# STUDY OF THE CARDIOVASCULAR EFFECTS OF PLATELET-ACTIVATING FACTOR

A Thesis
presented to the
University of Manitoba

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

by

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ISBN 0-315-76900-9

# STUDY OF THE CARDIOVASCULAR EFFECTS OF PLATELET-ACTIVATING FACTOR

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WEIMIN HU

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

This work is dedicated to
my parents, Zhijie Zhu and Chiwu Hu,
My wife, Xiaoli Lu,
and my son, Wenkun Hu,

# Abstract

Since the isolation of platelet-activating factor (PAF), the elucidation of its structure and its chemical synthesis, PAF has been demonstrated to possess potent cardiovascular effects. In this investigation, the hypotensive action of PAF was compared to that of some structurally similar compounds, and the coronary vascular effects of PAF in the perfused heart was characterized and its mechanism of action was examined.

Palmitoyl carnitine and lysophosphatidylcholine are structurally similar to PAF and accumulate in the ischemic myocardium. The first part of the study was designed to investigate the effects of palmitoyl carnitine, lysophosphatidylcholine and structurally related compounds on the blood pressure of anesthetized rats and compare them with the well known hypotensive effect of PAF. Palmitoyl carnitine was about 1000 times less potent than PAF in lowering the blood pressure. Lysophosphatidylcholine and other structurally related compounds had no significant hypotensive action. The hypotensive effect of palmitoyl carnitine was not blocked by a PAF antagonist. Therefore, its hypotensive action was not mediated through the same site or mechanism as PAF.

The second part of the study is to examine the coronary vascular effects of PAF in the perfused heart. A biphasic response characterized by an initial vasodilation followed by a vasoconstriction was observed after a bolus injection of PAF in the perfused rat heart. The coronary vascular effects of PAF were influenced by the starting perfusion pressure, the amount of PAF administered, the presence of albumin in the PAF stock solution and the animal species. The fact that a second injection of PAF fails to cause vasoconstriction can be explained by desensitization of the PAF

receptor and/or depletion of vasoactive mediators released by PAF.

The mechanisms of the coronary vascular effects of PAF in the perfused rat heart were examined using a number of pharmacological antagonists and inhibitors. Selectivity for the coronary vasodilator and vasoconstrictor effects of PAF was observed with different PAF antagonists. CV-6209 selectively blocked the vasodilator effects of PAF and a higher concentration was required to block the vasoconstrictor effect. The other PAF antagonists (FR-900452, WEB 2086 and BN-50739) showed selectivity for blocking the vasoconstrictor effect of PAF. A combination of low concentrations of CV-6209 and FR-900452 or WEB 2086 was effective in blocking both the vasodilator and vasoconstrictor effects of PAF. These results support the hypothesis that the functionally opposite effects of PAF in the perfused heart are mediated by different PAF receptor subtypes. The activation of the first subtype of the PAF receptor produces vasodilatation while the activation of the second subtype of PAF receptor produces vasoconstriction. The cyclooxygenase inhibitor, indomethacin, did not modify the coronary vascular effects of PAF. However, L-649,923 (a leukotriene antagonist) and MK-886 (a leukotriene synthesis inhibitor) eliminated both the vasodilator and vasoconstrictor effects of PAF. When leukotrienes were administered by bolus injection in the perfused rat heart, leukotriene $B_4$ ($LTB_4$) produced vasodilation while $LTC_4$ and $LTD_4$ produced vasoconstriction. Therefore leukotrienes are capable of mimicking the individual components of the biphasic response to PAF. The concentrations of leukotrienes from the effluent of perfused heart measured by radioimmunoassays were significantly increased after an injection of PAF. Pretreatment of the isolated heart with the PAF antagonist, CV-6209, abolished the increases in leukotrienes induced by PAF. The results suggest that the coronary vascular effects of PAF are mediated by lipoxygenase products, leukotrienes, in the isolated perfused rat heart.

The properties of PAF-released vasoactive substances and the interplay of these substances were studied with a cascade perfusion model using two perfused rat hearts. After an injection of PAF to the first heart, the effluent caused vasoconstriction in the second (recipient) heart. Pretreatment of the recipient heart with the leukotriene receptor antagonist, L-649-923, significantly attenuated but did not abolish the vasoconstrictor effect of the effluent. The remaining vasoconstrictor component was abolished by pretreating the first heart with the cyclooxygenase inhibitor, indomethacin. Pretreatment of both hearts with L-649,923 or of the first heart with the leukotriene synthesis inhibitor, MK-886, completely abolished the vasoconstrictor effect of the effluent. The results suggest that, upon injection of PAF, both leukotrienes and prostaglandins are released from the first heart into the effluent. In addition to directly mediating part of the vasoconstrictor effect in the effluent, leukotrienes also modulate the production and/or release of cyclooxygenase products in the effluent by a receptor mediated mechanism.

The challenge of future PAF research is to learn the molecular mechanism(s) of its action in relation to the various biological responses that it initiates.

# Acknowledgements

I would like to express my sincere thanks and appreciation to Dr. Ricky Y.K. Man for sharing with me his wisdom, for his continued advice and excellent supervision and for his enthusiastic encouragement throughout my years as a graduate student. Many thanks to professors, faculty and staff for their help and the knowledge I have gained from them. I would especially like to thank Dr. Patrick Choy and Dr. Deepak Bose who gave me their invaluable advice and encouragement and always be there when I needed. I would also give my grateful thanks to members of our laboratory, Mrs. Anne Blanchard, Ms. Ila McNicholl and Dr. Thomas Mock, for their excellent technical assistance and advice, for sharing some good times and memorable experiences. Thanks are also extended to fellow students for their friendship and interesting discussions on various aspects of science and life in general. My special thanks are also given to (soon-to-be) Dr. Mark D'Almeida for his friendship and helps in the past few years. Finally, I would like to thank my family for their loves, fully support, patience and understanding.

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# ABBREVIATIONS

| | |
|---|---|
| ANOVA | analysis of variance |
| ATP | adenosine 5'-triphosphate |
| ADP | adenosine 5'-diphosphate |
| cAMP | adenosine 3',5' cyclic-phosphate |
| cGMP | guanosine 3',5' cyclic-phosphate |
| cm | centimeter |
| CoA | Coenzyme A |
| dpm | disintegrations per minute |
| g | gram |
| *g* | gravity |
| G protein | guanine nucleotide regulatory protein |
| GTP | guanosine 5'-triphosphate |
| GTPase | guanosine triphosphatase |
| hr | hour |
| Ig E | immunoglobin E |
| LPC | lysophosphatidylcholine |
| $LTB_4$ | leukotriene $B_4$ |
| $LTC_4$ | leukotriene $C_4$ |
| $LTD_4$ | leukotriene $D_4$ |
| lyso-PAF | 1-*O*-alkyl-2-*sn*-acyl-glycero-phosphorylcholine |
| M | molar |
| mg | milligram |
| min | minute |
| ml | milliliter |
| mm | millimeter |

| | |
|---|---|
| mM | millimolar |
| Ni | inhibitory guanine protein |
| Ns | stimulatory guanine protein |
| nM | nanomolar |
| nmol | nanomole |
| PAF | platelet activating factor, 1-*O*-alkyl-2-*sn*-acetyl-glycero-phosphorylcholine |
| $PGE_2$ | prostaglandin $E_2$ |
| $PGI_2$ | prostacycline |
| rpm | rotations per minute |
| S.E. | standard error |
| $TxA_2$ | thrombixane $A_2$ |
| $TxB_2$ | thrombixane $B_2$ |
| $\mu$Ci | microCurie |
| $\mu$g | microgram |
| $\mu$l | microliter |
| $\mu$M | micromolar |
| $\mu$mol | micromole |

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# Introduction

## 1. Historical background

Between 1966 and 1969 evidence was obtained for complement-independent, antigen-induced activation of rabbit leukocytes that in some way resulted in histamine release from platelets (Barbaro and Zvaifler, 1966, Siraganian and Oliveira, 1968, Siraganian and Osler, 1969). Subsequently, Henson (1969, 1970) proposed that a "fluid-phase mediator" from leukocytes of immunologically sensitized rabbits might be involved. In 1977, Benveniste *et al.* first named the factor as platelet-activating factor (PAF) and provided evidence that it was a lipid like molecule (Benveniste *et al.*, 1977). In the autumn of 1979, three independent laboratories (Hanahan and Pinckard, 1979, Blank *et al.*, 1979, and Benveniste *et al.*, 1979) described the chemical structure of platelet-activating factor as a phospholipase $A_2$-sensitive phospholipid and identified this unique phospholipid as 1-*O*-alkyl-2-*sn*-acetyl-3-glycero-phosphorylcholine (fig. 1). Both PAF-acether (ace for acetate and ether for the alkyl bond) and AGEPC (acetyl glycerol ether phosphoryl choline) have been used in the literature to denote PAF.

## 2. Biochemistry of PAF

PAF is a chiral and unsymmetrically substituted D-glycerol derivative and an unique class of choline phosphoglyceride with several features. The $C_1$ position of glycerol must be in an ether linkage with a fatty alcohol. There are an acetate group at the $C_2$ position and the phosphocholine head group at the $C_3$ position, both of which are needed for the highest potency. Its chemical structure is different from naturaly occurring phosphatidylcholine which contains 1,2-diacylglycerol and phosphocholine at $C_3$. As an ether phospholipid, its structure is closely related to the naturally occurring

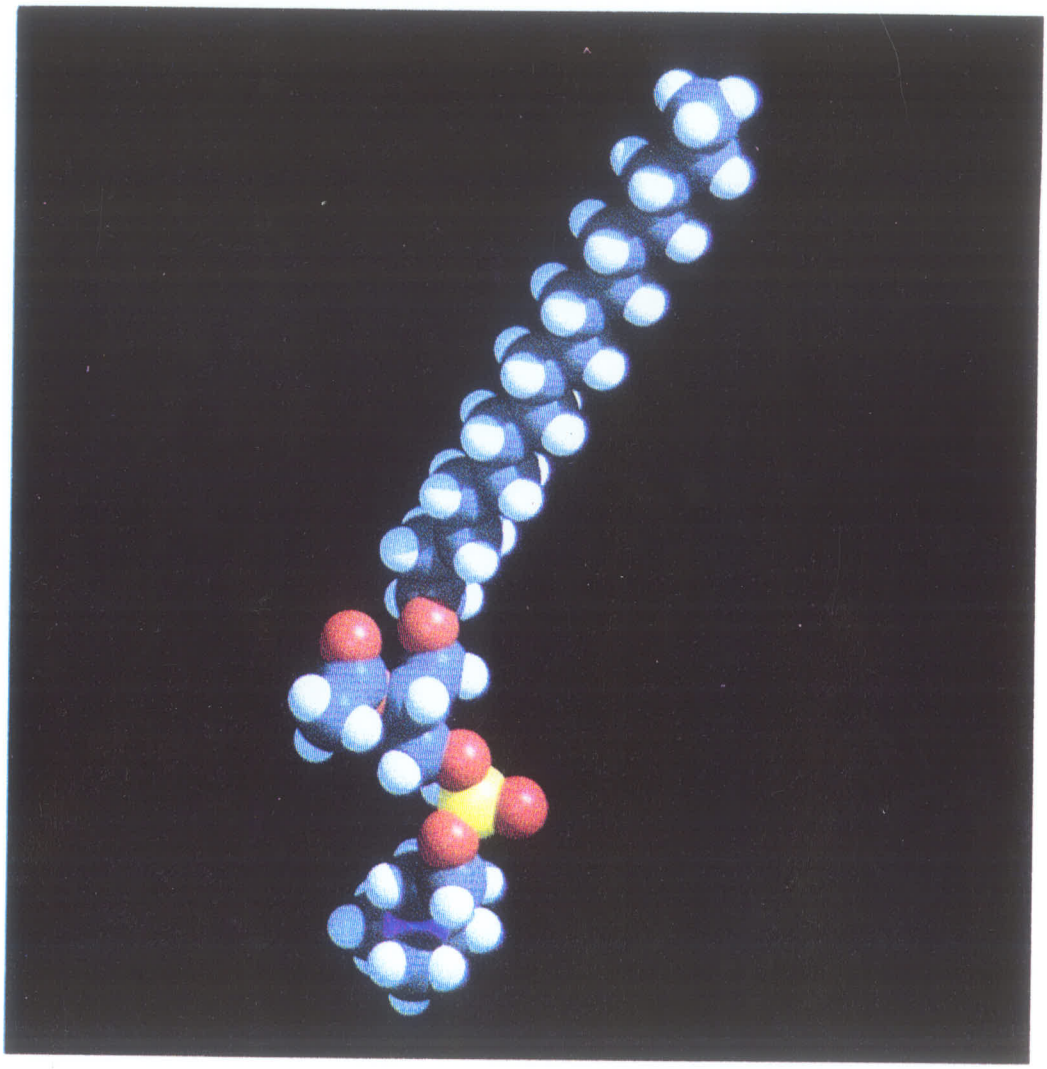


FIG. 1. **Structure of platelet-activating factor.** PAF is a choline phosphoglyceride with several features that are required for optimal biological activity. The *sn*-1 position of glycerol must be in an ether linkage with a fatty alcohol. The phosphocholine head group (*sn*-3) also is needed for the highest potency. Finally, at the *sn*-2 position there is acetate.

plasmalogens (Hanahan *et al.*, 1980). Thus, plasmalogens served as convenient chiral precursors for the preparation of PAF by catalytic reduction of the $C_1$-vinyl ether side-chain and acetylation of $C_2$-hydroxyl group.

## 2.1. Biosynthesis of PAF

PAF synthesis can be demonstrated upon the appropriate stimulation of a diversity of cells, including rat and mouse peritoneal macrophages (Mencia-Huerta *et al.*, 1979), mouse bone marrow-derived mast cells (Mencia-Huerta *et al.*, 1983, Benhamou *et al.*, 1986), rat kidney cells (Pirotzky *et al.*, 1984a), human cultured lymphoid cell lines (Bussolino *et al.*, 1984), endothelial cells (Camussi *et al.*, 1983, Prescott *et al.*, 1984, Bussolino *et al.*, 1986), human and rabbit neutrophils (Lynch *et al.*, 1979), natural killer cells (Malavasi *et al.*, 1986), and platelets (Chignard *et al.*, 1979).

A number of studies have demonstrated that lyso-PAF (1-alkyl-GPC) is a possible precursor of PAF. The synthesis of PAF upon cell stimulation was generally believed to involve a deacylation of the precursor molecule 1-alkyl-2-acyl-GPC by a phospholipase $A_2$ ($PLA_2$) and a subsequent acetylation of the lyso-PAF at the $C_2$ position to the active PAF molecule by a specific acetyltransferase (Snyder, 1985). The activation of phospholipase $A_2$ is calcium dependent and, generally, agonists which stimulate calcium mobilization induce the formation and release of PAF. Contrary to some mediators, PAF is not stored in the cells but is present in the form of the inactive precursor 1-alkyl-2-acyl-GPC as part of the membrane structure. Upon cell stimulation, phospholipase $A_2$ cleaves phosphlipids at the $C_2$ position leading to the release of fatty acids and the concomitant formation of lysophospholipid derivatives.

Acetyltransferase is the limiting step for the formation of PAF. Most PAF metabolizing enzymes (phospholipase $A_2$, acetyltransferase and acyltransferase) are located in the membranes except acetylhydrolase which is located in the cytosol (Braquet *et al.*, 1987).

PAF can also be synthesized through an alternative pathway by which a phosphocholine group is transferred into 1-*O*-alkyl-2-*sn*-acetyl-glycerol by the CDP-choline cholinephosphotransferase. This pathway has been characterized in numerous tissues, such as in rat spleen, human neutrophils and rabbit platelets. This reaction is catalyzed by a specific CDP-choline cholinephosphotransferase different from that involved in the conversion of diacylglycerol into phosphatidylcholine (Rennoij and Snyder, 1981). The formation of the substrate alkylacetylglycerol is the limiting factor of this pathway. The cholinephosphotransferase activity appears to be independent of cell stimulation and may occur in the absence of calcium (Satouchi *et al.*, 1984). The physiological significance of this pathway remains unclear.

## 2.2. Metabolism of PAF

Inactivation and conversion of PAF into its precursor by a deacylation-reacylation reaction is catalyzed by acetylhydrolase and acyltransferase. The degradation of PAF is ensured by acetylhydrolase, a highly active enzyme which converts PAF into lyso-PAF by removing the acetyl group from the $C_2$ position (Blank *et al.*, 1982, Alam *et al.*, 1983 and Braquet and Godfroid, 1987). This enzyme is present in the intracellular and extracellular compartments. Its intracellular form is found in the cytosolic fraction of various cells and tissues, whereas the extracellular form is recoverable from plasma. The properties of the plasma enzyme are similar to those of the cytosolic enzyme except that the former is resistant to the action of

proteases. In contrast to phospholipase $A_2$, acetylhydrolase cleaves only the short chain fatty acids esterified at the $C_2$ position of phospholipids and is calcium independent.

Whatever its route of formation, lyso-PAF like other lysophospholipids is cytotoxic and possesses detergent-like properties (Weltzien, 1979). Its elimination is achieved by an acylation system which introduces a long chain fatty acid into the $C_2$ position of lyso-PAF and the resultant 1-alkyl-2-acyl-GPC then becomes an integral part of the membrane (Touqui *et al.*, 1985). With exogenous lyso-PAF, only a small amount is converted to PAF. This suggests that acyltransferase has a higher affinity for lyso-PAF and/or a greater rate of reaction than acetyltransferase.

The metabolism of PAF *in vivo* has been studied by a number of investigators. Blank *et al.* (1981a) described the metabolism of intravenously injected [$^3$H] labeled PAF in rats. The PAF was initially cleared from the circulation rapidly ($t_{1/2}$ 30 seconds) and after 10 minutes only 10-20% of the radioactivity was present in the plasma compartment. The radioactivity rapidly accumulated in the lung, liver, spleen and kidney. Ten minutes after the injection, 46-72% of the radioactivity in these tissues was still associated with PAF itself, 11-21% with lyso-PAF and 12-27% with 1-alkyl-2-acyl-GPC. Lartigue-Mattei *et al.* (1984) confirmed the rapid clearance of radiolabeled PAF administered in rabbits. However, these authors also demonstrated rapid plasma protein binding (>97% of bound). They also found that PAF and lyso-PAF equilibrated in plasma (10% PAF, 90% lyso-PAF) between 30 minutes and 6 hours after injection.

## 2.3. Relationship between PAF synthesis and arachidonate metabolism

Free arachidonic acid is the substrate for the synthesis of prostaglandins, leukotrienes and thromboxane $A_2$. Recent reports also indicate that arachidonic acid may represent one of the major fatty acids incorporated into 1-alkyl-2-acyl-GPC during the deacylation-reacylation cycle of biosynthesis and inactivation of PAF (Chilton *et al.*, 1983, Kramer *et al.*, 1984, Malone *et al.*, 1985 and Robinson *et al.*, 1985). In addition, it has been suggested that 1-alkyl-2-acyl-GPC is both the precursor of PAF and an important source of arachidonic acid. The arachidonic acid can be made available by a phospholipase C-catalyzed conversion of phosphatidylinositol (Berridge, 1984) and hydrolysis of the resulting diacylglycerol by a diacylglycerol lipase. However, arachidonic acid can also be released by a direct phospholipase $A_2$-catalyzed hydrolysis of the 2-acyl group of phospholipids. This raises the possibility that upon cell activation the PAF precursor molecule, 1-alkyl-2-acyl-GPC, also provides arachidonic acid.

The above hypothesis has been proposed by many authors (Alam *et al.*, 1983, Chignard *et al.*, 1984, Chilton *et al.*, 1983b, 1984, Swendsen *et al.*, 1983, Sugiura *et al.*, 1983a, Alonso *et al.*, 1986 and Leslie and Detty, 1986) and is based upon three main experimental findings. Firstly, several cell types show a large enrichment of arachidonic acid in 1-alkyl-2-acyl-GPC. For instance, in rabbit alveolar macrophages 39% of the 2-acyl chains of 1-alkenyl-2-acyl-GPC are arachidonic acid, whereas only 17% and 80% of the 2-acyl chains of 1-alkenyl-2-acyl-GPC and diacylglycerol, respectively, carry this polyunsaturated fatty acid. The 1-alkyl-2-arachidonyl-GPC represents 29% of the total arachidonic acid associated with phosphatidylcholine plus phosphatidylethanolamine in this cell type (Sugiura *et al.*, 1983a). In rabbit and human platelets (Mueller *et al.*, 1983) the 1-alkyl-2-acyl-GPC is similarly enriched in

arachidonic acid, as 21% and 44% of the 2-acyl chains of 1-alkyl-GPC are arachidonic acid, respectively. In rat alveolar macrophages 35% of the phosphatidylcholine is 1-alkyl-2-acyl-GPC and 30% of the acyl chains of this compound are arachidonic acid, whereas diacyl-GPC contains only 12% arachidonic acid in the sn-2 position (Albert and Snyder, 1983b). Secondly, several cells have been reported to rapidly metabolize exogenously added PAF or lyso-PAF to 1-alkyl-2-acyl-GPC containing arachidonic acid in the $C_2$-position and to rapidly incorporate exogenously added, radiolabeled arachidonic acid into the acyl group of 1-alkyl-2-acyl-GPC. In rabbit platelets after 10 minute of incubation, 85% of the 2-acyl chains of the 1-alkyl-2-acyl-GPC derived from exogenously added PAF consist of arachidonic acid (Homma *et al.*, 1987). In rabbit and human neutrophils, following 20 minute of incubation with PAF or lyso-PAF, 15% and 75-80% of the 2-acyl chains consist of arachidonic acid, respectively (Chilton *et al.*, 1983a,b). A similar preferential incorporation of arachidonic acid into lyso-PAF has been demonstrated in human platelets (Kramer *et al.*, 1984), rat alveolar macrophages (Robinsion *et al.*, 1985) and rabbit peritoneal neutrophils (Swendsen *et al.*, 1986). The endogenous arachidonic acid mainly originates from phosphatidylcholine by a CoA-independent transacyclase reaction (McKean *et al.*, 1982, Collard *et al.*, 1984). Thirdly, cells radiolabeled with 1-alkyl-2-($^3$H)-arachidonyl-GPC subsequently release the label upon stimulation. This has been shown in rat and guinea pig alveolar macrophages (Albert and Snyder, 1984, Bachelet *et al.*, 1986), rabbit neutrophils (Swendsen *et al.*, 1983) and human monocyte-derived macrophages (Leslie and Detty, 1986).

The concept of a common precursor for PAF synthesis and arachidonic acid metabolism is attractive and is substantiated by subcellular localization of the enzymes in human blood platelets. McKean *et al.* (1986) demonstrated a 4-fold enrichment of an acyltransferase that preferentially incorporates arachidonic acid and linoleic acid into

lyso-PAF in the intracellular membranes of these cells. This intracellular membrane fraction also contains a higher proportion of choline-containing phosphoglycerides than the surface membrane (Lagarde *et al.*, 1982) and is enriched in 1-alkyl-2-acyl-GPC (McKean *et al.*, 1986) and a phospholipase $A_2$ that preferentially hydrolyzes 2-arachidonyl-phosphatidylcholine (Lagarde *et al.*,1982). However, the intracellular platelet membrane is also enriched in cyclo-oxygenase and thromboxane synthetase (Carey *et al.*, 1982). Lipoxygenase is mainly localized in the intracellular platelet membrane (Lagarde *et al.*, 1984). Thus, several enzymes involved in PAF and arachidonic acid metabolism appear to be localized in the same cellular compartment. However, further studies are required to establish the role of the PAF precursor in arachidonic acid metabolism.

2.4. Cell sources of PAF

*a*. Platelets. Chignard *et al.* (1979 and 1980) demonstrated that PAF is released from platelets stimulated with the ionophore A 23187, thrombin or collagen and suggested that PAF mediates the "third pathway of aggregation", since neither its production nor its effects are blocked by aspirin or by adenosyl diphosphate (ADP) scavengers.

*b*. Leukocytes. PAF and lyso-PAF are released from stimulated animal or human polymorphonuclear leukocytes. Monocytes, macrophages, mast cells and eosinophils also release PAF upon stimulation with the $Ca^{2+}$ ionophore A 23187, Zymosan particles or IgE.

*c*. Endothelial cells. Stimulated with thrombin or with an anti-factor VII antiserum, human endothelial cells in culture release PAF (Camussi *et al.*, 1987).

Interleukin-1 also induces the release of prostacyclin and PAF from human endothelial cell cultures.

