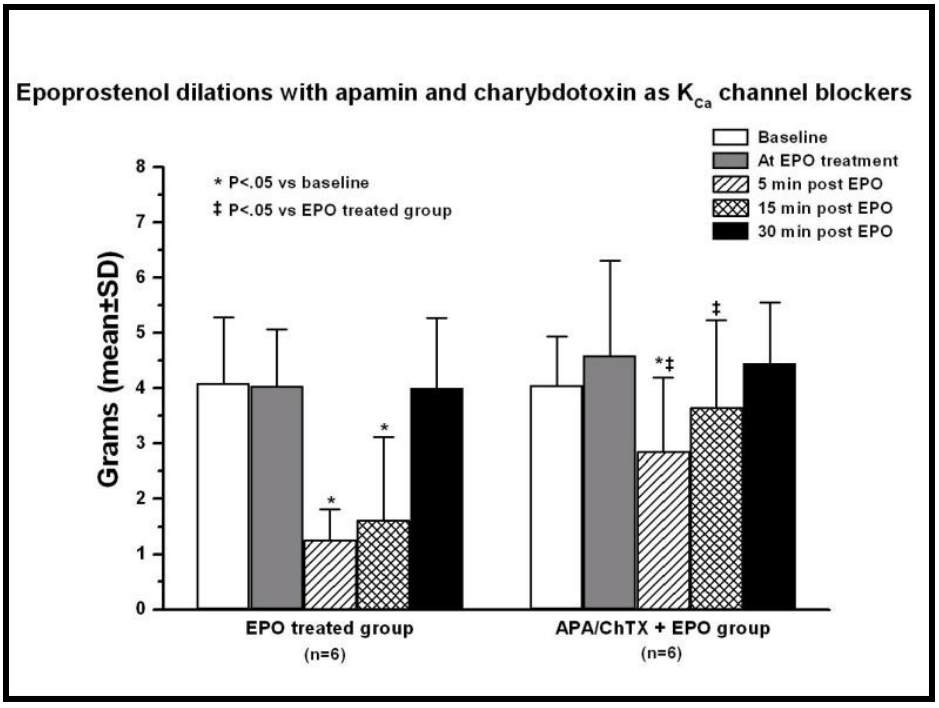


Figure 18. The PGI_2 analogue EPO produced a pronounced vasodilation which was inhibited by the combination of SK_{Ca} and IK_{Ca} channel blockers. The PGI_2 analogue EPO produced a fast and pronounced vasodilation which was inhibited by the combination of SK_{Ca} and IK_{Ca} channel blockers APA/ChTX. The maximal blocking effect is seen at 5 minutes post EPO.

3.1.6. Experiments to show that SK_{Ca} and IK_{Ca} are activated in the canine VSML.

Since the preceding results showed that SK_{Ca} and IK_{Ca} were involved in POX's vasodilation, we wanted to establish whether these channels were activated in the VSML. In one set of experiments, we tested the effects of two different K^+ channel activators in the denuded endothelium arterial preparation. We found that the specific SK_{Ca} activator, SKA-31, generated a marked decrease in the vascular tone of the denuded endothelium carotid artery rings. Furthermore, we observed that SKA-31 induced vasodilation was attenuated by the specific SK_{Ca} channel blocker APA. Mean values are shown in Figure 19.

