THE ROLE OF PLATELET ACTIVATING FACTOR IN THE MECHANISM OF NITRIC OXIDE SYNTHASES AFTER REGIONAL MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

by

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Abstract

Myocardial infarction is one of the leading-causes of death in North America. Standard treatment for myocardial infarction consists of reperfusion of the ischemic area. However, several life-threatening events occur after reperfusion is established. Evidence suggests that platelet activating factor and nitric oxide, two inflammatory mediators, have a main role during this myocardial ischemia-reperfusion injury. However, the mechanism through which these mediators exert their effects as well as a potential relationship between both, during regional myocardial ischemia-reperfusion injury, need to be investigated.

The effect of PAF in modulation of NO-releasing enzymes (iNOS, eNOS), severity of cardiac contractile depression and myocardial infraction lesion in the late (48 h post-ischemic reperfusion) phase of MIR injury were investigated. An *in-vivo* rabbit model underwent 30 min of regional myocardial ischemia followed by 48 h of reperfusion and treated, during the ischemic period, with a PAF antagonist (TCV-309). It was found that PAF up-regulates iNOS expression and down-regulates eNOS expression in the ischemic-reperfused heart. Furthermore, PAF induced myocardial contractile depression and a more severe infarct lesion at 48 hours post-ischemia reperfusion. These effects were associated to iNOS expression, indicating that NO is an important mechanism through which PAF exerts its negative effects on myocardial infraction and cardiac contractility.

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Dedication

For my parents, Lucia and Humberto, who have given me life, love, support and an example during all these years.

Acknowledgements

1. INTRODUCTION

Myocardial infarction (MI) is one of the most important causes of death in North America. In Canada, 20% of all deaths are due to ischemic heart disease (Fig. 1) and half of them are attributable to MI[1]. Standard treatment for MI is based on reperfusion of the ischemic area. This reperfusion can be obtained by different therapeutic methods, such as fibrinolytic agents, angioplasty or coronary artery bypass. Ultimately, once reperfusion is established, total survival of patients suffering of MI improves. A paradigm exists in that a significant mortality rate appears during reperfusion[2]. An important advance in improving patient recovery can be reached by understanding the mechanisms underlying the pathological processes that occur during myocardial post-ischemic reperfusion.

During reperfusion, the reintroduction of oxygenated blood into the ischemic region returns the affected tissue to its regular aerobic metabolism. Reperfusion also triggers the inflammatory cascade stimulating the formation of inflammatory mediators and oxygen-derived reactive species (ODRS). These inflammatory mediators (including ODRS) exert further damage to the ischemic tissue[3,4]. Recently, among these inflammatory mediators, platelet activating factor (PAF) and nitric oxide (NO) have attracted attention due to their roles in the mechanism of myocardial ischemia-reperfusion (MIR) injury.

Important clinical consequences observed during MIR are cardiac dysfunction and the severity of myocardial injury. The role of inflammatory mediators in these clinical consequences, particularly PAF and NO, is a broad field that remains to be investigated.

In the next pages, the pathogenesis of myocardial ischemia-reperfusion injury will be reviewed, focusing on the roles of the inflammatory mediators PAF and NO.

1.1. Myocardial Ischemia-Reperfusion Injury

1.1.1. Myocardial Ischemia

Important reduction or total interruption of blood supply will cause an ischemic period in an affected cardiac zone which ultimately could lead to MI. The affected cardiac region will suffer from a shortage of oxygen and nutrients essential for normal functionality and tissue survival. Lack of oxygen interrupts mitochondrial oxidative phosphorylation, switching the cellular energy production to anaerobic glycolysis. Lactic acid is a glycolysis product and its accumulation in the anaerobic cells can cause acidosis and cellular death. Furthermore, anaerobic metabolism cannot provide as much energy as aerobic, which compromises ATP supply.

ATP is important to cellular homeostasis. ATP is the energetic supply to ionic pumps in the cellular membranes and is essential for maintaining membrane integrity. During ischemia, lack of ATP brings an ionic imbalance with important ionic movements, intracellular influx of sodium and extracellular efflux of potassium, and as a consequence intracellular edema and damage to the myocardial sarcolemma[5]. These alterations in ionic concentrations modify membrane polarization, activating voltage-dependent Ca^{2+} channels and subsequent intracellular movement of calcium[6]. This increment in calcium inside the cell activates intracellular Ca^{2+}-dependent proteases and phospholipases, causing further cellular damage[7]. The extension of the damage observed during ischemic injury of the heart directly influences the severity of its physiologic effects. If the ischemic area is so extensive that the non-ischemic heart cannot compensate, regional ischemia could results in a drop on myocardial contractility, and as a consequence a reduction in volume circulating and systemic blood pressure. In addition, if heart conduction pathways are affected, arrhythmias could develop[5].

1.1.2. Myocardial Reperfusion

Reintroduction of oxygenated blood into the ischemic/infarcted myocardial region due to reperfusion treatment initiates dissimilar processes. In the first instance, cells reactivate their

regular metabolism, but a second mechanism activates the inflammatory cascade and ODRS production, resulting in further tissue damage.

ODRS are molecules or fragments of molecules with unpaired electrons in their outermost orbits. Among ODRS there are superoxide anion ($^{.}O_2^{-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{.}OH$), peroxynitrite ($ONOO^{-}$) and NO^{8-10}. Due to their high reactivity, ODRS produced during reperfusion can peroxidate the phospholipid component of cellular membranes, commencing necrotic changes in the ischemic-reperfused myocardium[11-14]. Moreover, inflammatory cascade initiated by lipid peroxidation and activated phospholipases leads to the release into the circulation of inflammatory mediators such as thromboxanes, leukotrienes, prostaglandins and PAF[15,16]. In fact, it has been proven that PAF is produced by ODRS in isolated hearts[17].

PAF is an inflammatory mediator released during reperfusion. PAF has been associated with diverse alterations observed during MIR. Among these alterations, PAF has been related with a reduction in myocardial contractility[18-24], coronary lumen[19,20,25] and an increment in infarct severity[20,22,23,26-33].

1.2. Platelet Activating Factor (PAF)

1.2.1. Structure and Properties

PAF (1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) (Fig 2a) is a phosphatidylcholine analogue with a long chain alkyl ether at position 1 and an acetate ester at position 2[34-36]. PAF belongs to a family of biologically active, structurally related alkyl phosphoglycerides, and may function as an intra or extracellular messenger[37]. Its name is based on its first effects observed on platelet aggregation after binding its platelet receptors. However, it is well known that PAF has platelet-independent effects such as activation of the inflammatory

cascade[38,24], direct negative inotropic effect on cardiomyocites[39,40], and stimulation of smooth muscle contraction[41-43].

Different cellular types produce PAF, including polymorphonuclear leukocytes, monocytes, macrophages, endothelial cells, cardiomyocytes and platelets[44]. Furthermore, most of the cells that produce PAF also possess PAF receptors[37]. PAF is synthesized from glycerol lipids through two principal pathways (Fig 3). The *de-novo* route is responsible for producing PAF in normal conditions. This pathway involves the acetylation of 1-*O*-alkyl-*sn*-glycerol to 1-*O*-alkyl-2-acetyl-*sn*-glycerol and further dephosphorylation through a phosphohydrolase, ending with the effect of a CDP-cholinephosphotransferase (Fig 3)[45]. On the other hand, the *remodeling* pathway is the most important during pathological processes[45-47]. In this, phospholipase A_2 (PLA_2) is activated and it hydrolizes the *sn*-2 arachidonate from alkyl choline phosphoglycerides, particularly from membranes producing 1-*O*-alkyl-*sn*-glycero-3-phosphocholine ("lyso-PAF") and free arachidonate (Fig 3)[48,49]. Next, lyso-PAF is converted to PAF when acetate is added to the *sn*-2 position. This step is mediated by a Ca^{2+}-dependent acetyltransferase[50,51]. Lyso-PAF can be produced via a CoA-independent transacylase route too[37]. This route accounts for the simultaneous PAF synthesis and mobilization of arachidonic acid. At the end of these pathways, PAF needs an ether linkage at the *sn*-1 position of the glycerol backbone, a short acyl chain, usually an acetyl residue, at the *sn*-2 position, and the polar head group of choline or ethanolamine at the *sn*-3 position for exerts full potency[37]. Once PAF is produced, it exerts its effects through either intracellular[52] or extracellular[53] membrane receptors. PAF receptors can be found in smooth muscle cells[54], cardiomyocytes[55], neutrophils[56,57], monocytes-macrophages[58,59], eosinophils[60], endothelial cells[61] and the central nervous system[62-64]. PAF receptor (PAFr) belongs to the 7-membrane spanning G-protein coupled receptor superfamily[65]. Interestingly, PAFr activates PLA_2 through G-proteins[66], ERK 1/2[67], MAPK[68] or PTK-dependent[69] mechanisms. Once activated,

PLA$_2$ hydrolyses phospholipids from cellular membranes and releases arachidonic acid (AA). AA can be metabolized to active eicosanoids, such as prostaglandins, thromboxanes, leukotrienes, as well as PAF[16]. Furthermore, PLA$_2$ is a key-point for the remodeling pathway as mentioned above. This suggests a positive feedback between PAF and PLA$_2$. PAF activates phospholipase C (PLC) via G proteins, leading to both the activation of protein kinase C (PKC) and the release of intracellular calcium stores[37].

The enzyme responsible for PAF regulation is PAF acetylhydrolase (PAF-AH)[70,71]. PAF-AH cleaves the short acyl chain at the *sn*-2 ester bond and reforms the biologically inactive lyso-PAF (Fig 2b). Once formed, lyso-PAF can be metabolized via CoA-independent transacylase, acyl-CoA acyltransferase or lysophospholipase D[72]. PAF-AH has been detected in blood, blood cells and several tissues[73,74]. It has been found that PAF-AH is produced by the heart and cardiomyocytes, being capable of inactivating PAF[37].

1.2.2. PAF Antagonists

1.2.2.1. Summary of PAF Antagonists

Two main categories of specific PAF antagonists exist: charged and non-charged compounds[75]. Charged compounds are structurally similar to PAF and can be subdivided into open and closed-chain analogues.

Open chain analogues: The first specific PAF antagonist described was CV-3988[76], an open chain analogue which blocks PAF binding to its receptors[77].At high doses, it has agonist properties[78]. Using CV-3988 as a template, a second open-chain PAF antagonist was developed, CV-6209, with 100-fold more potent activity against platelet aggregation and PAF-induced hypotension[79]. Recently, a new open-chain specific PAF antagonist, TCV-309, was developed by Takeda Chemical Industries (Japan). TV-309 will be described in more detail below.

Closed chain analogues: These antagonists are called closed chain due to a reorganization in either the glycerol or phosphate portion of the PAF molecule into a cyclic structure. SRI 63-072, SRI 63-675 and SRI 62-441 are part of this group. These compounds have similar PAF antagonistic properties as open-chain analogues[80].

Non-charged PAF Antagonists: These are cyclic analogues of PAF without an electrical charge. They are organized according the number of rings in their structure.

1.2.2.2. Properties of PAF Antagonist TCV-309

TCV-309 is a charged, open chain analog of PAF, which inhibits the binding of [^{3}H]-PAF at specific receptor sites without affecting phospholipase A_2, cyclooxygenase, TxA_2 synthase, or 5- or 12-lipoxygenase pathways[81]. TCV-309 specifically reduces hypotension caused by PAF without an effect on hypotension caused by acetylcholine, bradykinin or histamine[82]. TCV-309 does not have a hemolytic effect, as does CV-3988 and CV-6209[83,84].

1.2.2.3. Pharmacokinetics of TCV-309

The half-life of TCV-309 ($t_{1/2alfa}$ and $t_{1/2beta}$) after I.V. administration has been shown to be 0.1 and 1.4 h in rats and 0.2 and 2.1 h in dogs, respectively[85]. In rats and dogs, most of the TCV-309 administered was eliminated from the body within 48 h, with the majority (>70%) appearing in the feces via the hepatobiliary route[85].

1.2.3. Cardiovascular Effects of PAF

PAF has been shown to cause similar cardiovascular dysfunction as that occurs during myocardial ischemia-reperfusion, such as myocardial depression[86-90], increase in capillary permeability[91-97], coronary artery vasoconstriction[86,98-100], and pulmonary hypertension[86-89], among others.

1.2.3.1. Effects of PAF on Myocardial Contractility

It is well known that PAF exerts a negative myocardial contractility effect. At the cellular level, PAF has shown a direct negative inotropic influence on rat cardiomyocytes[39,40]. On

human[16,101] and guinea-pig[102] cardiac muscle preparations, PAF exerts a concentration-dependent reduction in contractility. Finally, in isolated hearts[98-100,103-109] and *in vivo*[110-112] models, PAF infusions cause myocardial contractile depression.

1.2.3.2. Effects of PAF on Capillary Permeability

PAF increases cardiac capillary permeability[91-97]. In isolated and perfused coronary vessels, PAF induces a concentration-dependent increase in albumin permeability[97] and, in isolated hearts, PAF increases capillary permeability to albumin, inducing organ edema[95,96].

1.2.3.3. Effects of PAF on Coronary Vascular Resistance

In ex-vivo models, PAF has demonstrated a dose-dependent effect. In isolated rat hearts, using a low dose bolus of PAF (1-100 pmol), coronary vasodilation or initial vasodilation followed by vasoconstriction appeared[113,114], but when PAF was applied as an infusion, it produced an increment in coronary vascular resistance[104,115]. Moreover, PAF generated a concentration-dependent increment of coronary vascular tone, in isolated swine hearts[99,100,103,116-118]. Interestingly, when PAF was administered in a model using crystalloid-perfused isolated rabbit hearts, it did not produce significant changes in coronary vascular resistance[119,120]. When it was administered to rabbit hearts perfused with crystalloid and platelets, the coronary blood flow decreased[121].

Administration of PAF in *in-vivo* models has resulted in different effects based on the species used. In swine, intracoronary infusion of PAF induced an initial vasodilation followed by a decrease in coronary blood flow[110,122]. In canine models, PAF has produced controversial effects. PAF has produced coronary vasoconstriction[123], vasodilation[124] or a biphasic vasodilation/vasoconstriction effect[125]. Interestingly, PAF was shown to be a vasodilator in intact canine coronary arteries, but to increase coronary vascular resistance when the endothelium was injured, such as after ischemia[126].

1.2.3.4. Effects of PAF on Pulmonary Circulation

PAF has been shown to produce pulmonary hypertension or augmentation in pulmonary vascular resistance in rabbits[127,128], dog[116,129], and swine[122]. For example, an intravenous administration of a synthetic compound similar to PAF led to a brief period of increase in total pulmonary resistance, as well as other alterations that were compared similar to IgE anaphylaxis in the rabbit[128]. In a canine model, PAF induced a transient increase in pulmonary artery pressure during the first 30 seconds after its administration, followed by an elevation in pulmonary vascular resistance at 90 seconds[116]. Finally, in a swine model, PAF infusion induced a 5- to 120-fold increase in pulmonary vascular resistance leading to right ventricle failure[122]. These effects were related to thromboxane A2 release.

1.2.3.5. Effects of PAF on Systemic Circulation

PAF causes systemic hypotension in several species including guinea pig[130], rat[131,132], rabbit[127,128], dog[133,116], and swine[122]. When administered intravenously, PAF has a hypotensive response with the following characteristics: it is dose dependent, its onset is rapid, its maximum effect is reached within the first minute, and its recovery is also dose dependent[37].

1.2.4. Role of PAF in MIR Injury

It has been observed that cultured endothelial cells and cardiomyocytes synthesize PAF after prolonged hypoxia[134] and that once heart is reperfused PAF is released from the ischemic tissue[19,32,135]. PAF has been founded to peak in the blood stream at 15 min in *ex vivo* rabbit and *in vivo* rat[32] and at 3 h in *in vivo* sheep[135] MIR models. In this last study, at 6 h after reperfusion was established, there was not a significant amount of PAF detectable. Furthermore, in humans undergoing reperfusion therapy, higher levels of PAF have been detected in their blood compared with patients under placebo treatment[136,137].