3. Structure-activity relationship

Numerous studies with PAF analogs have been done to establish the relationship between specific structural features of the molecule and its various biological activities. Several systems have been used to test the relative potencies of PAF analogs. Such systems include platelet aggregation and secretion, neutrophil degranulation, bronchoconstriction and induction of hypotension. However, the relative potencies obviously do not have to be the same in each test system.

PAF analogs that have been studied extensively include stereoisomers, variations of the 1-*O*-alkyl, 2-acetyl, 3-phosphoryl and 3-choline groups, as well as the glyceryl backbone and positional isomers. Each feature of the structure of PAF is important for optimal biological activity and the responses are stereospecific. Natural PAF has the R conformation at $C_2$. The S isomer has 1/600 the activity in rabbit platelet aggregation (Heymans *et al.*, 1981a,b), 1/4,000 the activity in rabbit platelet secretion (Blank *et al.*, 1982) and 1/2,000 the activity in rabbit platelet desensitization studies (Lalau-Keraly *et al.*, 1983). Thus a stereo-specific receptor is presumed to be involved in the PAF-induced stimulation of cells. The length of the 1-*O*-alkyl chain is also of critical importance, i.e., the $C_{14}$ to $C_{18}$ chains have optimal biological activity (Braquet and Godfroid, 1986). Satouchi *et al.* (1981a) demonstrated a 3 to 6 fold higher activity of the 1-*O*-hexadecyl relative to the 1-*O*-octadecyl (the natural presented form of PAF) compounds in rabbit platelet secretion. It was also noted in the original reports on PAF structure that 2-lyso-PAF has negligible, if any, biological activity (Blank *et al.*, 1979, Demopoulos *et al.*, 1979). Substitution of the 2-acetyl group by

other groups results in a significant reduction of activity. Satouchi *et al.* (1981a) investigated the effect of modification of the polar head group at the 3-position of the PAF molecule on rabbit platelet aggregation and secretion. Reduced potencies relative to PAF of 2.5, 20, 2200, 3800 and 4600-fold were obtained for phosphoryl-dimethylethanolamine, -monomethylethanolamine, -ethanolamine, ethanol and phosphoric acid in the 3-position, respectively. Increasing the chain length between the choline and phosphate groups by insertion of methylene group also results in a gradual loss of potency to induce rabbit platelet aggregation and secretion (Tokumura *et al.*, 1985) or hypotension (Wissner *et al.*, 1986). The chain length of the glyceryl backbone of the PAF molecule is also important. For instance, insertion of a methylene group between $C_1$ and $C_2$, or between $C_2$ and $C_3$, as well as the addition of methyl groups to $C_1$ and $C_2$, results in a reduced capacity to induce rabbit platelet aggregation and hypotension (Wissner *et al.*, 1985). It can be concluded that the modification of any part of the PAF molecule generally results in a severely decreased biological activity.

## 4. Pathophysiological and biological effects of PAF

### 4.1. Systemic effects of PAF

Systemic administration of PAF by intravenous injection to different animal species is followed by marked hypotension, pulmonary hypertension, increased resistance of airways (i.e., bronchoconstriction), increased vascular permeability, thrombocytopenia, neutropenia and death (Braquet *et al.*, 1987). Even though PAF does not activate rat platelets *in vitro*, *in vivo* thrombocytopenia accompanies hypotension, increased vascular permeability and a paradoxical leukocytosis is observed (Martins *et al.*, 1987).

Systemic administration of PAF has direct and indirect effects. In both rabbits and guinea pigs, systemic administration of PAF induces lung injury and it is believed to be caused by the immediate and delayed effects of PAF. When PAF is infused into the guinea pig, platelet and leukocyte counts drop approximately at the same time, the latter more intensively than the former. Depending on the rate of infusion of PAF and whether or not occuring hypertension following hypotension, bronchoconstriction may be absent possibly because the reduced bronchoconstriction is unaccompanied by the compensatory hypertension which usually is due to the catecholamines secreted as a reaction against asphyxia. The interactions between PAF and the various protease systems involved in inflammation and shock (kallikrein, complement, fibrinolysis, coagulation) have been studied. Emeis and Kluft (1985) have demonstrated that PAF (as well as other mediators) induces the secretion of tissue-type plasminogen from rat vessel wall, which was suppressed by phospholipase and lipoxygenase inhibitors under conditions in which cyclooxygenase inhibitors were inactive. Recent studies have shown that, after injection of PAF, plasma protease activity is significantly increased during the first 20 min following the challenge.

The effects of PAF are markedly dependent upon the animal species and the administration route (Vargaftig *et al.*, 1983). For instance, aerosolized PAF administered to guinea pigs produces bronchoconstriction, which is markedly tachyphylactic and can be blocked by aspirin and develops slowly. The accompanying hypotension is aspirin resistant and may be due to PAF leakage to the periphery. Bonnet *et al.* (1983) demonstrated that, under specific conditions, aspirin may reduce bronchoconstriction caused by intravenous administration of PAF, possibly by blocking arachidonic acid metabolism. A lipoxygenase dependence of the PAF effect may operate in mice, since both the leukotriene antagonist compound FPL 55712 as well as

lipoxygenase inhibitors protected against PAF-induced death. The mechanism of the systemic effects of PAF was studied mostly in guinea pigs and rabbits and has not been fully elucidated. Bronchoconstriction induced by PAF injected i.v. at 20 to 60 ng/kg to guinea pigs is accompanied by hypotension, thrombocytopenia, leukopenia (predominantly neutropenia) and increased vascular permeability. Bronchoconstriction depends on platelet participation, most probably upon secretion of mediators which are cyclooxygenase independent (Vargaftig *et al.*, 1980), whereas vascular permeability (systemic extravasation observed as increased hematocrit) is at least partially mediated by leukocytes (Handley *et al.*, 1984). Furthermore, PAF increases vascular permeability and induces systemic hypotension in rats, even though their platelets are unresponsive to PAF. In the guinea pig, the combined intradermal injection of PAF and $PGE_1$ enhances plasma protein extravasation, but prostaglandins reduce the number of platelets accumulating *in situ*. Platelets are directly involved with PAF-induced bronchoconstriction upon i.v. administration to guinea pigs. Given to rabbits, PAF induces hypotension, thrombocytopenia, neutropenia, bradycardia and release of platelet factor 4 and thromboxane. Immediately after its injection to rabbits, platelets and neutrophils aggregate throughout the pulmonary microvessels and small muscular arteries and bronchioles contract.

## 4.2. Cardiac and vascular effects of PAF

The cardiovascular effects of PAF have been extensively studied in recent years. Hypotension, decreased coronary flow and decreased cardiac contractility are the hallmarks of cardiovascular effects of PAF. PAF has been shown to produce strong and long-lasting hypotension in various animal species, e.g. normotensive and spontaneously hypertensive rats, rabbits, guinea pigs and dogs (Tanaka *et al.*, 1983). This action of PAF was thought to be endothelium-dependent (Kamitani *et al.*, 1984,

Kasuya *et al.*, 1984, Shigenobu *et al.*, 1985, 1987). In one study, Kamata *et al.* (1989) found that relatively low concentrations of PAF ($10^{-9}$-$10^{-7}$ M) produced endothelium-dependent relaxation of the rat aorta in the presence of bovine serum albumin. It is generally accepted that the aorta offers little resistance to flow. It is obvious that the contribution of vessels of smaller diameter to peripheral vascular resistance is much greater. Extending the effect of PAF in aorta to that in small vessels, they concluded that this vasodilator action of PAF at low concentrations might be the mechanism of its hypotensive action *in vivo*. The vasorelaxant action of PAF was also examined in perfused mesenteric vascular beds and mesenteric artery strips isolated from rats (Chiba *et al.*, 1990). PAF at about $3X10^{-12}$ M caused a dose-dependent vasodilation of norepinephrine-contracted mesenteric vascular bed and phenylephrine-contracted mesenteric artery strips, and higher concentrations of PAF were required to relax the aorta and pulmonary arteries. PAF-induced relaxation of mesenteric artery was dependent on the presence of endothelium and were inhibited either by hydroquinone or methylene blue, which inhibit the action of endothelium-derived relaxing factor (EDRF) or by L-canavanine, which inhibits the formation of nitric oxide from L-arginine. Phospholipase $A_2$ inhibitors such as quinacrine and ONO-RS-082 abolished the relaxation induced by acetylcholine but did not affect that induced by PAF. Thus, PAF induces a vasorelaxation by releasing EDRF from endothelial cells, as does acetylcholine, although the pathway to produce the substances by PAF may be different from that by acetylcholine (Chiba *et al.*, 1990). The relatively slow development of maximal systemic blood pressure reduction may also be due to a gradual decline in cardiac output and loss of plasma volume. Evidence for PAF-induced loss of intravascular fluid in the baboon has been presented by McManus *et al.* (1981). The observations by Handley *et al.* (1987) have indicated that injected [$^3$H]PAF disappeared initially fairly rapidly from the primate circulation, with a $t_{1/2}$ of

30-40 seconds. This seems to suggest that the continued presence of PAF in the blood is not required for the development of peak hemodynamic changes.

PAF induces a dysfunction resembling that observed in cardiac anaphylaxis. Levi *et al.* (1984) reported that PAF was released by antigen-challenged hearts and was a mediator of contractile failure, coronary constriction and arrhythmias which characterized cardiac anaphylaxis. A number of investigators have studied the cardiovascular effects of PAF in the coronary circulation. However, the results of these studies were divergent. PAF was shown to cause vasoconstriction in isolated perfused guinea pig heart (Levi *et al.*, 1984, Piper and Stewart, 1987) and in the perfused rat heart (Piper and Stewart, 1986). Sybertz *et al.*, (1985) observed that intracoronary injection of PAF in anesthetized dog decreased coronary flow. In contrast, Jackson *et al.*, (1986) reported that intracoronary injection of PAF in the anesthetized dog produced only increases in coronary flow and this effect was reduced after the removal of circulating platelets. Other studies reported a biphasic effect, i.e. an increase in flow followed by a decrease in flow after intracoronary injection of PAF (Feuerstein *et al.*, 1984, Mehta *et al.*, 1986, Erza *et al.*, 1987 and Fieldler *et al.*, 1987). One possible explanation that may account for some of the diverse cardiovascular effects of PAF in the heart is that the vasodilator response may require interactions in the intact animal and is therefore not observed in the isolated perfused heart. Coronary vasoconstriction induced by PAF and antigen in isolated perfused hearts was ascribed to the PAF-induced release of leukotriene $C_4$ ($LTC_4$), thromboxane $A_2$ ($TxA_2$) and prostaglandins. Piper and Stewart (1986) believed that the cardiovascular effects of PAF are mediated by other vasoactive substances, such as, prostaglandins and leukotrienes.

In a study of the negative inotropic effect of PAF on non-coronary perfused human right atrial pectinate muscles paced at constant rate, Robertson *et al.*, (1987) found that the potent negative inotropic effect of PAF on human myocardium was independent of coronary flow changes and was not modified by atropine, indomethacin and leukotriene receptor antagonists but was abolished by PAF receptor antagonists. They concluded that the negative inotropic effect of PAF was mediated by specific receptors and involved neither cholinergic mechanisms nor arachidonate metabolites. Studies on the isolated guinea pig and human papillary muscles have shown that PAF exerted a direct negative inotropic effect on the cardiac muscle coupled with a reduction in the action potential duration (Alloatti *et al.*, 1986). In the presence of platelets, the electrical and mechanical alterations were significantly worsened and PAF induced a biphasic effect characterized by a transient positive inotropism followed by a dose-dependent decrease in coronary flow, negative inotropism, reduction of action potential duration and conduction arrhythmias in ischemic-reperfused rabbit heart (Montrucchio *et al.*, 1989). The effects of PAF on $Na^+$-dependent calcium uptake in myocardial sarcolemmal vesicles were examined in order to clarify its mechanism of inotropic action on the heart. PAF significantly inhibited $Na^+$-$Ca^{2+}$ exchange. The passive permeability of sarcolemmal vesicles to $Ca^{2+}$ and the passive $Ca^{2+}$ binding to the cardiac sarcolemma were markedly elevated after PAF treatment (Meng *et al.*, 1990). As the concentration of PAF they used was very high (20-40 $\mu$M), the role of $Na^+$-$Ca^{2+}$ exchange in the negative inotropic effect of PAF on the myocardium is questionable.

PAF has been found to induce myocardial dysfunction, coronary vasoconstriction and arrhythmias in the post-infarcted rabbit isolated heart (Mickelson *et al.*, 1988). Recently, PAF was detected in the effluent blood from the coronary sinus in patients with coronary artery disease undergoing atrial pacing (Montrucchio *et*

*al.*, 1986) and was found to be released in significant amounts during the initial reperfusion of the ischemic-reperfused rabbit heart (Montrucchio *et al.*, 1989). There is also some direct evidence for the release of PAF from the ischemic myocardium of baboons (Annable *et al.*, 1985). The extracardiac PAF release is closely associated with ischemic symptoms in patients with coronary artery disease. The role of PAF in acute myocardial ischemia was studied by the ligation of the left main coronary artery in anesthetized rats (Stahl *et al.*, 1988). PAF significantly increased coronary perfusion pressure and induced an increase in cardiac permeability when fluorescein isothiocyanate bovine albumin was used as a marker. These data indicate that PAF is an important mediator of ischemic damage in rat acute myocardial ischemia. Moreover, the extension of ischemic damage may be enhanced by the increase in cardiac permeability induced by PAF. Therefore, PAF may be an important causative agent of ischemic cardiac dysfunctions.

The PAF-induced myocardial dysfunction, coronary vasoconstriction and arrhythmias can be inhibited by PAF receptor antagonists, such as, BN-52021, CV 6209, kadsurenone. PAF antagonists have been used to prevent the effects of PAF in a number of experimental conditions.

In isolated perfused rat lungs, PAF induced pulmonary vasoconstriction, which was accompanied by release of leukotrienes, was inhibited by the putative lipoxygenase inhibitor, diethycarbamazine (Voelkel *et al.*, 1982). Furthermore, PAF elicited the formation of leukotrienes from cat chopped pulmonary and vascular tissues and from rat and guinea pig chopped lung tissues (Lefer *et al.*, 1984). When administered via intravenous infusion to rabbits, PAF induced acute transient pulmonary hypertension (Ohar *et al.*, 1990). Chronic intravenous PAF infusion into rabbits for 4 weeks induced physiologic and morphologic changes and pulmonary hypertension

characterized by increased pulmonary arterial pressure and total pulmonary resistance and by enlargement of the right ventricle, shortening of the internal elastic lamina, intimal thickening and a decreased number of small pulmonary arteries. Systemic PAF infusion was associated with a profound reduction of renal blood flow and glomerular filtration. PAF also exerted a direct effect on kidney functions, since indomethacin protected against the reduction in systemic blood pressure but did not antagonize the fall in renal plasma flow and in glomerular filtration. The mechanisms of the different effects of PAF on systemic and coronary, pulmonary and renal microcirculation were not completely clear but a local role for vasoconstrictors, such as leukotrienes, prostaglandins and angiotension II, was suggested.

### 4.3. Effects of PAF on other systems

PAF may be involved with a variety of pathophysiological conditions, including arterial thrombosis, acute inflammation, acute allergic diseases, endotoxic shock, and transplant rejection. PAF has also been shown to exert physiological effects in the early pregnancy and to exert a beneficial effect in cancer chemotherapy.

#### 4.3.1. The role of PAF in thrombosis

Platelet activation by PAF is not inhibited by ADP-scavenging agents nor by inhibitors of cyclooxygenase. Accordingly, a so-called "third pathway of platelet aggregation" was postulated. This pathway is independent from the cyclooxygenase metabolites and granular ADP (Kinlough-Rathbone *et al.*, 1977). Chignard *et al.* (1979, 1980) found that PAF is secreted into the supernatants by rabbit platelets stimulated with the $Ca^{2+}$ ionophore A23187, thrombin, or collagen. The secreted PAF is suggested to be the actual mediator of platelet activation. PAF is also released by human platelets stimulated with a calcium ionophore or with thrombin (Chignard *et*

*al.*, 1983), particularly when its degradation and subsequent incorporation into the platelet membranes as an acylated derivative are prevented by an acetylhydrolase inhibitor (Touqui *et al.*, 1985). R (but not S) PAF stimulated guanosine triphosphatase (GTPase) activity in platelet membranes in a dose dependent manner. This stimulation is inhibited by BN 52021 and by related analogs and kadsurenone. PAF also stimulates inositol metabolism in platelets and may operate through a guanine nucleotide regulatory protein, distinct from the stimulatory (Ns) or inhibitory (Ni) guanine nucleotide regulatory protein, which is stimulated by cholera or pertussis toxins, respectively (Houslay *et al.*, 1986 and Hwang *et al.*, 1986). In contrast, in human neutrophils, the effects of PAF are inhibited by pertussis toxin, suggesting an action on the Ni subunit. *In vitro* study, the activation of receptor leads to enzyme secretion and formation of superoxide and a platelet thrombus, which was invaded and surrounded by leukocytes and spreaded over the adjoining vacuolized endothelium (Bourgain *et al.*, 1985). PAF also alters the molecular organization of cytoskeletal proteins which controls endothelial permeability. Human endothelial cells stimulated by PAF retract and lose reciprocal contact, while stress fibers disappear or become less regular. Such impairments lead to blob formation. PAF and epinephrine induce aggregation of human platelets in a synergistic manner (Vargaftig *et al.*, 1982). This may be relevant to the interactions of epinephrine and PAF, released at the same time under different conditions such as stroke and different forms of shock. PAF antagonists suppress aggregation induced by the combination of PAF and epinephrine.

### 4.3.2. Acute inflammation

PAF increases vascular permeability (Pirotzky *et al.*, 1985). This effect is accompanied by platelet accumulation, but does not appear to be platelet mediated in the guinea pig, rabbit or rat, in which the role of neutrophils was also ruled out. The

intradermal injection of PAF in the rat is followed by increased vascular permeability, edema, vascular lesions and thrombi. An important finding is the presence of platelet thrombi, even though rat platelets are refractory to the direct effect of PAF *in vivo* and *in vitro*. Systemic PAF induces hemoconcentration, i.e., a generalized increase in vascular permeability, probably due to a direct or indirect (cell-mediated) effect on the endothelium (Handley *et al.*, 1984). PAF induces rat paw edema which is antagonized by dexamethasone but not by indomethacin or piroxicam (Cordeiro *et al.*, 1987). PAF antagonists inhibit PAF-induced edema. Endogenous production of PAF in close proximity to microvascular endothelial cells appears to be an important step. The intradermal injection of PAF in humans is said to induce a biphasic inflammatory response, with acute and late-onset components, reminiscent of the response to moderate doses of allergen in sensitized individuals (Archer *et al.*, 1984). Endothelial swelling and a perivascular infiltrate of mononuclear cells and neutrophils are accompanied by early intravascular accumulation of neutrophils and late (24 h) appearance of lymphocytes and histiocytes.

### 4.3.3. Asthma and systemic anaphylaxis

Asthma is characterized by a variable and reversible airflow obstruction and by bronchial hyper-responsiveness, an excessive airway narrowing in response to a variety of apparently unrelated stimuli. There is now abundant experimental evidence that inflammation of the airways may lead to bronchial hyper-responsiveness, which is a characteristic feature of asthma. The involvement of PAF with asthma is suggested by its ability to induce bronchoconstriction, lung inflammation and hyper-responsiveness. Another possible involvement of PAF with asthma concerns its ability to potentiate bronchoconstriction by other agents (Morley *et al.*, 1984). PAF induced bronchial hyper-reactivity is similar to antigen-induced changes in airway reactivity observed in

asthmatics. Animals made hyper-reactive to exogenous spasmogens by prior treatment with PAF also showed a reduction in sensitivity to ß-adrenergic agents. Hyper-reactivity may result from a nonspasmogenic effect of PAF involving edema, platelet and eosinophilic infiltration and epithelial damage (Lellouch-Tubiana *et al.*, 1985). PAF may be a potent amplifier of eosinophil response, since it is highly chemotactic and induces the release of cationic proteins, such as the major basic protein, which in turn, increase bronchial hyper-reactivity. Finally, PAF increases mucus output and alters its physical properties. Whether these changes are a direct effect of PAF or are secondary to the release of cytotoxic substances, such as the major basic protein and to infiltrating eosinophils is not yet known.

4.3.4. Endotoxic and allergic shock

Shock is characterized by systemic hypotension, pulmonary hypertension, endothelial dysjunction (stretched pore phenomenon) and stimulation of different plasma systems (kallikrein, fibrinolysis and clotting) and circulating cells (erythrocyte sludge, stimulation of leukocytes and platelets). It is difficult to envision that this very complex event can be accounted for by a single primary mediator. There are many analogies between endotoxic shock and the effects of systemic PAF in the dog and rat (Bessin *et al.*, 1983). It has been demonstrated that the PAF antagonists improved survival and prevented or corrected endotoxic shock triggered by i.p. injections of endotoxin in the rat which was accompanied by the appearance of PAF in the peritoneal and spleen cells of the intoxicated animals. PAF antagonists prevented PAF and endotoxin-induced hypotension in the rat to a similar extent but animals made tolerant to endotoxin still respond to subsequent PAF injections. The involvement of PAF in endotoxemia has been recently confirmed by using isolated guinea pig lung parenchymal strips from both normal and endotoxin-treated animals. In the latter, a

significant desensitization to PAF in comparison to the control group was seen. Endotoxic shock is a very complex event and there are notable species differences among various experimental models. Since PAF may have different receptors, on the same or on different cell types, it is not surprising that a single autocoid antagonist should fail to prevent all of its effects.

4.3.5. Transplant rejection

PAF may be involved in renal transplant rejection since it is released during kidney hyperacute allograft rejection (Ito *et al.*, 1984). This phenomenon is a humoral-mediated event caused by preformed antibodies involving complement activation and intravascular platelet aggregation. However, this type of reaction is rarely seen in patients receiving organ transplants, as most patients experience acute cell-mediated rejection (Hall *et al.*, 1984). Lymphocyte and monocyte infiltration and increasing numbers of platelets adhered to the graft vascular endothelial cells are readily observed. In the irreversible rejection, platelet/endothelial cell aggregates are very prominent. The involvement of platelets in transplant rejection provided the rational for trying PAF antagonists in organ transplantation. Treatment of rat cardiac allograft recipients with PAF antagonist, BN-52021 alone or in combination with either azathioprine or cyclosporine significantly delayed graft rejection. The combination of azathioprine and PAF antagonist was more effective in prolonging graft survival than the conventional immunosuppressive combination of azathioprine and prednisolone.

4.3.6. Kidney diseases and renal immune disorders

PAF induced the release of thromboxane and prostaglandins from primary cultures of human (Ardaillou *et al.*, 1985) or rat (Schlondorff *et al.*, 1986) glomerular

mesangial cells. This effect was associated with a shape change of mesangial cells which was similar to that observed with angiotensin II and was consistent with contraction of the cells (Ardaillou *et al.*, 1985). PAF also dose dependently stimulated the formation of reactive oxygen species from cultured mesangial cells and caused a decrease in the planar surface of the glomeruli. These effects were inhibited by PAF antagonists. Pirotzky *et al.*, (1984) showed that PAF was released by isolated perfused rat kidneys and glomeruli, as well as by suspensions of medullary cells but not by tubules upon stimulation by the calcium ionophore A 23187 or antigen from immunized rats. Systemic PAF infusion was associated with profound reduction of renal blood flow, glomerular filtration and urinary sodium excretion, possibly due to the fall of systemic blood pressure (Bessin *et al.*, 1984). PAF also exerted a direct effect on kidney functions, since indomethacin protected against the reduction in systemic blood pressure but did not antagonize the fall in renal plasma flow and in glomerular filtration nor urinary sodium excretion (Hebert *et al.*, 1987).

Evidence for PAF involvement in renal immune injury has been provided by the observations that PAF was released during kidney hyperacute allograft rejection (Ito *et al.*, 1984). It has been proposed that PAF participated in glomerular immune complex deposition of experimental serum sickness and in systemic lupus erythematosus (Cammsui *et al.*, 1981). In these experiments, the following changes were observed: (a) accumulation of platelets and neutrophils with aggregation and degranulation in glomerular capillaries; (b) loss of glomerular fixed negative charges due to binding of cationic proteins released from platelets and neutrophils; and (c) transient (hours) and mild proteinuria.

One recent study has shown that PAF pretreatment in anesthetized dogs practically abolished natriuresis and diuresis induced by synthetic atrial natriuretic

factor (Thievant *et al.*, 1987). In contrast, the PAF antagonist BN 52021, which is devoid of effects per se on diuresis and electrolyte excretion, potentiated the effects of atrial natriuretic factor and captopril.