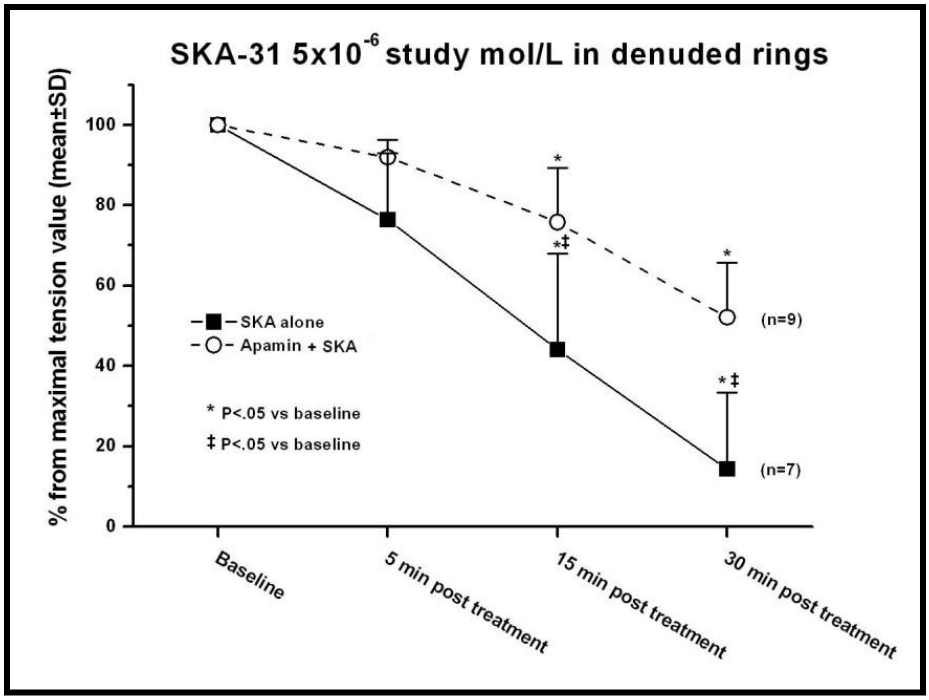


Figure 19. SK_{Ca} can be activated in the VSML of canine tissue to promote vasodilation. In a denuded endothelium arterial ring preparation, SKA-31 activates specifically SK_{Ca} channels to promote a marked and statistically significant ($p<0.05$) vasodilation. The activation of these channels is further shown by the blocking effect seen with the SK_{Ca} inhibitor APA.

Using an identical approach, we utilized the IK_{Ca} activator 1-EBIO to determine if this group of channels was capable of inducing vasodilation in the endothelial denuded preparation. As seen in Figure 20, 1-EBIO produced a significant reduction in vascular tension of the denuded endothelium arterial segments. The response to 1-EBIO was attenuated with the use of ChTX, further confirming the involvement of the IK_{Ca} channel in the preparation. See Figure 20.

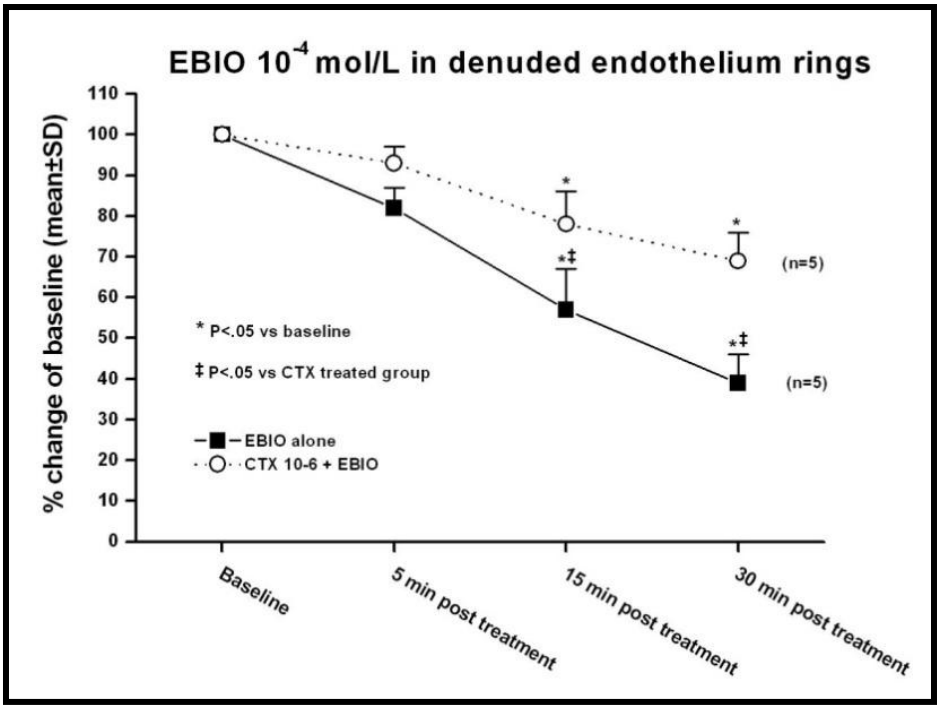


Figure 20. IK_{Ca} channels can be activated in the VSML to promote vasodilation. Similar to the previous figure, EBIO-1 activates IK_{Ca} channels to significantly decrease the measured tension of the rings. The activation of these channels is further shown by the blocking effect seen with the IK_{Ca} inhibitor ChTX.

3.2 Mass spectroscopy experiments

To further assess whether the PGHS enzyme was the link between POX and the generation of arachidonic metabolites, we used wave-scan spectroscopy to determine whether POX administration transformed native PGHS enzyme to its derivatives as an indication of PGHS conversion.