The role of PAF during MIR has been studied by our group[138-140] and others[18,33,95,135,141-143] and although not all the studies have been supportive, there is strong evidence that PAF,

during MIR, plays an important negative influence by generating myocardial contractility depression[18-24], coronary vasoconstriction[19,20,25], pulmonary hypertension[138] and a higher severity of myocardial damage[20,22,23,26-33].

1.2.4.1. Myocardial Contractility

Results have shown that PAF antagonism protects against myocardial contractile depression during MIR. When administrated before onset of ischemia, PAF antagonists BN 50739, TCV-309, L-659 989, SDZ 63675 and CV-3988 shielded against myocardial depression in isolated rat hearts, isolated rabbit hearts, *in-vivo* sheep, rabbit and swine models, respectively[144,19,20,22,24]. PAF antagonists have been applied immediately before onset reperfusion and they improved cardiac contractility in MIR models too. For example, in isolated[18] and *in vivo*[29] rabbit hearts, PAF antagonists SDZ 63-675 and WEB 2170 improved cardiac contractility. In addition, when PAF-AH (which inactivates PAF) has been administered before reperfusion is established it improves cardiac contractility in an *in vivo* rabbit model of MIR[23].

However, other studies of MIR under PAF antagonism treatment have not obtained a significant improvement in cardiac contractility. In *in vivo* canine models, RP 59227[26], BN 52021[30], CV-3988[30], WEB 2086[25] and TCV-309[145] did not improve cardiac contractility and neither did WEB-2170[33] in an *in vivo* cat model.

1.2.4.2. Capillary Permeability

In isolated rat hearts, capillary permeability was increased during ischemia-reperfusion. This result was attenuated by treatment with the PAF antagonist CV-6209[95].

1.2.4.3. Coronary Circulation

In MIR models, PAF has shown the same controversial effects on coronary vascular resistance that PAF has produced in non-ischemic models. PAF antagonist TCV-309 produced an increment in coronary blood flow during MIR at 1 h[19] and 5 h[140] reperfusion in

isolated rabbit hearts perfused with diluted blood[19]. L-659 989 had the same effect at 15 to 30 min reperfusion in an *in vivo* sheep model[20], as did WEB-2086 at 24 h reperfusion in an *in vivo* dog model[25]. On the other hand, other studies have not reported a significant difference in coronary blood flow in MIR models treated with PAF antagonists. These studies were performed in *in-vivo* canine[26,30], *in-vivo* rabbit[22] and isolated rat heart[146,147] MIR models. Regarding time of drug administration, this has been controversial since some experiments were performed with PAF antagonist applied prior to ischemia[19,26,30,146,147], while others received it prior to reperfusion[20,22,25], and, in one case, it was applied at both times[140].

1.2.4.4. Pulmonary Circulation

We previously observed that PAF protects against pulmonary hypertension during MIR using an *in vivo* swine model[138]. In a swine model of 60 minutes of regional ischemia followed by 6 hours of reperfusion, pulmonary artery pressure was significantly increased at 30 minutes after ischemia.

1.2.4.5. Systemic Circulation

Despite the hypotensive effect of PAF administration[116,122,127,128,130-133], some controversy exists among the studies of PAF influence in systemic blood pressure during MIR injury.

PAF antagonism has protected against systemic hypotension during MIR when administered prior to ischemia in rabbit[22,27] and swine[138]. There has been protection against systemic hypotension in an *in vivo* rabbit model of 40 min ischemia and 24 h reperfusion when PAF antagonist was applied 5 min prior reperfusion[22,29]. Conversely, there was no beneficial effect on systemic blood pressure during MIR with PAF antagonists administered prior to, or during ischemia, in the *in-vivo* dog[26,30,141] and rabbit[28] models.

1.2.4.6. Infarct Severity

Many studies have proven that PAF plays an important role in heart infarction severity as a result of MIR. This role has been detected using *in-vivo* dog[22,26,-30], rat[31,32], sheep[20] and cat[33]

models, blocking PAF effects before or during the ischemic period. In addition, the severity of myocardial infarct lesions were reduced when PAF-AH was administered before reperfusion in the *in vivo* rabbit model[23]. Only in one study, using a canine model, PAF antagonism did not modify myocardial infarction[141].

1.3. Nitric Oxide Synthase (NOS)-Generated Nitric Oxide (NO)

1.3.1. Structure and Properties of NO

NO is an inorganic radical with an unpaired electron in its most external orbit[148]. NO was first discovered to be a potent vasodilator, but it has shown different effects depending on its concentration. At low concentrations NO activates guanylate cyclase producing cGMP. On the other hand, high concentrations of NO can act as free radical species. At high levels, NO is highly reactive and it can cause direct lipid peroxidation on cellular membranes, or indirect damage via $\cdot OH$ or $ONOO^-$ production through its reaction with $\cdot O_2^-$. NO is metabolized by forming complexes with ions or macromolecules and binding with O_2 to form the inactive metabolites NO_2 and NO_3[3].

1.3.2. Nitric Oxide Synthase (NOS)

The enzyme Nitric Oxide Synthase (NOS) produces NO from the aminoacid L-arginine[149]. Two subtypes of NOS can be detected in the heart: endothelial (eNOS) and inducible NOS (iNOS).

eNOS is found in the endothelial cells of coronary arteries. eNOS is Ca^{2+} dependent and is constitutivly expressed and activated instantaneously by physical stimulation and/or by activation of its specific receptor[149-151]. Once it is activated, eNOS produces NO in a low range (picomolar levels) and is controlled by a negative feed-back by NO[149]. At this level, NO is responsible for maintaining appropriate coronary vascular tone, as well as inhibiting

platelet aggregation, and platelet and neutrophil adhesion to endothelium by binding to Fe^{2+} in the heme group of enzymes and proteins[149,152,153].

In the heart, iNOS has been found in macrophages and cardiomyocytes. iNOS is transcriptionally upregulated in pathological conditions such as MI without reperfusion or MIR, therefore, needing several hours to be expressed. iNOS has been detected significantly upregulated at 48 hours after reperfusion is established[154]. Once activated, iNOS produces high levels of NO (nanomolar levels). At these high levels, NO, and subsequent products of its reaction with other ODRS (such as $ONOO^-$), can produce direct lipid peroxidation of the cellular membranes as explained above[149]. After this, lipid peroxidation releases AA and other inflammatory mediators, including PAF, which increases and prolongs tissue damage. In addition, NO can compete with O_2 on the mitochondria, prevailing in the production of O^{2-}, which can react with NO to produce $ONOO^-$, leading to an irreversible inhibition of the respiratory chain[149].

1.3.2.1. Role of eNOS-Generated NO in MIR Injury

Evidence suggests that eNOS has a beneficial effect during MIR. Antagonists of NOS have been administrated to animals during MI and MIR resulting in myocardial contractility reduction and coronary vascular tone increase[155-159]. When a specific iNOS antagonist has been used, leaving eNOS to exert its effects, both myocardial contractility and blood flow improved[160,161].

1.3.2.2. Role of iNOS-Generated NO in MIR Injury

Evidence suggests that iNOS has a negative contribution during MIR injury. iNOS activity and its subsequent products $NO/ONOO^-$ are increased in MI and MIR[154,162-167].

In a model of MI, without reperfusion, iNOS activity was significantly increased at 2 days after infarction with a higher myocardial production of NO. In the presence of iNOS

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inhibitors, levels of iNOS and NO decreased. Furthermore, both myocardial contractility and coronary blood flow improved[161].

Using models of MIR, iNOS has been significantly increased at 5 h reperfusion, after 20 min of regional ischemia, when ischemic versus non-ischemic areas were compared[154]. In one study, after 30 min of regional ischemia, followed by 48 h reperfusion, an iNOS inhibitor increased myocardial contractility, blood flow, and reduced infarct size in an *in-vivo* rabbit model[167].

1.4. Relationship of PAF and NO

There is some evidence that PAF and NO are related by function.

In pulmonary circulation, some studies have suggested a relationship between PAF and NO. Either exogenous PAF infusion or endogenous PAF released through sepsis has induced iNOS activity in lung, which was attenuated using a PAF antagonist[131]. In a canine pulmonary model, pulmonary hypertension was caused by PAF infusion within 10 min. This change in pressure was potentiated by preventing NO release[168]. It has been observed that blockage of NO production prevents PAF-induced microvascular permeability[169,170]. Moreover, in papillary muscle preparations, PAF infusion has promoted NO release and similar protective effects against contractile depression were obtained with both PAF and NOS antagonists[171].

During MIR, we previously observed that after 5 h post-ischemia reperfusion, PAF blocked eNOS upregulation without affecting iNOS expression in an *ex-vivo* model of global ischemia[140]. Interestingly, in this study, both myocardial contractility and coronary blood flow were negatively affected by PAF. All this information suggests that both PAF and NO have important roles during MIR injury. Furthermore, there is some evidence suggesting that PAF and NO may be related. Additional investigation of their roles during regional MIR

injury could lead to a better understanding of such pathology. Previously, we demonstrated that PAF could induce its negative effects during MIR, in part by eNOS modulation, after 5 h reperfusion. However, the relationship of PAF and iNOS during regional MIR remains to be investigated.

1.5. Rationale, Hypothesis and Aims

1.5.1. Rationale

There is a lack of knowledge on the mechanism through which PAF exerts its effect on MIR injury which needs to be investigated. In particular, understanding the mechanism through which PAF exerts its negative effects in cardiac contractility and severity of MI lesion could lead to a deeper knowledge of MIR injury and subsequent treatment of this pathology.

Evidence suggests that NO may also has an important role in MIR injury. Moreover, there is some evidence suggesting a link between PAF and NO. The extent as well as the mechanism of this relationship, is not established. Using a clinically relevant regional MIR injury model to further study this relationship would lead to a better understanding of the pathogenesis of MIR injury and to the development of therapeutic strategies towards MIR injury treatment.

1.5.2. Hypotheses

Generally, we hypothesize that one of the mechanisms responsible for the late (24-48 h) morbidity and mortality, after myocardial ischemia-reperfusion injury, is modulation of nitric oxide enzymes by PAF.

It is hypothesized that one of the mechanisms responsible for the negative inotropic effect in the late (48 h post-ischemia reperfusion) phase of MIR injury is due to modulation of NO-generating enzymes by PAF.

A second hypothesis is that at 48 h post-ischemia reperfusion, modulation of NO-releasing enzymes by PAF is responsible for the severity of MI lesion.

1.5.3. Objective

The objective of this study is to determine the effect of PAF in modulation of NO-generating enzymes (iNOS, eNOS), severity of cardiac contractile depression, and MI lesion in the late (48 h post-ischemia reperfusion) phase of MIR injury.

1.5.4. Aims

(a) To investigate myocardial iNOS/eNOS gene expressions after 48 h of post-ischemia reperfusion.

(b) To inspect iNOS protein expression in the ischemic-reperfused heart after 48 h reperfusion.

(c) To evaluate the contribution of PAF to iNOS/eNOS gene expression at 48 h in the pre-ischemic reperfused myocardium.

(d) To investigate the influence of PAF on iNOS protein expression in the ischemic-reperfused myocardium.

(e) To determine the contribution of eNOS and iNOS to the PAF-related effects on regional MIR injury after 48 h reperfusion.

II. MATERIALS AND METHODS

2.1. Animal Care

All animal care was performed according with the "Guidelines of the Canadian Council on Animal Care" under the acceptance and supervision of the Animal Care Committee of the University of British Columbia

2.2. Rationalization of Experimental Design

2.2.1. Animal Model

Important considerations exist regarding animal species used as MIR model. Studies in which a PAF antagonist was administered in a model of coronary occlusion in dogs showed minimal beneficial effects, unlike other reports on different animal models. A primary reason for this is likely the extensive collateral circulation in the canine heart that can provide substantial blood flow to the heart during periods of coronary artery ligation, thereby reducing ischemic damage to the heart and potentially limiting the release of and damage induced by PAF.

Rabbit was chosen as a model based on its similarities to humans. Both species lack the development of collateral circulation in response of ischemia[172]. In addition, both humans and rabbits are deficient in xanthine oxidase, an important enzyme in the pathway of ODRS formation[3]. Finally, rabbits are more cost-effective than a larger animal model.

2.2.2. PAF Antagonist

The WEB-class of PAF antagonists have been shown to differ from other PAF antagonists in their effects. WEB-class antagonists have stimulated, or inhibited physiological function, depending on their concentration and tissue type. For example, in isolated rat hearts, low concentrations of WEB 2086 and WEB 2170 blocked the vasoconstrictor effects of PAF. At moderate concentrations, these agents alone (in the absence of PAF) had stimulatory effects

in increasing coronary vascular resistance[105]. In tissue preparations, incubation with WEB 2086 improved cardiomyocyte contractility[39]. Consequently, studies using the WEB class of PAF antagonists must be interpreted with some reservation, with the understanding that this class of PAF antagonists can have different effects, dependent upon the dose used.

TCV-309 (Takeda Chemical Industries, Osaka, Japan) is an open-chain selective PAF antagonist described in detail in section 1.2.2. TCV-309 has been used previously to induce PAF antagonism by a dose of 0.1 mg/kg[85]. Using this dose, TCV-309 administered by slow I.V. infusion over 30 min inhibited the effects of PAF on platelet aggregation and blood pressure by 97% after 1 h post-infusion and 70% at 4 h. We have applied a similar dose of TCV-309 in MIR models with significant effects resulting from PAF antagonism.[138-140]. A single dose of TCV-309 used in the clinically relevant situation of infusion before onset of reperfusion could block PAF receptors in the early reperfusion period. In addition, we wanted to evaluate a PAF antagonist with a potential application in humans and TCV-309 has been proposed for clinical application.

2.2.3. Groups and Assessment Times

The surgical team was blinded by animal randomization. Animals were randomly assigned into three groups (Table 1). Sham group (n=7) underwent complete surgical protocol, but without coronary ligation, keeping the chest open for 40 min during the first surgical procedure. Sham group was included in order to compensate the effects of operative trauma, anesthesia and stress due to open chest surgery. Control (n=7) and treated (n=8) groups were both subjected to 30 min of regional myocardial ischemia of the free wall of the left ventricle induced by acute ligation of the distal half of the LAD coronary artery. Treated group received TCV-309 via slow I.V. infusion (2mL/min over 10 min) starting at 9 min after onset of ischemia. TCV-309 dose was 0.1mg/kg diluted in 20 mL 0.9% NaCl. Control group

received 20 mL 0.9% NaCl in similar conditions. All three groups were followed for 48 hours.

Positive dP/dt was monitored and recorded from the left ventricle at the following times: baseline (prior ischemia was established), at 10, 20 and 30 minutes of ischemia, and 2 min, 10 min and 48 h after reperfusion was instituted. After 48 h reperfusion, animals were euthanized and tissue samples obtained for subsequent analysis.

2.2.4. Operative Techniques

New Zealand white rabbits (3.5-4.5 kg) were anesthetized using ketamine induction (intramuscular injection, 20mg/kg) and 0.75 – 1.25% inhaled isoflurane thereafter. After an adequate anesthesic level was reached (confirmed by absence of pain reflex in limbs and eye blink response), animals were endotracheally intubated and mechanically ventilated with 45% oxygen. Respiration rate and tidal volume were adjusted at 10-12 breaths/min and 10-12 mL/kg, respectively. Electrocardiogram was attached and electrical cardiac activity and heart rate were monitored continuously. Body temperature was maintained at 37°C with a heating pad and monitored by a rectal temperature probe. The marginal ear artery and vein were cannulated for administration of fluids and pharmacological agents and for arterial blood pressure measurement.

Once animals were stabilized, a posterolateral thoracotomy was performed in the 6th intercostal space exposing the left thoracic cavity content. Left lung was carefully mobilized and retracted, the pericardium opened and cardiac apex exposed. Left anterior descending coronary artery (LAD) was identified and secured with a surrounding suture approximately at the middle of its trajectory. A Millar pressure transducer catheter (Millar Instruments, TX, USA) was inserted through an apex incision and advanced into the left ventricle for intraoperative measurement of ventricular pressure. The transducer was secure in place and connected to a monitoring computer with Sonographic software running (Northboro, MA).