4.3.7. Gastrointestinal system

PAF is the most potent ulcerogen yet described in the rat (Conzalez-Crussi and Hsuen, 1983 and Rosam *et al.*, 1986). This effect is not mediated via platelets or cyclooxygenase products nor via histamine or adrenergic receptors. PAF-induced ulcerations mimic the gastrointestinal impairment obtained after endotoxin administration. The damage to the gastrointestinal tract was characterized by vascular congestion extending throughout the mucosa and sometimes to the submucosa. As with endotoxin, PAF also induces hemorrhagic damage in the stomach, small intestine and in the distal colon. The strongest evidence for a role for PAF in endotoxin-induced gastrointestinal necrosis is that at doses which inhibit PAF-induced gastrointestinal damage, PAF antagonists, BN 52021, CV 3988 and RO 19-3704 inhibited endotoxin-induced gastrointestinal ulcerations. In contrast, PAF does not appear to be involved in gastric hypersecretion, in pylorus-ligated rats, and in aspirin-, phenyl-, or butazone-induced gastric damage, since BN 52021 only afforded a mild or no protection under conditions where ranitidine was effective (Braquet *et al.*, 1987).

4.3.8. Central nervous system

A potential role for PAF in the central nervous system was suggested by the discovery that triazolobenzodiazepines (alprazolam or triazolam) and some benzodiazepines antagonize PAF effects (Page *et al.*, 1983). PAF or PAF-like phospholipids may be involved in cell-to-cell interactions in mature brain. The

antagonism of this process may contribute to the therapeutic effects of triazolobenzodiazepines. In the developing nervous system, PAF may play a role in cell differentiation (Palma-Carlos *et al.*, 1986). One observation supporting this suggestion is the finding of a neuronal dysfunction in individuals with Zellweger syndrome, a genetic disorder in which there is a lack of enzymes needed for the synthesis of ether-phospholipids (Kelly, 1983). This possibility is supported by the fact that the brain contains relatively high levels of enzymes for PAF metabolism. Recent studies of Kornecki *et al.* (1986) demonstrated that the PAF induced growth arrest and morphological differentiation of the cloned neuronal cell line NG 108-15. This effect is concentration and time dependent. Only when the dose of PAF is increased above levels that induce maximal differentiation, does cytotoxicity appear. That PAF is cytotoxic to neural cells raises the possibility that PAF may be one of the factors responsible for the irreversible neuronal degradation associated with spinal cord injury, trauma and stroke. PAF antagonists may enhance neuronal recovery after brain ischemia since in anesthetized dog with multifocal ischemia, kadsurenone enhanced early postischemic recovery. Similar results were recently observed with BN 52021 in a related model of brain ischemia in gerbils.

4.3.9. Immune response

PAF may regulate lymphocyte functions either (a) indirectly since PAF produces leukotriene formation in lungs, which are potent modulators of lymphocyte function (Rila-Pleszczynski *et al.*, 1984) or (b) directly by acting on T-cell and macrophage functions. When PAF ($10^{-12}$ M) was added to adherent rat spleen monocytes stimulated with lipopolysaccharide, it induced a significant increase in interleukin-1 synthesis and release (Pignol *et al.*, 1987). In contrast, at $10^{-7}$ M PAF, a decrease in both interleukin-1 synthesis and release was observed. BN 52021 and

related antagonists significantly reversed these effects. Such PAF-induced interleukin-1 synthesis and release may explain the antitumor activity of 1-*O*-alkyl-lysophospholipids (Berdel, 1986) whose effects may partly be mediated by the generation of highly tumouricidal immune-competent cells from the monocyte-macrophage lineage, which are able to produce a large amount of PAF upon stimulation. A positive feedback between interleukin-1 and PAF may be thus involved in the amplification of the immune response. Several cyclooxygenase metabolites of arachidonic acid, in particular prostaglandins of the E series, can exert a powerful suppressive effect on lymphocyte proliferation. Indeed, while indomethacin has no significant effect *per se*, its concomitant use in the proliferation assay completely prevented the PAF-induced suppression of lymphocyte proliferation, indicating that PAF may exert its effect through some cyclooxygenase metabolites, e.g., $PGE_2$ (Pignol *et al.*, 1987).

4.3.10. Pregnancy and ovoimplantation

PAF may be the first physiological signal produced by the embryo for maternal recognition of pregnancy. It is present in human amniotic fluid and the enzymes involved in PAF metabolism are found in human amnion tissue where PAF is synthesized (Billah *et al.*, 1985). PAF is also present in the rabbit uterus during early pregnancy in concentrations well above those required to initiate cutaneous permeability (Angle *et al.*, 1985). The correlation between the inability of cultured embryos to produce PAF and to result in pregnancy suggests that PAF production is a prerequisite for pregnancy and that it could be used as a marker for embryo viability. In mammals, the success of implantation requires a rapid metabolic burst (blastocyte activation). Embryo-derived PAF may promote platelet activation and subsequent release of factors which stimulate the blastocyte activation and consequent implantation (O'Neill *et al.*, 1985). Indeed, blastocyte activation is promoted by platelet-dependent serum factors.

Much attention has centered on an "early pregnancy factor" (EPF) which can be detected in the serum of mice, sheep and humans within the first 24 hours of fertilization. Recent work suggests that embryo-derived PAF may be this "ovum factor" (O'Neill *et al.*, 1985). Indeed, synthetic PAF induces the expression of EPF within an hour of injection in mature female mice at all stages of the oestrus cycle except for metaoestrus.

### 4.3.11. PAF-related phospholipids in cancer chemotherapy

Phospholipid analogs of PAF, the alkyllysophospholipids, may provide a new approach to cancer chemotherapy (Berdel, 1986). These ether lipids possess an unusually broad range of biological activities including macrophage activation, malignant cell differentiation and direct cytotoxicity, all thought to be membrane mediated. Unlike most antitumour agents, these analogs do not appear to have a direct effect on DNA synthesis or function and are nonmutagenic. The methoxy analog of PAF, one of the most potent alkyllysophospholipids, is strongly associated with surface and intracellular membranes. This product inhibits the uptake of essential nutrients (choline, palmitic acid) by the HL-60 cells. An inverse relationship appears to exist between alkyl ether phospholipids exhibiting PAF activity (hypotensive, inflammatory and allergic reactions) and those possessing selective antitumour properties.

## 5. PAF antagonists

### 5.1. Nonspecific inhibition of PAF

Drugs which interfere with the rise in intracellular calcium also interfere with the cell response to PAF *in vitro* and *in vivo*, These include agents which act directly,

such as calcium channel antagonists, calmodulin inhibitors, calcium chelators and local anaesthetics (Hartung, 1983 and Khan *et al.*, 1985), or indirectly by modulating the level of cyclic nucleotides, e.g., prostaglandin $I_2$ ($PGI_2$) or $PGE_1$ (Bussolino *et al.*, 1980) and $ß_2$-agonists, such as salbutamol (Baranes *et al.*, 1986) for cAMP, the molsidomine metabolite, Sin-1, for cyclic guanosine monophosphate cGMP (Chignard *et al.*, 1985) and phosphodiesterase inhibitors (Vargaftig *et al.*, 1984). A similar nonspecific inhibition was observed with inhibitors of phospholipase (Bourgain *et al.*, 1985) and antagonists of thromboxane and leukotrienes (Chilton *et al.*, 1982). The *in vivo* anaphylactic-like effects of PAF in the guinea pig are not inhibited by anti-$H_1$ or anti-$H_2$ agents, except when they are combined with aspirin, indomethacin, or salicylic acid, which are inactive alone (Vargaftig *et al.*, 1982).

## 5.2. Specific inhibition of PAF.

Specific PAF antagonists are useful tools for defining the biological roles of PAF and conformational properties of PAF receptor sites. They can be conveniently classified into four different groups as follows.

### 5.2.1. Structural analogs of PAF.

*a. Nonconstrained backbone.* In this series, the antagonists are derived directly from the PAF framework. The first compound described in this family was CV 3988 which incorporates an octadecyl carbamate in position 1, a methylether in position 2 and thiazolium ethyl phosphate in position 3. CV 3988 is an orally active and potent antagonist for the specific binding of [$^3$H] PAF to human, rabbit and guinea pig platelets. A weak agonistic activity was found only at high concentrations. A new analog CV 6209 has a N-acetyl carbamyl methyl pyridinium side-chain in place of the

phosphorylcholine group. It is about 80 times more potent than CV 3988 *in vitro* and *in vivo*, but is poorly absorbed orally. (Terashita *et al.*, 1986). Replacement of the phosphoryl ethyl thiazolium moiety of CV 3988 with a heptamethylene thiazolium at $C_3$ yielded another group of antagonists among which Ono 6240 is the most potent (Myiamoto *et al.*, 1985). Ono 6240 inhibits PAF-induced guinea pig platelet aggregation, hypotension and bronchoconstriction in rats and guinea pigs. A similar approach was followed by the group at Hoffmann La Roche, leading to potent antagonists, such as Ro 19-3704, Ro 19-1400, Ro 18-8736 and Ro 18-7953. These antagonists can inhibit PAF-induced aggregation of rabbit, human and guinea pig platelets, as well as bronchoconstriction, hypotension, thrombocytopenia, leukopenia and vascular permeability in the guinea pig.

*b. Constrained backbone.* Moderately active PAF antagonists are produced from cyclization of the PAF framework, such as the Sandoz piperidine-derived SRI 63-073 (Lee *et al.*, 1985). SRI 63-073 synthesis was designed by combining thiamine phosphate (which displays a modest PAF-inhibitory effect *in vivo*) with a modified PAF framework. The potency of these compounds is relatively low.

*c. Tetrahydrofuran derivatives.* Sandoz has also synthesized a series of tetrahydrofuran-related PAF antagonists related to the PAF framework (Jaejji *et al.*, 1984). The most potent, SRI 63-441 (Alonso *et al.*, 1982), is a specific inhibitor of PAF-induced human platelet aggregation and protectes rats from the development of PAF-induced hemorrhagic lesions in the gastric mucosa. It also attenuated endotoxin-induced lung injury in rats and improved coronary flow during cardiac anaphylaxis. The two critical requirements to be achieved in this series are: (a) good oral absorption and (b) lack of partial agonism. Furthermore, the effects of these antagonists on enzymes of PAF metabolism (acetylhydrolase, acyltransferase) need clarification.

5.2.2. Natural products.

The most promising chemical series of PAF inhibitors include natural compounds isolated from Chinese or Brazilian plants (terpenes and lignans) and from various bacterial strains (gliotoxins).

*a. Terpenes.* A family of potent PAF antagonists is formed by *ginkgolides A, B, C, M* and *J* (respectively, BN 52020, BN 52021, BN 52022, BN 52023 and BN 52024) (Anderson *et al.*, 1983) and terpenes isolated from the Chinese tree *Ginkgo biloba L* (Braquet, 1984 and Braquet *et al.*, 1985). Of these, BN 52021 is the most effective. BN 52021 and related antagonists inhibit PAF binding to rabbit and human washed platelets and human leukocytes. BN 52021 displaced [$^3$H]PAF from its receptor in a manner similarly to nonlabeled PAF in both human and rabbit platelets. The inhibitory activity is not removed by washing platelets suggesting the binding is irreversible. BN 52021 is also an effective antiaggregating agent. This effect is specific to PAF, since no antagonism of other aggregating agents (ADP, collagen, arachidonic acid, thrombin and A23187) was noted and since BN 52021 does not inhibit arachidonic acid metabolism nor the binding of a large variety of mediators (thromboxane A, leukotriene C4 and others) (Braquet *et al.*, 1985). Finally, a significant decrease in PAF-induced $Ca^{2+}{}_i$ measured by the fluorescent probe Quin-2 was recorded in rabbit platelets treated by BN 52021 which inhibits PAF binding to its receptor and the subsequent cellular response mediated by activation of the phosphatidyl inositol cycle and $Ca^{2+}$ mobilization via a guanine nucleotide regulatory protein (Avdonin *et al.*, 1985). In contrast, BN 52021 does not antagonize phorbol myristate acetate induced protein kinase C and phosphorylation. BN 52021 inhibits PAF-induced ion transport impairment in both macrophages and guinea pig ventricular

muscle fibers. BN 52021 dose dependently inhibits the enhancement of cytotoxicity due to PAF on Schistosoma mansoni coated with both complement ($C_3b$) and specific antibody (IgG) (McDonald *et al.*, 1986). More interestingly, BN 52021 and the related *ginkgolides* decrease the IgE-mediated cytotoxicity of human eosinophils. These data suggest that BN 52021 may interfere with the amplification of allergic reactions and inflammatory responses associated with helminthic infections.

*b. Lignans.* Lignans and neolignans are an immense chemical family formed biogenetically by oxidative dimerization of hydroxyalkoxyphenylpropane (Rao, 1978). The term lignan is traditionally reserved for compounds in which the precursors are linked by ß-carbons of each lateral chain, whereas the term neolignan is applied to products in which dimerization intervenes on carbons other than ß-carbons.

*(i). Benzofuranoid neolignan.* A neolignan isolated by the Merck group from *Piper futokadsurae*, a plant used in Southern China as antirheumatic and antiallergic, was the first natural product discovered to act as a potent inhibitor of [$^3$H]PAF binding to a rabbit platelet membrane preparation. It was named kadsurenone and was shown to be a specific and potent inhibitor of PAF-induced platelet aggregation. Kadsurenone inhibits PAF-induced aggregation of human neutrophils (Shen *et al.*, 1985). In a Langendorff perfused guinea pig heart perfused at constant pressure, kadsurenone (1 $\mu$M) effectively antagonized the PAF-induced decrease in contractility and coronary flow (Levi *et al.*, 1984).

*(ii). Substituted furanoid lignans.* Several structures in lignan series are also potent PAF antagonists. This is especially the case with tetrahydrofuran-derived compounds, such as L-652,731, which are orally active and several times more potent than kadsurenone *in vitro* and *in vivo* (Hwang *et al.*, 1985). L-652,731 reverses

hypotension, extravasation, neutropenia and release of lysosomal enzymes in rats by either i.v. or p.o. administration.

*c. Gliotoxin and related products.* Very recently, PAF antagonists were found to be produced by the fermentation of different fungi and microorganisms. These products are derived from bisdithiobis (methylthio) gliotoxin which was first isolated from the wood fungus *G. delquescens*. Most of these products possess the dialkylthiopiperazinedione skeleton. The most potent antagonists are FR-900452 (S.phacofaciens) and FR-49175 (P. tertikowskii). FR-49175 (0.1 mg/Kg, i.v.) significantly inhibited PAF-induced bronchoconstriction in guinea pigs. However, it did not prevent PAF-induced hypotension in rats or vascular permeability increase in mice and failed to inhibit immune anaphylaxis in guinea pigs. In contrast, FR-900452 is a potent inhibitor of endotoxin-induced thrombocytopenia and prevents PAF-induced hypotension.

5.2.3. Synthetic structures.

Very few products of synthetic origin have been described as having specific PAF-antagonistic properties. 48740 RP, a (3-pyridyl)-1H,3H-pyrrolo(1,2-c)thiazole derivative, was found to inhibit PAF-induced human and rabbit platelet aggregation (Sedivy *et al.*, 1985). This inhibition is not selective, since 48740 RP interferes with platelet aggregation by arachidonic acid, collagen and thrombin at high concentrations. 48740 RP ( 10 mg/kg i.v.) is a competitive and full antagonist of PAF binding sites and inhibits PAF-induced hypotension, thrombocytopenia, hemoconcentration and hyperfibrinolysis in the rabbit or in the rat.

5.2.4. Pharmacological agents.

Several products belonging to well-known pharmacological classes show selective antagonism against PAF.

*a. Triazolobenzodiazepines.* Triazolobenzodiazepines are classical psychotropic agents. Kornecki *et al.* (1985) have shown that alprazolam and triazolam are potent inhibitor of PAF-induced human platelet activation. Other benzodiazepines, e.g., diazepam or chlordiazepoxide, are not active. These effects were specific to PAF, since the response of human platelets to ADP, thrombin, epinephrine, collagen, arachidonic acid and the calcium ionophore A23187 was not inhibited. The structure-activity relationship in this chemical series has been published by the Boehringer research group (Casals-Stenzel *et al.*, 1986). The triazole ring is required for inhibition of PAF action. The synthesis of a new triazolobenzodiazepine was recently accomplished by the introduction of a hydrophilic side chain to a triazolobenzodiazepine to produce WEB 2086, which inhibits PAF-induced human platelet and neutrophil aggregation. Aerosols of WEB 2086 also inhibit the bronchial and circulatory effects of i.v. PAF infusion in guinea pigs (Casals-Stenzel *et al.*, 1986). WEB 2086 is effective against several models of anaphylaxis and antagonizes endotoxin-induced shock and PAF-induced gastric ulcerations (Casals-Stenzel *et al.*, 1986).

*b. Calcium channel blocking agents.* Recent reports have shown that certain calcium channel blocking agents, such as gallopamil (D 600) and diltiazem, are PAF inhibitors. Conversely, the compounds of the 1,4-dihydropyridines serirs are only weak inhibitors (Tuffin and Wade, 1985). Recent work by Valone (1987) showed that inhibition of binding of [$^3$H]PAF by diltiazem and verapamil results from competitive

and noncompetitive mechanisms. This effect is mediated by allosteric mechanisms similar to those described for calcium channel blockers and adrenergic receptors. Recently, Hwang *et al.* (1987) described a new dual antagonist L-652,469 isolated from the methylene chloride extracts of the buds of Tussilago fara L. This compound inhibits the [$^3$H]PAF-specific binding to rabbit platelet membranes and the specific binding of $Ca^{2+}$ channel blockers (e.g., [$^3$H]nitrendipine) in cardiac sarcolemmal vesicles.

## 6. PAF receptors

### 6.1. Presence and characteristics of PAF receptors

One of the early findings was that PAF, a phospholipid, acts via receptors on the membranes of responsive cells. The involvement of specific receptor(s) was first suggested by the demonstration that only the naturally occurring stereoisomer (R) stimulated various PAF responses (Heymans *et al.*, 1981 and Vargaftig and Benveniste, 1983). Additional data corroborated these findings: very low concentrations (usually lower than 0.1 nM) are necessary to trigger biological effects; specific desensitization takes place after tissue exposure to PAF; and there is specific inhibition by PAF antagonists. The existence PAF receptors has recently been confirmed by binding experiments using [$^3$H]PAF and the $K_d$ values usually are between $10^{-10}$ and $10^{-9}$ M. High affinity receptors have been described in many cells and tissues, such as, platelets, polymorphonuclear leukocytes, monocytes and macrophages and lung, kidney, brain and the cardiovascular system (Snyder, 1987). The receptor(s) are stereospecific and the extent of binding correlates well with the responses by whole cells. The affinity and number of these receptors are interrelated with the tissue and

species specificity. Rat platelets are not aggregated by PAF *in vitro*. This is explained by the fact that they do not have high affinity receptors (Inarrea *et al.*, 1984).

One difficulty in studies of PAF receptor is that PAF is amphiphilic. This results in high levels of nonspecific binding of PAF to membranes. Attempts to solubilize the receptor with detergent have been severely limited since PAF is readily incorporated into mixed micelles. Another problem is that most types of responsive cells have only several hundred to several thousand receptors. Recently, Nishihira *et al.* (1985) isolated the PAF receptor from human platelets and identified it as a protein with a molecular weight of 160,000. Unfortunately, more detailed biochemical characterization of the PAF receptor is hampered by the lack of a consistent procedure to solubilize the membrane binding protein and to prepare high titer specific antibodies to PAF (Nishihira *et al.*, 1984). At present, the PAF receptor has not been purified or characterized at the molecular level. However, binding assays may be improved by the recent development of radiolabeled PAF receptor antagonists that have less nonspecific binding (Dent *et al.*, 1989). Hwang (1988) found that the rank order of potency for several antagonists was different in human neutrophils and platelets and that monovalent cations had different effects in the binding to the two cells types. From his results and those of others it appears likely that there are least two receptors. Additionally, recent reports suggest that two types of cultured cells will be good models. Kupffer cells, a liver macrophage, have almost 11,000 PAF receptors/cell and their turnover can be analyzed over prolonged periods (Chao *et al.*, 1989). When HL-60 cells differentiate they change from expression of few receptors to about 6000/cell (Vallari *et al.*, 1990). This receptor may be further studied by expression cloning.

6.2. The signal transduction of PAF receptor.

A putative conformation of PAF platelet membrane binding sites can be deduced on the basis of the data obtained with agonists and antagonists (Godfroid and Braquet, 1986 and Braquet and Godfroid, 1987). The lipophilic moiety is essential for both agonistic and antagonistic activities. The long fatty chain of PAF deeply enters the membrane in a hydrophobic area, e.g., hydrophobic lipid-lipid or lipid-protein interactions. After binding to its receptor, PAF might indirectly influence the conformation of the unknown target sited within the membrane by an electronic charge transfer from the ether function, by modification of the fluidity around the part of the targets included in the bilayer and /or by deranging the external protein-phospholipid polar head interactions. The receptor protein may, in turn, activate the guanyl nucleotide regulatory protein via GTP hydrolysis.

PAF receptors are thought to exert their signal via a G protein since the binding of PAF to membranes stimulates GTPase activity (Hwang *et al.*, 1986 and Houslay *et al.*, 1990). Phospholipase C is then stimulated with phosphodiesterase cleavage of inositol phospholipids, especially of phosphatidyl inositol-4,5-bisphosphate ($IP_2$), into inositol-1,4,5-triphosphate ($IP_3$) which induces $Ca^{2+}$ mobilization from internal pools. Diacylglycerol is also produced which activates protein kinase C. Both increased $[Ca^{2+}]_i$ and protein kinase C activation mediate the cellular response. Activation of protein kinase C also leads to the breakdown of phosphatidylcholine, yielding phosphatidic acid and diacylglycerol. PAF antagonists, which inhibit PAF binding to its receptor, antagonize all the events of the signalling process.

Receptors for PAF undergo rapid desensitization in response to occupancy. Activation of protein kinase C by other routes also down regulates the PAF receptor

(O'Flaherty *et al.*, 1989 and Yamazaki *et al.*, 1989), implying that the response to PAF may be mediated by protein kinase C. If so, this could indicate a complex role for protein kinase C; it appears to transmits signals in response to PAF and then shuts off subsequent responses. Receptors reappear on the cell surface by a process that requires synthesis of new receptors (Chao *et al.*, 1989).

6.3. The possible role as an intracellular messenger.

PAF was originally described as a soluble factor in blood, so it is apparent that some cells secrete it following synthesis. However, it has been found that the PAF made by endothelial cells is not secreted (McIntyre *et al.*, 1985, 1986). This observation and those of others, stimulated a reexamination of the secretion of PAF. Many types of cells secrete only a fraction of the PAF produced and the percentage of PAF secreted varies with different conditions (Cluzel *et al.*, 1988). These findings have led to the suggestion that PAF may serve at times as an intracellular messenger (Lynch and Henspn, 1986, Stewart *et al.*, 1990 and Worthen *et al.*, 1988). Studies to test this hypothesis have been hampered by the lack of a potent selective inhibitor of PAF synthesis, but this remains an interesting and potentially important possibility. A second possibility is that PAF on the surface of cells can function as an intercellular messenger and such a role has been described in endothelial cells and neutrophils (Vercellotti *et al.*, 1989 and Zimmerman *et al.*, 1990).

# Thesis objectives

PAF is an unique species of phospholipid. Since the isolation of PAF, the elucidation of its structure and chemical synthesis of PAF, its biological and pathophysiological actions have been extensively studied. The original idea to examine the cardiovascular effects arose from our long standing interest in the cardiovascular effects of phospholipids. We initiated the study with the investigation of the hypotensive effect of PAF and compared it with the effects of its phospholipid structural analogs, palmitoyl carnitine, lysophosphatidylcholine, and related compounds on the blood pressure in anesthetized rats. The diverse cardiovascular effects of PAF in the coronary circulation have been shown in a number of studies *in vivo* and *in vitro* (Demopoulos *et al.*, 1979, Hanahan *et al.*, 1986, Braquet *et al.*, 1987). A systematic study of the actions of PAF on coronary circulation was therefore initiated. The initial objective of the study was to characterize the coronary vascular responses to PAF, followed by an examination of the mechanisms of its cardiovascular effects in the isolated perfused heart. We propose that the opposite functional vasodilator and vasoconstrictor effects of PAF are mediated by different PAF receptor subtypes. Subsequently, we tried to define the possibility of existence of the different PAF receptor subtypes in the coronary vasculature using a pharmacological approach. Since it has been demonstrated that the coronary vascular effects of PAF are mediated by other vasoactive substances, such as, leukotrienes and prostaglandins (Piper and Stewart, 1986), it is necessary to clarify the interactions of PAF-induced vasoactive compounds and interactions of PAF with these compounds in the isolated perfused rat heart. Thus, we developed a cascade perfusion model with isolated hearts to study this objective.