We measured PGHS change in absorption at 411 nm. This represents the absorbance peak obtained with the incubation of the enzyme and haematin 1 µM (.4 ± 0.02). During placebo administration, the enzyme's spectrophometric peak was consistently stable at 5, 10 and 15 min post incubation (see Figure 21A). On the contrary, when we added POX 10^{-6} mol/L to the solution there was sudden and brisk drop in the observed PGHS peak of absorption (.4 ± 0.02 to .34 ± 0.03). Furthermore, the decrease was even more pronounced at 5, 10 and 15 minutes post POX instillation as seen on Figure 21B.

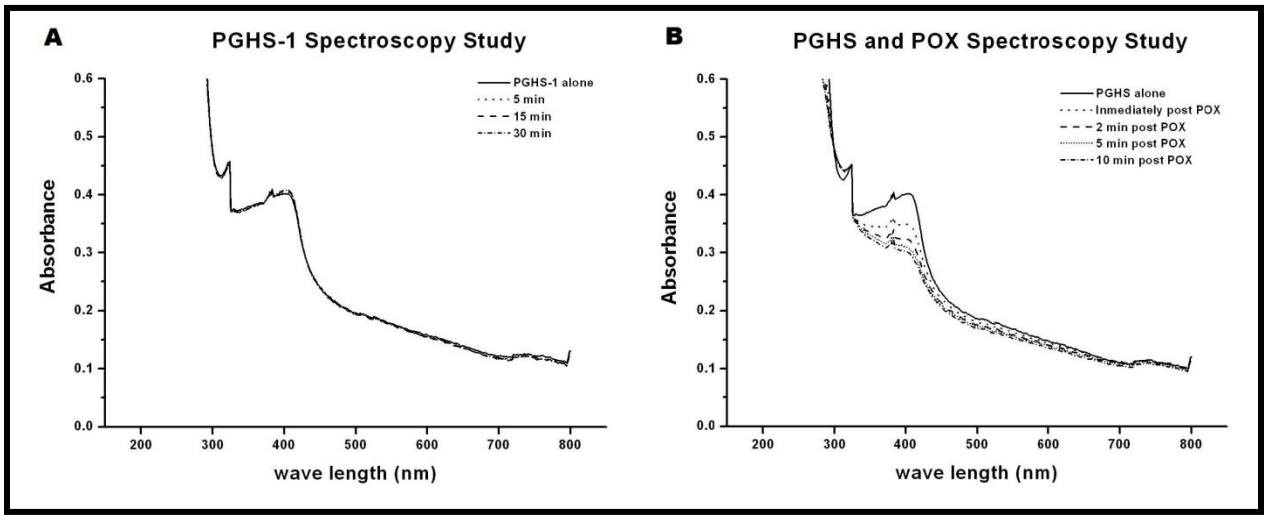


Figure 21. Mass spectroscopy studies using PGHS and POX. A) The native PGHS-1 enzyme absorbance peak is seen at about 400 nm. B) Adding POX to PGHS produces a decrease in the peak of the enzyme immediately following the instillation. The decrease in absorbance peak continues even after 10 minutes of POX's addition to the cuvette.

3.3 HAVSMC preparation

Finally, to further confirm that POX could act at the cellular level, we cultured, preconstricted and treated with POX several HAVSMC. The results show that phenylephrine produced a consistent and strong contraction of the cultured vascular cells. After the cells were treated with POX, we observed a statistically significant ($p < 0.05$) lengthening of the same vascular cells. See Figure 22.