Acute regional myocardial ischemia was induced by occlusion of the LAD. Both ends of the LAD suture were passed through a rubber tube. The rubber tube was pulled down and secured in place with a mosquito clamp interrupting blood flow distally. The rubber tube and suture were secured in place with a silk tie to the mosquito clamp. After the heart underwent 30 min of regional ischemia, LAD was released, reperfusing the ischemic area. Subsequently, the left lung was reexpanded, chest wall closed by conventional surgical protocol, and a chest tube left into chest cavity in order to prevent potential tension pneumothorax. The animals were maintained under anesthesia and artificial ventilation until physically stable, then weaned off ventilation and anesthesia and monitored for 2 days.

Forty-eight hours following the initial surgery animals underwent similar anesthetic and surgical protocols as previously described including assessment of ventricular function. Subsequent measurements of cardiovascular indices were recorded.

2.2.5. Tissue Processing

Following last dP/dt recording, Miller catheter was pulled out from left ventricle and cardiac apex incision closed. Animals were euthanized through a pentobarbital overdose. Immediately, descending aorta was clamped and a small incision was performed closely above vascular clamp. A Foley catheter was inserted through aortic incision and advanced into the heart in order to identify left cardiac cavities. The heart was excised out from chest cavity and irrigated with 150 ml of 0.9% NaCl solution at room temperature and 80 mmHg perfusion pressure. Once excised and irrigated the heart was evaluated, identifying left ventricle free wall and infarcted area. Heart was serially sectioned in a plane parallel to the atrioventricular groove in slices of approximately 5 mm thickness. The distal third slide from the apex was immersed in 70% ethanol for 4 h, transferred to 10 % buffered formalin for 24 h and embedded in paraffin. The infarcted area was excised from subsequent slices and divided. Equal segments were stored in two ways for further testing. Segments were either

snap frozen in liquid nitrogen and stored at − 70°C or placed in cryogenic mold containing OCT (Optimal Cutting Temperature) compound (Sakura Finetechnical Co, Tokyo, Japan), snap frozen in liquid nitrogen and stored at − 70°C.

2.2.6. Indices of Assessment

2.2.6.1. eNOS/iNOS mRNA Expression

Messenger RNA expression was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) technique.

Tissue Homogenization: Sixty to 100 mg of heart tissue was homogenized with 2 ml homogenization buffer (RNAzol; Tel-Test, inc., Friendswood, Texas, USA) at approximately 8000 rpm using a homogenizer (Biospec Products, inc., Racine, WI, USA).

RNA Extraction: (a) Bulk Extraction: RNA was extracted by adding 200 μL chloroform to the homogenized tissue sample and setting it on ice for 15 min. Samples were centrifuged at 14000 rpm at 4°C for 15 min to separate RNA from DNA and protein. *(b) RNA Precipitation:* The aqueous layer (containing the RNA) was removed and mixed with 500 μL isopropanol. The mixture was set on dry ice for 45 min and then centrifuged at 14000 rpm at 4°C for 10 min. *(c) RNA Wash:* After removal of supernatant, 500 μL 75% EtOH was mixed with each sample and then centrifuged at 12000 rpm at 4°C for 10 min. The supernatant was removed and the RNA pellet was air dried for 5-8 min. 50 μL DEPC water (sterile, distilled water containing 500 μL diethyl pyrocarbonate per 500 mL water; Sigma-Aldrich Co., Oakville, Ont., Canada) and 1 μL RNase inhibitor (Ambion, Inc. Austin, Texas, USA) were mixed with each sample.

UV Spectrophotometry: 2 μL of each sample was added to 998 μL DEPC water and the absorbance at 260 and 280 nm was measured. The ratio of absorbance at 260:280 nm was recorded to verify RNA purity. RNA concentration in each sample was calculated using the following formula:

[mRNA] (µg/mL) = A_{260} x 40 µg/mL (RNA conversion factor) x 1000 µL/2 µL (dilution factor)

The volume of sample required to obtain 5 ug RNA for the DNase reaction was calculated using the following formula:

$$\frac{5\ µg}{\text{concentration (µg/µL)}} = \text{vol sample required (µL)}$$

Gel Electrophoresis: In order to check for the integrity of extracted RNA, 2 µL of sample was loaded onto a 1% agarose gel, which was made up of 50 mL TAE (tris-acetate/EDTA) buffer, 0.5 g agarose, and 10 µL ethidium bromide (EtBr). The gel was immersed in TAE buffer, containing 0.7% volume EtBr, and run at 100 V for 20 min using the Easy-Cast electrophoresis system. After this, the agarose gel was placed in a transilluminator (Stratagene, La Jolla, CA, USA). Images of the RNA bands within the gel were recorded using Eagle Sight™ Software (Stratagene, La Jolla, CA, USA).

DNase Reaction: The following were added to 5 µg samples of RNA: 6 µL 10x PCR Buffer (Sigma-Aldrich Co., Oakville, Ont., Canada), 3 µL 0.1 M $MgCl_2$, 2.4 µL 25 mM deoxynucleotide triphosphate (dNTP; Promega, Madison, WI, USA), 1.5 µL RNase inhibitor (Ambion Inc., Austin, TX, USA) and 1.5 µL DNase I (Boehringer Mannheim, Laval, Quebec, Canada). DEPC water was used to dilute each sample to a total volume of 55 µL. Samples were then loaded into a PCR machine (Perkin Elmer, Norwalk, CT, USA) and held at 37°C for 45 min, 99°C for 5 min, and 4°C for at least 5 min.

Reverse Transcription Reaction: 3 µL random primer (Life Technologies, Burlington, Ont., Canada) and 2 µL Superscript II RT (reverse transcriptase) (200 units/µL; Life Technologies, Burlington, Ont., Canada) were added to each sample with the exception of the "-RT" internal control sample which received 2 µL DEPC water instead of Superscript II RT. Samples were mixed, centrifuged for 5 sec on a mini centrifuge (National Labnet Co.,

Woodbridge, NJ), and incubated in the PCR machine at 25°C for 10 min, 42°C for 5 min, 99°C for 5 min and 4°C for a minimum of 5 min.

Polymerase Chain Reaction: 5 µL of each cDNA sample was mixed with compounds as described in Table 2. These samples were loaded into the PCR machine and were initially exposed to 15 min at 95°C, followed by 36 cycles of 1 min each at 95°C, 1 min at 55°C, and 1 min at 72°C. At the end of the cycles, the samples were held at 72°C for 7 min, 99°C for 5 min, and then 4°C until the samples were removed from the PCR machine. Following amplification, 10 µL loading dye (0.25% xylene cyanol, 15% Ficoll in DEPC water) was added to each sample tube and mixed.

Agarose Gel: A 2% agarose gel was prepared as described in the *Gel Electrophoresis* section. Twenty µL of each sample was added to its corresponding well. Five µL of the DNA marker Hind III λ (New England Biolabs, Inc., Mississauga, Ont., Canada) was added to one well in each row. Electrophoresis was performed at 100 V for 30 min.

Quantification of Gene Expression: Agarose gels were placed inside a transilluminator (Stratagene, La Jolla, CA, USA). Images were recorded at varying light exposure times. The DNA band intensity was quantified using Eagle Sight™ Software (Stratagene, La Jolla, CA, USA).

PCR Primers: The forward and reverse eNOS and iNOS primers were obtained from Dr. Bruce McManus' Lab (Professor, Dept. Pathology & Laboratory Medicine, University of British Columbia). eNOS primer sequences were based on homologous regions of the mouse and human gene sequences. iNOS primer sequences were based on the rabbit gene sequence. Gene sequences were available from GenBank. The primer sequences are shown in Table 3.

Internal Controls: (a) –RT: The objective was to include this control to ensure that the DNase I was effective at removing all DNA from the extracted RNA. A random sample of extracted RNA from a tissue sample was used, beginning at the DNAase reaction step. This

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sample was treated as all other samples, except that it received 2 μL DEPC water instead of 2 μL Superscript II RT in the RT reaction step. *(b) –cDNA:* This control was included in order to determine if there was any DNA contamination in the samples during the protocol. It was included starting in the PCR step of the reaction (refer to Table 2) and was treated equally to other samples, except that it received 4 μL DEPC and 1 μL 25 mM dNTP, instead of 5 μL cDNA.

Verification of Primer Specificity: To confirm iNOS and eNOS amplification by their respective primers, amplified PCR products were isolated and purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Canada). The purified PCR products were sequenced and found to correspond to the authentic sequences of eNOS and iNOS cDNA.

Optimization of Cycle Repeats to Linear Range: Reaction products accumulate during a PCR reaction at a rate dependent on the amplification efficiency. The linear range of the reaction occurs during the period of the PCR in which the amplification efficiency is at its maximum and remains constant over a number of cycles. At some point during the reaction, the amplification efficiency falls and the rate of product accumulation slows or "plateaus". The linear range was determined for iNOS, eNOS and 18S primers by removing samples from the PCR machine after 28, 30, 32, 34, 36, 38, 40 and 42 cycles and quantifying the amplified DNA. Graphs were plotted for product versus number of cycles, and the linear range of amplification was determined. All PCR reactions were performed within the linear range of the primers.

18S Ribosomal RNA (rRNA) as an Internal Standard: "Multiplex" RT-PCR technique was used in order to compensate variations in RNA quality, quantification errors, and random tube-to-tube variations in both RT and PCR reactions. Multiplex PCR consists in using 2 primer sets in a single PCR – one to amplify the cDNA of interest, and the second to amplify an endogenous control, and the gene of interest is normalized against the amplified control.

For this, the endogenous control must be in the same linear range as the RNA under study. Ambion's Competimer™ technology (Ambion Co., Austin, TX, USA) was chosen to modulate the amplification efficiency of a PCR template. Ambion's primers and competimers are supplied separately as 5 μM stocks of forward and reverse primers mixed in a 1:1 ratio. The 18S competimers block the DNA extension. By mixing 18S primers with increasing amounts of 18S competimers, the overall PCR amplification efficiency of 18S cDNA can be reduced without the primers becoming limiting and without loss of relative quantification.

Optimization of Ratio of 18S Primers:Competimers: The mRNA for each gene is present in unique concentrations in tissue, depending on species, tissue type, and treatment. Consequently, during the DNA quantification procedure, the light exposure time for recording of band density will be unique, depending on the quantity of DNA. It is important to establish a ratio of primer:competimer so that exposure times will be similar to that for the DNA of each gene being studied. This will avoid under- or over-exposure of 18S primer:competimer, which will reduce variability, thereby improving accuracy. A control PCR experiment was performed using primer:competimer ratios of 1:9, 1:14, and 1:19. The band densities were then compared to the band densities of DNA generated using eNOS and iNOS primers at identical cycle numbers. The ratio that gave the most similar band density to eNOS and iNOS (1:9) was chosen for all experiments.

2.2.6.2. iNOS Immunofluorescence Detection

iNOS was detected qualitatively by fluorescence immunoreactivity analysis. Analysis of specimens was performed in a blind fashion with respect to group allocation.

Frozen OCT embedded samples were sectioned at five μm using a Cryostat, mounted on slides, and fixed with cold 2% paraformaldehyde in PBS for 20 min, washed alternately with PBS and PBS containing 0.1 mol/liter of glycine and then incubated for 30 min in blocking

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buffer (PBS containing 0.5% BSA and 50 μg/ml normal goat IgG). Slides were washed for 5 min in PBS. After washing, samples were incubated with iNOS antibody.

Primary antibody, a monoclonal anti-iNOS mouse IgG1 antibody (BD Transduction, Can), was diluted in blocking buffer (1:50 dilution). Slides were incubated with primary antibody dilution for overnight at 4 °C. Negative control slides were incubated in blocking buffer without containing anti-iNOS antibody. After washing three times for 5 minutes in PBS, samples were incubated in a secondary antibody dilution for 4 h at room temperature. Alexa Fluor 488 conjugated goat anti-mouse IgG antibody (Molecular Probes, USA), diluted 1:200 in blocking buffer was used as secondary antibody. Slides were washed for 5 minutes three times in PBS and mounted with Dako Fluorescence Mounting Medium (Mississauga, ON). Slides were examined and images were captured using a Nikon Eclipse TE200 microscope and Hamamatsu Orca100 camera.

2.2.6.3. iNOS and PAF-AH Western Blotting Analysis

iNOS and PAF-AH protein expression was quantitatively detected by Western Blotting and normalized against the internal control actin to account for differences in protein loading as described previously[73].

Homogenization: 0.4-0.6 g tissue was minced and placed in 2 mL lysis buffer (50 mM Tris-Cl, 1% Triton-X-100, 10% glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na orthovanadate, 1 mM Na molybdate, 0.01 mg/mL aprotinin, and 40 μg/mL PMSF). Tissue was homogenized on ice at 20-30% of maximum speed for approximately 30 seconds, using a homogenizer manufactured by Polytron Inc. (Switzerland). The homogenate was then spun down (1400 rpm, 4°C for 10 min) in a microcentrifuge (Heraeus Instruments, USA). The supernatant was removed and frozen at -70°C.

Protein Assay: Homogenate supernatant samples were assayed for protein concentration using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and an ELISA plate reader

(Biotek Instruments, Winooski, VT, USA). Briefly, 10 µL of 1:5 diluted homogenate supernatant was added to each well in an ELISA plate, 200 µL of the kit reagent was then added to each well. The solutions were then incubated at 37°C for 30 min. The absorbance at 550 nm (A_{550}) was then measured for each sample using the ELISA plate reader. A standard curve was created by measuring A_{550} at known concentrations of BSA (0.0325 – 2 mg/mL), using similar methods as outlined above. The curve was then plotted, and the formula for the line was used to calculate the concentration of each sample. Samples were subsequently diluted to 1 mg/mL with lysis buffer.

Western Blot Analysis: (a) Protein Separation: One hundred µL of 100 mg/ml sample supernatant was combined with 95 µL 2x sample buffer and 5 µL mercaptoethanol (see Table 4 for ingredients). Samples were heated at 90°C for 5 min. A gel-running stand was then filled with running buffer (see Table 4 for ingredients). Ten percent acrylamide gels (see Table 4 for ingredients) were then placed in a rack within the running buffer. 40 µL of sample buffer/supernatant mix was added to each well within the gels. The running apparatus was set at 80 V power and run for 20 min, followed by 120 V for about 20 min. *(b) Protein Transfer:* The gel and a nitrocellulose membrane were positioned beside each other, placed in a running stand, filled with transfer buffer (see Table 4 for ingredients), and set at 100 V power for 1 h. *(c) Antibody Incubation:* The membrane was removed and placed in Tris-buffered saline (pH 7.4) containing 5% skim milk powder for 2 h at room temperature to block non-specific binding. Following this, membranes were incubated in a primary antibody solution containing 5% skim milk, at 4°C overnight with gentle shaking. Anti-iNOS mouse IgG1 (BD Transduction, Can), antiPAF-AH goat IgG (Santa Cruz Biotechnology, Inc., USA) and anti-actin goat IgG (Santa Cruz Biotechnology, Inc., USA) were used as primary antibodies in 1:1000 dilutions. After overnight incubation the blots were rinsed extensively with blotting buffer and then incubated with a 1:5000 dilutions of secondary antibodies in

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PBS, 0.05% Tween, 5% skim milk powder, and shaken for 30 min at room temperature. Anti-mouse IgG goat IgG-HRP (Molecular Probes, USA) and anti-goat IgG donkey IgG-HRP (Santa Cruz Biotechnology, Inc., USA) were used as secondary antibodies. The membranes were then rinsed in 50 mM Tris/Cl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20. *(d) ECL Detection:* Proteins were detected using the enhanced chemiluminescence (ECL) detection system (Amersham, IL., USA). *(e) Quantification:* The membranes were placed in a film cassette for 5 min and the film was developed thereafter. Bands density was quantified using a computer equipped with BioRad Quantity One software (BioRad, Ont., Canada).

2.2.6.4. Myocardial Contractility

Global myocardial function was assessed by measuring the change in pressure in relation to time (dP/dt). dP/dt is considered to be a particularly accurate measurement of global myocardial function due to independence from preload and afterload. dP/dt values were obtained through the means described in section 2.2.3 and 2.2.4.