# Part 1

## Effects of platelet-activating factor on arterial blood pressure in the rat - A comparison with palmitoyl carnitine and structural analogs

## Abstract

Palmitoyl carnitine and lysophosphatidylcholine have been implicated in the generation of cardiac arrhythmias in the ischemic myocardium. These amphiphilic compounds are structurally similar to platelet-activating factor (PAF). The present study compared the hypotensive effect of these compounds to PAF in the anesthetized rat. Palmitoyl carnitine was about 1000 times less potent than PAF in lowering the blood pressure. Lysophosphatidylcholine and other structurally related compounds had no significant hypotensive action. CV 3988, a PAF antagonist, blocked the hypotensive action of PAF but had no effect on the hypotensive action of palmitoyl carnitine. This suggested the effect of palmitoyl carnitine was not associated with the same site or mechanism as PAF. The results also ruled out the involvement of prostaglandin formation and of the sympathetic nervous system since indomethacin, phenoxybenzamine and propranolol did not affect the hypotensive action of palmitoyl carnitine. In addition, it is unlikely that palmitoyl carnitine exerted its effect by a direct membrane perturbing action because lysophosphatidylcholine, which possesses similar amphiphilic properties, does not share the same hypotensive effect.

## Introduction

Palmitoyl carnitine and lysophosphatidylcholine have been reported to accumulate in the ischemic myocardium (Idell-Wenger *et al.*, 1978; Shaikh and Downar, 1981; Corr *et al.*, 1982; Man *et al.*, 1983). Exogenous lysophosphatidylcholine and palmitoyl carnitine have been shown to produce electrophysiological abnormalities in isolated cardiac preparations (Corr *et al.*, 1981). Palmitoyl carnitine and lysophosphatidylcholine are structurally similar to platelet-activating factor (PAF) (Figure 2). PAF has been shown to be a potent mediator of platelet aggregation (Benveniste, 1974). In addition, PAF has been implicated in allergic reactions, immunological responses, septic shock and a variety of other reactions (Hanahan, 1986; Braquet *et al.*, 1987). PAF is a unique species of ether-linked choline phospholipid with an acetyl group at the *sn*-2 position (Demopoulos *et al.*, 1979; Benveniste *et al.*, 1979). The antihypertensive polar renomedullary lipid has been shown to have the same chemical structure as PAF and possesses a potent hypotensive effect (Blank *et al.*, 1979; Prewitt *et al.*, 1979). In a recent study, PAF, lysophosphatidylcholine and palmitoyl carnitine were found to produce similar electrophysiological alterations in canine Purkinje fibers and guinea pig papillary muscles (Nakaya and Tohse, 1986). Although the hypotensive effects of PAF have been well documented, little information is available concerning the effects of palmitoyl carnitine, lysophosphatidylcholine and their structural analogs on arterial blood pressure. The present study was designed to investigate the effects of palmitoyl carnitine, lysophosphatidylcholine and structurally related compounds on the blood pressure of anesthetized rats and to compare them with the well known hypotensive effect of PAF.

## Methods and Materials

### *Materials*

The following chemicals were obtained from Sigma Chemical Company: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), lysophosphatidylcholine (from egg yolk), palmitoyl carnitine, carnitine chloride, bovine serum albumin, albumin-agarose, palmitic acid, glycerophosphocholine, propranolol and indomethacin. CV 3988 was kindly provided by Dr. M. Nishikawa (Takeda Chemical Industries, Ltd.). Phenoxybenzamine was supplied by Smith Kline and French Laboratories. All solutions were prepared fresh daily. Indomethacin was dissolved in ethanol. All other compounds were dissolved in saline.

### *Preparatory procedures*

Long Evans rats were anesthetized with pentobarbital 50 mg/kg body weight i.p. A tracheotomy was performed, and the trachea was intubated. The carotid artery was cannulated. The cannula was connected to a pressure transducer to record the mean arterial blood pressure on a paper chart recorder. The jugular vein was cannulated for the administration of the test compounds and drugs. Additional anesthetic was given to maintain anesthesia when required. The lead II electrocardiogram was also monitored.

### *Experimental protocol*

Animals were divided into groups with a matching average weight (280 $\pm$ 15 g). This eliminated the need to adjust the amount of the test compound and the volume

for injection because of the possible variation in the weight of each animal group. To investigate the hypotensive effect of PAF, 0.1 ml of a solution containing 0.025, 0.05 or 0.1 $\mu$g of PAF was administered intravenously as a bolus injection. These amounts of PAF are equivalent to 0.09, 0.18 and 0.36 $\mu$g/kg or 0.16, 0.33 and 0.65 nmol/kg respectively. Following the injection of PAF, 0.2 ml of saline was used to clear the cannula. To examine the hypotensive effect of lysophosphatidylcholine and palmitoyl carnitine, a 0.1 ml solution containing various amounts of the test compound (maximum 200 $\mu$g or 710 $\mu$g/kg) was injected, followed by 0.2 ml saline. The effects of 200 $\mu$g of glycerophosphocholine, palmitic acid and carnitine were also studied in a similar fashion. Saline injections (0.3 ml total volume) were used to serve as controls. Phenoxybenzamine (7.0 mg/kg) and propranolol (3.6 mg/kg) were given to produce $\alpha$ and $\beta$ receptor blockade. Receptor blockade was tested by the administration of phenylephrine and isoproterenol. The hypotensive effect of the test compound was measured before and 15 min after receptor blockade. In separate experiments, the hypotensive effect of the test compound was measured before and 30 min after the administration of 10 mg/kg indomethacin. The PAF antagonist, CV 3988, was infused over a 5 min period to give a total dose of 10 mg/kg. After the blood pressure had stabilized, the hypotensive effect of the test compounds was measured. To test the effect of binding of the test compound to proteins, 5 mg of bovine serum albumin was added to the test solution before injection. This amount produced a concentration of albumin similar to that in normal serum (5 g/100 ml). Bovine serum albumin covalently attached to 4% beaded agarose was used to compare the binding of palmitoyl carnitine and lysophosphatidylcholine to albumin. After the appropriate incubation period, the sample was centrifuged to separate those bound to albumin (the pellet) and those remaining in the supernatant. The amount of palmitoyl carnitine or lysophosphatidylcholine in the supernatant was determined as a function of time. Values were expressed as mean $\pm$ standard deviation. The students' t test for paired

and unpaired data were used where appropriate. A probability ($P$) value $< 0.05$ was considered significant.

## Results

*Hypotensive effects of palmitoyl carnitine, PAF and their structural analogs*

PAF produced a dose-dependent decrease in mean arterial blood pressure. Palmitoyl carnitine (100-200 $\mu$g) also produced a significant hypotensive effect. However, the hypotensive effect of palmitoyl carnitine was more transient than PAF (usually lasted about a min or less). Lysophosphatidylcholine (200 $\mu$g) had no significant effect on mean arterial blood pressure. The effects of PAF, palmitoyl carnitine and lysophosphatidylcholine on the mean arterial blood pressure are summarized in Table 1. Control injections of saline had no effect. Structurally related compounds such as glycerophosphocholine, palmitic acid and carnitine (all 200 $\mu$g) were tested for comparison. Only palmitic acid produced a significant, though sometimes variable, decrease in blood pressure (Table 1). Since only palmitoyl carnitine showed a consistent and significant hypotensive action, it was decided that further comparison with PAF would be made with palmitoyl carnitine only.

*Effect of the PAF antagonist, CV 3988, on the blood pressure response*

The effects of the specific PAF antagonist, CV 3988 (10 mg/kg) on the hypotensive response are depicted in Figure 3. The hypotensive effect of the lower dose of PAF was completely abolished by CV 3988. With the higher dose of PAF, CV 3988 significantly reduced the peak effect of PAF on the mean blood pressure. In addition, CV 3988 also diminished the duration of action of PAF. However CV 3988 had no significant effect on the hypotensive action of palmitoyl carnitine (Figure 3).

*Effect of adrenergic receptor blockade on the blood pressure response*

Phenoxybenzamine and propranolol were used to compare the effects of $\alpha$ and $\beta$ receptor blockade on the hypotensive effect of PAF and palmitoyl carnitine. After the administration of phenoxybenzamine and propranolol, mean arterial blood was reduced from 116 $\pm$ 21 to 90 $\pm$ 15 mm Hg (n=22). Adrenergic receptor blockade did not significantly affect the hypotensive effect of PAF and palmitoyl carnitine. The results are summarized in Figure 4.

*Effect of indomethacin on the blood pressure response*

The role of prostaglandins in the hypotensive effect of PAF and palmitoyl carnitine was examined by treatment with the cyclooxygenase inhibitor indomethacin (10 mg/kg). Indomethacin decreased the maximum hypotensive effect of 0.025 and 0.05 $\mu$g PAF (Figure 5). However, only the hypotensive effect of 0.05 $\mu$g PAF was significantly reduced by indomethacin. The hypotensive effect of palmitoyl carnitine was not significantly affected by indomethacin (Figure 5). To investigate the effect of the solvent, 0.1 ml of ethanol was injected in 3 separate experiments. Pretreatment with ethanol had no effect on the resting blood pressure, and the response to PAF and palmitoyl carnitine was not affected (data not shown).

*Effect of albumin on the blood pressure response*

The effects of binding the test compounds to proteins were examined by the addition of 5 mg albumin to the solution prior to injection. The results are summarized in Figure 6. The maximum hypotensive effect of PAF was not altered. However, the hypotensive effect of palmitoyl carnitine was significantly attenuated in the presence of

albumin ($P < 0.001$). The binding of palmitoyl carnitine and lysophosphatidylcholine (0.1 $\mu$M) to albumin covalently attached to agarose (1.36 mg or 0.02 $\mu$M protein) was also tested. After 1 min of incubation, no significant difference in the amount of palmitoyl carnitine and lysophosphatidylcholine bound to albumin was detected (52 and 50% bound respectively, n=3). Similar results were observed after 5 and 10 min of incubation.

## Discussion

From the present study it is clear that palmitoyl carnitine, though much less potent than PAF, possesses a hypotensive effect. The amount of palmitoyl carnitine required to produce significant hypotension was 200 $\mu$g (710 $\mu$g/kg or 1.6 $\mu$mol/kg). The calculated concentration of long chain acyl carnitine in the cytosol of normal rat hearts is 0.025 mM (Idell-Wenger *et al.*, 1978). Hearts perfused with buffer containing 1.2 mM palmitate bound to 3% bovine serum albumin had a cytosolic concentration of 0.38 mM long chain acyl carnitine. After 20 min of ischemia, cytosolic long chain acyl carnitine increased to 1.95 mM (Idell-Wenger *et al.*, 1978). In view of the high intracellular concentration of long chain acyl carnitine in the presence of elevated palmitate levels and the accumulation of long chain acyl carnitine under ischemic conditions, a substantial amount of palmitoyl carnitine may be present in the circulation and may produce those cardiovascular effects. However, the possible plasma concentration of palmitoyl carnitine under various pathophysiological conditions is not known.

The amounts of PAF used in the present study (0.09, 0.18 and 0.36 $\mu$g/kg) were comparable to these used in earlier studies. Caillard *et al.* (1982) showed PAF (0.06 - 0.50 $\mu$g/kg i.v.) caused a dose-related reduction in blood pressure in normotensive and spontaneously hypertensive rats. Lai *et al.* (1983) demonstrated a potent hypotensive effect of PAF (0.03 - 10.0 $\mu$g/kg i.v.) in rats. They also concluded that the hypotensive action of PAF was not the result of cholinergic, $\beta$-adrenergic or histaminergic receptor interaction. The administration of PAF (1 $\mu$g/kg i.v.) had been shown to produce a sustained hypotension resembling that of endotoxin shock (Terashita *et al.*, 1985). The same study also showed that CV 3988 (0.05 - 1 mg/kg) was able to reverse the hypotensive action of PAF and endotoxin. Since PAF receptors

are absent in rat platelets (Hanahan, 1986; Braquet *et al.*, 1987; Inarrea *et al.*, 1984), the use of rats in the present study as well as in the previous studies would eliminate the complication of platelet aggregation in these animals.

English and Toth (1988) reported that PAF still induced systemic hypotension in pithed and vagotomized rats and this hypotensive effect of PAF could be blocked by WEB 2086, a specific PAF receptor antagonist. They concluded that peripheral mechanisms are likely to be at least partially responsible for mediating the hemodynamic effects of PAF. One possible peripheral mechanism is an increase the diameter of arterioles. Microscopic observations on the exteriorized mesenteric circulation of anesthetized rats demonstrated an increase in mean arteriolar diameter after PAF perfusion or local PAF application (Lagente *et al.*, 1988).

Studies on receptor binding characteristics of PAF have shown a very high degree of structural specificity. The ether bond at the *sn*-1 position and a short acyl group at the *sn*-2 position are required for agonistic activities (Godfroid and Braquet, 1986). Hence lyso PAF (removal of the 2 acetyl group) is devoid of any significant biological activity. Although palmitoyl carnitine is structurally similar to PAF, palmitoyl carnitine does not possess an ether bond nor a short acyl group (see Figure 2). It is therefore unlikely that the action of palmitoyl carnitine is mediated through a mechanism similar to PAF. The use of the PAF antagonist CV 3988 enabled us to further determine whether the action of palmitoyl carnitine was mediated via the same site or mechanism as PAF. The lack of any effect by CV 3988 on the action of palmitoyl carnitine suggests that palmitoyl carnitine exerts its hypotensive effect through a different site or mechanism. The absence of any inhibitory effect on the hypotensive action of palmitoyl carnitine by indomethacin, phenoxybenzamine and propranolol indicates that the production of prostaglandins or the activation of $\alpha$ and $\beta$

adrenergic receptors are unlikely to have an important role for the action of palmitoyl carnitine.

PAF, palmitoyl carnitine and lysophosphatidylcholine all have a charged choline head group and a long chain non-polar group. At high concentrations, the amphiphilic nature of these compounds can lead to interaction with the cell membrane and produce cellular perturbations. Indeed, the presence of micromolar concentrations of lysophosphatidylcholine and palmitoyl carnitine have been shown to produce electrophysiological abnormalities (Corr *et al.*, 1981) and membrane fluidity changes (Fink and Gross, 1984). Since albumin has a high affinity for amphiphilic lipid compounds, the elimination of the hypotensive effect of palmitoyl carnitine by albumin is compatible with this assumption. Our data also showed palmitoyl carnitine and lysophosphatidylchoine bound to albumin at similar rates. One difficulty with this concept is that palmitoyl carnitine and lysophosphatidylcholine had been demonstrated to produce similar electrophysiological alterations (Corr *et al.*, 1981; Nakaya and Tohse, 1986) suggesting the two compounds have similar membrane perturbing properties. However, only palmitoyl carnitine showed a hypotensive effect in the present study while lysophosphatidylcholine had no significant effect. It is therefore unlikely that palmitoyl carnitine exerted its effect by a direct membrane perturbing action. The mechanism of action of palmitoyl carnitine on blood pressure remaines to be defined.

Endothelium-dependent relaxation of vascular smooth muscle by lysophosphatidylcholine has been reported *in vitro* (Saito *et al.*, 1988). However, this relaxation is not mediated by the release of endothelium-derived relaxing factor (nitric oxide) and corresponded to the cyclic GMP level in rabbit thoracic aorta (Menon *et al.*, 1989). These investigators suggest that the weak detergent action of

lysophosphatidylcholine on the membrane dynamics of the endothelial cells is responsible for the production of cyclic GMP resulting in vascular relaxation (Saito *et al.*, 1988). Whether palmitoyl carnitine possesses endothelium-dependent relaxant properties *in vitro* remains to be determined. However, our results indicate that palmitoyl carnitine but not lysophosphatidylcholine showes a significant hypotensive effect *in vivo*.

In conclusion, our results showed that PAF has more potent hypotensive properties than palmitoyl carnitine. The hypotensive effect of palmitoyl carnitine was not mediated through the same site or mechanism as PAF. In addition, it is unlikely that palmitoyl carnitine exerted its effect by a direct membrane perturbing effect due to its amphiphilic nature. Finally, we have also ruled out the possible indirect effect of palmitoyl carnitine on blood pressure mediated through the secondary formation of prostaglandins and the activation of the sympathetic nervous system. The possibility that palmitoyl carnitine has a direct vasodilatory action cannot be ruled out. However, such an action of palmitoyl carnitine has not been reported and remains to be elucidated. At present, it is not clear whether palmitoyl carnitine may have any role in the regulation of blood pressure especially under conditions where palmitoyl carnitine is known to accumulate such as ischemia and hypoxia.

Table 1

**Effects of PAF, palmitoyl carnitine and structurally related compounds on the mean arterial blood pressure**

| Test compound | Amount ($\mu$g) | Change in mean blood pressure (mm Hg) | % change from control | n |
|---|---|---|---|---|
| Saline | | + 4 ± 10 | + 5 ± 11 | 10 |
| PAF | 0.025 | - 15 ± 12** | - 16 ± 11 | 4 |
| | 0.05 | - 35 ± 8** | - 29 ± 8 | 4 |
| | 0.1 | - 56 ± 15** | - 46 ± 9 | 5 |
| Palmitoyl carnitine | 100 | - 12 ± 5* | - 10 ± 3 | 5 |
| | 200 | - 25 ± 14** | - 20 ± 10 | 5 |
| Lysophosphatidylcholine | 100 | - 8 ± 3 N.S. | - 8 ± 2 | 3 |
| | 200 | - 6 ± 2 N.S. | - 6 ± 2 | 3 |
| Palmitic acid | 200 | - 14 ± 12** | - 13 ± 10 | 7 |
| Carnitine | 200 | - 8 ± 2 N.S. | - 7 ± 2 | 4 |
| Glycerophosphocholine | 200 | - 4 ± 1 N.S. | - 4 ± 1 | 4 |

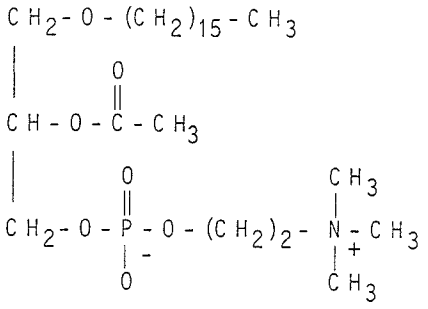
The test compound was given as a 0.1 ml iv injection and this was followed by a 0.2 ml saline injection. The peak change in mean arterial blood pressure was recorded.

Values represent mean ± S.D., n = number of experiments.

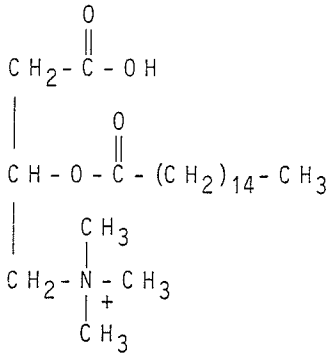
N.S. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, when compared with saline controls.

The mean arterial blood pressure in all groups prior to the injection of the test compounds was 114 ± 16 mm Hg (range 107-121).

A



B



C

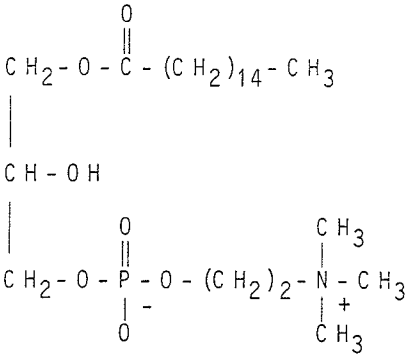


**Figure 2.** Chemical structures of platelet-activating factor (A), palmitoyl carnitine (B) and lysophosphatidylcholine (C).

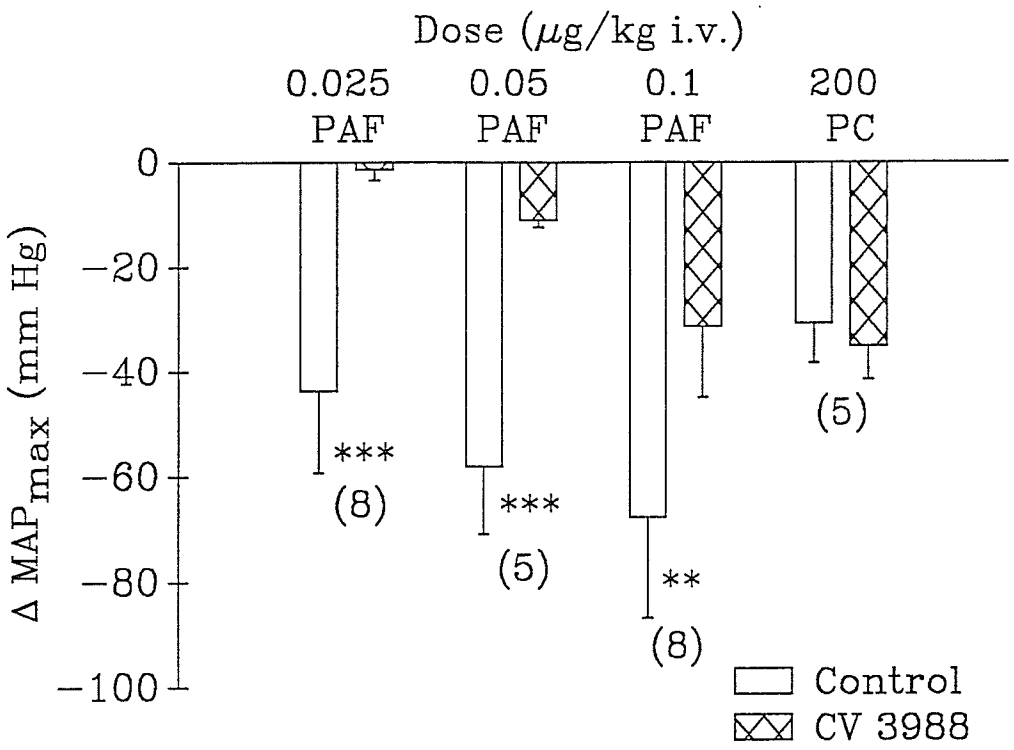



**Figure 3.** The effect of CV 3988 on the hypotensive action of platelet-activating factor (PAF) and palmitoyl carnitine (PC). Values represent mean ± S.D., n=number of experiments. ** $P < 0.01$, *** $P < 0.001$.

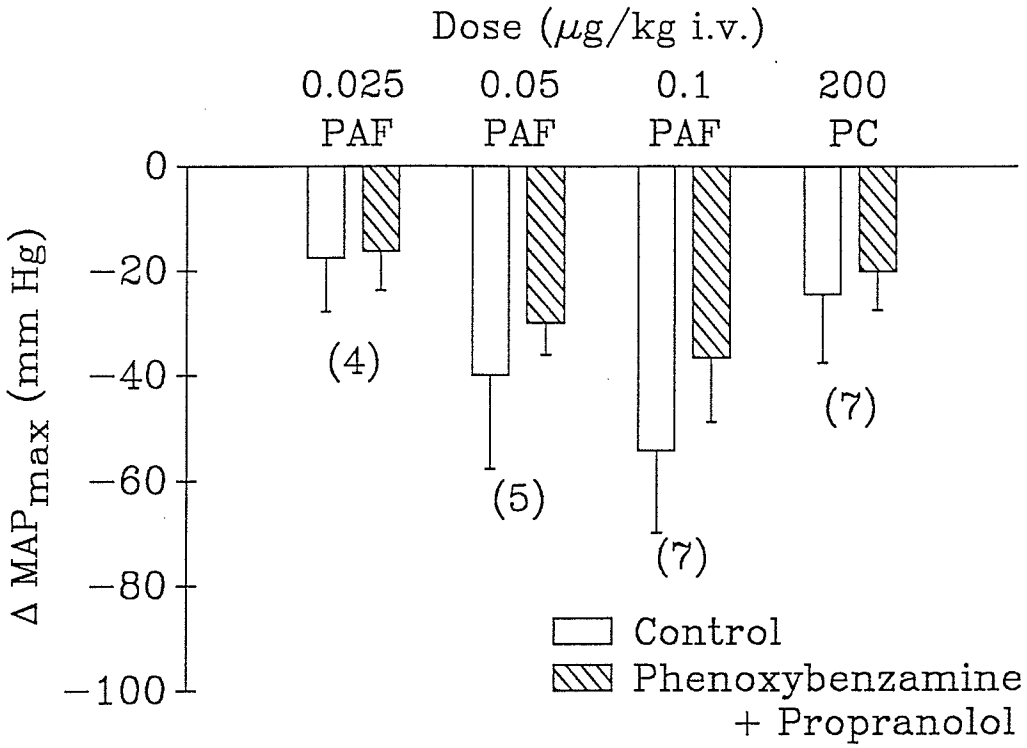



**Figure 4.** The effect of phenoxybenzamine and propranolol on the hypotensive action of platelet-activating factor (PAF) and palmitoyl carnitine (PC). Values represent mean ± S.D., n=number of experiments.