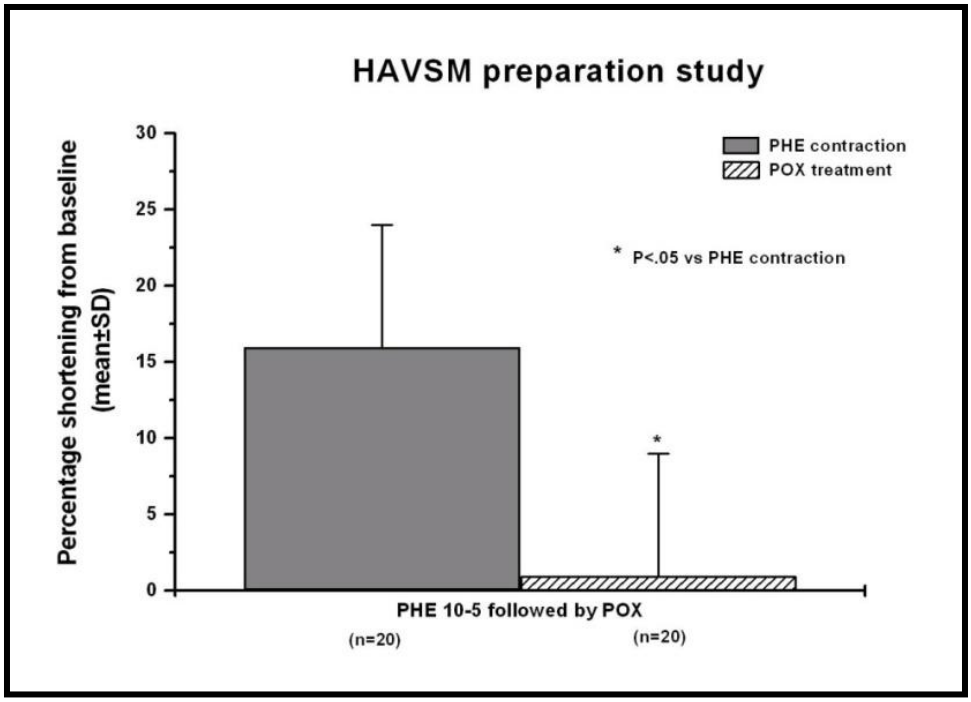


Figure 22. POX produces lengthening of pre-constricted HAVSMC. In HAVSMs, POX produces a significant lengthening of previously phenylephrine -contracted vascular cells. POX is able to return the cultured cells almost to their baseline length.

Discussion

ROS generation is increased in SS and these mediators have the capability of inducing multiple deleterious effects during sepsis[12,18]. Among ROS, H_2O_2 has well known cell signaling[9] and vascular tone modulation effects[10-14]. In previous experiments, we have shown that H_2O_2 could be involved in the cardiovascular collapse of an experimental model of sepsis by activating H_2O_2 dependent pathways[15,16]. H_2O_2 is one of the most important ROS involved in the generation of HP's. HP's are highly oxidizing molecules which are capable of generating cellular damage[12] and that have been shown to be increased in animal models and human studies of SS[20,23-25]. The purpose of this study was to determine whether HP's could decrease vascular tone in a carotid artery ring preparation, as a surrogate for the systemic vasodilation that occurs in SS. To examine the former question, we used the HP, POX. Our choice for this particular molecule was based on several reasons: a) in our previous research we showed that POX, was capable of producing myocardial depression (another important feature in SS) in a right ventricle *in vitro* assay due to the generation of the catalase derivative compound I[7]; b) POX reaction with catalase generates a stable form of compound I[60], which has high oxidizing and cell signaling properties[7,8]; c) POX structure is similar to H_2O_2[9,18,21].

In the present study we showed that POX produced a consistent and significant vasodilation in a phenylephrine pre-constricted carotid ring arterial preparation. We tested two different concentrations of POX solution based on previous work by Jones and Middlemiss[60]. These authors found that a solution of POX at around 1.25 to 2.5 x 10^{-4} mol/L coupled with catalase at 2 x 10^{-9} mol/L generated optimal concentrations of HP's and catalase derivatives. Furthermore,

the level of HP's has been measured in some animal models of sepsis[24,25] and found to be increased with concentration ranging between 10^{-3} and 10^{-4} mol/L, which confirmed that the final concentration chosen (2.5×10^{-4} mol/L) for the remaining experiments was comparable to what occurs in models of sepsis and SS. As mentioned before, the POX molecule is similar to H_2O_2; however, POX possesses a hydroxyl group attached to an O_2 radical which may be relevant in the type of pathway activated by POX. After the initial results with POX in the arterial preparation, we wanted to replicate the results in isolated myocytes. Interestingly, we found that in phenylephrine pre-constricted HAVSMCs, POX lengthened the previously constricted HAVSMCs almost to the baseline level. It is noteworthy that in this group of experiments, we used a lower POX concentration because we did not want to produce any damage to the cells with a higher concentration of the agent. Due to the former, we did not see any macroscopic abnormalities in the isolated myocytes. Based on the results of both types of experiments, we were confident that POX's effect was uniform and significant.