2.2.6.5. Histologic Evaluation

An experienced pathologist, blind to group allocation, examined hematoxylin and eosin-stained sections of areas of infarction. The following histologic changes associated with MIR injury were searched: notably coagulation or contraction band necrosis, polymorphonuclear (PMN) infiltration, edema, and hemorrhage[173]. Microscopic fields of the infarct areas at 200x original magnification were used for assessment. The following scales were used for scoring. I) for PMN infiltration: 0 = no increase in leukocyte presence within capillaries, 1+ = occasional PMNs identified within capillaries, 2+ = frequent clusters or linear aggregates of PMNs marginating within capillaries, 3+ = obvious extravasation of PMNs into the perivascular space, 4+ = extensive, diffuse PMN extravasation; II) for interstitial edema: 0 = no increase in interstitial widening, 1+ = patchy, mild increase in perivascular spaces between muscle bundles, 2+ = diffuse, mild interstitial widening or very focal severe

widening of interstice, 3+ = generalized severe interstitial widening; III) for interstitial hemorrhage: 0 = no hemorrhage, 1+ = focal identification of small clusters of scattered individual red blood cells within the interstices, 2+ = patchy deposits of space-expanding red blood cell aggregates, and 3+ = diffuse interstitial hemorrhage; IV) for coagulation or contracting band necrosis: presence or absence.

2.2.7. Statistical Analyses

Cardiovascular function was compared using percentage of change values rather than raw values in order to eliminate any difference occurred by chance. Statistical differences of the three groups at each time point of dP/dt assessment, mRNA expression ratios, and immunoblot densitometry ratios were statistically compared between groups using a one-way ANOVA and Bonferroni post-hoc test. The semi-quantitative comparison of histological analysis was analyzed using the Kruskal-Wallis χ^2 test and the Mann-Whitney test for pair-wise comparisons. Finally, associations of iNOS and eNOS band density ratios with other results were searched using Pearson's correlation. Differences were considered significant if p value was less than 0.05; beta error was established on 0.20 and power on 0.80.

III. RESULTS

3.1. Role of PAF in iNOS mRNA Expression

Results of RT-PCR described in Figure 4 and Table 5 show a significant decrease ($p<0.01$) in the level of iNOS mRNA expression in treated animals in comparison to control group. The level of iNOS mRNA in untreated animals was also higher than sham operated group, however, this difference was not significant. Actual band densities (ratio of iNOS:18S) were as follows ($\pm$S.E.M.): Sham 1.261 ± 0.129, Untreated 1.483 ± 0.216, PAF Antagonist 0.803 ± 0.072.

3.2. Role of PAF in iNOS Protein Detection

3.2.1. iNOS Immunofluorescence

iNOS presence among animals compared by immunostaining shows an enhanced signal of iNOS from MIR regions of untreated animals with a uniform distribution (Fig 5). Animals that were under PAF blockage expressed a minimal presence of iNOS in their MIR region, although it was not as low as non-MIR animals.

3.2.2. iNOS Western Blotting

Results of western blotting analysis described in figure 6 and table 5 show a significant presence ($p<0.000$) of iNOS protein in the infracted myocardial tissue of untreated animals in comparison to both sham and treated animals. The presence of iNOS protein in treated animals was not different from sham operated animals. Actual band densitometry ratios (iNOS:actin) were as follows ($\pm$S.E.M.): Sham 0.94 ± 0.071, Untreated 1.544 ± 0.036, PAF Antagonist 0.96 ± 0.054.

3.3. Role of PAF in eNOS mRNA Expression

As demonstrated in Figure 7 and Table 5, RT-PCR detected a significant increase of eNOS mRNA expression in treated animals compared to both untreated (p<0.02) and sham operated animals (p<0.04). Ischemia-reperfusion did not alter eNOS mRNA expression in the untreated group compared with sham-operated animals. Actual band densities (ratio of eNOS:18S) were as follows (±S.E.M.); Sham 1.102±0.043, Untreated 1.033±0.045, PAF Antagonist 1.744±0.262.

3.4. PAF-AH Protein Detection

As demonstrated in Figure 8 and Table 5, the presence of the main enzyme responsible for PAF degradation, PAF-AH, in the ischemic-reperfused areas was similar among the three groups. Actual band densities (ratio of PAF-AH:actin) were as follows (±S.E.M.): Sham 1.15±0.076, Untreated 0.97±0.192, PAF Antagonist 1.3±0.113.

3.5. Effects of PAF on Myocardial Contractility

Results of percentage of change in positive dP/dt are described in Table 6 and Figure 9. Cardiac function declined in both MIR groups at 10 minutes of ischemia and continued decreasing during the entire ischemic period until the onset of reperfusion. Untreated group was 19-32% lower than baseline, whereas treated animals were 10-32%. This decline through the entire ischemic period was significant (p<0.04) for both groups against sham-operated animals. During the early reperfusion period both MIR groups showed a recovery. At 2 minutes, positive dP/dt values presented an increase, but both ischemic-reperfused groups continued to be significantly lower (p<0.05) than the sham operated group (23-26% from baseline). However, at 10 minutes, these differences were not significant (10-19% from baseline).

Nevertheless, at 48 hours reperfusion, untreated animals were 34% below baseline. This contractility index was significantly lower (p=0.03) than the one from animals under PAF antagonism (11% from baseline). Animals who received TVC-309 showed similar values than sham-operated animals (18% from baseline).

3.6. Morphologic Analysis

Representative samples of ischemic-reperfused myocardium of treated and untreated animals are showed in Figure 10 and ranges are described in Table 7. Both ischemic-reperfused groups showed PMN infiltration, edema and interstitial hemorrhage. However, PAF caused significant PMN infiltration (p=0.002) and edema (p=0.003) in the infarcted zone of control animals compared against animals under PAF blockage. Moreover, PAF induced significant differences concerning contraction band necrosis, PMN infiltration, edema and hemorrhage (p<0.02 for all four variables) between ischemic-reperfused myocardium samples under non-PAF blockage and non-MIR hearts. There was no significant difference between sham-operated and treated animals.

3.7. Correlations

Finally, several associations between variables were identified (Table 8). As you can expect, iNOS mRNA and protein expressions were positively associated (r=0.444, p<0.05, 2-tailed). iNOS gene expression was negatively associated with +dP/dt raw values (r=-0.494) (p<0.05, 2-tailed, Figure 11) and iNOS protein expression was negatively associated with percentage of change of +dP/dt (r=-0.443) (p<0.05, 2-tailed, Figure 11).The presence of iNOS in ischemic-reperfused tissue was strongly associated with pathologic variables of tissue damage: PMN infiltration (r=0.909) edema (r=0.875) and hemorrhage (r=0.772) (p<0.01, 2-

tailed, Figure 12). Significant associations between eNOS mRNA expression and values

from +dP/dt or variables of tissue damage were not identified.

IV. DISCUSION

4.1. Effects of PAF on iNOS during MIR injury

Our results show that PAF upregulates iNOS expression during MIR. Other evidence does exist that iNOS is upregulated during MIR. In an *in-vivo* rat model, involving 20 min of regional ischemia followed by 5 h reperfusion[154], iNOS activity was increased 7-fold in the ischemic-reperfused *versus* nonischemic-reperfused area. Furthermore, in a recent *in-vivo* rabbit model of 30 min regional ischemia, followed by reperfusion, iNOS activity did not significantly increase in ischemic-reperfused area versus that of sham-operated animals until 48 h reperfusion[167]. Our previous experience in an *ex vivo* model of global myocardial ischemia is that after 30 min of ischemia and 5 h reperfusion, iNOS mRNA expression was not increased[140]. Consequently, this suggests that iNOS is upregulated during MIR injury. As iNOS is transcriptionally regulated, it may not be overexpressed in the ischemic-reperfused myocardium during the early reperfusion, but at the late phase of MIR injury.

There are some studies that show a relationship between PAF and NO. PAF-induced delayed hypotension is inhibited by N^{ω}-nitro-L-arginine, which blocks the production of nitric oxide from NOS[131]. *In-vitro* studies have shown the involvement of the nitric oxide pathway in PAF-induced relaxation of rat thoracic aorta, evidenced by N^{ω}-nitro-L-arginine abrogating the effects of PAF[174]. Furthermore, an iNOS inhibitor was shown to improve coronary artery flow rate following 30 min reperfusion in rabbit heart[160]. Finally, in papillary muscle preparations, PAF has been related to NO production[171]. These results suggest that some of PAF-related effects may be mediated by NO production.

Previously our group found that PAF did not modulate iNOS mRNA expression at 5 h of myocardial post-ischemia reperfusion. This can be expected due to the necessary transcriptional expression of iNOS, therefore, it suggests that iNOS-generated NO does not participate in PAF-related effects during the early phase of MIR[140].

Our current results show that iNOS-generated NO may participate in the PAF-related effects during the late reperfusion period of regional MIR injury. PAF upregulates after 30 min of regional ischemia and 48 h reperfusion iNOS expression in the ischemic-reperfused heart. Heart samples from an ischemic-reperfused region showed an upregulation of iNOS, in both its gene and protein expressions. This iNOS regulation was induced by PAF. Interestingly, iNOS protein was significantly over-expressed in samples from animals under non-PAF antagonism compared against both PAF antagonism and non-MIR groups. iNOS gene expression was significantly upregulated between treated and untreated animals only. This observation, added to the weak correlation (r=0.444) between iNOS mRNA and protein ratios, suggests a post-transcriptional regulatory effect on iNOS during the ischemic-reperfusion period.

One mechanism through which PAF may regulate iNOS is via phosphoinositide 3-kinase gamma (PI3Kγ). PAFr, a G-protein-coupled receptor, signals through activation of PI3Kγ. PI3Kγ can activate Akt/PKB and the latter is able to phosphorylate NOS[175]. Other mechanisms could be by interaction with other cytokines. PAF can cause the release of tumor necrosis factor-α (TNF-α) from cultured monocytes[176] and TNF-α is able to induce iNOS in isolated cardiomyocytes[177]. Furthermore, several cytokines can activate nuclear factor-kappaB, resulting in an upregulation of iNOS gene[149]. Possible sources of iNOS in the ischemic-reperfused heart are cardiomyocytes and infiltrating macrophages. Increase of iNOS in the ischemic-reperfused myocardium can lead to a locally elevated NO production. High levels of NO produced by iNOS can damage local myocardium by lipid-peroxidation of the cell wall, or activation of inflammatory cells, and release of inflammatory mediators causing further damage.

These findings suggest that iNOS could be a mechanism through which PAF exerts its local damage to the ischemic-reperfused heart and cardiac contractility depression during the late-phase of MIR injury.

4.2. Effects of PAF on eNOS during MIR Injury

Our current results show that PAF downregulates eNOS mRNA expression during MIR injury. There is some evidence that eNOS could be affected during ischemic processes. The effects of hypoxia on eNOS in non-pulmonary endothelial cells have been studied and the results are controversial. For example, in bovine aortic endothelial cells, upregulation of eNOS mRNA and protein expression occurred in cells incubated at low oxygen tension[178]. Also, it has been seen that eNOS protein is upregulated in cerebral blood vessels during cerebral ischemia[179]. Conversely, other studies found reductions of eNOS expression in human umbilical vein endothelial cells and bovine aortic endothelial cells exposed to low oxygen tension[180,181]. Moreover, in pulmonary circulation, expression of eNOS has been studied in *in-vitro* pulmonary endothelial cells under hypoxia. In human primary pulmonary artery endothelial cells[182], cultured porcine pulmonary artery endothelial cells[183], and bovine pulmonary artery endothelial cells[184], hypoxia has been shown to reduce eNOS mRNA and/or amount of eNOS protein. Interestingly, the effects of PAF on both pulmonary, and coronary vascular tones, are similar.

PAF may affect coronary vascular tone in the early reperfusion phase by interfering with NO production, as NO is a main mediator responsible for vascular tone and the main enzyme responsible for NO generation, in the vessels endothelium, is eNOS. In a MIR model, PAF modulated eNOS gene expression at 5 h of reperfusion, reducing it in the ischemic-reperfused heart[140]. This observation was accompanied with a reduction of coronary blood

flow and cardiac contractility. Our current results show that PAF modulates eNOS gene expression during the late phase of MIR injury too.

Other PAF-related effects can influence eNOS-coronary expression. In isolated non-ischemic heart models, PAF has showed to induce a dose-dependent increment in coronary vascular resistance[99,100,103,116-118], mediated by its effect on calcium mobilization due to inositol trisphophate and membrane-channel regulatory effects[102,185]. Furthermore, PAF has been shown to be a powerful stimulant of neutrophil chemotaxis and adhesion to endothelial cells[186] and aggregation of platelets[187]. This accumulation of cells in the coronary circulation can reduce blood flow into coronary vessels[188]. By these mechanisms, PAF could decrease coronary flow rate following ischemia and reperfusion[20,33]. According to Poiseuille's law, this can decrease shear stress in the vessel walls. Increased shear stress has been demonstrated to upregulate eNOS mRNA expression in endothelial cells[189] and evokes release of NO in the rabbit coronary vascular bed[190]. In the absence of PAF-related coronary blood flow reduction, eNOS-generated NO can cause vasodilation, improving blood flow to the heart (and reducing neutrophil adhesion and platelet aggregation).

Other mediators could be part of PAF influence on eNOS. During reperfusion, mediators are released which can promote either increased (e.g. endothelin-1)[131] or decreased (e.g. PAF) eNOS mRNA expression. High levels of PAF are released immediately upon reperfusion. PAF can cause the release of tumor necrosis factor-α (TNF-α) from cultured monocytes[176] and TNF-α can decrease expression of eNOS in endothelial cells[189]. It is suggested that by removing a possible inhibitory effect of PAF on eNOS mRNA expression utilizing a PAF antagonist, the balance shifted towards an increase in eNOS mRNA expression. This provides one explanation as to how a blockage of PAF receptors with TCV-309 could result in increased eNOS expression.

Other possible mechanism involves the effects of oxidized low-density lipoproteins (LDL). PAF activates neutrophils and monocytes to generate superoxide radicals and other reactive oxygen species. Reactive oxygen species can oxidize LDL and high levels of oxidized LDL have been shown to reduce the expression of eNOS[191].

It should be mentioned that although TCV-309 has been well established to be a selective antagonist for PAF, its effects on eNOS mRNA expression have not been studied. Consequently, it is possible that the increase in eNOS mRNA was achieved through actions of the agent independent of the PAF receptor.

In the present study, eNOS mRNA expression was not correlated either with cardiac contractility or variables of damage on the infarcted region. This suggests that PAF does not need eNOS-generated NO to exert its effects on these parameters during the late phase of MIR injury. PAF may induce cardiac depression and induce damage to the ischemic-reperfused tissue by different mechanisms, which is explained below. Furthermore, as eNOS does not need to be transcriptionally regulated, its direct beneficial effects by releasing NO into the coronary lumen could have happened earlier than 48 h. Our findings on eNOS regulation in the late phase could be due to an effect of PAF, As mentioned above this PAF-related effect does not need eNOS-generated NO in order to be exerted during the late phase of MIR injury.

This evidence suggests that PAF modulates eNOS-generating NO during the late reperfusion period of MIR injury, but eNOS-generated NO is not an important mechanism through which PAF exerts its effects on cardiac contractility and damage to the ischemic-reperfused myocardium.

4.3. PAF Effects on Myocardial Contractility

At the late-phase of MIR injury, PAF is responsible for cardiac contractility depression. PAF has been shown to exert a negative effect in cardiac contractility in non-ischemic reperfused models. In tissue preparations, PAF has shown a negative effect on cardiac contractility. Results from isolated cardiomyocytes suggest that PAF has a direct effect on the reduction in contractility[16,40]. In addition, PAF induced a decrease in myocardial contractility in isolated rat[104-108] and guinea pig[98-100,103,109] hearts. This effect has been shown to not be related to arachidonic acid derivates[103].