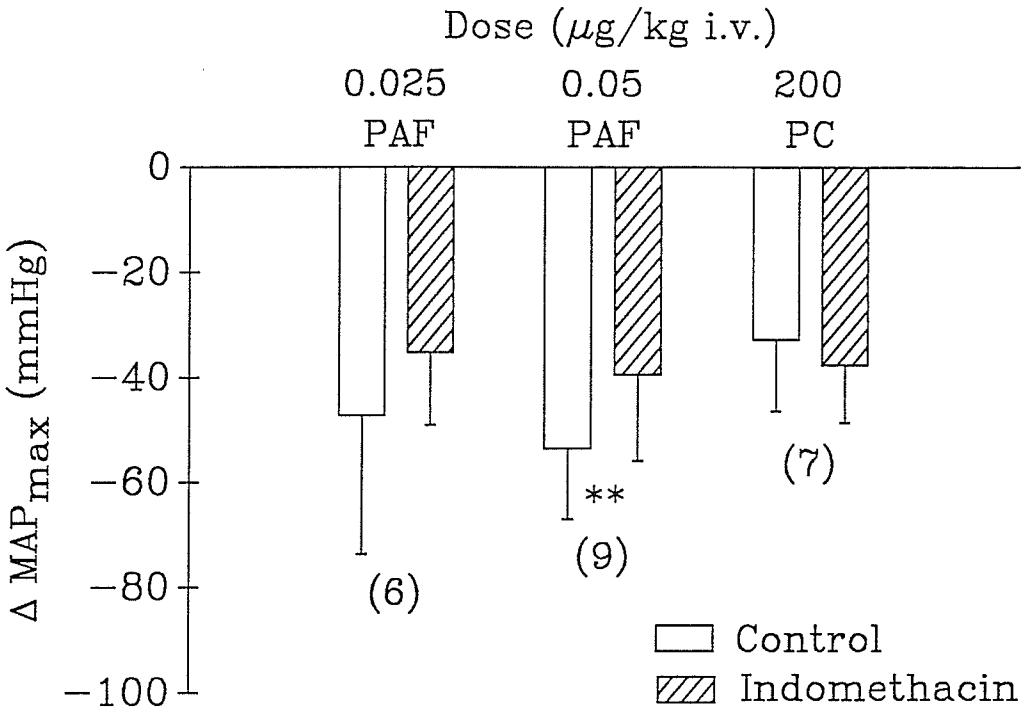



**Figure 5.** The effect of indomethacin on the hypotensive action of platelet-activating factor (PAF) and palmitoyl carnitine (PC). Values represent mean ± S.D., n=number of experiments. *** $P < 0.001$.

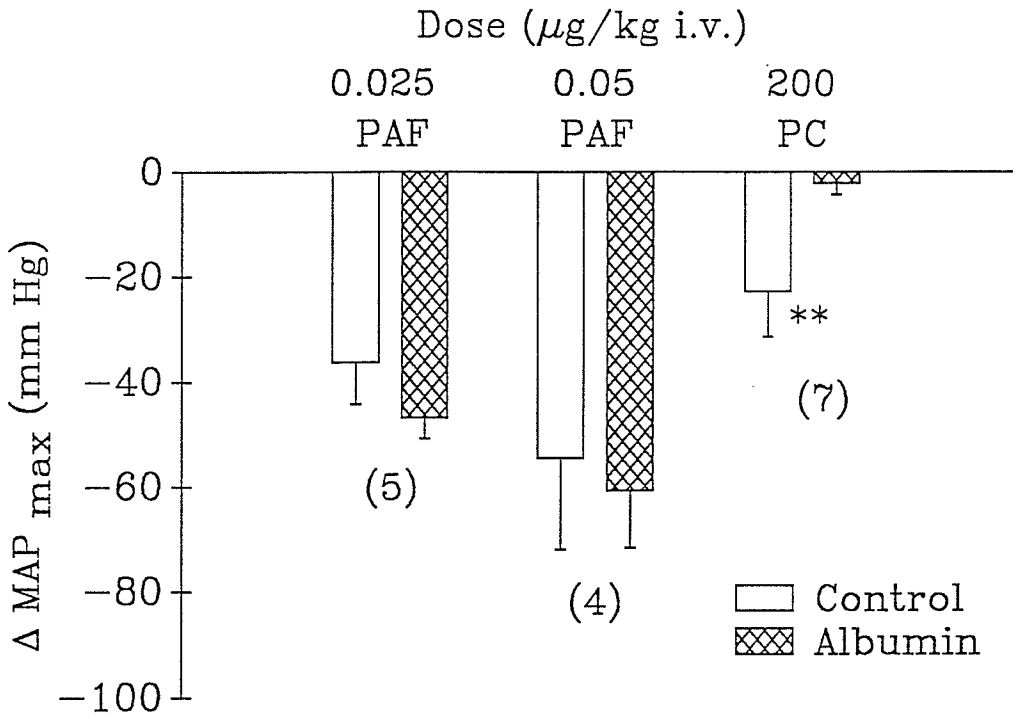



**Figure 6.** The effect of albumin on the hypotensive action of platelet-activating factor (PAF) and palmitoyl carnitine (PC). Values represent mean ± S.D., n=number of experiments. ** $P < 0.01$.

# Part 2

## Characterization of the coronary vascular responses to platelet-activating factor in the isolated perfused heart

## Abstract

The coronary vascular responses to platelet-activating factor (PAF) were studied in isolated perfused hearts. Constant flow rates were maintained and the changes in perfusion pressure were recorded following bolus injections of PAF. The effect of PAF on the perfusion pressure was variable and depended on certain experimental conditions. When the starting baseline pressure was 65-75 mm Hg, bolus injections of PAF resulted in an initial decrease followed by an increase in perfusion pressure. However the decrease in perfusion pressure was less pronounced when the starting baseline pressure was 40 mm Hg. Similar coronary vascular responses to PAF were observed in perfused rat hearts under either constant flow rate or constant pressure. The coronary vascular response to PAF was dependent on the amount of PAF injected. Injections of 10 fmol - 1 pmol of PAF prepared in a solution containing bovine serum albumin resulted in decreases in perfusion pressure that were not followed by increases. A biphasic response, consisting of an initial decrease followed by an increase in perfusion pressure was observed following injections of 10 and 100 pmol of PAF. Only increases in perfusion pressure were recorded following injections of 1000 pmol of PAF. The response to injection of PAF was also dependent on whether it was prepared in a solution containing serum albumin. Repeated injections of PAF showed tachyphylaxis. The results from repeated injections of PAF indicate that depletion of vasoactive mediators induced by PAF or receptor desensitization can explain the mechanism of the failure of a second injection of PAF to initiate a vasoconstrictor response in the perfused heart. The coronary vascular effects of PAF were also tested in perfused guinea pig hearts to uncover any species dependence of the action of PAF. Unlike in perfused rat hearts, only a dose-dependent vasoconstrictor response was observed following a bolus injection of 1 fmol to 10 pmol PAF in perfused guinea-pig

hearts. This study demonstrates that it is possible to observe vasodilation, vasoconstriction and biphasic responses to PAF in the isolated rat heart model.

## Introduction

Since the isolation of PAF, the elucidation of its structure and the chemical synthesis of PAF (Benveniste *et al.*, 1979; Demopoulos *et al.*, 1979; Hanahan *et al.*, 1980), its vasoactive properties have been extensively studied. The diverse cardiovascular effects of PAF in coronary circulation have been described in a number of studies *in vivo* and *in vitro* (Demopoulos *et al.*, 1979; Hanahan *et al.*, 1986; Braquet *et al.*, 1987). In isolated perfused hearts, both a decrease in coronary flow under constant pressure perfusion (Levi *et al.*, 1984) and an increase in perfusion pressure under the condition of constant flow (Piper and Stewart, 1986, 1987) were observed, indicating a vasoconstrictor effect. In intact animals, the effects of intracoronary injection of PAF were inconsistent. Feuerstein *et al.* (1984) observed a biphasic effect of PAF on coronary flow (an increase followed by a decrease). Sybertz *et al.* (1985) only observed a decrease in coronary flow by PAF, while Jackson *et al.* (1986) found an increase in coronary flow. Several subsequent studies showed a biphasic response of coronary flow (Mehta *et al.*, 1986; Ezra *et al.*, 1987; Fieldler *et al.*, 1987).

Several possible explanations may account for some of the diverse vascular effects of PAF in the heart. Firstly, the vasodilation response requires interaction in the intact animal and is therefore not observed in the isolated perfused heart. This can explain why only a vasoconstriction response was observed in studies using perfused hearts. In addition, the experimental model used such as constant flow or constant pressure perfusion and the animal species may also account for some of the observed differences. Secondly, PAF, like many amphiphilic lipids, has a tendency to adhere to the container surface. The procedures used to prepare small quantities of PAF, especially in the pmol range, are important. The use of serum albumin in some studies can eliminate this potential problem. Thirdly, natural PAF exists mainly as a mixture

of hexadecyl- and octadecyl-acetyl-glycero-phosphocholine. It has been demonstrated that the hexadecyl-form of PAF is more potent than the octadecyl-form in causing platelet activation and aggregation. Thus some of the differences may be caused by the use of synthetic PAF with different proportions of alkyl groups.

The initial objective of this study was to characterize the coronary vascular responses to PAF under various experimental conditions in the isolated perfused rat heart and the interplay of these conditions on the coronary vascular effects of PAF. The conditions studied were the effect of the perfusion model (using constant flow rate compared to using constant perfusion pressure), the starting baseline perfusion pressure, the preparation of PAF with an albumin-containing solution, and the effect of various amounts of PAF on the vascular response. The possible differences between PAF prepared from tissue extracts and synthetic PAF in the hexadecyl-form were also examined in this study.

In the second part of this study, two other objectives were examined. It has been reported in *in vitro* studies that repeated injections of PAF could not elicit a vasoconstriction response (Piper and Stewart, 1986, 1987). The possible explanations include desensitization of PAF receptors, depletion of vasoactive mediators induced by PAF, or loss of vascular smooth muscle response to PAF or its mediators. In this study, we attempted to further investigate the possible mechanisms for the lack of coronary vascular response to repeated injections of PAF. The platelets of various animal species have been reported to have different sensitivities to PAF (Namm *et al.*, 1982). Therefore, we also investigated the effects of PAF in guinea-pig hearts to determine whether a similar coronary vascular response could be observed following bolus injections of PAF.

## Methods and Materials

*Heart perfusion*

Male Sprague-Dawley rats (250-300 g) and Hartley guinea-pigs (300-400 g) were euthanized by cervical dislocation 15 min after heparin (1000 u/kg, i.p.) was administered. Their hearts were rapidly excised and placed in ice-cold Krebs-Henseleit solution oxygenated with 95% $O_2$ - 5% $CO_2$. The Langendorff technique was used to perfuse hearts with Krebs-Henseleit solution which was maintained at $37\pm0.5°C$ and oxygenated with 95% $O_2$-5% $CO_2$. The composition of Krebs-Henseleit solution was as follows (mM): NaCl 118, KCl 4.7, $NaH_2PO_4$ 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25 and glucose 5.5. The heart was allowed to beat spontaneously and was perfused at a constant flow rate using a roller pump or under a constant perfusion pressure. The perfusion pressure was measured by attaching a pressure transducer (Bentley Trantec Model 800) to a side arm of the aortic cannula. To measure changes in flow rate under constant pressure perfusion, the flow rate was measured using an electromagnetic flow meter (Carolina Medical). Both perfusion pressure and flow rate were recorded on a Gould chart recorder and monitored on a digital display of the perfusion pressure and flow rate. The procedures to determine the coronary vascular effects of PAF in the perfused rat heart had been described as previous section.

*Drug preparation and administration*

Hearts were stabilized for 20 min using Krebs-Henseleit solution. In some experiments, the flow rate was adjusted to obtain a perfusion pressure of 65-75 mm Hg during the stabilization period and was maintained constant for the rest of the experiment. In experiments in which the perfusion pressure was maintained by

constant, the pressure was set by the height of the perfusate column and was set at 70 mm Hg. PAF, 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (prepared from bovine heart, Sigma Chemical Co.), was prepared in saline (0.9% NaCl) solution containing 0.25% bovine serum albumin (Sigma Chemical Co.). For comparison, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (Sigma Chemical Co.) prepared in saline solution containing 0.25% bovine serum albumin was also used. The leukotriene, $LTC_4$, (Merck Frosst Canada Inc.), was diluted in saline to the appropriate concentration. All stock solutions were made fresh daily and refrigerated between experiments. All bolus injections of PAF and $LTC_4$ were given in a volume of 0.1 ml over a one second period, into the perfusion line 5-6 cm proximal to the aortic cannula. In some experiments, a second injection was administered 10 min after the first injection. Instantaneous changes in perfusion pressure or flow rate and the time at which maximum changes occurred were recorded.

In some experiments a second injection was administered after 10 min. In another series of experiments, 15 nmol PAF/ml or 1 nmol PAF/ml in saline containing 0.25% bovine serum albumin were continuously infused into the perfusion line at a rate of 0.04 ml/min for 20 min.

*Statistical analysis*

The data were analysed by two-tailed unpaired or paired Student's t-tests, or one way analysis of variances where appropriate. A P value less than 0.05 was considered to be significant. Duncan's multiple range test was used to determine significant differences between groups. Values are reported as means and standard deviations of the means unless otherwise noted.

## Results

*The effect of the baseline perfusion pressure on the coronary vascular response to PAF.*

The results presented in Table 2 illustrate the effects of bolus injections of 1.0 nmol of PAF on the perfusion pressure of rat hearts which were perfused at two different starting pressures. An initial decrease in perfusion pressure was observed after the injection of PAF and was more pronounced ($P < 0.05$) at the higher starting perfusion pressure. There was no significant difference in the subsequent increase in perfusion pressure or in the time this peak occurred in the two groups. Saline injection did not produce any significant change on the perfusion pressure. The starting pressure therefore was important in obtaining a biphasic response to PAF. Subsequent experiments were conducted at starting pressures of between 65 and 75 mmHg.

*The effects of various amounts of PAF and albumin on the coronary vascular response.*

The effects of various amounts of PAF prepared in saline or saline with 0.25% bovine serum albumin on the perfusion pressure of rat hearts are illustrated by the results presented in Table 3. As with saline alone, injections of saline with 0.25% bovine serum albumin did not produce any significant change on the perfusion pressure. When PAF was prepared in saline without albumin, amounts of less than 10 pmol PAF had no effect on the perfusion pressure (data not shown). Injection of 10 pmol PAF produced only a slight decrease in perfusion pressure which was not followed by an increase in perfusion pressure (Table 3A). The decrease in perfusion pressure following an injection of 10 pmol of PAF peaked at $32.2 \pm 10.1$ sec, which was significantly slower than the results obtained with 100 and 1000 pmol PAF injections.

When PAF was prepared with albumin, it was possible to inject 10 fmol to 1 pmol PAF and elicited a significant decrease in perfusion pressure which was not followed by an increase in perfusion pressure (Table 3B). Injections of 10 and 100 pmol of PAF produced decrases in perfusion pressures which were followed by increases in perfusion pressures. Injections of 1000 pmol PAF prepared in albumin solution did not elicit a decrease in perfusion pressure. The time at which the increases in perfusion pressure peaked were significantly later following injection of 10 pmol PAF prepared in albumin solution, compared with the times obtained with injections of 100 and 1000 pmol of PAF (Table 3B).

To test for the recovery of PAF prepared in saline or saline with 0.25% bovine serum albumin, $^{3}$H-labelled PAF was added before the dilution procedure. The recoveries of PAF based on the radioactivity after dilution to stock solutions for injection of 1000, 100 and 1 pmol of PAF were 57.8±27.8, 39.5±19.5 and 25.8±18.0% when prepared in saline and 95.5±7.5, 90.7±11.0 and 91.3±13.4% when prepared in saline with 0.25 bovine serum albumin (values represent mean ± S.D., n=6). The results showed significant reduction of recovery when PAF was prepared in the absence of albumin.

For comparison, injections of 100 pmol of the hexadecyl-form of PAF prepared in albumin solution were tested. A similar biphasic response was observed (initial decrease, -9.1±7.3 mm Hg and the subsequent increase, 36.8±16.1 mm Hg, n=9). The times of peak changes were 11.7±2.8 and 64.7±25.4 sec respectively. These values were not statistically significant from the values obtained with 100 pmol of PAF prepared from bovine hearts (see Table 3B).

*Comparison of the coronary vascular responses to PAF in rat hearts using constant flow and constant pressure perfusion*

Comparison of the parameters of constant flow and constant pressure perfusion is shown in Table 4 and Table 5. There was no significant difference in the flow rate required to attain a control pressure between 65-75 mm Hg using constant flow perfusion from that using a constant perfusion pressure of 70 mm Hg. Bolus injections of 1 pmol PAF produced only vasodilatation (a decrease in perfusion pressure and an increase in perfusion flow rate in hearts using constant flow and constant pressure perfusion respectively). With a larger amount of PAF (100 pmol, bolus injection), a biphasic response characterized by an initial vasodilatation followed by a vasoconstriction was observed. These results are summarized in Table 4 and 5. The time to reach a peak response for the initial vasodilation and the subsequent vasoconstriction when 100 pmol PAF was administered were similar in hearts perfused under constant flow and constant pressure conditions ($P > 0.05$).

*The effects of repeated injections of PAF in rat hearts*

After a bolus injection of 100 pmol PAF in the perfused rat heart, a second injection of 100 pmol PAF did not produce any significant change in the perfusion pressure as reported previously (Piper and Stewart, 1986). Figure 7 shows the coronary vascular effect of PAF was transient and the perfusion pressure returned to baseline level within 10 min (67 $\pm$ 3 mm Hg before PAF injection and 69 $\pm$ 6 mm Hg 10 min after PAF injection, n=8). The second injection of PAF was therefore given after 10 min of the first injection in the rest of this study. The coronary vascular responses to a second injection of PAF were dependent on the amount of PAF given in the first injection. These results are summarized in Table 6. When 1 pmol PAF was

administered first, a second injection of 10 or 100 pmol PAF resulted in an initial vasodilatation followed by a vasoconstriction. After an initial injection of 1 pmol PAF, the vasodilatation produced by 10 pmol PAF was significantly greater while the vasoconstrictor response was greatly reduced (compare data in Table 6 A and B). The vasoconstriction produced by a second injection of 100 pmol PAF was also reduced when compared to that produced by an initial injection of 100 pmol PAF (22.1 $\pm$ 6.2, n=9 and 29.1 $\pm$ 12.4, n=11 respectively, $P > 0.05$). When 10 pmol PAF was given in the first injection, a second injection of 10 pmol PAF did not produce a significant vasoconstrictor response while a second injection of 100 pmol PAF resulted in a significant vasoconstriction (Table 6). However, the vasoconstriction produced by a second injection of 100 pmol PAF was reduced (18.8 $\pm$ 10.3, n=6, $P < 0.05$ when compared to the effect of an initial injection of 100 pmol PAF). In contrast, if 100 pmol PAF was given in the initial injection, a second injection of PAF (100 and 10,000 pmol) did not elicit any significant vasoconstriction (Table 6).

To determine whether the tachyphylaxis after an injection of 100 pmol PAF was time-dependent, in separate experiments a second injection of 100 pmol PAF was given after 60, 90 or 120 min following a first injection of 100 pmol PAF. The vasodilatation and vasoconstriction by a second injection of 100 pmol PAF was 6.0 $\pm$ 1.7 and 0 mm Hg respectively after 60 min (n=3), 7.7 $\pm$ 2.1 and 0.7 $\pm$ 1.2 mm Hg respectively after 90 min (n=3) and 5.7 $\pm$ 2.1 and 0.7 $\pm$ 0.6 mm Hg respectively after 120 min (n=3). These effects were not statistically different from those obtained with a second injection of 100 pmol PAF, 10 min after the first injection of 100 pmol PAF. The results indicate that there was no time-dependent recovery of the vasoconstrictor response to PAF.

*The coronary vascular responses to bolus injections of PAF and $LTC_4$ in the same isolated perfused rat heart*

To determine the response of the perfused rat heart to repeated exposure to different vasoactive compounds, PAF and $LTC_4$ were administered. The coronary vascular responses of the same hearts to PAF and $LTC_4$ are summarized in Table 7. There was no significant difference in the coronary vascular response to an injection of 100 pmol PAF in hearts with and without a previous administration of 50 pmol $LTC_4$. The vasoconstriction produced by 50 pmol $LTC_4$ was also similar in hearts with or without a previous administration of 100 pmol PAF (Table 7).

*The effects of continuous infusions of PAF on the perfusion pressure.*

Figure 8 illustrates the effects of continuous infusions of a high (600 pmol/min, Figure 8A) and low (40 pmol/min, Figure 8B) amount of PAF on the perfusion pressure. Within the first 30 seconds of infusion of either 600 or 40 pmol PAF/min a decrease in perfusion pressure was observed. This was followed by an increase in perfusion pressure which peaked at about 2 minutes. This increase in perfusion pressure was not sustained. After 10 minutes of infusion with 600 pmol PAF/min the perfusion pressure returned to the pre-infusion level and was not different from control. However as illustrated by Figure 8B the perfusion pressure, although it had declined from the peak value, was still significantly higher than control ($p<0.05$) after 20 minutes of infusion of 40 pmol/min. The time course of perfusion pressure changes with continuous infusion of PAF was slower than for bolus injection. Compared to the changes with continuous infusion of PAF in Figure 8, the perfusion pressures before and after 5 and 10 min of bolus injections of 100 pmol PAF were $65.8\pm3.6$, $67.7\pm7.5$ and $65.7\pm6.8$ mm Hg respectively ($n=12$).

*The effect of PAF in perfused guinea pig hearts*

To determine the coronary vascular effect of PAF in another animal species, experiments were conducted using guinea pig hearts. The results of bolus injections of PAF in guinea-pig hearts using constant flow perfusion are summarized in Table 8. An injection of PAF ranging from 1 fmol to 1 pmol resulted in only a vasoconstrictor response. No vasodilator response was observed with any of the amounts of PAF tested in perfused guinea pig hearts. The magnitude of the vasoconstriction was dose-dependent. Serial dilution was used to obtain very small amount of PAF for injection. The recovery after the dilution was determined by using [$^3$H]PAF and the accuracy of recovery was limited by the specific radioactivity of the PAF used (81 Ci/mmol). The lowest amount of PAF that we were able to measure recovery reliably was 1 fmol. Hence further dilution to obtain a quantity smaller than 1 fmol was not used

## Discussion

Either constant flow or constant pressure perfusion of isolated hearts are feasible in the traditional Langendorff perfusion model. If the flow was maintained constant, any change in vascular resistivity was reflected by a change in the perfusion pressure. This allowed us to continuously monitor pressure changes. This protocol eliminated the need to measure flow during constant pressure perfusion by collecting the effluent over a period of time. Moreover, the collection technique would not detect small changes in flow over a short period of time. The use of constant flow and measuring the perfusion pressure changes had been employed successfully in the studies of Piper and Stewart (1986, 1987). Alternatively, perfusion pressure can be maintained constant and changes in vascular resistance can be measured as changes in flow rate. However, changes in vascular resistance by a vasoactive compound, in particular intense vasoconstriction, could produce relative ischemia which might change the subsequent coronary vascular response. In *in vivo* studies where a small amount of PAF was administered by an intracoronary injection, the perfusion pressure or the systemic blood pressure was usually not affected although the alterations in vascular resistance would result in changes in coronary blood flow. Hearts perfused under a constant pressure would mimic more closely the situation of *in vivo* studies. Since the coronary vascular responses to PAF were similar under constant flow and constant pressure perfusion, it suggests that different modes of perfusion are not a factor in the diverse results of previous studies on the coronary vascular effects of PAF.

The coronary vascular responses to PAF in the isolated perfused heart have been described by previous investigators as vasoconstriction (Levi *et al.*, 1984; Piper and Stewart, 1986, 1987). When PAF was administered by intracoronary injection in intact anesthetized animals, vasoconstriction (Sybertz *et al.*, 1985), vasodilation (Jackson *et*

*al.*, 1986) and biphasic responses (Feuerstein *et al.*, 1984) were observed. In the present study, we demonstrated using the same animal model, that it was possible to produce vasodilation, vasoconstriction and biphasic responses to PAF. Small amounts (10 fmol to 1 pmol prepared in albumin solution) of PAF administered by a bolus injection resulted in a vasodilatory response which was not followed by vasoconstriction. Intermediate amounts (10 and 100 pmol) of PAF produced an initial vasodilation followed by a vasoconstriction response. Large amounts (1000 pmol) elicited only vasoconstriction. A biphasic response was also observed following the bolus injection of 100 pmol of the synthetic hexadecyl-form of PAF.