After observing the consistent and reproducible decrease in vascular tension generated by POX in the arterial and the effective lengthening of the HAVSMCs in the culture preparation, we investigated the pathway involved in the effect. We examined the involvement of the usual cellular signaling mechanisms that produce vasodilation in the VSML. As a first step, we demonstrated that POX action was independent of endothelium integrity. We functionally assessed the absence of the endothelial layer by the application of acetylcholine. On this note, Furchgott et al., showed that the endothelium was necessary for the relaxation produced by acetylcholine in isolated rabbit arteries[19]. Moreover, in the following years, it was recognized

that the endothelium was one of the most important factors regulating vascular tone by the production and release of NO as well as other factors such as PGI_2, adenosine and others[19,35]. Nonetheless, other studies have shown that there are agents capable of producing endothelium independent vasodilations, one of these mediators is Lzm-S[7,8]. In concordance with the previous statement, we showed that POX's induced decrease in vascular tone is not related to the production of NO (a primarily endothelial generated substance) since the NOs inhibitor $_{L}$-NMMA did not inhibit the vasodilatory response. Even though, there is evidence that suggests that ROS, and in particular H_2O_2 could activate different NOs to generate vascular relaxations[18], nevertheless, other authors have demonstrated that ROS may activate other mechanisms to produce vasodilation that do not involve the production and release of NO[8,18]. Our results indicate that POX causes relaxation by a NO independent mechanism.

We also investigated the role of cGMP and cAMP in POX's action since these two intracellular messengers are frequently involved in physiologic vascular relaxation[40,63]. GC and AC are the step-limiting enzymes in both pathways that when activated, produce cGMP and cAMP respectively which in turn stimulate protein kinases to produce vasodilation[63]. In the VSML, one of the main stimuli for the production of cGMP is the interaction of NO with its specific G-protein coupled receptor, which then decreases vascular tone[62,63]. In our bioassay, we used selective and irreversible inhibitors of both enzymes and found no blocking effect to POX's generated relaxation. These results are not surprising considering that NO was not relevant in POX's action, thus, the NO-cGMP pathway is not affected by POX.

Another common pathway that produces vasodilation is the AA-PGHS axis. This pathway generates AA metabolites such as PGs that are capable of decreasing vascular tone[64]. Besides the known physiologic effects of this pathway, it has been shown that conditions such as sepsis and SS enhance the production of PGs, especially PGI_2 which may have an important role in the decrease in systemic arterial resistance observed in SS[32]. Moreover, other authors have demonstrated that H_2O_2 can enhance the production of PGs in multiple pathophysiological conditions[37,65]. Due to these reasons, we used the classic PGHS (COX-1) inhibitor indomethacin to test the importance of this axis in our preparation. We demonstrated that indomethacin significantly blocked POX's induced vasodilation at high and low concentrations (from 10^{-5} to 10^{-8} mol/L), which would suggest that PGHS is activated in POX's response even at low drug concentrations (10^{-8} mol/L). Furthermore, we showed that POX produces an increase in the major metabolite of PGI_2, 2, 3-dinor-6-keto $PGF_{1\alpha}$, after 30 minutes of exposure of the arterial rings to POX, thus providing more evidence for the involvement of PGHS.

The specific effect of POX in PGHS seems to be related to the structural and cellular biology of the PGHS enzyme. As it is well known, PGHS (COX-1 and COX-2 being generic names) is the key enzyme in prostanoid biosynthesis catalizing two sequential reactions: a cycloxygenase reaction which catalyzes the oxygenation of AA to yield prostaglandin G_2 (PGG_2) and a peroxidase reaction to convert PGG_2 to prostaglandin H_2 (PGH_2)[66,67]. It is a complex mechanism in which both activities occur at distinct but structurally and functionally interconnected sites. The activity of the peroxidase portion of PGHS is a vital step in the activation of the enzyme's COX motif[69]. The peroxidase reaction of PGHS occurs at a heme-containing active site located near

the protein surface[70]. The first step in the reaction requires a HP and involves the formation of oxidized intermediates (similar to the catalase derivative compound I) ending with the formation of a Tyrosine385 radical that acts as a transducer to activate COX catalysis[66,67]. Kulmacz et al., demonstrated that several HPs were effective activators of PGHS at the peroxidase site favoring catalysis of the enzyme and prostanoid conversion[70]. However, no previous studies have addressed the question of whether POX could be a good substrate for PGHS. Our spectrophotometric analysis showed that POX quickly transformed PGHS to its oxidized heme-containing intermediates demonstrated by the sudden decrease in the absorbance peak of the native enzyme, proving that POX may be a good substrate for PGHS. Although, we recognize that more analysis is needed in order to determine the exact nature and kinetics of the interaction between POX and PGHS, we believe that this study shows that POX is capable of stimulating effectively the PGHS enzyme.

We also wanted to determine if POX had any effect in the other portions of the AA-PGHS pathway since other studies have shown that ROS and in particular H_2O_2 stimulate the activation of PLA_2 and the stimulation of the LO and EPO enzymes to increase the production of AA derivatives[64,65,67]. Nonetheless, it is interesting to observe that our results suggest that POX appears to exert a very specific effect at the PGHS since there was no inhibition of its action when we used PLA_2, LO and EPO inhibitors.