In MIR models, PAF has been found to be responsible for the cardiac contractility depression observed during MIR injury. In regional *in vivo* swine[138] and global *ex vivo* rabbit[140] models of MIR, PAF contributes to myocardial contractile depression in the heart along the initial 5 h reperfusion[140] and it has increased the requirement for inotropic support. These results are supported by other published MIR studies including those in isolated rabbit heart[18,19], *in-vivo* rabbit[22], *in-vivo* rat[32,95], *in-vivo* sheep[20] and *in-vivo* swine[24] using various PAF antagonists such as SDZ 63-675[18,22], WEB 2170[18], L-659 989[20,32], TCV-309[19], CV-3988[24] and CV-6209[95]. Our current results shows that PAF is responsible for the contractile depression observed during the late phase of MIR as well.

However, some MIR *in-vivo* studies have failed to demonstrate an effect of PAF antagonism on myocardial contractility. These studies have been performed in canine models [25,26,30,145] and cat[33]. Studies in which a PAF antagonist was administered in a model of coronary occlusion in dogs showed minimal beneficial effects, unlike other reports on different animal models. A primary reason for this is likely the extensive collateral circulation in the canine heart that can provide substantial blood flow to the heart during periods of coronary artery ligation, thereby reducing ischemic damage to the heart, and potentially limiting the release of and the damage induced by PAF. The study using an *in-vivo* cat model used the PAF antagonist WEB-2170[33]. The WEB-class of PAF antagonists have been shown to differ from

other PAF antagonists in their effect. WEB-class antagonists have stimulated or inhibited physiological function depending on their concentration. For example, in isolated rat hearts, low concentrations of WEB 2086 and WEB 2170 blocked the vasoconstrictor effects of PAF. At moderate concentrations these agents alone (in the absence of PAF) had stimulatory effects in increasing coronary vascular resistance[105]. In tissue preparations, incubation with WEB 2086 improved cardiomyocytes contractility[39]. Consequently, studies using the WEB class of PAF antagonists must be interpreted with some reservation, with the understanding that this class of PAF antagonists can have different effects, depending upon the dose used.

PAF can exert its negative contractile effect on the ischemic-reperfused heart by different mechanisms. Using non-ischemic models, studies indicate that PAF can act directly on myocytes to reduce contractility. For example, experimental models where neurohumeral and hemodynamic influences were eliminated, such as human cardiac muscle strips [16,101], paced non-coronary perfused guinea pig left atrium and ventricular papillary muscle[102,192], and isolated rat myocytes[39], have shown that PAF causes a direct negative inotropism, which is blocked by specific PAF antagonists. Studies have demonstrated that rat myocytes exposed to hypoxia-reperfusion release PAF[193] and the PAF receptor gene has been detected and cloned in cardiomyocytes[55].

PAF can regulate cardiomyocytes contractility via calcium mobilization. PAF mediates the release of internal calcium stores via PLC[37]. PAF increases intracellular calcium via mobilization of intracellular stores by inositol trisphophate and influx of extracellular calcium through membrane-channel regulation[102] either directly by PAF or indirectly by lipooxygenase-derived metabolites.

PAF releases arachidonic acid via PLA_2[37] and arachidonic acid metabolites may contribute, in part, in the contractility effect of PAF. These effects may be cyclooxygenase and lipoxygenase mediated. Negative inotropic PAF effects have been attenuated by

indomethacin, suggesting a role of cyclooxygenase-derived metabolites[16], but only during the late reperfusion period[127]. TxA2 is released following infusion of PAF in isolated rat hearts[194] and *in-vivo* swine[110], and it has been shown to impair ventricular contractility[195]. Arachidonic acid metabolites may be influenced, in part, by the effect of PAF on reduced contractility.

PKC has been found to modulate myocardial contractile depression of PAF in a number of studies [40,196,197]. For example, in isolated rat myocytes, PAF was shown to decrease contractility (prevented by PAF antagonist BN 50739). However, in PKC-depleted cells, PAF had no effect on contractility[40]. Furthermore, it was found that inhibition of PKC by calphostin C improved contractility and reduced arrhythmias in ischemic-reperfused isolated rat hearts[197]. PI3Kγ was shown to participate in PAF myocardial contractility effects. PI3Kγ is activated by G-protein coupled receptors (such as PAFr) and, in PI3Kγ-deficient mice, the cardiodepressant effect of PAF was not present[175].

Some results show that NO can participate in the contractile-depressive effect of PAF. In papillary muscle preparations, PAF induced NO production and PAF-induced contractile depression was blocked by NOS inhibitor[171]. During the early phase of MIR, it has been observed that PAF blocked eNOS expression, caused contractile depression, and diminished coronary blood flow[140]. Our current results show that PAF modulates both eNOS and iNOS expressions during the late phase of MIR. There was no strong correlation found between iNOS expression and contractile depression. As mentioned, NO can participate in PAF-induced contractile depression and iNOS is responsible for generation of NO in significant amounts. As mentioned previously and will be explained below, PAF can exert its contractile effects during MIR by several mechanisms different from NO production and this can explain why a strong correlation between cardiac contractility and NOS expression was not found.

Outside its direct influence on cardiomyocytes, a reduction in myocardial contractility can be due to the influence of PAF on myocyte death, microvascular permeability, coronary vasoconstriction, capillary plugging, pulmonary vasoconstriction and arrhythmias.

Firstly, PAF has been shown to cause a significant reduction in systemic arterial blood pressure (ABP)[116,122,127,128,130-133] and in MIR models, PAF significantly decreased ABP during reperfusion[22,27,138,139]. This reduction in arterial blood pressure could decrease flow directed into the coronary arteries due to a reduced after-load on the left heart. As the coronary ostia are located in the aorta, reduced after-loads could decrease the filling pressure of the coronary arteries, reducing perfusion into the affected region.

Secondly, PAF can cause right ventricular overload, increased right atrial pressure, and reduced filling of the left ventricle due to its effects on increasing pulmonary vascular resistance. Some studies have proven that PAF causes pulmonary hypertension[116,122,127-129]. For example, PAF has been shown to increase pulmonary vascular resistance in isolated blood-perfused canine lungs[198] and similar effects on pulmonary vascular pressure have been due to PAF in an *in-vivo* regional model of MIR[138]. Furthermore, infusion of PAF at concentrations that caused a marked increased (up to 120-fold) in pulmonary vascular resistance was accompanied with reductions in cardiac output and systemic hypotension[122]. It appears that much of the effect of PAF on increasing pulmonary vascular resistance is mediated via TxA_2 production. A TxA_2 analogue (U 46619) has induced similar changes to pulmonary vascular resistance as PAF. Furthermore, either indomethacin-induced inhibition of TxA_2 production, or a TxA_2 synthase inhibitor (OKY-046), significantly reduced the pulmonary vasoconstrictive effects of PAF[122].

The high afterload on the right ventricle, caused by pulmonary hypertension, can lead to a substantial reduction in right pump function which results in a reduction in end-diastolic

pressure in the left ventricle, and underfilling of the left heart, thereby compromising cardiac output and systemic blood pressure.

Thirdly, PAF can reduce contractility by increasing coronary vascular resistance. An increase in coronary vascular tone will reduce coronary blood flow to the myocardium and, therefore, reduce the supply of oxygen and nutrients to the heart required for contraction and, in this way, reduce contractility.

When PAF has been infused either in a fast[104] or slow[106,108] infusion, it induced an increase in coronary perfusion pressure. In isolated non-ischemic heart models, PAF has been shown to induce a dose-dependent increment in coronary vascular resistance[99,100,103,116-118], and during MIR, PAF reduced coronary blood flow in ex-vivo rabbit hearts exposed to global MIR[140], and this is in agreement with other reports in in-vivo sheep[20] and in-vivo dog[25] models.

The effect of PAF on coronary blood flow could be due to two PAF-induced mechanisms. Effect of PAF on vascular wall tone is mediated by its effect on calcium mobilization by inositol trisphophate and membrane-channel regulation[102,185]. The effect of PAF on coronary vascular tone during MIR can be related, in part, to cycloxygenase and lipoxygenase metabolites as coronary vasoconstriction has been induced by TXA_2[111]. TXA_2 has been found to be released from the heart following PAF infusion[110,194]. Besides inducing an increment in coronary vascular resistance, PAF has been related to myocardial contractility depression[111,195].

Other studies in in-vivo dog[26,30], rabbit[22], and in isolated rat hearts[146,147] have shown no significant effects of PAF antagonist on coronary flow. These discrepancies may relate to differences in species, the absence of blood in isolated crystalloid-perfused heart systems, or corporeal influences on the heart of the in-vivo system.

Two of the studies that did not find protective effects of PAF antagonist on myocardial contractility, or coronary vascular resistance during MIR, were performed on canine models.

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Canine has an extensive collateral circulation in the heart, therefore the myocardial damage may be less severe and the recovery may be better in this specie compared to others. In studies of isolated crystalloid-perfused rat hearts[146,147], that reported no protective effects of PAF antagonists on coronary vascular resistance, the absence of blood in the perfusion system may have been a source for no difference between groups. Blood cells such as macrophages, basophils and neutrophils, as well as platelets, release vasoactive substances, including PAF and they can respond to PAF and cytokines. Consequently, by having blood absent from the system, the vasoactive substances released from the tissue may have contributed less to increasing vascular resistance than would have been the case in the presence of blood.

In an *ex-vivo* rabbit heart study of global MIR[140] changes in coronary vascular flow rate were fairly subtle between the untreated group and the group treated with PAF antagonist; probably more subtle than its effects on other parameters. These borderline effects support why some studies have found reduced coronary vascular resistance with PAF antagonist and others did not.

A canine study demonstrated that ischemic-induced endothelial damage can contribute to increased sensitivity of cardiac function to PAF. It was found that exogenous administration of PAF in a perfused canine heart caused no vasoconstriction[142]. However, following ischemia-reperfusion in this study, the same concentration of PAF significantly reduced coronary blood flow rate. The vasodilator response to acetylcholine (an endothelium-dependent vasodilator substance) was reduced by ischemia-reperfusion. The differential effect of PAF and acetylcholine were attributed to changes in the functional integrity of the coronary endothelial cells due to ischemia-reperfusion.

Fourthly, PAF increases microvascular permeability in the heart, which results in myocardial edema. Using scanning electron and transmission microscopy, Northover showed that PAF

causes changes in the shape of endothelial cells that may give rise to the increase in vascular permeability. This shape change has been shown to be associated with an influx of Ca^{2+} into the endothelial cells[199]. Intracellular calcium increment can activate PLA$_2$, which may lead to the dose-dependant PAF synthesis of prostacyclin and TxA$_2$ involved in PAF-induced permeability changes[37]. Protein kinase C (PKC) appears to be important for the PAF-induced increase in microvascular permeability, as PKC inhibitors were shown to block this effect of PAF administration in the hamster cheek pouch[200]. Myocardial edema may cause an increase in myocardial tissue pressure, thereby inhibiting the ability of myocytes to relax following contraction (i.e., stiffening of the heart)[94], leading to an increase in end-diastolic pressure and reduction in contractility.

Fifthly, PAF can contribute to myocardial cell death during MIR. This has been demonstrated as PAF blockage during MIR, which caused less severe infarct lesions in several studies[20,22,26-33]. The mechanism through which PAF can modulate myocardial infarction will be explained below. In this way, PAF can reduce the number of viable myocytes, thereby reducing contractility.

In addition, disruption of membrane integrity causes dissipation of ion gradients, and an influx of Ca^{2+} into the cell[96], resulting in tonic contraction of myocytes, thereby inhibiting relaxation. Additionally, the rise in intracellular concentration of calcium activates proteases and phospholipases than can amplify damage to cell structures[7]. All of these can result in the loss of contractile ability.

Finally, ionic currents can contribute to PAF-induced depression in contractility. In ventricular myocytes, it has been shown that PAF infusion shortens the action potential duration[201] which could contribute to reduced contractility. One group has shown that PAF decreased the slow Ca^{2+} current[102], whereas another group has reported the opposite, in that PAF increased Ca^{2+} influx via slow Ca^{2+} channels[202]. In guinea pig papillary muscle

myocytes, PAF was shown to reduce intracellular sodium activity (a^i_{Na}) (which is the intracellular potential measured with a Na^+-sensitive microelectrode)[192]. The reduction in a^i_{Na} can lead to the loss of intracellular Ca^{2+} via Na/Ca exchange with subsequent compromise of cardiac contractility. It has been shown that PAF-induced negative inotropism is correlated with a decrease in systolic intracellular calcium concentration during systole[203].

All this information, in addition to our current results, supports that PAF is responsible for cardiac contractility depression during the late-phase of MIR injury. NO-generated iNOS could participate in this contractility depression induced by PAF by its direct tissue damage, but PAF can influence cardiac contractility by several other mechanisms.

4.4. Effects of PAF on Myocardial Infarct Damage

Our current results show that PAF contributes to the severity of myocardial infarction in the late phase of MIR, and an important mechanism is via NO-generated iNOS. Other studies have demonstrated that PAF, during MIR, modulates the infarct lesion[20,22,26-33]. During MIR injury, several PAF-effects can influence the severity of myocardial infarction.

First, PAF modulates capillary permeability. It has been found that PAF induced a concentration-dependent increase in the permeability coefficient of albumin in isolated perfused swine coronary vessels[97]. Furthermore, in isolated rat and guinea pig hearts, it was observed that PAF significantly increased cardiac capillary permeability using fluorescein isothiocyanate bovine albumin as a marker of permeability, and histological measures of edema[95,96]. Using scanning electron and transmission microscopy, Northover showed that PAF causes changes in the shape of endothelial cells which may give rise to the increase in vascular permeability. This shape change has been shown to be associated with an influx of Ca^{2+} into the endothelial cells[199]. Influx of calcium can activate PLA_2, which may lead to the

production of prostacyclin and TXA$_2$, and it is related to PAF-induced permeability changes[37].

Protein kinase C (PKC) appears to be important for the PAF-induced increase in microvascular permeability as PKC inhibitors were shown to block this effect of PAF administration in the hamster cheek pouch[200]. PTK activation via PAFr may be important for the enhanced capillary permeability produced by PAF. It has been identified that PAF alters the shape of endothelial cells by rearrangement of the cytoskeleton[204,205]. This change in endothelial configuration can explain the increased microvascular permeability observed in respond to PAF[206,207]. Focal adhesion kinase (FAK) is an intracellular PTK involved in cytoskeleton assembly which is activated by PAF[208,209]. FAK has been detected in endothelial cells[209] and this PTK can explain the effects of PAF in the endothelium and, ultimately, capillary permeability[68]. This increase in capillary permeability could allow a neutrophil infiltration into ischemic-reperfused myocardium, however, further steps modulated by PAF participate in its effect on myocardial tissue damage during MIR injury.

PAF has been shown to be a powerful stimulant of neutrophil chemotaxis and adhesion to endothelial cells[186] and PAF also stimulates the aggregation of platelets[187]. The aggregated cells, and those attached to the vessel wall, can physically block or slow down blood flow in the coronary vessels[188], diminishing the delivery of nutrients to the damaged zone and increasing cell death extension. Furthermore, neutrophil chemotaxis and adhesion are the first steps for neutrophil migration into the ischemic-reperfused tissue. During inflammation, an initial rapid step is activation of endothelial cells which changes their surface properties to become adhesive for leukocytes. In the first few minutes after activation of the endothelial cells, they express an adhesion protein, P-selectin, on their surface to which neutrophils and monocytes bind. Concurrently, the activated endothelial cells also synthesize PAF, which is directed to the cell surface, but is not excreted. The leukocytes that have been tethered by P-

selectin are then activated by PAF, suffer an influx of calcium[37]. This step induces tight binding of leukocytes to the vascular wall and their subsequent emigration and priming for secretion of their granular contents[210].

MIR leads to activation of neutrophils, which promotes the expression of adhesion molecules on both neutrophils and the vascular endothelium. This attracts neutrophils to the surface of the endothelium and initiates a cascade of cell-cell interactions, leading first to adherence of neutrophils to the vascular endothelium, followed later by transendothelial migration and direct interaction with myocytes[188]. Restriction of neutrophil infiltration by using antibodies which block either P-selectin on the endothelium or L-selectin on the neutrophils has been shown to reduce tissue damage during reperfusion[211,212]. Furthermore, PAF antagonists have been able to block neutrophil migration[213] and exogenous PAF administration has promoted transendothelial migration[214]. Recently, it has been shown that integrins are upregulated by PAF and that the β_1-integrins are involved in PAF-induced leukocyte locomotion in extravascular tissue[215].