The initial vasodilation response to PAF was prominent when the baseline starting perfusion pressure was between 65-75 mm Hg. With a perfusion pressure of 40 mm Hg, the vasodilation response was not evident. This effect can be due to the lack of pressure-induced arterial tone at the lower perfusion pressure. These results may explain why this initial vasodilation response was not observed by Piper and Stewart (1986, 1987) since a perfusion pressure of 40 mm Hg was used in their studies. A 2 min collection period of the effluent to determine coronary flow under constant pressure perfusion (Levi *et al.*, 1984) may not have detected the initial vasodilation response since it was brief (usually peaked at less than 20 sec). Continuous flow measurements were made in all intact animal studies and the perfusion pressure would be above 75 mm Hg. The detection of the initial dilation response would be expected. As suggested from the results of the present study, the diversity in the responses to intracoronary injections of PAF was dependent on the amount of PAF used and the inclusion of serum albumin in the preparation of PAF. Our results indicated that if serum albumin is not included in the solution when using small amounts of PAF, significant reduction of the response may occur.

In this study, and in the studies of Piper and Stewart (1986, 1987), repeated injections could not elicit a vasoconstriction response in the isolated perfused heart. It has been suggested that the vasoactive effects of PAF may be mediated via the release of leukotrienes or prostaglandins (Piper and Stewart, 1987), substances which may not be replenished in the isolated heart. The possible explanations also include desensitization of PAF receptors and loss of vascular smooth muscle response to PAF or its mediators. In this study, we found that the vasoconstrictor response to a second injection of PAF was dependent on the amount of PAF given in the second injection as well as the amount of PAF in the initial injection. If the initial injection of PAF was small (1-10 pmol), a second injection of a small amount of PAF (10 pmol) produced only a small vasoconstrictor response while 100 pmol PAF could still produce a significant vasoconstrictor response. However, the presence of a larger amount of PAF (100 pmol) in the initial injection would abolish or greatly diminish the vasoconstrictor response to PAF irrespective of the amount of PAF in the second injection. This indicates that the exposure to a large amount of PAF (100 pmol) in the initial injection of PAF may cause receptor desensitization. This leads to a reduction or elimination of subsequent response to PAF in the perfused rat heart. The exposure to a smaller amount of PAF may produce only partial or incomplete desensitization. Thus a reduction of the coronary vascular effect to a second injection of PAF would be expected. However, repeated intracoronary injections of PAF produced similar vascular responses in *in vivo* studies (Feuerstein *et al.*, 1984; Mehta *et al.*, 1986; Erza *et al.*, 1987; Fiedler *et al.*, 1987). Therefore, receptor desensitization does not occur or occurs slowly in *in vivo* studies. It is also possible that the production of the vasoactive compounds fails to recover after an initial injection of PAF *in vitro* studies, i.e, in the isolated perfused heart, while production of the vasoactive compounds can recover quickly *in vivo* to enable the heart to respond to subsequent injections of PAF. This explanation is also supported by the decline in the response to PAF during

continuous infusion. It has been well documented that eicosanoids, including leukotrienes, are not stored in tissues and cells. They are synthesized *de novo* in response to various stimuli. The rate limiting factor for their synthesis is the availability of arachidonic acid. So the key step of synthesis of PAF-induced vasoactive compounds, such as leukotrienes, is that phospholipase $A_2$ cleaves phospholipids at the $C_2$ position leading to the release of arachidonic acid upon the stimulation. Failure of supply and depletion of arachidonic acid, a inactivation of the enzymes involved in the synthesis, or a combination of these factors could also affect the production of vasoactive compounds. Although the desensitization of PAF receptor is most likely the mechanism for the phenomenon of tachyphylaxis, the possibility of failure of reproduction of the vasoactive compounds can not be ruled out from present study.

It has been suggested that the coronary vasoconstrictor effect of PAF was indirectly mediated by the release of $LTC_4$ (Piper and Stewart, 1986), and leukotrienes including $LTC_4$ have been shown to possess vasoconstrictor properties. The role of $LTC_4$ in mediating the coronary vasoconstrictor effect of PAF was further confirmed by the detection and quantity of $LTC_4$ in the coronary effluent after the administration of PAF in the perfused heart (Piper and Stewart, 1986). In order to determine whether the reduced response to a second injection of PAF was due to an altered coronary vascular response to leukotrienes, the effect of $LTC_4$ was examined. Our results indicated that there was no change in the coronary vascular responses to PAF or $LTC_4$ which were injected into the same perfused rat heart irrespective of the injection order of these two compounds. In spite of a significant loss of vasoconstrictor response to a second administration of PAF after a first injection of PAF in the perfused rat heart, our data show that the heart was still capable of generating a normal vasoconstriction with other vasoconstrictive compounds such as $LTC_4$. Therefore, the mechanism for a

reduction or elimination of the PAF response after an initial injection of PAF can not be due to a failure of the coronary vascular smooth muscle to constrict in response to $LTC_4$.

In the experiments with perfused guinea-pig heart, PAF only elicited a vasoconstrictor response and the vasodilator response was not observed. The lowest amount of PAF tested (1 fmol) was still capable of producing a significant vasoconstrictor response in the perfused guinea pig heart. However, the results from the present study show that 10 pmol of PAF was needed to elicit a significant coronary vasoconstrictor effect in the rat heart. These results are consistent with the report that the guinea-pig heart was more sensitive to the cardiac action of PAF than the rat heart (Letts and Piper, 1983; Lefer and Roth, 1985). In this regard, rat platelets had been shown to be insensitive to PAF due to a lack of PAF receptors while platelets from other animals including rabbit, dog, guinea-pig and sheep were sensitive to PAF (Namm *et al.*, 1982; Cargill *et al.*, 1983). Our results suggest that animal species differences may be a contributing factor to the reported diversity coronary vascular responses to PAF.

With respect to the coronary vasoconstriction produced by PAF, the role of leukotrienes, $LTC_4$ and $LTD_4$, was confirmed by the release of $LTC_4$-like bioactivity in the coronary effluent and the quantitation of $LTC_4$ by radioimmunoassay (Piper and Stewart, 1986). The initial vasodilation produced by PAF was not observed in many studies and it is not clear what might be the underlying mechanism. Prostaglandins (such as, prostacyclin) and some other leukotrienes (such as, $LTB_4$) can cause coronary vasodilation. However, their possible role in PAF-induced coronary vascular responses will be investigated and discussed in the following sections.

In conclusion, this study demonstrates the effects of PAF on the coronary vasculature and the conditions which precipitate diversities in the response. The diverse coronary vascular responses to PAF can also be observed between various animal species, but are not due to the different perfusion models in isolated heart studies. The results suggest that it is necessary to study carefully the dose response to PAF. In addition, PAF should be prepared in a solution containing serum albumin to ensure that the desired amounts of PAF are delivered to the system under study. The data also suggest that the depletion of vasoactive mediators induced by PAF and/or receptor desensitization may be the mechanism(s) by for the failure vasoconstrictor response after repeated injections of PAF in perfused hearts. However, the mechanism(s) for the desensitization of the PAF receptor or for the depletion of vasoactive mediators after exposure to PAF remains to be elucidated.

**Table 2**

**The effects of bolus injection of 1.0 nmol PAF on the perfusion pressure of rat heart perfused at different starting pressure**

| Initial pressure (mm Hg) | Decrease in perfusion pressure | | Increase in perfusion pressure | | Flow rate | heart rate (beats/min) | n |
|---|---|---|---|---|---|---|---|
| | Maximum (mm Hg) | Time (s) | Maximum (mm Hg) | Time (s) | (ml/min/g heart wt) | | |
| 42.5 ± 2.7 | -2.5 ± 2.2 | 11.4 ± 2.2 | 25.2 ± 11.1** | 90.0 ± 16.4 | 6.7 ± 1.5 | 263 ± 21 | 6 |
| 74.3 ± 4.0 | -8.8 ± 6.3**b | 13.0 ± 4.2 | 31.6 ± 11.4** | 80.0 ± 17.8 | 14.9 ± 2.9b | 296 ± 43 | 8 |

PAF was prepared in 0.1 ml of 0.9% NaCl without albumin; results are expressed as mean ± SD.

a One value was excluded because there was no decrease in perfusion pressure.

b $P<0.05$ when compared with the results of the group with a starting pressure of 42.5 mm Hg.

** $P<0.01$, compared to the pre-injection values.

**Table 3**

**The effects of various amounts of PAF prepared in saline or saline with 0.25% bovine serum albumin on the perfusion pressure of rat hearts**

| Amount of PAF (pmol) | Control Pressure (mm Hg) | Decrease in Perfusion Pressure | | Increase in Perfusion Pressure | | n |
|---|---|---|---|---|---|---|
| | | Maximum (mm Hg) | Time (s) | Maximum (mm Hg) | Time (s) | |
| (A) Prepared in saline without albumin | | | | | | |
| 10 | 68.5 ± 2.6 | -6.3 ± 1.7** | 32.2 ± 10.1 [a] | N.C. | N.C. | 4 |
| 100 | 68.4 ± 3.0 | -9.0 ± 6.2** | 15.0 ± 4.4 | 21.5 ± 8.1** | 87.7 ± 7.4 | 6 |
| 1000 | 74.3 ± 4.0 | -8.8 ± 6.3** | 13.0 ± 4.2 | 31.6 ± 11.4** | 80.6 ± 17.8 | 8 |
| (B) Prepared in saline with 0.25% bovine serum albumin | | | | | | |
| 0.001 | 68.0 ± 1.7 | -2.0 ± 1.7 | 25.0 ± 5.0 | N.C | N.C. | 3 |
| 0.01 | 68.6 ± 5.1 | -4.6 ± 1.2** | 17.1 ± 6.9 | N.C. | N.C. | 8 |
| 0.1 | 71.5 ± 4.7 | -6.1 ± 1.8** | 16.0 ± 3.8 | N.C. | N.C. | 7 |
| 1.0 | 69.6 ± 3.9 | -9.0 ± 7.1** | 21.6 ± 8.9 | N.C. | N.C. | 18 |
| 10 | 67.9 ± 3.2 | -7.6 ± 6.9** | 15.8 ± 7.2 | 19.5 ± 13.8** | 86.2 ± 17.5 [a] | 18 |
| 100 | 65.8 ± 3.6 | -7.9 ± 6.1** | 10.1 ± 4.2 | 25.7 ± 12.3** | 63.9 ± 23.4 | 12 |
| 1000 | 71.0 ± 1.4 | N.C. | N.C. | 33.0 ± 16.0** | 45.0 ± 10.6 | 5 |

[a] $P < 0.01$ when compared with the results of the 100 and 1000 pmol groups.

* $P < 0.05$, ** $P < 0,01$, compared to the pre-injection values.

N.C. denotes no detectable change.

**Table 4**

**The effects of PAF under constant flow perfusion** [a]

| Amount of PAF | Control flow rate (ml/min/g heart) | Decrease in perfusion pressure | | Increase in perfusion pressure | | n |
|---|---|---|---|---|---|---|
| | | Maximum (mm Hg) | Time to peak (s) | Maximum (mm Hg) | Time to peak (s) | |
| 1 pmol | 12.8 ± 1.7 | -6.3 ± 2.5 | 22 ± 7 | - | - | 6 |
| 100 pmol | 12.1 ± 0.9 | -6.0 ± 4.1 | 9 ± 3 | 27.5 ± 8.2 | 60 ± 15 | 6 |

[a] Rat hearts were perfused as described in Materials and Methods. In these experiments, the flow rate was kept constant and changes in perfusion pressure were detected by a pressure transducer. Values represent mean ± S.D. - denotes no detectable change.

**Table 5**

**The effects of PAF under constant pressure perfusion** [a]

| Amount of PAF | Control flow rate (ml/min/g heart) | Increase in perfusion flow | | Decrease in perfusion flow | | n |
|---|---|---|---|---|---|---|
| | | Maxmum (ml/min/g heart) | Time to peak (s) | Maximum (ml/min/g heart) | Time to peak (s) | |
| 1 pmol | 13.0 ± 3.7 | 2.3 ± 1.2 | 27 ± 7 | - | - | 6 |
| 100 pmol | 12.8 ± 2.8 | 2.0 ± 1.4 | 13 ± 4 | -6.1 ± 2.5 | 45 ± 10 | 6 |

[a] Rat hearts were perfused as described in Materials and Methods. A constant perfusion pressure of 70 mm Hg was used in these experiments and changes in flow rate were detected by an electromagnetic flow meter. Values represent mean ± S.D. - denotes no detectable change.

**Table 6**

**The effects of a second bolus injection of PAF on the perfusion pressure of rat hearts** [a]

| Injection | Amount of PAF (pmol) | Control pressure (mm Hg) | Decrease in perfusion pressure | | Increase in perfusion pressure | | n |
|---|---|---|---|---|---|---|---|
| | | | Maximum (mm Hg) | Time to peak (s) | Maximum (mm Hg) | Time to peak (s) | |
| (A) | | | | | | | |
| First injection | 1 | 70 ± 4 | -5.3 ± 4.0 | 23 ± 5 | - | - | |
| Second injection | 10 | 71 ± 1 | -12.7 ± 7.4 [b] | 25 ± 5 | 8.0 ± 9.8 [c] | 94 ± 8 | 3 |
| First injection | 1 | 70 ± 2 | -6.8 ± 3.8 | 25 ± 13 | - | - | |
| Second injection | 100 | 68 ± 4 | -5.8 ± 4.1 | 10 ± 3 | 22.1 ± 6.2 [d] | 65 ± 29 | 9 |
| (B) | | | | | | | |
| First injection | 10 | 67 ± 1 | -8.3 ± 2.9 | 14 ± 5 | 20.9 ± 9.9 | 95 ± 10 | |
| Second injection | 10 | 66 ± 2 | -6.0 ± 2.2 | 11 ± 3 | 1.5 ± 2.1 [d] | 93 ± 12 | 9 |
| First injection | 10 | 70 ± 2 | -8.3 ± 9.3 | 15 ± 4 | 25.0 ± 10.6 | 83 ± 15 | |
| Second injection | 100 | 70 ± 2 | -6.5 ± 6.2 | 15 ± 8 | 18.8 ± 10.3 | 83 ± 21 | 6 |
| (C) | | | | | | | |
| First injection | 100 | 67 ± 3 | -5.4 ± 3.9 | 10 ± 5 | 28.3 ± 12.6 | 76 ± 19 | |
| Second injection | 100 | 69 ± 6 | -4.1 ± 4.1 | 26 ± 17 | 1.3 ± 1.9 [d] | *e* | 7 |
| First injection | 100 | 67 ± 1 | -6.0 ± 8 | 11 ± 3 | 30.5 ± 9.3 | 53 ± 3 | |
| Second injection | 10,000 | 71 ± 4 | -5.5 ± 3.3 | 20 ± 4 | 3.3 ± 1.5 [c] | 71 ± 12 | 4 |

[a] Hearts were perfused using a constant flow rate. Values represent mean ± S.D. - denotes no detectable change.
[b] $P < 0.05$, [c] $P < 0.01$ and [d] $P < 0.001$ when compared to the values after the first injection of PAF.
[e] Increases in perfusion were small and the time to peak effect cannot be accurately measured.

**Table 7**

**The effects of injection order of PAF and $LTC_4$ on the perfusion pressure of rat hearts** [a]

| Compound | Control perfusion pressure (mm Hg) | Decrease in perfusion pressure | | Increase in perfusion pressure | | n |
|---|---|---|---|---|---|---|
| | | Maximum (mm Hg) | Time to peak (s) | Maximum (mm Hg) | Time to peak (s) | |
| First injection PAF (100 pmol) | 69 ± 3 | -12.7 ± 12.5 | 13 ± 6 | 22.9 ± 9.0 [b] | 76 ± 22 | |
| Second injection $LTC_4$ (50 pmol) | 69 ± 6 | - | - | 41.0 ± 31.2 [c] | 9 ± 1 | 3 |
| First injection $LTC_4$ (50 pmol) | 69 ± 6 | - | - | 41.2 ± 26 | 17 ± 11 | |
| Second injection PAF (100 pmol) | 69 ± 4 | -5.0 ± 4.2 | 10 ± 1 | 29.4 ± 24.4 | 44 ± 21 | 5 |

[a] Hearts were perfused using a constant flow rate. Values represent mean ± S.D. - denotes no detectable change.

[b] No statistical difference in the increase in perfusion pressure was detected when PAF was injected first or when PAF was injected second, 10 min after a first injection of $LTC_4$.

[c] No statistical difference in the increase in perfusion pressure was detected when $LTC_4$ was injected first or when $LTC_4$ was injected second, 10 min after a first injection of PAF.

**Table 8**

**The effects of a bolus injection of PAF in perfused guinea pig hearts** [a]

| Amount of PAF | Control perfusion pressure (mm Hg) | Decrease in perfuson pressure | | Increase in perfusion pressure | | n |
|---|---|---|---|---|---|---|
| | | Maximum (mm Hg) | Time to peak (s) | Maximum (mm Hg) | Time to peak (s) | |
| 1 fmol | 73 ± 1 | - | - | 8.2 ± 2.7 [b] | 64 ± 37 | 5 |
| 10 fmol | 72 ± 2 | - | - | 12.0 ± 5.3 [b] | 83 ± 33 | 10 |
| 1 pmol | 73 ± 2 | - | - | 21.4 ± 7.9 [b] | 56 ± 35 | 5 |

[a] Guinea pig hearts were perfused using a constant flow rate as described in Materials and Methods. Values represent mean ± S.D. - denotes no detectable change.

[b] $P < 0.05$ when compared to controls (injections of 0.1 ml saline).

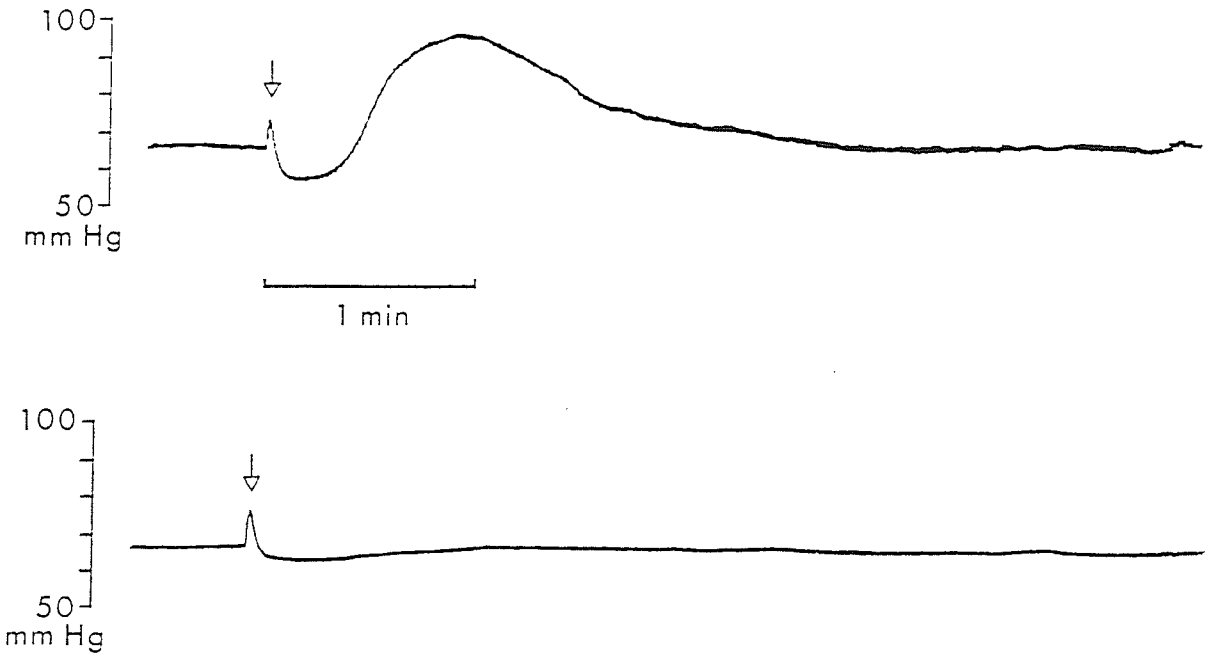



**Figure 7.** Original tracings showing the changes in perfusion pressure in a perfused rat heart when 100 pmol PAF was injected first (top tracing) and 10 min later (bottom tracing). Arrow indicates the time when 100 pmol PAF was given as a bolus injection (a small injection artifact is displayed on the tracing). A first injection of 100 pmol PAF produced an initial vasodilatation followed by a vasoconstriction (top tracing). Perfusion pressure returned to preinjection level in approximately 5 min. No significant change in perfusion pressure was observed when a second injection of PAF was administered 10 min later (bottom tracing).

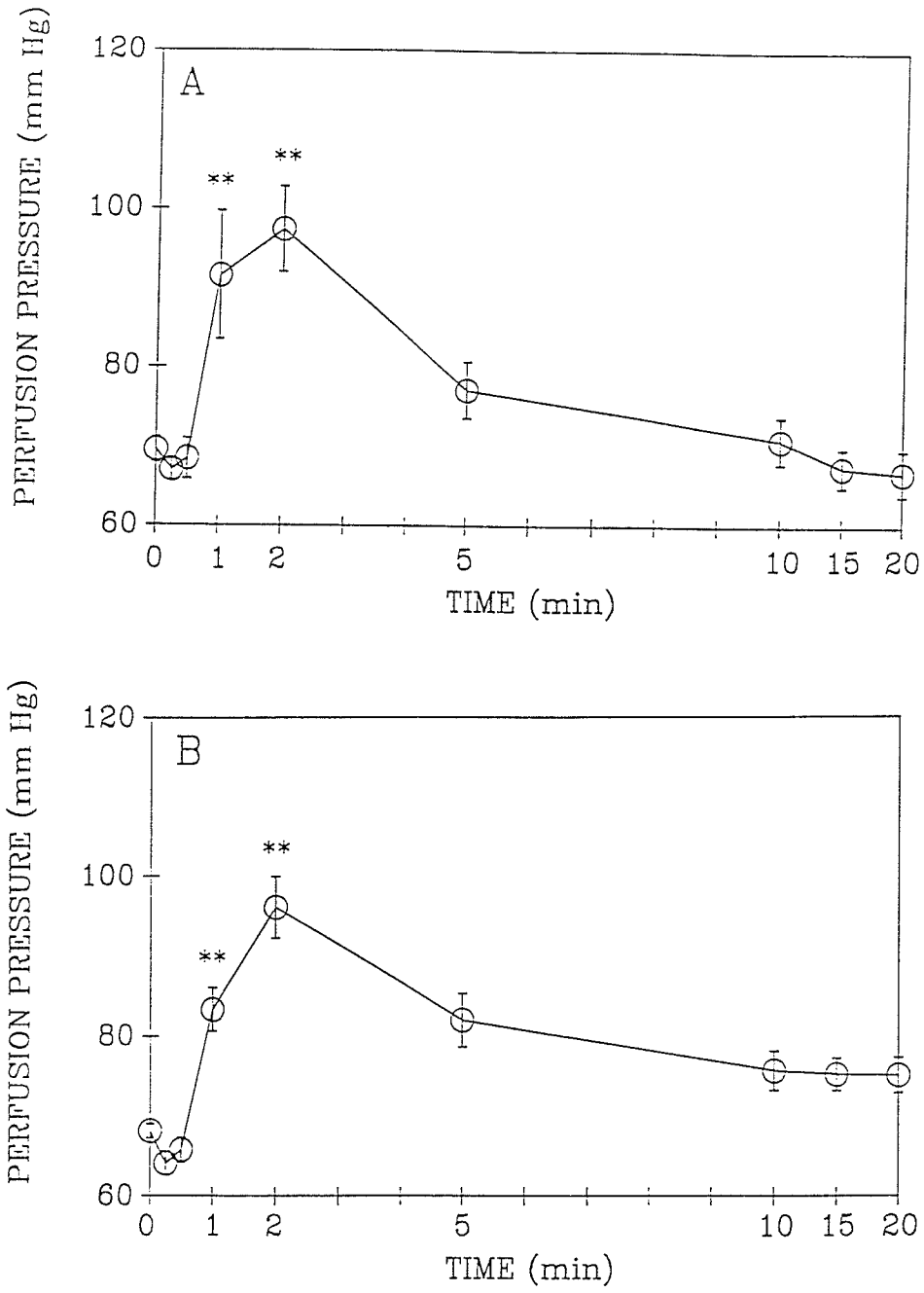



**Figure 8.** The effects of continuous infusions of PAF on the perfusion pressure of perfused rat hearts. Panel A represents the effects of an infusion of 600 pmol PAF/min (n=8). Panel B represents the effects of an infusion of 40 pmol PAF/min (n=12). In both panels values are expressed as means ± standard error of the means, * represents $P < 0.05$, and ** represents $P < 0.01$ when compared to the initial perfusion pressure. PAF was prepared in a saline solution containing 0.25% bovine serum albumin.