In recent years, there has been a greater recognition for the role of K^+ channels in the normal regulation of vascular tone and in multiple diseases[50,51]. SS is one of the conditions where it has been postulated that excessive activation of K^+ channels produce VSMC hyperpolarization and

systemic vasodilation, thus decreasing systemic vascular resistance and contributing to the generation of hypotension[52]. Besides the usual vasodilation pathways, we wanted to examine the role of K^+ channels in POX's induced vascular relaxation. As a first step to accomplish this we used a high concentration of KCl to induce vasoconstriction and compared it to the usual phenylephrine response of the arterial rings. We showed that POX was unable to decrease the measured tension of arterial rings constricted with high concentrations of KCl as compared to the pronounced vasodilation seen with the phenylephrine pre-contracted rings. The contractile response after KCl has a physiological explanation; by adding to the arterial bath a high concentration of KCl (25 mmol/L) we induced a high extracellular concentration of K^+ which moved the normal equilibrium potential (E_K) of the cellular membrane (usually around -85 mV for a ~5 mmol/L extracellular K^+ concentration) to the physiologic membrane potential (-40 to -60 mV), promoting the inactivation of K^+ channels and in consequence generating a vasoconstriction by an increase in the intracellular concentration of calcium (Ca^{2+}), either by releasing it from the sarcoplasmic reticulum or by the opening of voltage dependent Ca^{2+} channels to promote a quick influx into the cell[51, 61].

POX's inability to produce vasodilation suggested that K^+ channels participate in its action. As mentioned before, four different types of K^+ channels have been identified in VSMC: a) Ca^{2+} activated K^+ channels (K_{Ca}), b) voltage activated K^+ channels (K_V), c) ATP-sensitive K^+ channels (K_{ATP}) and inward rectifier K^+ channels (K_{IR})[34, 50, 51]. Furthermore, evidence from a few studies using animal models of SS appeared to point at the K_{ATP} channel as the most important culprit in the excessive K^+ channel activation seen in this condition[53]. On the other hand, small clinical

studies in SS that tried to inhibit the K_{ATP} channel with glyburide were not successful[52,54]. The absence of clinical response to glyburide may have been related to several methodological flaws of the trials and to the fact that the K_{ATP} channels may not be the only ones activated in SS[53,54]. On this note, in a peritonitis induced animal model of SS, Kuo et al found that several types of K^+ channels were involved in the excessive vascular hyperpolarization seen after 18 hours of sepsis[56]. Our results in which we used POX in the arterial preparation confirm the observations from the clinical and animal studies since glyburide was not able to inhibit the decrement in tension induced by POX. Similarly, the K_V channel blocker 4-AP was not effective in blocking POX's response. The lack of inhibition with the K_V blocker suggests that despite being widely distributed in VSMCs[51], they are not involved in the relaxation produced by POX. In unpublished observations, we were able to determine that POX is not effective in activating whole-cell K_V currents in human embryonic kidney (HEK) cells using the patch-clamp technique. However, more research is needed in this interesting topic. On the other hand, we showed that the K_{IR} blocker, Ba^{2+} inhibited POX's vasodilation. K_{IR} channels are abundant in small resistance arteries[51] where they are activated by membrane hyperpolarization and in response to moderate increases in the extracellular K^+ concentration (to 10-15 mM), features that distinguishes them from the other K^+ channels and allows cells to regulate hyperpolarization, and in the case of VSMCs, generate vasodilation[74]. Thus, the blockade of this channel may be relevant in vasodilatory conditions such as SS. Nevertheless, there are no studies addressing the role of K_{IR} channels in sepsis.

Besides the K_{IR} channels, we also found that subtypes of the K_{Ca} channels may be stimulated by POX. Indeed, a combination of SK_{Ca} (APA) and IK_{Ca} (ChTX) channel blockers significantly abolished the vasodilation induced by POX, despite the low drug concentrations tested. It is also compelling to note that we could not inhibit POX's effect with the individual use of the aforementioned agents in the preparation. In addition, other authors have demonstrated (using electrophysiological techniques) that there is coexistence of K_{Ca} currents in VSMCs[71], so, it would appear that different subtypes of K_{Ca} channels become activated simultaneously to produce hyperpolarization in VSMCs and as a consequence vascular relaxation. The effects obtained with the combination of APA and ChTX were consistent and significant; however, the current evidence in SK_{Ca} and IK_{Ca} channel physiology postulates that both of these channels, and in particular the SK_{Ca} channel, are preferentially expressed in the endothelial cells of the vasculature[50]. Furthermore, the evidence for a functional role of SK_{Ca} channels in VSMCs is limited. Similarly, in healthy VSMCs, IK_{Ca} channels either do not exist or they are poorly expressed[50]. Nevertheless, there are studies that have shown the existence of these channels in VSMCs. A current sensitive to APA was detected in the study by Gebremedhin et al. using VSMCs[70]; meanwhile, Tharp et al found that in proliferating or damaged vascular myocytes, the expression of the IK_{Ca} channel increases drastically[72]. Adding to the current controversy, most of the studies related to the expression or the functional status of a particular K_{Ca} channel have been done on cells from small animal species such as rats or mice; thus, the role of these channels in humans or large animals is still a matter of extensive research[50,51].