Once into the ischemic-reperfused myocardium, neutrophils release several inflammatory mediators, inducing tissue damage. PAF can activate neutrophils and monocytes to generate superoxide radicals and subsequently other ODRS, including NO[216,217]. ODRS are known to oxidize LDL lipids leading to cell wall damage. PAF receptor-mediated stimulation of G-proteins leads to phospholipase A_2 activation, followed by the release of arachidonic acid, which is further metabolized into eicosanoids. Eicosanoids can damage the cells. PAF has been demonstrated to contribute to the generation of ODRS and activation of proteases, which can directly damage cell membranes including those in the vascular wall[218-220]. During post-ischemic reperfusion, activated neutrophils can induce tissue injury by generating ODRS, proteases, arachidonic metabolites (including eicosanoids and PAF), and other proinflammatory mediators[188]. Both, ODRS and inflammatory mediators released during

reperfusion, have influence on neutrophils and coronary endothelium. They stimulate adhesion molecules expression triggering the neutrophil-endothelium interaction[188]. The activated neutrophils migrate transendothelially into the myocardium during reperfusion[221],and once they are into the cardiac tissue, these leukocytes can liberate further ODRS, proteolytic enzymes and more inflammatory mediators, damaging the myocardium. Administration of a PAF antagonist (SDZ 63-675 or WEB 2170) was shown to decrease neutrophil infiltration into the ischemic-reperfused region of the rabbit heart[29], to diminished infarct severity, and improve cardiac function. Additionally, as mentioned, activated neutrophils release PAF[188] which can further augment damage through the many mechanisms mentioned in sections 1.2.2. and 1.2.3. This suggests a possible positive feedback mechanism that may lead to an increase in injury: PAF is known to activate neutrophils which leads to an increased production of PAF, causing further damage.

Our results demonstrate that iNOS is an important mechanism through which PAF generates its effects on myocardial infarct lesion. Both cardiomyocytes and neutrophil-infiltrating myocardium express iNOS. iNOS is transcriptionally upregulated and releases NO at high levels during inflammatory processes such as MIR injury. At these levels, iNOS-generated NO participates as an ODRS, reacting with the phospholipid component of membranes, causing lipidperoxidation and cell wall destruction, extending the myocardial damage. Furthermore, NO can react with superoxide anion, producing peroxynitrite, another harmful ODRS. Peroxynitrite is released from ischemic-reperfused hearts and its formation has been abolished by NOS inhibitors, confirming that iNOS-released NO participates in its formation[222]. In addition, NO can interfere with the respiratory chain in the mitochondria, leading to inhibition of respiration and irreversible cellular damage[149].

During the late phase of MIR, iNOS is upregulated at 48 h in the ischemic-reperfused myocardium. Once expressed, iNOS releases NO at high levels in the ischemic-reperfused

tissue. At these levels, NO can directly, and indirectly, cause tissue damage, releasing PAF[17], extending the tissue injury by the mechanisms discussed above and participating in PAF-related effects during MIR, particularly inducing a more severe tissue damage during the late phase of MIR.

Therefore, it can be summarized that NO-generated iNOS is an important mechanism through which PAF modulates its effects on the severity of the myocardial infract lesion.

V. SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

5.1. Summary

During MIR, an inflammatory process is initiated which causes the activation or release of many mediators and signal transduction molecules including PAF, NO, endothelin-1, tumor necrosis factor-α, thromboxanes, leukotrienes, caspases, MAP kinases and others. Studies have suggested that one of these mediators, PAF, exerts the following actions during MIR: coronary vasoconstriction, myocardial contractile depression, arrhythmias, systemic hypotension, pulmonary hypertension, myocyte cell death, increased capillary permeability and neutrophil infiltration.

It is important to elucidate the processes that occur during MIR, and to identify the molecules that exert damage during this time. In the literature, the protective effects of a specific PAF receptor antagonist administered at a clinically relevant time (i.e., immediately prior to reperfusion) have been proven in our laboratories and by other investigators. Furthermore, the relationship of PAF to other inflammatory mediators has also been investigated. However, the role of NO in the mechanism of MIR injury, as well as its relation to PAF is not clear.   We hypothesized that PAF exerts its effects on negative inotropism and severity of MI lesion at 48 h post-ischemia reperfusion by modulation of NO-releasing enzymes. The objective of the present investigation was to determine the effect of PAF in modulation of NO enzymes (iNOS, eNOS), severity of cardiac contractile depression, and MI lesion in the late (48 h post-ischemic reperfusion) phase of MIR injury. Methods used for this purpose consisted of: an *in-vivo* model of regional MIR, during 30 minutes ischemia and 48 h reperfusion Cardiac contractility performance was evaluated by measurement of +dP/dt in the left ventricle. At the end of this period gene expressions of eNOS and iNOS were determined from the ischemic-reperfused region. Furthermore, iNOS and PAF-AH protein

expression were also determined. Finally, severity of the MIR lesion was evaluated by histopathologic analysis.

Results of this protocol clearly demonstrated that after regional MIR injury, PAF modulated the presence of iNOS and eNOS in the ischemic-reperfused region. In fact, when the effect of PAF was blocked by the specific PAF receptor antagonist (TCV-309), the amount of iNOS mRNA as well as iNOS protein was significantly decreased. This decline correlated with the functional performance of the heart measured by +dP/dt and the morphological changes in the ischemic-reperfused myocardium, measured by leukocyte infiltration, edema, and hemorrhage.

These results prove that one of the mechanism responsible for the negative inotropism, and the tissue damage in the late (48 h) phase of MIR injury, is due to the modulation of iNOS by PAF.

The research performed for this dissertation has furthered knowledge on the mechanism through which PAF exerts its damage during the late phase of myocardial ischemia-reperfusion. Also, it brought forward new evidence that protection against the effects of PAF in a clinically relevant model of MIR that improves recovery from the injury. Although further investigation could be performed, such as confirming iNOS influence on myocardial infraction extension by an iNOS-specific inhibitor, it is hoped that this experiment has enhanced the knowledge on the role of PAF in MIR injury, from the mechanisms of action through to the clinical potential of inhibiting PAF during MIR. It is a goal that the current contribution will be of assistance in developing novel therapeutic interventions that would favorably influence the mortality and morbidity associated with acute myocardial infarction particularly, and ischemic cardiovascular disease generally.

5.2. Possible Future Directions for Research

Although the *in-vivo* effects of PAF antagonist on MIR injury are now better understood, there remains much that can be studied concerning the mechanism of action of PAF in the heart. Elucidation of these mechanistic pathways would lead to better understanding of the specific role of PAF in MIR injury and could identify other potential pharmacological targets for inhibiting the injury associated with PAF and MIR. Furthermore, understanding the role of PAF in relation to the other mediators involved in MIR injury could add to more knowledgeable decisions on which mediators would be most beneficial to block during MIR. Finally, I believe that the research findings are at a stage that we can consider performing a clinical trial on the protective effects of PAF antagonism and iNOS inhibition against MIR injury.

5.3. Conclusions

(a) PAF upregulates iNOS mRNA expression at 48 h of regional myocardial post-ischemia reperfusion.

(b) PAF increases iNOS protein expression at 48 h of regional myocardial post-ischemia reperfusion.

(c) PAF induces an eNOS mRNA downexpression during the late phase of myocardial ischemia-reperfusion.

(d) PAF induces myocardial contractile depression at the late phase of regional MIR injury.

(e) PAF contributes to the severity of myocardial infarction at the late phase of regional MIR.

(f) At 48 h post-ischemia reperfusion, an important mechanism through which PAF exerts its effects on myocardial contractility, and tissue damage is by modulation of iNOS-generated NO.

(g) This may explain, in part, the etiology of morbidity and mortality associated with myocardial reperfusion therapy.

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Group	Treatment	Surgical Intervention
Sham		Instrumentation only
Control	Untreated (NaCl 0.9%)	30 min regional ischemia
Treated	PAF antagonist TCV-309 (0.1 mg/kg)	followed by 48 h reperfusion

Table 1. Experimental groups, treatments, and surgical interventions in an *in vivo* rabbit model of regional myocardial ischemia.

	Volume (µL)			
	INOS/eNOS	18S	-RT Sample	-cDNA Sample
cDNA	5	5	5	0
10x buffer (Qiagen, Austin TX, USA)	9.5	9.5	9.5	9.5
25 mM $MgCl_2$ (Qiagen)	1	1	1	1
25 mM dNTP	0	0	0	1
Taq polymerase (5 units/µL; Qiagen)	0.5	0.5	0.5	0.5
Forward primer	1	1	0	0
Reverse primer	1	1	0	0
18 s alternate primer pair (Ambion Co., Austin, TX, USA)	0	0.4	0.4	0.4
18 s alternate competimer (Ambion, Co., Austin, TX, USA)	0	3.6	3.6	3.6
DEPC water	82	82	80	84

Table 2. Volumes (µL) of compounds added to sample tubes for the PCR reaction to measure

iNOS/eNOS gene expression in *in vivo* rabbit model of regional myocardial ischemia-

reperfusion. "-RT" and "-cDNA" are internal control samples.

Primer	Sequence
Forward eNOS primer	5'-TGG CTG CCG ATG CTC CCA AC – 3'
Reverse eNOS primer	5' –TTG TCA CCT CCT GGG TGC GC – 3'
Forward iNOS primer	5' –GTC AGC CCT CAG AGT ACA AC – 3'
Reverse iNOS primer	5' –TGA CAC TCC GCA CAA AGC AG –3'

Table 3. Primer sequences for detection of eNOS and iNOS in *in vivo* rabbit model of regional myocardial ischemia-reperfusion.

Reagent	Amount	Compound
2x Sample Buffer	0.6 mL	1M Tris HCl, pH 8.0
	5.4 mL	dH_2O
	2.0 mL	10% SDS
	1.0 mL	Glycerol
	250 µL	1% bromophenol blue
Running Buffer	6.05 g	Trizma Base, pH 8.8
	28.8 g	Glycine
	1980 mL	dH_2O
	20 mL	10% SDS
10% Acrylamide Gel	3.6 mL	resolving gelbuffer
	2.5 mL	30% acrylamide
	2.7 mL dH_2O	75 µL 10% APS
	7.5 µL	Temed
Resolving Gel Buffer	900 mL	dH_2O
	90.8 g	Trizma base, pH 8.8
	2.0 g	SDS
Transfer Buffer	6.05 g	Trizma Base, pH 8.8
	28.8 g	Glycine
	1600 mL	dH_2O
	400 mL	methanol

Table 4. Reagents used in Western blotting for detection of iNOS and PAF-AH in *in vivo* rabbit model of regional myocardial ischemia-reperfusion.

	Sham	Untreated	PAF Antagonist	p value
eNOS (rt-pcr)	*1.102±0.043	°1.033±0.045	*,°1.744±0.262	*0.036, °0.02
iNOS (rt-pcr)	1.261±0.129	*1.483±0.216	*0.803±0.072	*0.009
iNOS (w-b)	*0.94±0.071	*,°1.544±0.036	°0.96±0.054	*0.000, °0.000
PAF-AH (w-b)	1.15±0.076	0.97±0.192	1.3±0.113	

Table 5. Comparison of band density ratios obtained by RT-PCR (rt-pcr) and Western Blotting

(w-b) of eNOS (endothelial nitric oxide synthase), iNOS (inducible nitric oxide synthase) and

PAF-AH (platelet-activating factor acetylhydrolase). Significant differences (p values)

correspond to each pair marked in the same row.

Time	SHAM dP/dt	UNTREATED dP/dt	PAF ANTAGONIST dP/dt
Baselime	1481±119	1698±118	1706±141
10 min isch	1574±119	1365±102	1546±155
20 min isch	1433±116	1218±103	1336±108
30 min isch	1493±109	1151±105	1147±58
2 min rep	1410±125	1293±176	1313±104
10 min rep	1376±114	1406±145	1384±121
48 h rep	1159±29	1119±100	1510±119

Table 6. Positive dP/dt (mmHg/sec) in *in vivo* rabbit hearts, in response to 30 min of regional ischemia followed by 48 h reperfusion. Groups received PAF antagonist TCV-309, vehicle, or were sham-operated, ± values represent standard error of the mean.

	Contraction band necrosis	PMN infiltration	Edema	Interstitial hemorrhage
SHAM	0-1	0-1	0-1	0-1
CONTROL	1	3-4	1-2	1-3
PAF ANTAGONIST	0-1	0-1	0-1	0-2

Table 7. Range of scores of histopathologic changes in tissue samples of the ischemic-reperfused region of an in-vivo rabbit model of regional myocardial ischemia-reperfusion. Sham n=7, Control n=7, PAF antagonist n=8.

	iNOS RT-PCR		iNOS WB		eNOS RT-PCR	
	r	p	r	p	r	p
raw +dP/dt	-0.494	0.019	-0.232	0.298	0.216	0.334
% +dP/dt	-0.214	0.339	-0.443	0.039	0.384	0.078
Contraction Band	0.076	0.756	0.424	0.07	-0.041	0.868
PMN Infiltration	0.359	0.131	0.909	0	-0.262	0.279
Edema	0.318	0.184	0.875	0	-0.38	0.108
Hemorrhage	0.373	0.116	0.772	0	-0.087	0.722
iNOS RT-PCR	1		0.444	0.039	-0.357	0.103
iNOS WB	0.444	0.039	1		-0.403	0.063
eNOS RT-PCR	-0.357	0.103	-0.403	0.063	1	

Table 8. Associations obtained by Pearson's correlation between NOS ratios and values of

cardiac contractility and infraction lesion; r values represent correlation coefficient, p values

represent significance of correlations.
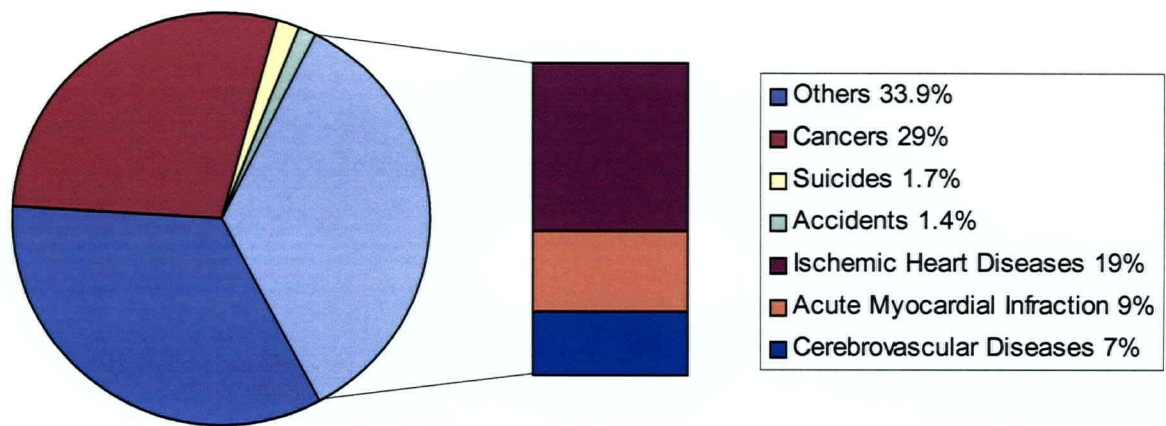
Figure 1. Leading causes of death, percentage of deaths, Canada, 2002. Source: Statistics Canada.

$$\underset{\underset{O^-}{|}}{\overset{O}{\overset{\|}{\underset{}{}}}}$$

$$CH_3\overset{O}{\overset{\|}{C}}O\overset{\overset{\displaystyle CH_2O(CH_2)_{15\text{-}17}CH_3}{|}}{CH}$$
$$CH_2O\overset{O}{\overset{\|}{P}}OCH_2CH_2N^+(CH_3)_3$$
$$\underset{O^-}{\overset{|}{}}$$

A.

$$\overset{\displaystyle CH_2O(CH_2)_{15\text{-}17}CH_3}{|}$$
$$\overset{\displaystyle CH_2}{|}$$
$$CH_2O\overset{O}{\overset{\|}{P}}OCH_2CH_2N^+(CH_3)_3$$
$$\underset{O^-}{\overset{|}{}}$$

B.
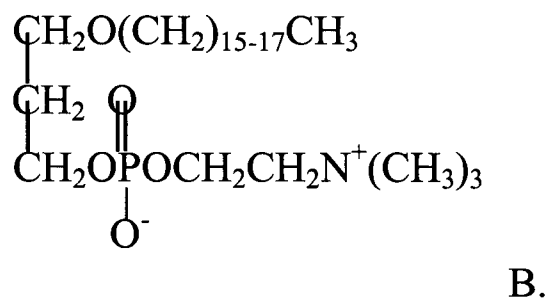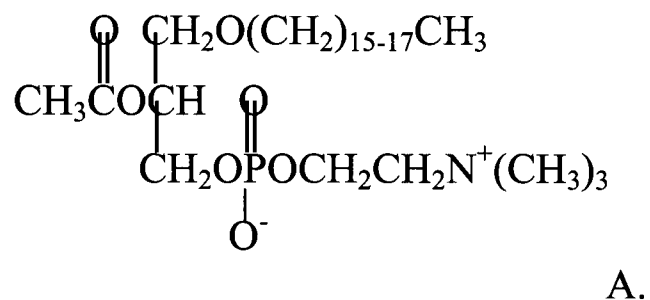
Figure 2. A. Chemical structure of platelet activating factor. B. Chemical structure of lyso-platelet activating factor.