# Part 3

# Mechanisms of the Coronary Vascular Effects of Platelet-activating Factor in the Perfused Rat Heart

**Abstract**

The results from the previous section demonstrated that bolus injections of platelet-activating factor (PAF) in the perfused rat heart resulted in coronary vasodilatation, vasoconstriction and the combination of both depending on the amount of PAF that was injected. In the present study, the mechanisms of these coronary vascular effects of PAF in the perfused rat heart were investigated with a number of pharmacological antagonists and inhibitors.

Selectivity for blocking the coronary vasodilator and vasoconstrictor effects of platelet-activating factor (PAF) in the perfused rat heart was observed with different PAF antagonists. CV-6209 showed selectivity for blocking the vasodilator effect of PAF and a higher concentration (10 fold) was required to block the vasoconstrictor effect. The remaining PAF antagonists (FR-900452, WEB 2086 and BN-50739) showed selectivity for blocking the vasoconstrictor effect of PAF (10, 200 and 1000 fold respectively). A combination of low concentrations of CV-6209 (10 nM) with FR-900452 (5 $\mu$M) or WEB 2086 (0.5 $\mu$M) was effective in blocking both the vasodilator and vasoconstrictor effects of PAF. These results support the hypothesis that the functionally opposite effects of PAF in the perfused heart are mediated by different PAF receptor subtypes.

The cyclooxygenase inhibitor, indomethacin, did not modify the coronary vascular effects of PAF. However L-649,923 (a leukotriene antagonist) and MK-886 (a leukotriene synthesis inhibitor) eliminated both the vasodilator and vasoconstrictor effects of PAF. The concentrations of leukotrienes from the effluent of perfused heart measured by radioimmunoassay were significantly increased after an injection of PAF. Pretreatment of isolated heart with PAF antagonist, CV-6209, abolished the increase in

leukotrienes induced by PAF. When leukotrienes (LT) were administered by bolus injection in the perfused rat heart, $LTB_4$ produced vasodilatation while $LTC_4$ and $LTD_4$ produced vasoconstriction. L-649,923 blocked both vasodilator and vasoconstrictor effects of the leukotrienes tested. The results suggest that lipoxygenase products are responsible for both the vasodilator and vasoconstrictor actions of PAF in the coronary vasculature of the perfused rat heart while the cyclooxygenase products do not play a significant role.

## Introduction

The effects of PAF on the coronary circulation have been examined. However, the results of these studies are inconsistent. In the previous section, it has been demonstrated that depending on the amount of PAF, bolus injections of PAF could produce vasodilatation (10 fmol - 1 pmol PAF), vasoconstriction (1 nmol PAF) and a biphasic response (10 and 100 pmol PAF) in perfused rat hearts (see results of Part 2). It has been hypothesized that the functionally opposite effects of PAF in the perfused heart are mediated by different PAF receptor subtypes. The activation of the first subtype of PAF receptor produces vasodilatation while the activation of the second subtype of PAF receptor produces vasoconstriction.

The studies of Piper and Stewart (1986, 1987) clearly identified the presence of 6-keto-$PGF_{1\alpha}$, thromboxane $B_2$ and leukotriene-like bioactivity in the effluent of the perfused heart after PAF administration. However, only a vasoconstrictor response was observed in these studies. Hence, it is not clear what role these vasoactive compounds will have in mediating the effects of PAF, especially the vasodilator response. In the present study, the mechanisms for coronary vasodilator, vasoconstrictor and biphasic responses to PAF were examined. The coronary vascular responses to PAF were determined in the absence and presence of PAF antagonists. The cyclooxygenase inhibitor (indomethacin), the leukotriene antagonist (L-649,923, Jones *et al.*, 1986) and the leukotriene synthesis inhibitor (MK-886, Gillard *et al.*, 1989; Rouzer *et al.*, 1990) were tested for their ability to inhibit the coronary vascular effects of PAF. The concentrations of leukotrienes in the effluent from perfused rat heart were measured by specific radioimmunoassay. The coronary vascular effects of leukotrienes and their ability to mimick the coronary vascular effects of PAF were also examined.

## Methods and Materials

*Rat heart perfusion*

Following cervical dislocation, hearts from Sprague-Dawley rats (250 - 350 g) were rapidly excised and placed in cool Krebs-Henseleit solution (4°C) oxygenated with 95% $O_2$ - 5% $CO_2$. The solution had the following composition (mM): NaCl 120, $NaH_2PO_4$ 1.18, $MgSO_4$ 1.18, KCl 4.76, $CaCl_2$ 1.25, $NaHCO_3$ 25.0, and glucose 5.5. The aorta was cannulated for coronary perfusion. The heart was allowed to beat spontaneously. The temperature of the perfusate was maintained at 37 $\pm$ 0.5°C and the coronary flow was controlled by a roller pump. The perfusion pressure was measured by a pressure transducer attached to a side arm of the aortic cannula. The perfusion pressure was recorded on a Gould chart recorder and monitored on a digital display.

*Drug preparation and administration*

Hearts were equilibrated with Krebs-Henseleit solution for 20 min. During the equilibration period, the flow rate was adjusted to obtain a control perfusion pressure of 65 - 75 mm Hg. This perfusion pressure range was selected based on our previous experience that the vasodilator response was more prominent under this condition than at lower perfusion pressures (see results in Part 2). In experiments which required a drug pretreatment, 10 min of stabilization with Krebs-Henseleit solution was followed by 10 min of pretreatment with Krebs-Henseleit solution containing a PAF antagonist, or a leukotriene antagonist, and continued throughout the administration of PAF. The cyclooxygenase inhibitor, indomethacin, was added to the perfusion solution at the start of the stabilization period and the effect of PAF injection was tested after 45 min of

perfusion in the presence of indomethacin. All stock solutions were made fresh daily and kept at 4°C between experiments. The leukotriene antagonist, L-649,923 (sodium (ß*S**, *R**)-4-(3-4(-acetyl-4-hydroxy-2-propylphenoxy)-propylthio)-*r*-hydroxy-ß-methylbenzenebutanoate, Merck Frosst Canada Inc.) and the leukotriene synthesis inhibitor, MK-886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid, Merck Frosst Canada Inc.) were dissolved in distilled water and then diluted in Krebs-Henseleit solution to a concentration of 0.1 - 5 $\mu$M and 1 $\mu$M respectively. CV-6209 (2[N-acetyl-N-(2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl)-aminomethyl]-1-ethylpyridinium chloride) was dissolved in saline (0.9% NaCl) containing 0.25% bovine serum albumin (BSA, Sigma Chemical Co.). WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]-triazolo-[4,3-a][1,4]-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone) was dissolved in saline. BN-50739 (tetrahydro-4,7,8,10 methyl-(chloro-2 phenyl)-6[dimethoxy-3,4-phenyl)thio]methythiocarbonyl-9 pyrido[4'3'-4,5]thieno[3,2-f]triazolo-1,2,4[4,3-a]diazepine-1,4) was dissolved in dimethyl sulfoxide (DMSO) and slowly added to warm (50°C) Krebs-Henseleit solution while stirring constantly to a maximum final concentration of 10 $\mu$M in 0.4% DMSO. BN-52021 (9H-1,7a-(epoxymethano)-1H,6aH-cyclopenta(c)furo(2,3-b)furo-(3',2':3,4)cyclopenta-(1,2-d)furan-5,9,12-(4H)-trione, 3-tert-butylhexahydro-8-methyl, Institut Henri Beaufour, France) was dissolved in dimethyl sulfoxide (DMSO) and slowly added to warm (50°C) Krebs-Henseleit solution while stirring constantly to a final concentration of 30 $\mu$M in 0.4% DMSO. FR-900452 (1-methyl-3-(1-(5-methylthiomethyl-6-oxo-3-(2-oxo-3-cyclopenten-1-ylidene)-2-piperazinyl)ethyl)-2-indolinone, Fujisawa Pharmaceutical Co., Japan) was dissolved in ethanol then diluted in Krebs-Henseleit solution to a final concentration of 5 $\mu$M in 0.1% ethanol. Indomethacin (Sigma Chemical Co.) was dissolved in 0.1 M $Na_2CO_3$ solution and diluted in Krebs-Henseleit solution to a final concentration of 2.8 $\mu$M. Experiments were also conducted to test the effects of 10

min pretreatment with Krebs-Henseleit solutions containing 0.4% DMSO and 0.1% ethanol on the coronary vascular response to PAF. PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (prepared from bovine heart, Sigma Chemical Co.) was prepared fresh daily in saline (0.9% NaCl) containing 0.25% bovine serum albumin (Sigma Chemical Co.). Only one injection of PAF was given to each heart. Bolus injections of PAF were given in a volume of 0.1 ml and over a one second period, into the perfusion line 5-6 cm proximal to the aortic cannula. Changes in perfusion pressure and the time at which maximum changes occurred were recorded. The leukotrienes (LT), $LTB_4$, $LTC_4$ and $LTD_4$ (Merck Frosst Canada Inc.), were diluted daily in saline (0.9% NaCl) to the appropriate amounts for bolus injections of 0.1 ml.

*Radioimmunoassay for leukotrienes*

Leukotrienes were determined by a competitive binding radioimmunoassay using a commercially available kit from Amersham International (U.K.). Samples of effluent from perfused isolated rat heart for periods of 0-1, 1-2, 2-5 and 5-10 min after bolus injection of 100 pmol of PAF were collected into test tubes. The hearts were pretreated with the PAF antagonist, CV-6209 (100 nM), or the leukotriene synthesis inhibitor, MK 886 (10 $\mu$M) as described in the previous section. The test tubes containing samples were capped and stored at -20°C. The concentration of leukotrienes in the effluent from perfused rat heart was determined without prior extraction or purification.

Leukotriene $B_4$: Briefly, 100 $\mu$l of effluent from perfused rat heart was mixed with 100 $\mu$l of [5, 6, 8, 9, 11, 12, 14, 15 (n)-$^3$H] leukotriene $LTB_4$, followed by 100 $\mu$l of specific antiserum to the leukotrienes and 100 $\mu$l of assay buffer. The mixture was incubated overnight at 4°C. Then 200 $\mu$l of dextran-coated charcoal was added to

the mixture. Each tube was mixed well immediately after the addition of dextran-coated charcoal and all tubes were left at 25°C for 5 min to remove unbound leukotrienes. After centrifugation at 2,000 g for 10 min at 4°C, the supernatant was decanted into 10 ml of aqueous scintillation fluid and the radioactivity measured in a ß-scintillation counter (Beckmann). Known amounts of $LTB_4$ were used to construct a standard curve and the concentration of $LTB_4$ in each sample was determined from the standard curve. The sensitivity of the $LTB_4$ assay was 1.6 pg/tube.

Leukotriene $C_4$: 100 $\mu$l of effluent from perfused rat heart was mixed with 100 $\mu$l of [5, 6, 8, 9, 11, 12, 14, 15 (n)-$^3$H] leukotriene $C_4$, followed by 100 $\mu$l of specific antiserum to the leukotrienes and 100 $\mu$l of assay buffer. The mixture was incubated overnight at 4°C. Then 500 $\mu$l of dextran-coated charcoal was added to the mixture in an ice-bath for 10 min to remove unbound leukotrienes. After centrifugation at 2,000 g for 10 min at 4°C, the supernatant was decanted into 10 ml of aqueous scintillation fluid and the radioactivity measured in a ß-scintillation counter (Beckmann). The concentration of $LTC_4$ in each sample was determined from the standard curve. The sensitivity of the $LTC_4$ assay was 8 pg/tube.

Leukotriene $C_4/D_4/E_4$: 100 $\mu$l of effluent from perfused rat heart was mixed with 100 $\mu$l of [14, 15 (n)-$^3$H] $LTC_4$, followed by 100 $\mu$l of specific antiserum to the leukotrienes and 100 $\mu$l of assay buffer. The mixture was incubated overnight at 4°C. Then 250 $\mu$l of dextran-coated charcoal was added to the mixture in an ice-bath for 15 min to remove unbound leukotrienes. After centrifugation at 2,000 g for 15 min at 4°C, the supernatant was decanted into 10 ml of aqueous scintillation fluid and the radioactivity measured in a ß-scintillation counter (Beckmann). The concentration of leukotrienes in each sample was determined using a standard curve. The sensitivity for $LTC_4$ in this assay was 12.5 pg/tube. The cross-reactivity for $LTD_4$ was 181.8% and

the cross-reactivity for $LTE_4$ was 92.7%. Calculation of $LTD_4$ is that $LTD_4$ concentration equals the value obtained from $LTC_4$ standard curve divided by % cross-reactivity of $LTD_4$ and then multiplying by 100%.

*Statistical Analysis*

Data were analyzed by the Students t-test and analysis of variance (ANOVA) followed by Duncan's test where appropriate. Values are expressed as means $\pm$ standard deviation (SD) and $P < 0.05$ was considered statistically significant.

## Results

*The effects of adding various pharmacological agents to Krebs-Henseleit solution on the perfusion parameters of rat hearts.*

In order to examine the mechanism of the coronary vascular effects of PAF in the perfused rat heart, pretreatments with various pharmacological agents were used. Table 9 summarizes the effects of various additions to Krebs-Henseleit solution on some of the baseline parameters. The flow rate was adjusted in these experiments to attain a perfusion pressure between 65 - 75 mm Hg. The flow rates in the presence of ethanol, DMSO, indomethacin, L-649,923 and MK-886 were not significantly different from the flow rate of the group perfused with normal Krebs-Henseleit only. The perfusion pressure of the group with BN-52021, although significantly higher than the group perfused with Krebs-Henseleit, was not different from the corresponding group receiving the vehicle 0.4% DMSO. However, the perfusion pressure and heart rate of the group with indomethacin was higher than the corresponding group perfused with normal Krebs-Henseleit only.

*The initial results with the PAF antagonists, FR-900452 and BN-52021, on the response of rat heart to PAF.*

The effects of 1 and 100 pmol PAF on the perfusion pressure were determined in hearts pretreated with the PAF antagonists, FR-900452 and BN-52021. The results are summarized in Table 10. Bolus injections of 1 pmol PAF in hearts perfused with normal Krebs-Henseleit solution or with the vehicles 0.4% DMSO and 0.1% ethanol resulted in decreases in perfusion pressure which peaked within approximately 30 s. FR-900452 (5.0 $\mu$M) and BN-52021 (30 $\mu$M) had no effect on the perfusion pressure

response to 1 pmol PAF (Table 10). Bolus injections of 100 pmol PAF resulted in biphasic responses in the control group as well as in groups where 0.1% ethanol or 0.4% DMSO was added to Krebs-Henseleit solution. In these experiments, the initial decrease in perfusion pressure was followed by an increase in perfusion pressure which peaked at approximately 1 min after the bolus injection. Both FR-900452 (5.0 $\mu$M) and BN-52021 (30 $\mu$M) abolished the increase in perfusion pressure. FR-900452 and BN-52021 appeared to enhance the vasodilator component of the response to 100 pmol PAF in these groups (approximately two times greater than their control groups, $P < 0.05$).

These concentrations of FR-900452 and BN-52021 have been demonstrated to antagonize the platelet aggregating effect of PAF (Okamoto *et al.*, 1986; Nunez *et al.*, 1986). Higher concentrations of these compounds were not tested in these initial experiments due to their limited solubility in aqueous solution, the need to use solvents as vehicles and the limited availability of larger quantities of these compounds.

*Further study on the effects of PAF antagonists, CV-6209, FR-900452, WEB 2086 and BN-50739 on the response of rat hearts to PAF.*

The availability of other PAF antagonists and also a larger quantity of FR-900452 enabled us to examine the effects of pretreatment with various concentrations of PAF antagonists on the coronary vascular effects of 100 pmol PAF in the perfused rat heart. Pretreatment with low concentrations of the PAF antagonist CV-6209 (10 and 50 nM) were effective in blocking the vasodilator effect of 100 pmol PAF and a higher concentration (100 nM) was required to abolish the vasoconstrictor effect (Table 11). Pretreatment with the other PAF antagonists showed a reverse pattern. The vasoconstrictor effect of PAF was abolished by a low concentration of FR-900452 (5

$\mu$M), WEB 2086 (0.5 $\mu$M) and BN-50739 (0.01 $\mu$M) while higher concentrations (50 $\mu$M FR-900452, 100 $\mu$M WEB 2086 and 10 $\mu$M BN-50739) were required to completely abolish the vasodilator effect (Table 11). When the vasoconstrictor effect of PAF was abolished by a low concentration of FR-900452, WEB 2086 or BN-50739, a significantly greater vasodilator effect was observed. Pretreatment with 0.1% ethanol and 0.4% DMSO (the vehicle for FR-900452 and BN-50739 respectively) did not alter the effects of 100 pmol PAF. Bolus injections of 0.1 ml saline did not show any detectable coronary vascular effect. A combination of low concentrations of CV-6209 (10 nM) with FR-900452 (5 $\mu$M) or WEB 2086 (0.5 $\mu$M) was effective in blocking both the vasodilator and vasoconstrictor effects of 100 pmol PAF in the perfused rat heart (Table 12). Using the data from Table 11, approximately 10 fold selectivity for blocking the vasodilator effect of PAF than for the vasoconstrictor effect of PAF was observed for CV-6209 while a 10, 200 and 1000 fold selectivity for blocking the vasoconstrictor effect was observed for FR-900452, WEB 2086 and BN-50739.

*The effects of indomethacin, L-649,923 and MK-886 on the response of rat hearts to PAF.*

The addition of indomethacin, 2.8 $\mu$M, to Krebs-Henseleit solution did not significantly affect the response to bolus injections of 100 pmol PAF (Table 13). The coronary vascular effects PAF were also examined in the presence of the leukotriene antagonist, L-649,923 (0.1 - 5 $\mu$M). The results are summarized in Table 13. Statistically significant reduction of the decrease in perfusion pressure by PAF was observed with 1 and 5 $\mu$M of L-649,923 while significant reductions of the increases in perfusion pressure were observed with 0.1 - 5 $\mu$M of L-649,923. When the highest concentration of L-649,923 (5 $\mu$M) was used, both the initial decrease and the

subsequent increase in the perfusion pressure were abolished. MK-886 (1 $\mu$M) also significantly attenuated the changes in perfusion pressure produced by PAF (Table 13).

*The effects of $LTC_4$, $LTD_4$ and $LTB_4$ on the perfusion pressure of rat hearts.*

The results of bolus injections of $LTC_4$ and $LTD_4$ are summarized in Table 14. Only rapid increases in perfusion pressure (within 15 s) were observed. Statistical analysis showed that the increase in perfusion pressure following $LTC_4$ was dose-dependent ($P < 0.001$). The increase in perfusion pressure following bolus injections of $LTD_4$ was also dose-dependent ($P < 0.01$). The results of bolus injections of $LTB_4$ are summarized in Table 14. The decrease in perfusion pressure in the presence of $LTB_4$ was dose-dependent ($P < 0.05$).

The effects of bolus injections of $LTB_4$, $LTC_4$ and $LTD_4$ were studied in hearts pretreated with 5 $\mu$M L-649,923 and compared with the effects on the perfusion pressure in the absence of L-649,923 as shown in Tables 14. Pretreatment with of L-649,923 (5 $\mu$M) significantly attenuated the decrease in perfusion pressure following bolus injections of 100 pmol $LTB_4$ (12.4 $\pm$ 10.6 vs 1.4 $\pm$ 1.0 mm Hg, n=10 and 9 in the absence and presence of L-649,923 respectively, $P < 0.01$). The increases in perfusion pressure following bolus injections of 100 pmol $LTC_4$ and 100 pmol $LTD_4$ were also attenuated in hearts pretreated with 5 $\mu$M L-649,923 (70.0 $\pm$ 9.1 vs 3.2 $\pm$ 1.8 mm Hg, n=4 and 5 in the absence and presence of L-649,923 respectively for $LTC_4$, $P < 0.01$, and 39.8 $\pm$ 10.4 vs 5.5 $\pm$ 4.4 mm Hg, n=4 and 4 in the absence and presence of L-649,923 respectively for $LTD_4$, $P < 0.01$).

The effects of bolus injections of $LTB_4$, $LTC_4$ and $LTD_4$ were also studied in hearts pretreated with 5 $\mu$M FR-900452. Pretreatment with the PAF antagonists did not affect the vasoconsrictor response of the heart to 100 pmol $LTC_4$ (52.4 $\pm$ 5.6, and

56.5 ± 8.4 mm Hg, n=5 and 6 in the absence and presence of FR-900452 respectively) and 100 pmol $LTD_4$ (55.2 ± 5.7 and 56.5 ± 13.9 mm Hg, n=5 and 6 respectively). However, pretreatment with FR-900452 significantly attenuated the vasodilator response of the heart to 100 pmol $LTB_4$ (8.3 ± 2.5 and 2.4 ± 1.6 mm Hg, n=6 and 7 in the absence and presence of FR-900452 respectively, $P < 0.001$).

*Quantitation of leukotrienes in the effluent by radioimmunoassay.*

The effects of PAF antagonist, CV-6209, and leukotriene synthesis inhibitor, MK 886, on PAF-induced leukotriene release from isolated rat hearts are summarized in Table 15. The concentrations of leukotrienes in the effluent (baseline values) were determined in a control group in which the isolated rat heart was given a bolus injection of 0.1 ml saline. $LTB_4$ was undetectable and the amount of $LTC_4$ and $LTD_4$ were very low in this group. Significant amounts of leukotrienes ($LTB_4$, $LTC_4$ and $LTD_4$) were detected in the effluent after a bolus injection of 100 pmol PAF. Pretreatment of the perfused rat heart with CV-6209 or MK 886 significantly blocked the release of the leukotrienes into the effluent by PAF. The time course for the maximal release of leukotrienes into the effluent was determined at four seperate periods (0-1, 1-2, 2-5 and 5-10 min after an injection of PAF) and the results are shown in Table 15. The maximal release of $LTB_4$ was 398 pg/ml and was observed within 1 min after a bolus injection of PAF. The maximal release of $LTC_4$ and $LTD_4$ were 765 pg/ml and 1435 pg/ml respectively and occurred at 1-2 min after a bolus injection of PAF. The time course for the appearance of leukotrienes in the effluent is therefore similar to the time course for the vasodilator and vasoconstrictor effects of PAF (peak effect 20-30 s and 60-90 s respectively).

## Discussion

A bolus injection of 100 pmol of PAF was used in most experimental protocols in this study since this amount consistently produced a biphasic response, i.e. an initial vasodilatation followed by vasoconstriction. This enabled us to examine the effects of various pharmacological interventions on both the coronary vasodilatation and vasoconstriction by PAF in the same heart. As in previous studies using the perfused heart, a constant flow rate was used in the present study and changes in perfusion pressure was used as a reflection of the presence of coronary vasodilatation or vasoconstriction. Similar coronary vascular responses to a bolus injection of PAF under constant pressure perfusion were observed when the changes in coronary perfusion rate were measured by an electromagnetic flow probe.

Specific binding of radiolabelled PAF was first demonstrated in platelets (Valone *et al.*, 1982; Hwang *et al.*, 1983) and subsequently demonstrated in a number of tissues (see review of Braquet *et al.*, 1987). The binding of PAF showed the presence of high affinity sites, stereospecificity and saturation kinetics. These data suggested the presence of a specific PAF receptor on the cell membrane and the binding of PAF could be blocked by PAF antagonists (Braquet *et al.*, 1987; Saunders and Handley, 1987). The presence of multiple PAF receptors was suggested by the study of Hwang (1988). The availability of various PAF antagonists enabled us to examine whether the coronary vascular effects of PAF, specifically the vasodilator and vasoconstrictor effects, were mediated by different PAF receptor subtypes. The coronary vascular effects of PAF were therefore examined in the perfused rat heart pretreated with the PAF antagonists, CV-6209, FR-900452, BN-52021, BN-50739 and WEB 2086.