We were aware of the current uncertainties in this area, so we decided to clarify our results by showing that SK_{Ca} and IK_{Ca} could be activated in the VSML and not in the vascular endothelium. To do this, we used specific activators of the SK_{Ca} and IK_{Ca} (SKA-31 and EBIO-1 respectively) channel in arterial rings without endothelial layer to eliminate the contribution of the endothelium in K^+ channel activation. We were very careful in denuding the endothelial layer as well as asserting the absence of the endothelium with acetylcholine to ensure that the observed response was related to the action of the VSML. The results show that both activators promoted an important and significant vasodilation that could be attenuated with the use of a specific K^+ channel blocker further confirming the activation of the SK_{Ca} and IK_{Ca} channels. To our knowledge, this is the first report in the literature that provides evidence for the presence of these two subtypes of K_{Ca} channels in large animal species. Moreover, these findings give us an explanation for the result of POX and the SK_{Ca} and IK_{Ca} blockers.

Based on the previous findings, our results suggest that POX stimulates the PGHS enzyme to produce PGI_2 and is also capable of activating K_{IR} and SK_{Ca} and IK_{Ca} channels to decrease vascular tone. However, we wanted to examine if there was a link between these two mechanisms. Some studies have shown that PGs can activate K^+ channels in the arterial vasculature. Bouchard et al[75], showed that the infusion of different types of PGs to isolated coronary heart vessels and aortic rings from rats generated a profound coronary vasodilation that was attenuated with the use of the K_{ATP} channel blocker glyburide. Moreover, these authors found that iloprost, a PGI_2 analog, produced a more pronounced vasodilation in the aortic rings as compared to the other PGs, but glyburide was incapable of blocking the vascular relaxation. Based on the latter observation, the authors argued against the role of K_{ATP} channel

activation in their aortic ring preparation. In agreement with Bouchard et al, we also found that EPOP, another PGI_2 analog (ultra short half life), produced a prominent vasodilation; nevertheless, the decrease in tone in the arterial preparation was significantly inhibited with the pretreatment of a combination of SK_{Ca} and IK_{Ca} channels blockers (APA/ChTX). Consistent with this idea, Clapp et al showed in guinea-pig aortic rings that BK_{Ca} channels are involved in the vasorelaxation induced by the PGI_2 analogues iloprost and cicaprost[76]. To the best of our knowledge, there are no studies to determine the effects of K^+ channels in the vasodilation generated by EPO. Despite the latter and taking into consideration the current evidence; the results from our study also support the notion that PGs, and especially PGI_2, can activate SK_{Ca} and IK_{Ca} channels which provides an explanation for the mechanism of POX's induced vasodilation.

Based on the results, we concluded that POX is a good substrate for the peroxidase portion of the PGHS enzyme which in turn stimulates the generation of PGI_2. Then, this mediator activates SK_{Ca}, IK_{Ca} and K_{IR} channels to induce cell membrane hyperpolarization and consequently, vasorelaxation. In Figure 6 we can see the proposed mechanism in POX's decrease of vascular tension.