H₂C-O-FA   Remodeling

| Pathway

AA-CH

|

H₂C-℗–Choline

PLA₂   ↓ ↳AA

H₂C-O-FA

|

OH-CH

|

H₂C-℗–Choline

Acetyltransferase   ↓

     O H₂C-O-FA

     ‖ |

CH₃-C-O-CH   **PAF**

        |

   H₂C-℗–Choline

     ↑ *ChoP Tranferase*   De Novo

    O H₂C-O-FA   Pathway

     ‖ |

CH₃-C-O-CH

         |

     H₂C-**OH**

     ↑ *Phosphohydrolase*

    O H₂C-O-FA

     ‖ |

CH₃-C-O-CH

         |

     C-℗

     ↑ *Acetyltransferase*

   H₂C-O-FA

      |

   HO-CH

      |

   H₂C-℗
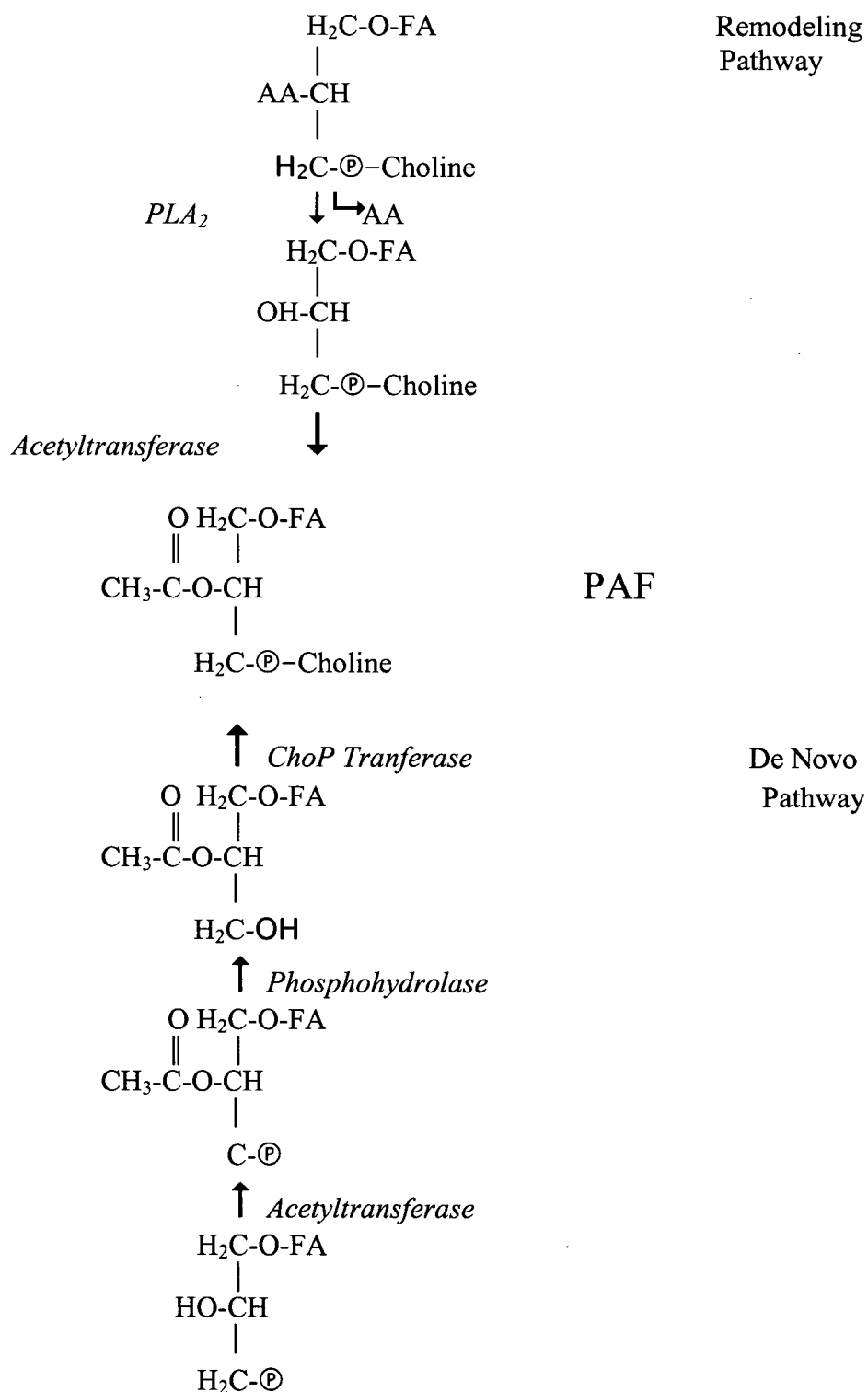
Figure 3. Biosynthesis pathways of PAF. Abbreviations are: ℗, phosphate group; PLA₂,

phospholipase A₂; ChoP Transferase, CDP-cholinephosphotransferase; AA, arachidonic acid or

arachidonate; FA, fatty acid.

iNOS
306 bp

18S

iNOS mRNA expression
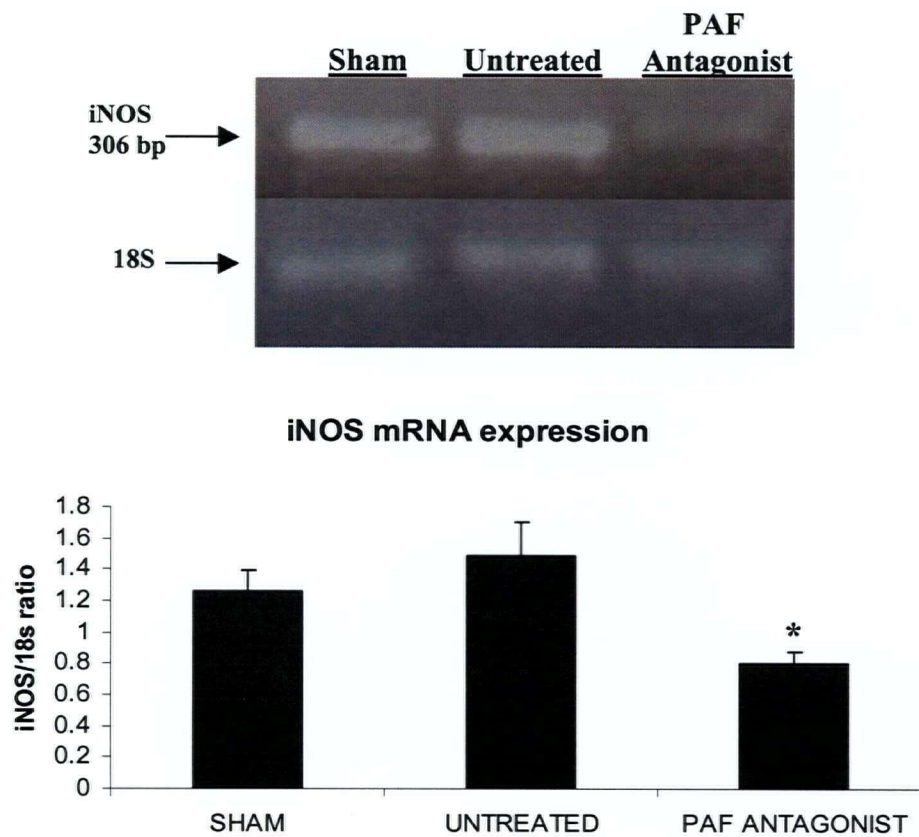
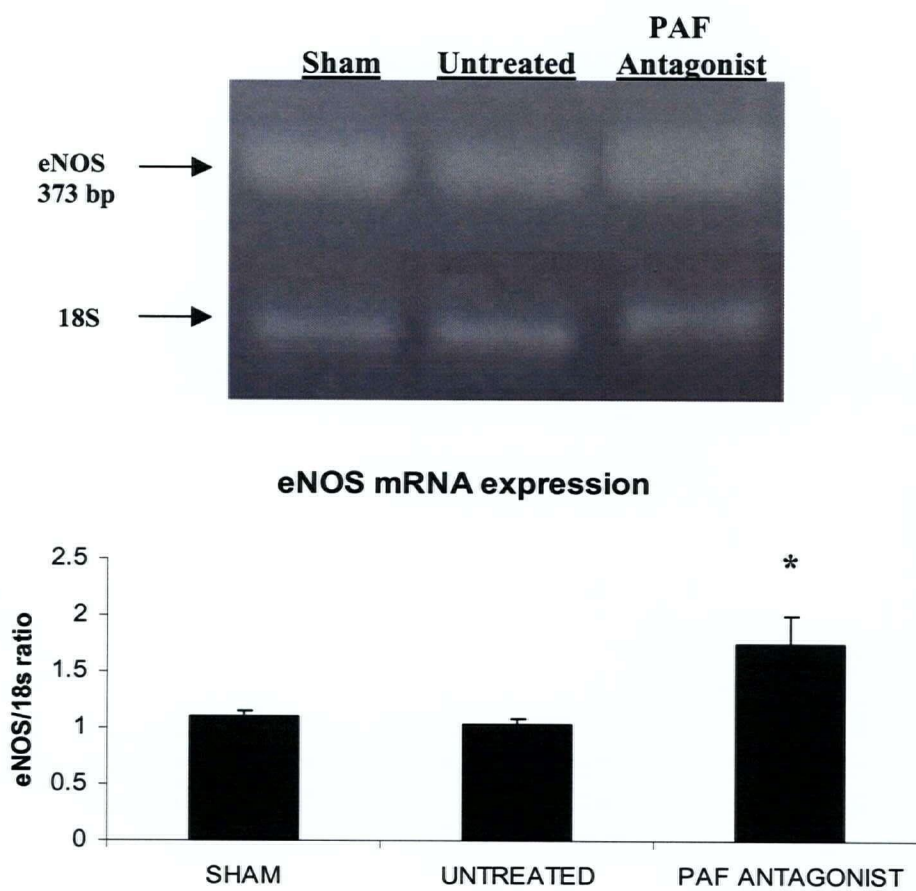Figure 4. inducible nitric oxide synthase (iNOS) mRNA expression measured by reverse transcription polymerase chain reaction (RT-PCR). * p<0.01 for PAF antagonist versus untreated; error bars represent standard error of the mean.
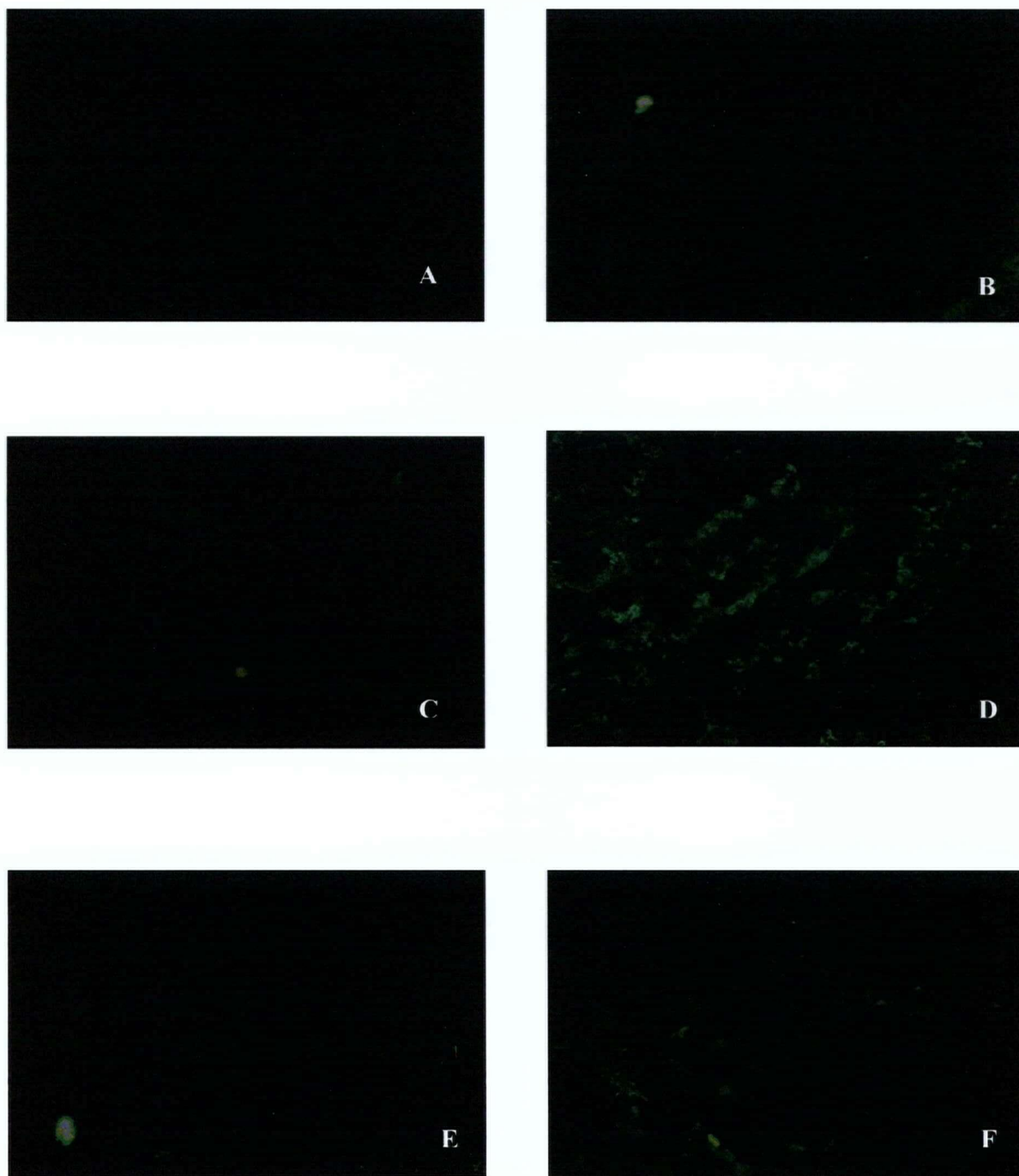
Fig 5. iNOS immunofluorescence detection in ischemic-reperfused heart samples. Figure B represents sample from sham group, figure D from control group and F form treated group. Images A, C and E are negative controls. Magnification 20x.
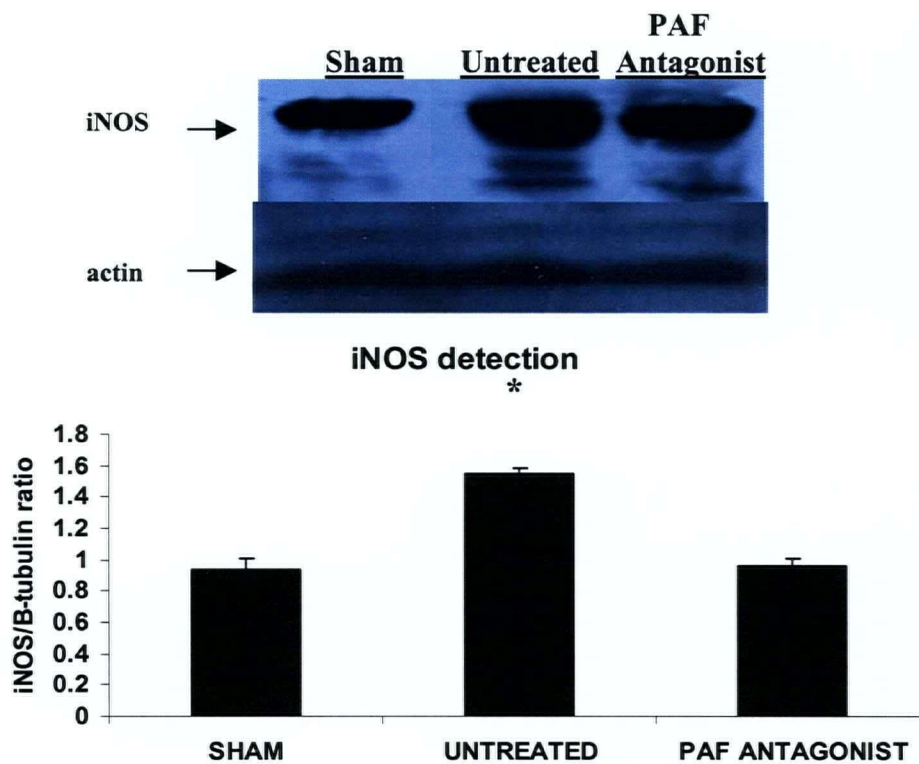
Fig 6. inducible nitric oxide synthase (iNOS) detection by Western blotting. * p<0.000 for

untreated against PAF antagonist and sham animals; error bars represent standard error of the

mean.