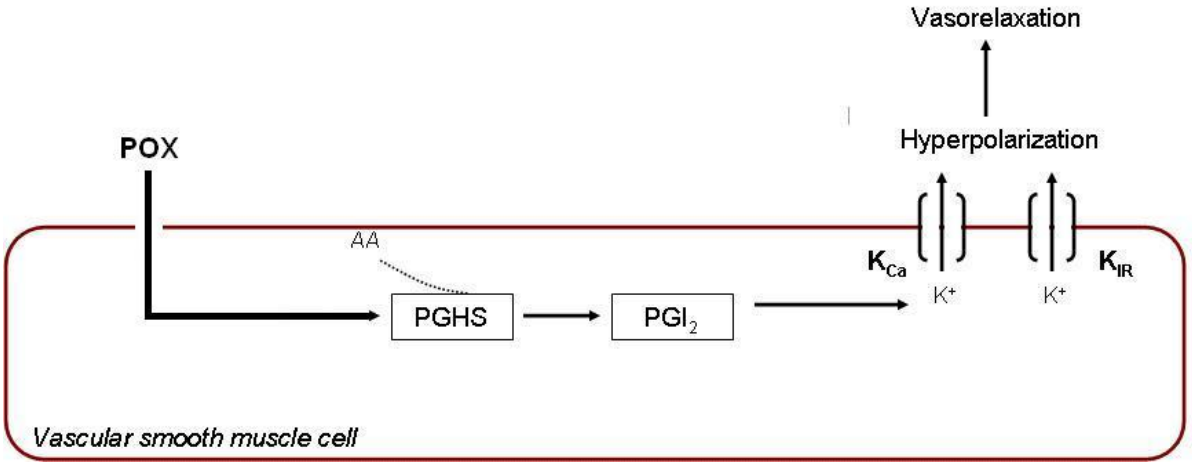


Figure 23. Proposed mechanism for POX's induced vasodilation. POX's induced vasodilation is related to the stimulation of the peroxidase portion of the PGHS enzyme which then produces PGI_2. PGI_2 is then capable of activating SK_{Ca} and IK_{Ca} channels to produce cell hyperpolarization and in consequence, vasodilation.

Our study addresses a novel mechanism of vasodilation in an arterial carotid ring preparation that may be relevant in SS. As previously exposed, HPs are elevated in SS where they can promote several deleterious effects at the cellular level and may enhance the organ failure encountered en this condition. Furthermore, we have shown that HPs are capable of promoting arterial vasodilation, a prominent feature of the disease that contributes to the development of systemic hypotension. Hence, HPs and their associated signaling mechanisms could become potential drug targets for the treatment of the cardiovascular collapse seen in SS.

Strengths and limitations of the study

We consider that the study has multiple strengths:

a) Research in the SS area is extremely important since this condition is one of the most important and difficult to treat entities in clinical medicine. Thus, more efforts are needed to address the mechanisms of this disease.

b) The study shows a novel mechanism for arterial vasodilation that could be important in SS. To the best of our knowledge, the finding that HPs are capable of generating arterial vasodilation has not been previously reported and warrants more research in the future.

c) We systematically ruled out the most common pathways for arterial vasodilation in our preparation that eventually led us to discover the effects of PGs and K^+ channels on POX's induced vascular relaxation. As a result of this systematic approach, we are confident that the exposed pathway reflects the actual effect.

d) Another novel finding in this study is the presence of SK_{Ca} and IK_{Ca} channels in VSMCs of a large animal. So far, these channels have been described mostly in the endothelial layer of small animal species. The fact that we found an effect of these channels in our preparation warrants more research in this topic.

e) We underline the importance of the VSML in the vasodilation of the arterial rings. Extensive research has been done to determine the importance of the endothelium in the systemic vasodilation that occurs in SS. Unfortunately, this line of study has not produced a clinically important pharmacologic agent. Thus, the discovery of new pathophysiological mechanisms is very important to find new therapeutic targets.

We also recognize that the limitations of the study are:

a) We were unable to obtain electrophysiological evidence (i.e. patch clamp techniques) of the activation of K^+ channels in VSMCs. We tried to learn the basics of K^+ channel currents using the patch clamp technique; unfortunately, we could not test the specific K_{Ca} channels involved in isolated HEK cells to substantiate the findings that we observed in the VSMC and isolated arterial preparation; therefore, there is a need for more research to expand the knowledge in this area.

b) Due to the importance of the SK_{Ca} and IK_{Ca} channels in our preparation, we wanted to confirm their existence in VSMCs of large species using one of the following methods:

    a. Treating the HAVSMCs culture method with the specific K^+ channel activators SKA-31 and 1-EBIO and determine cell lengthening.

    b. Performing expression studies on the HAVSMCs to reveal the existence of these channels.

    c. Utilizing specific antibodies to the desired K^+ channels in the organ bath.

    However, due to the lack of graduate study time, we focused on the more importance aspects of the POX pathway.

Future research

Given the limitations of the present study, we believe that it is necessary to address those issues in future experiments.

After the completion of the previously mentioned *in vitro* studies, it would be interesting to try the blockade of the pathway *in vivo* models of SS. We acknowledge that the discovery of a pathway becomes more relevant when the mechanism is tested in animal models of SS.

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