Fig 7. Endothelial nitric oxide synthase (eNOS) mRNA expression measured by reverse transcription polymerase chain reaction (RT-PCR). * p <0.04 for PAF antagonist compared against sham and p<0.02 against untreated; error bars represent standard error of the mean.

PAF degradation
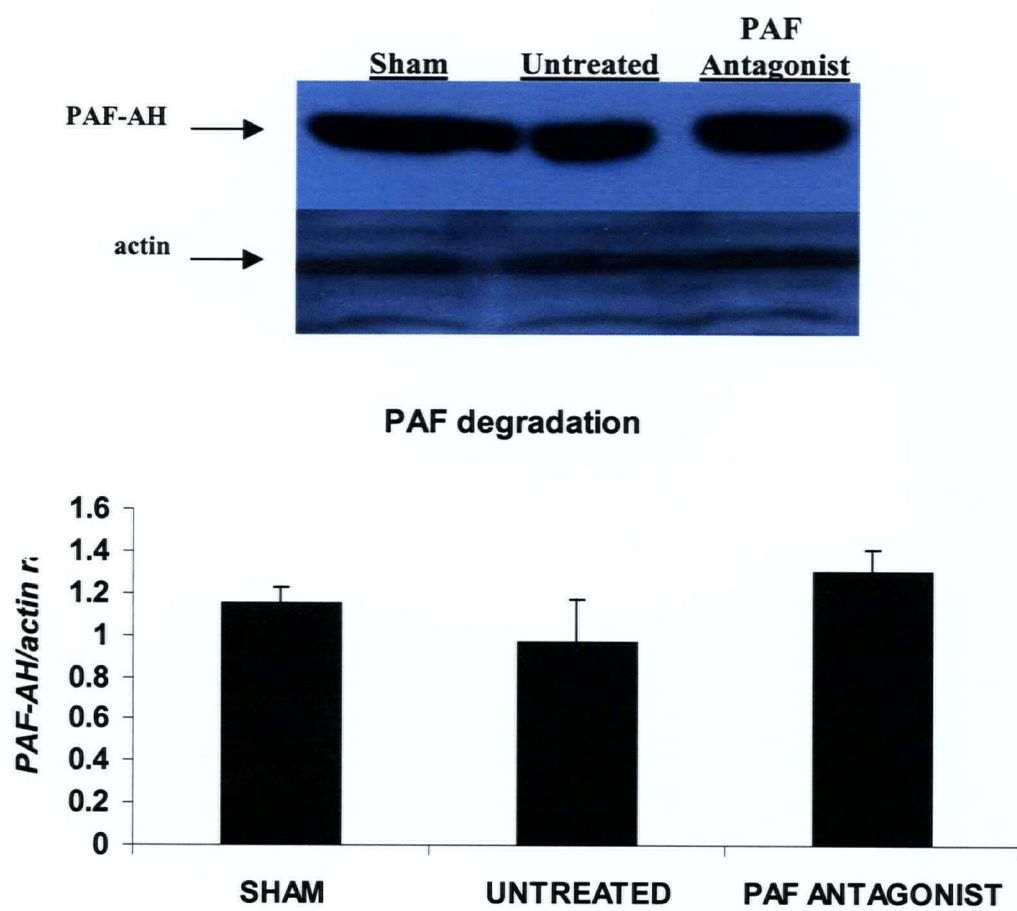
Fig 8. Platelet-activating factor acetylhydrolase (PAF-AH) detection by Western blotting; error bars represent standard error of the mean.
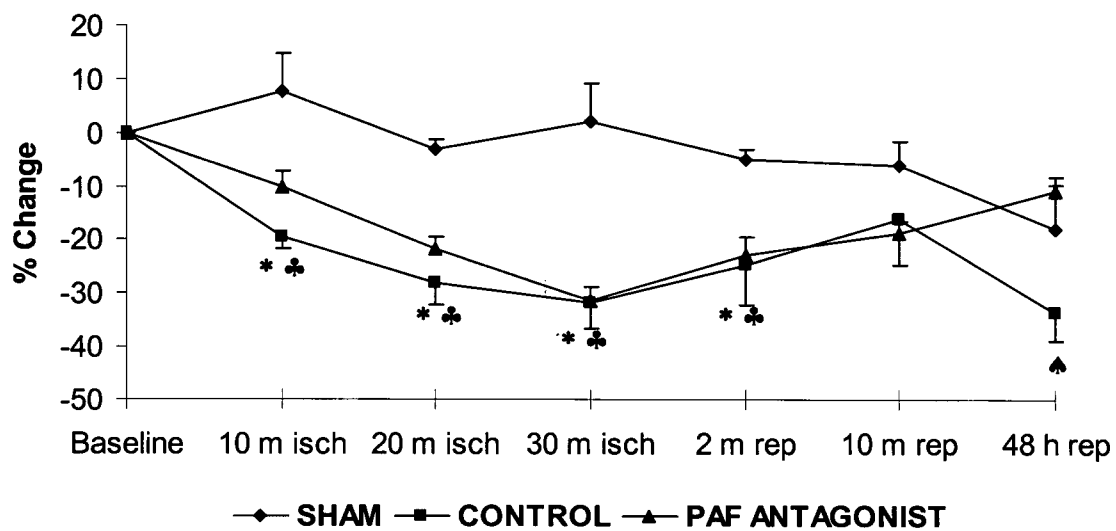
Figure 9. Percentage change of positive dP/dt in response to regional ischemia-reperfusion. X-axis represents 30 minutes ischemia followed by 48 hours reperfusion; error bars represent standard error of the mean. * $p < 0.05$ for untreated versus sham; ♣ $p < 0.05$ for treated versus sham; ♠ $p < 0.05$ untreated versus treated.
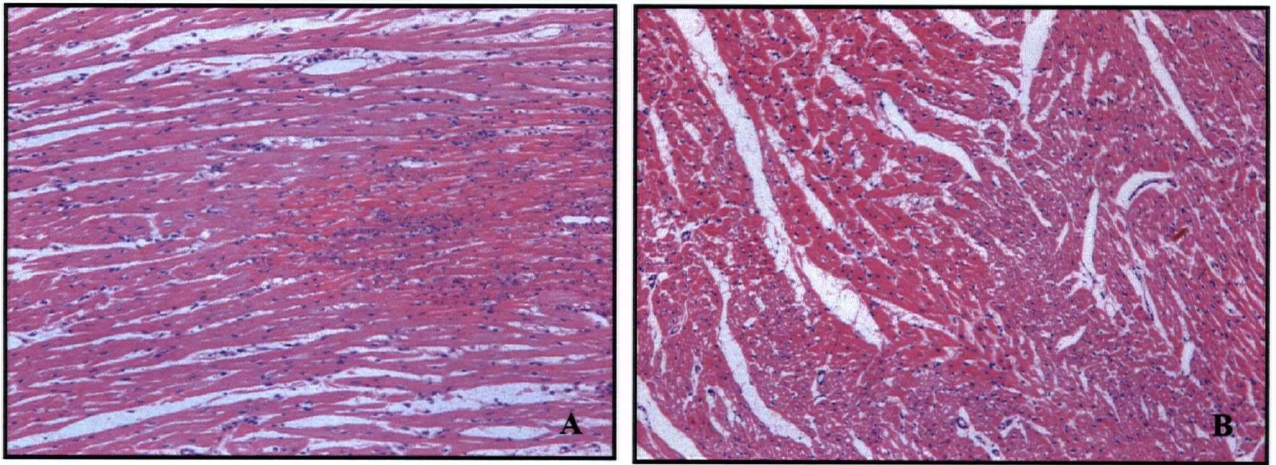
Figure 10. Hematoxylin-eosin staining in in-vivo rabbit hearts exposed to ischemia-reperfusion in the absence (picture A) or presence (picture B) of PAF antagonist TCV-309. Untreated hearts (picture A) presented hypereosinophilic zones, which corresponded to infracted areas, with edema and leucocyte infiltration. Hearts from treated animals (picture B) presented hypereosinophilic areas but with a less significant inflammatory reaction. The presence of nucleus reveals that, although the tissue was ischemic, it is not completely lost. Magnification is 100x.
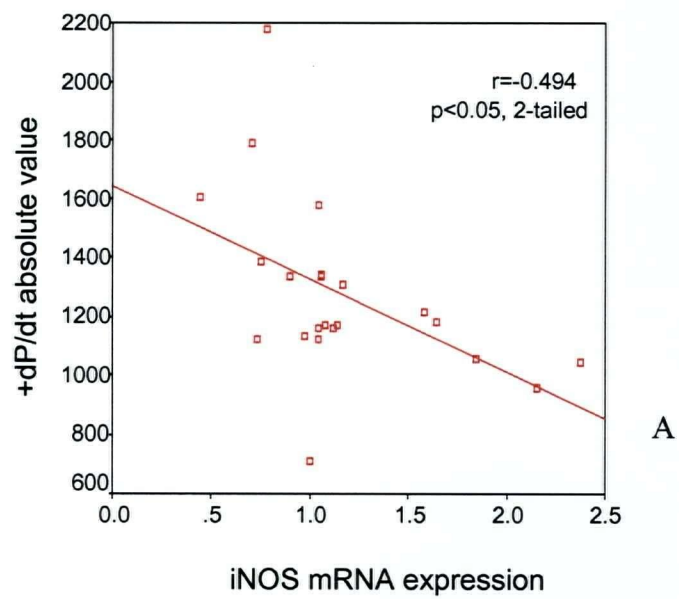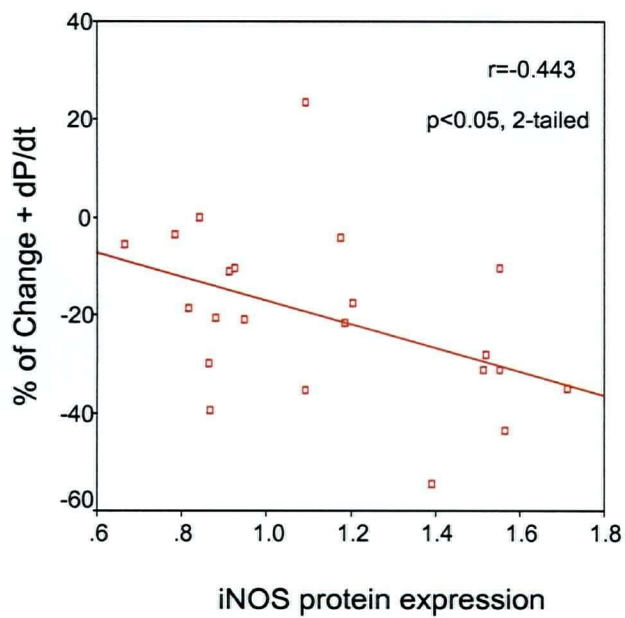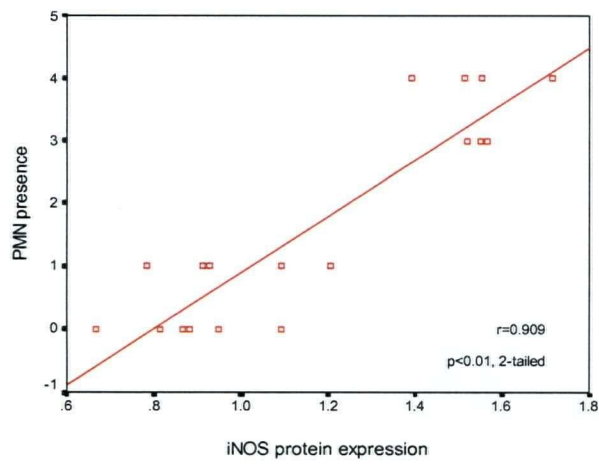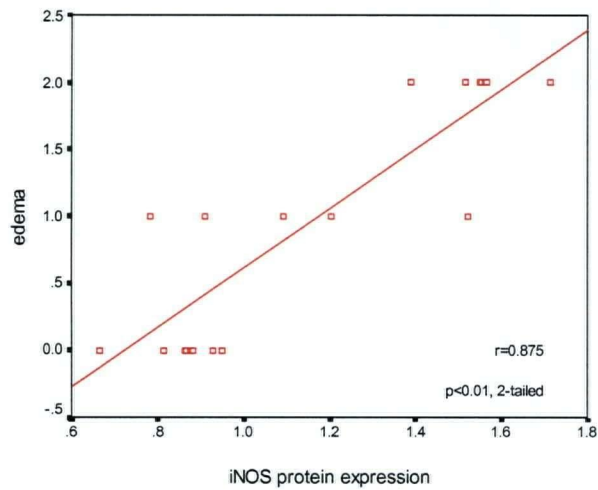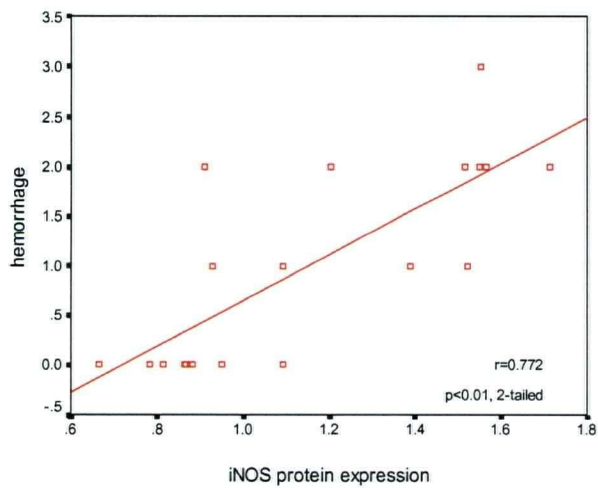
Figure 11. Associations between iNOS gene expression and +dP/dt raw data (A) and iNOS

protein expression and +dP/dt percentage of change (B).

Figure 12. Associations between iNOS protein expression and presence of leukocyte infiltration

(A), edema (B) and hemorrhage (C) in ischemic-reperfused in-vivo heart tissue.