The present study demonstrated for the first time that functionally opposite vasodilator and vasoconstrictor effects of PAF could be selectively abolished by different PAF antagonists in the perfused heart. These results are compatible with the concept of existence of PAF receptor subtypes. Selectivity for blocking the vasodilator effect of PAF was observed with CV-6209 and selectivity for blocking the vasoconstrictor effect was observed with FR-900452, WEB 2086 and BN-50739. The presence of PAF receptor subtypes can also explain the different time course and the threshold amount of PAF for the PAF-induced vasodilatation and vasoconstriction in the heart (see results in Part 2).

The existence of PAF receptor subtypes has been postulated in a number of studies using different cell types in the same or different animal species. Based on the lower affinity of kadsurenone for pig peripheral leukocyte aggregation than for PAF-induced chemiluminescence of guinea pig peritoneal macrophages (91 fold difference in $pA_2$ values), Lambrecht & Parnham (1986) suggested the presence of PAF receptor subtypes (named $PAF_1$ and $PAF_2$ receptors). Differences in potency (6-10 times) of ONO-6240 on PAF receptor binding and PAF-induced aggregation in human leukocytes and platelets also supported the presence of PAF receptor subtypes (Hwang, 1988). A dissociation between PAF-induced superoxide anion generation (high concentration of PAF, $\mu$M range), and intracellular degranulation and peroxidase release (low concentration of PAF, nM range) in guinea pig eosinophils was observed (Kroegel *et al.*, 1989). High and low concentrations of WEB 2086 were also required to block these PAF-induced changes. However, different ionic conditions were present in the test systems because PAF-induced superoxide production was $Mg^{2+}$ dependent while PAF-induced peroxidase release was $Ca^{2+}$ dependent. Hence, they concluded that their results could be explained on the basis of two PAF receptor subtypes or a single receptor that can exist in low and high affinity states (Kroegel *et al.*, 1989).

Indeed, Hwang *et al.*, (1989) demonstrated that the conformation and affinity of the PAF receptor in rabbit platelet membranes could be regulated by the ionic environment, including $Ca^{2+}$ and $Mg^{2+}$ concentrations.

However, PAF receptor conformational change cannot fully explain the data that showed selectivity of PAF antagonists on receptor binding under similar ionic environment (Hwang, 1988). Furthermore, the effects of PAF antagonists, WEB 2086, L-652,731 and BN-52021, on the PAF-induced chemiluminescence and prostacyclin generation by guinea-pig resident peritoneal macrophages and on pig peripheral blood leukocyte and platelet aggregation also showed significant differences in $pA_2$ values and the rank order of potency of PAF antagonists between guinea-pig macrophages, and pig platelets and leukocytes (Stewart & Dusting, 1988). In this study, PAF antagonists showed selectivity for blocking the vasodilator and vasoconstrictor effects of PAF in the perfused heart and thereby provided further evidence for the existence of PAF receptor subtypes. Unlike previous studies where receptor subtypes exist in different cell types, our data suggest that receptor subtypes can exist in the same organ and initiate opposite effects. However, the role of these functionally opposite PAF receptor subtypes in the heart and the corresponding vasodilator and vasoconstrictor effects in the regulation of coronary flow remains to be elucidated.

Our results demonstrated that if the action of PAF involved multiple receptor subtypes, much higher concentrations of a single PAF antagonist would be required. However, our data suggest that it is possible to combine low concentrations of two selective PAF antagonists to produce the same effect, thus eliminating the need for high concentrations. This may be particularly noteworthy if high concentrations of PAF antagonists are associated with undesirable side effects.

The role of arachidonic acid metabolites in the mediation of the coronary vascular effects of PAF was investigated by using a number of pharmacological agents. The biphasic coronary vascular response to a bolus injection of PAF in the perfused rat heart was blocked by the leukotriene antagonist (L-649,923) and leukotriene synthesis inhibitor (MK-886). This suggested that the effects of PAF were mediated by leukotrienes. In previous studies, the leukotriene antagonist FPL-55712, was shown to attenuate the vasoconstrictor response following injections of PAF (Piper and Stewart, 1986; Feuerstein *et al.*, 1984; Fielder *et al.*, 1987). However, the vasodilator effect, when observed, was not altered by FPL-55712 (Feuerstein *et al.*, 1984; Fielder *et al.*, 1987). It is not known whether the differences in the effectiveness of L-649,923 and FPL-55712 in inhibiting the vasodilator response is due to their selectivity for different leukotrienes. In the present study, bolus injections of $LTC_4$ and $LTD_4$ resulted in increases in perfusion pressure, which were blocked when the hearts were pretreated with 5 $\mu$M L-649,923. Bolus injections of $LTB_4$ caused decreases in perfusion pressure which were also blocked when the hearts were pretreated with L-649,923. Therefore, bolus injections of appropriate leukotrienes are capable of mimicking the individual components of the biphasic response to PAF.

The results of Piper and Stewart (1986, 1987) showed that indomethacin attenuated the increase in perfusion pressure in hearts following bolus injections of PAF. Also, indomethacin blocked the coronary constriction produced by intracoronary injection of PAF in the pig heart (Feuerstein *et al.*, 1984). These data suggest that the effects of PAF may be mediated by cyclooxygenase products. However, under our experimental conditions, pretreating the hearts with indomethacin had no significant effect on the vasodilator and vasoconstrictor responses following bolus injections of PAF. Our data therefore suggest that cyclooxygenase products are not important in the

mediation of the coronary vascular response of PAF and are contrary to previous results. This can be due to the differences in experimental conditions to those of Piper and Stewart (1986, 1987). The higher starting perfusion pressure used in the present study has been shown to have a more prominent vasodilator response (see results in Part 2) than the lower perfusion pressure used previously. Species difference (rat heart vs pig heart) may also account for the difference between our results and those of Feuerstein *et al.* (1984).

In previous studies, the increase in perfusion pressure following a bolus injection of PAF in the rat heart could not be repeated by a second injection (Piper and Stewart, 1986, 1987; Man *et al.*, 1990). In this study, we were able to observe increases in perfusion pressure following repeated bolus injections of $LTC_4$ or $LTD_4$ (results not shown). This indicates that the reproduction endogenous leukotrienes may be blocked following the first injection of PAF. However the blood vessels are still capable of responding to leukotrienes. This provides further indirect evidence to support the hypothesis that the PAF effects are mediated by the release of vasoactive compounds such as leukotrienes and the failure of production of these compounds limits the response to subsequent exposure to PAF.

The hypothesis that the coronary vascular effects of PAF are mediated by leukotrienes was confirmed by directly measuring leukotrienes from the effluent of perfused rat heart using radioimmunoassay. There is a positive relationship between the amount of leukotrienes ($LTB_4$, $LTC_4$ and $LTD_4$) in the effluent and the coronary vascular effect of PAF. Both PAF antagonist and leukotriene synthesis inhibitor abolished the PAF-induced release of leukotrienes. The time course for maximal releases of $LTB_4$ and $LTC_4$ and $LTD_4$ (within 1 min and 1-2 min respectively) was

compatible with the time course for the vasodilator and vasoconstrictor effects of PAF (peak effect 20-30 s and 60-90 s respectively).

The results of the present study can be explained on the basis of the presence of two different PAF receptors in the rat heart. The activation of the first type of PAF receptor leads to the formation of $LTB_4$ resulting in the observed coronary vasodilatation while the activation of the second type of PAF receptor leads to the formation of $LTC_4$ and/or $LTD_4$ resulting in coronary vasoconstriction. The leukotriene antagonist (L-649-923, 5 $\mu$M) and the leukotriene synthesis inhibitor (MK-886, 1 $\mu$M) were able to abolish the coronary vasodilator and vasoconstrictor actions of PAF due to the release of $LTB_4$, $LTC_4$ and $LTD_4$.

This hypothesis is compatible with the different time course of the vasodilator and vasoconstrictor effects of PAF. The use of specific $LTB_4$, $LTC_4$ and $LTD_4$ antagonists would enable us to further examine this hypothesis. Although the leukotriene antagonist L-649,923 showed specificity for $LTD_4$ in binding assay (Jones *et al.*, 1986), there may be a lesser selectivity of L-649,923 *in vivo* (Ford-Hutchinson, personal communication). This would account for our observation that 5 $\mu$M L-649,923 abolished both the vasodilator and vasoconstrictor actions of PAF. However, selectivity of L-649,923 can be detected when lower concentrations of L-649,923 (0.1 - 0.5 $\mu$M) were used. Substantial reduction of the coronary vasoconstrictor effect of PAF was observed while no significant effect on the coronary vasodilating action of PAF was detected (Table 13). Another consideration is that L-649,923 may have a non-specific effect on PAF receptors and therefore can inhibit the coronary vascular effect of PAF. However, L-649,923 (10 $\mu$M) does not affect PAF receptor binding assay in platelets (Ford-Hutchinson, personal communication). In addition, inhibition of leukotriene synthesis by MK-886 produced the same results as the leukotriene

antagonist. These data further reinforce the role of leukotrienes in both the vasodilator and vasoconstrictor effects of PAF.

Release of leukotriene-like bioactive substances by PAF was also reported in the effluent of perfused rat heart and it included $LTB_4$, $LTC_4$ and $LTD_4$ (Piper and Stewart, 1986). However, the source for the production of these leukotrienes by PAF has not been fully identified. Based on the relatively short time course for the occurrence of the coronary vascular response to PAF (20 - 60 s) and the detection of leukotrienes, the endothelium or the smooth muscle cells are likely sources as the target for PAF-induced production of these vasoactive lipoxygenase products. Perivascular mast cells have also been proposed as the source for the production of leukotrienes (Piper and Stewart, 1986). Since the PAF-induced coronary vasodilatation and vasoconstriction have different time courses, it is possible that $LTB_4$, and $LTC_4$ and $LTD_4$ are produced from different sources. The involvement of multiple cell types in the initiation of the complex coronary vascular effects of PAF is compatible with the different sensitivity of the vasodilator response to PAF (10 fmol - 1 pmol) as compared to the vasoconstrictor response (10 pmol or more) in the study of the section 2. However, the identification of the multiple PAF receptors in the rat heart and the possible involvement of different cell types in the generation of the vasoactive leukotrienes responsible for the vasodilator and vasoconstrictor effects of PAF remain to be elucidated.

**Table 9**

**The effects of adding various compounds to Krebs-Henseleit solution on the perfusion parameters of rat hearts**

| Addition to normal Krebs-Henseleit | Flow Rate (ml/min/g heart) | Control Pressure (mm Hg) | Control Heart Rate (beats/min) | n |
|---|---|---|---|---|
| None | 11.5 ± 1.7 | 67.7 ± 1.7 | 270 ± 39 | 9 |
| Ethanol 0.1% | 11.7 ± 3.2 | 67.8 ± 2.7 | 272 ± 46 | 12 |
| DMSO 0.4% | 11.9 ± 2.6 | 69.3 ± 2.6 | 289 ± 44 | 15 |
| FR-900452 5.0 μM | 11.9 ± 1.6 | 69.8 ± 2.5 | 252 ± 29 | 14 |
| BN-52021 30 μM | 12.7 ± 2.6 | 71.0 ± 2.6** | 281 ± 36 | 12 |
| Indomethacin 2.8 μM | 11.0 ± 1.1 | 72.4 ± 3.3** | 323 ± 50* | 7 |
| L-649,923 1 μM | 9.8 ± 1.9 | 66.8 ± 1.7 | 260 ± 42 | 6 |
| L-649,923 5 μM | 12.2 ± 1.4 | 68.0 ± 1.8 | 261 ± 40 | 6 |
| MK-886 1 μM | 11.4 ± 0.6 | 70.5 ± 5.8 | 293 ± 18 | 11 |

Values represent mean ± SD. ANOVA followed by Duncan's test was used to determine significant differences from the group perfused with normal Krebs-Henseleit solution. * represents $P < 0.05$; ** represents $P < 0.01$.

**Table 10**

**The effects of the PAF receptor antagonists, FR-900452 and BN-52021, on the coronary vascular response to bolus injections of 1 and 100 pmol PAF**

| Solution | Control Pressure (mm Hg) | Decrease in Perfusion Pressure | | Increase in Perfusion Pressure | | n |
|---|---|---|---|---|---|---|
| | | Maximum (mm Hg) | Time (s) | Maximum (mm Hg) | Time (s) | |
| A. Bolus injections of 1 pmol PAF. | | | | | | |
| Ethanol 0.1% | 68.3 ± 2.8 | -4.2 ± 5.3 | 25 ± 14 | - | - | 6 |
| FR-900452 5.0 μM | 71.0 ± 1.6 | -4.6 ± 1.5 | 14 ± 4 | - | - | 5 |
| DMSO 0.4% | 69.6 ± 2.8 | -9.5 ± 6.5 | 23 ± 9 | - | - | 8 |
| BN-52021 30 μM | 72.3 ± 2.0 | -4.6 ± 2.4 | 31 ± 9 | - | - | 7 |
| B. Bolus injections of 100 pmol PAF. | | | | | | |
| Ethanol 0.1% | 67.3 ± 2.7 | -5.0 ± 3.8 | 10 ± 4 | 21.3 ± 11.1 | 61 ± 17 | 6 |
| FR-900452 5 μM | 69.1 ± 2.8 | -12.4 ± 8.2 * | 22 ± 13 * | 2.2 ± 3.3 ** | [a] | 9 |
| DMSO 0.4% | 68.9 ± 2.5 | -5.7 ± 3.5 | 13 ± 6 | 20.0 ± 6.1 | 67 ± 19 | 7 |
| BN-52021 30 μM | 69.2 ± 2.4 | -14.4 ± 3.9 ** | 23 ± 6 * | 0 ± 0 ** | [a] | 5 |

Values represent mean ± SD. The Student's unpaired t-test was used for statistical comparisons between vehicle and treatment groups. * represents $P < 0.05$; ** represents $P < 0.01$. Time represents the time at which the maximum change occurred. [a] Since there was little or no effect of PAF injections in these groups, the time was not applicable.

**Table 11**

**Effects of the PAF antagonists, CV-6209, FR-900452, BN-50739 and WEB 2086, on the vasodilator and vasoconstrictor effects of 100 pmol PAF in the perfused rat heart**

| | Concentration | Peak vasodilator effect (mm Hg) [a] | Peak vasoconstrictor effect (mm Hg) [a] | n |
|---|---|---|---|---|
| CV-6209 | 0 | -8.4 ± 2.2 | 29.2 ± 5.8 | 5 |
| | 10 nM | -2.2 ± 1.3 ** [b] | 36.0 ± 9.7 | 5 |
| | 50 nM | -1.2 ± 2.1 ** | 31.0 ± 15.0 | 5 |
| | 100 nM | -2.4 ± 1.9 ** | 1.4 ± 1.9 ** [b] | 5 |
| FR-900452 | 0 | -7.8 ± 1.7 | 26.3 ± 9.0 | 6 |
| | 1 μM | -13.6 ± 4.7 * | 23.8 ± 11.8 | 5 |
| | 3 μM | -15.3 ± 2.7 * | 20.8 ± 5.8 | 6 |
| | 5 μM | -14.5 ± 7.6 * | NC [b] | 6 |
| | 30 μM | -7.5 ± 4.9 | NC | 6 |
| | 50 μM | -1.6 ± 1.5 * [b] | 0.8 ± 2.0 ** | 5 |
| WEB 2086 | 0 | -9.8 ± 3.9 | 24.3 ± 4.5 | 6 |
| | 0.1 μM | -15.0 ± 1.4 | 9.0 ± 3.6 ** | 4 |
| | 0.5 μM | -18.4 ± 3.9 * | NC [b] | 5 |
| | 1 μM | -24.8 ± 6.0 ** | NC | 6 |
| | 10 μM | -26.6 ± 5.5 ** | NC | 4 |
| | 30 μM | -11.0 ± 2.9 | 0.3 ± 0.5 ** | 5 |
| | 50 μM | -12.7 ± 4.0 | 0.3 ± 0.6 ** | 4 |
| | 100 μM | -2.5 ± 2.1 * [b] | 0.2 ± 0.4 ** | 6 |
| BN-50739 | 0 | -9.8 ± 4.6 | 23.4 ± 8.1 | 5 |
| | 0.001 μM | -11.7 ± 3.2 | 31.3 ± 2.1 ** | 5 |
| | 0.01 μM | -15.4 ± 4.0 * | 0.8 ± 1.8 ** [b] | 5 |
| | 0.1 μM | -18.0 ± 4.5 ** | 0.3 ± 0.7 ** | 8 |
| | 1 μM | -7.0 ± 1.4 | NC | 5 |
| | 5 μM | -3.0 ± 0.4 ** | NC | 5 |
| | 10 μM | -0.3 ± 0.6 ** [b] | 0.3 ± 0.5 ** | 5 |

Values represent mean ± standard deviation, n=number of experiments. NC denotes no detectable change in perfusion pressure. Statistical analyses were performed by analysis of variance followed by Duncan's test. * $P < 0.05$ and ** $P < 0.01$ when compared to the appropriate data in the absence of the PAF antagonist.

[a] With constant flow perfusion, decrease in perfusion pressure represents vasodilatation (negative value), and increase in perfusion pressure represents vasoconstriction.

[b] Since PAF antagonists did not produce a progressive transition of blocking the vasodilator or vasoconstrictor effects of PAF, the lowest concentration that reduced each effect completely was chosen for the calculation of selectivity for the vasodilator and vasoconstrictor effects.

**Table 12**

**Effects of the combination of PAF antagonists, CV-6209 with FR-900452 and WEB 2086, on the vasodilator and vasoconstrictor effects of 100 pmol PAF in the perfused rat heart**

| | Concentration | Peak vasodilator effect (mm Hg) | Peak vasoconstrictor effect (mm Hg) | n |
|---|---|---|---|---|
| CV-6209 + FR-900452 | 10 nM 5 $\mu$M | -1.7 $\pm$ 0.5 | NC | 6 |
| CV-6209 + WEB 2086 | 10 nM 0.5 $\mu$M | -0.2 $\pm$ 0.4 | 1.2 $\pm$ 1.3 | 5 |

Values represent mean $\pm$ SD. NC denotes no detectable change in perfusion pressure. Statistical analyses were performed by ANOVA followed by Duncan's test. All data were significantly different ($P < 0.001$) from data in the absence of any PAF antagonist (see values in Table 11).

**Table 13**

**The effects of indomethacin, L-649,923 and MK-886 on the coronary vascular response to bolus injections of 100 pmol PAF**

| Solution | Control Pressure (mm Hg) | Decrease in Perfusion Pressure | | Increase in Perfusion Pressure | | n |
|---|---|---|---|---|---|---|
| | | Maximum (mm Hg) | Time (s) | Maximum (mm Hg) | Time (s) | |
| Normal Krebs-Henseleit | 67.7 ± 1.7 | -8.9 ± 6.5 | 10 ± 5 | 27.1 ± 13.8 | 71 ± 22 | 9 |
| Indomethacin 2.8 μM | 72.4 ± 3.3 | -5.3 ± 3.0 | 11 ± 3 | 32.9 ± 10.6 | 49 ± 8 | 7 |
| L-649,923 0.1 μM | 68.3 ± 2.1 | -7.0 ± 2.2 | 11 ± 3 | 12.5 ± 7.2** | 88 ± 8 | 6 |
| L-649,923 0.5 μM | 67.8 ± 1.7 | -8.7 ± 1.9 | 11 ± 3 | 6.3 ± 4.4** | 99 ± 12 | 6 |
| L-649,923 1.0 μM | 66.8 ± 1.7 | -4.2 ± 3.1* | 13 ± 9 | 2.0 ± 2.7** | [a] | 6 |
| L-649,923 5.0 μM | 68.0 ± 1.8 | -1.8 ± 1.0** | 13 ± 12 | 1.0 ± 1.7** | [a] | 6 |
| MK-886 1 μM | 70.5 ± 5.8 | -3.5 ± 3.0* | 10 ± 7 | 2.7 ± 1.4** | 50 ± 22 | 11 |

Values represent mean ± SD. ANOVA followed by Duncan's test was used to determine which groups were significantly different from the group perfused with normal Krebs-Henseliet. * represents $P < 0.05$; ** represents $P < 0.01$. Time represents the time at which the maximum change occurred. [a] Since there was little or no effect of PAF injections in these groups, the time was not applicable.

Table 14

**The changes in the perfusion pressure of rat hearts following bolus injections of $LTC_4$, $LTD_4$ and $LTB_4$**

| | Control Pressure (mm Hg) | Changes in Perfusion Pressure | | n |
|---|---|---|---|---|
| | | Maximum (mm Hg) | Time (s) | |
| *$LTC_4$* | | | | |
| 1 pmol | 68.3 ± 3.8 | +6.4 ± 3.5** | 18 ± 9 | 8 |
| 10 pmol | 72.0 ± 2.1 | +23.8 ± 10.2** | 14 ± 6 | 6 |
| 50 pmol | 69.7 ± 1.5 | +58.2 ± 23.0** | 14 ± 1 | 6 |
| 100 pmol | 66.5 ± 1.3 | +70.0 ± 9.1** | 11 ± 1 | 4 |
| *$LTD_4$* | | | | |
| 1 pmol | 70.7 ± 5.1 | +7.3 ± 4.2 | 23 ± 11 | 3 |
| 100 pmol | 68.8 ± 2.2 | +39.8 ± 10.4** | 15 ± 10 | 4 |
| *$LTB_4$* | | | | |
| 1 pmol | 69.7 ± 3.4 | -0.7 ± 1.2 | - | 6 |
| 10 pmol | 71.2 ± 3.3 | -4.5 ± 6.4 | - | 6 |
| 100 pmol | 69.4 ± 3.5 | -12.4 ± 10.6* | 11 ± 4 [a] | 10 |

Values represent mean ± SD. The signs indicate increase or decrease in perfusion pressure. ANOVA was used for statistical analysis, and indicated that the changes in perfusion pressure by $LTC_4$, $LTD_4$ and $LTB_4$ were all dose-dependent. * represents $P < 0.05$ and ** represents $P < 0.01$ when compared with the pre-injection pressure. Time represents the time at which the maximum change occurred. [a] n=8, 2 hearts do not have a vasodilatation response to $LTB_4$.

**Table 15**

**Quantitation of leukotrienes ($LTB_4$, $LTC_4$ and $LTD_4$) by radioimmunoassays in the effluent of perfused rat hearts**

| Injection | Saline control | 100 pmol PAF | 100 pmol PAF | 100 pmol PAF | n |
|---|---|---|---|---|---|
| Pretreatment | none | none | CV-6209 (100 nM) | MK 886 (10 $\mu$M) | |
| *$LTB_4$* (pg/ml) | | | | | |
| 0-1 min | - | 398 ± 113 | 8 ± 5 | - | 4 |
| 1-2 min | - | 215 ± 155 | 11 ± 3 | - | 4 |
| 2-5 min | - | 258 ± 153 | 5 ± 4 | - | 4 |
| 5-10 min | - | 110 ± 77 | 3 ± 4 | - | 4 |
| *$LTC_4$* (pg/ml) | | | | | |
| 0-1 min | 45 ± 17 | 213 ± 51 | 23 ± 6 | 40 ± 34 | 4 |
| 1-2 min | 49 ± 21 | 765 ± 388 | 30 ± 10 | 75 ± 19 | 4 |
| 2-5 min | 33 ± 5 | 348 ± 175 | 20 ± 10 | 34 ± 14 | 4 |
| 5-10 min | 35 ± 6 | 63 ± 36 | 23 ± 6 | 33 ± 22 | 4 |
| *$LTD_4$* (pg/ml) | | | | | |
| 0-1 min | 33 ± 56 | 255 ± 118 | - | 25 ± 50 | 4 |
| 1-2 min | 37 ± 63 | 1435 ± 619 | - | 25 ± 50 | 4 |
| 2-5 min | - | 588 ± 433 | - | 38 ± 75 | 4 |
| 5-10 min | 37 ± 63 | 76 ± 35 | - | 25 ± 50 | 4 |

Values represent mean ± SD. n = number of samples. - denotes no detectable amount.

# Part 4

# Interaction of platelet-activating factor-released vasoactive substances in the perfused rat heart

**Abstract**

The coronary vascular effects of platelet-activating factor (PAF) have been studied and proposed to be mediated by the release of vasoactive substances. In this section, a cascade perfusion model using two perfused rat hearts was developed to investigate the properties of PAF-released vasoactive substances and the interplay of these substances. The properties of the vasoactive substances after an injection of PAF (100 pmol) in the perfused rat heart was examined by using the effluent from the first heart for perfusion of a second (recipient) heart. The presence of vasoconstrictor substances in the effluent was characterized by an increase in the perfusion pressure of the recipient heart.

Previous exposure of the recipient hearts to PAF (100 pmol) abolished the response of the heart to subsequent direct administration of PAF, but did not affect the response of the recipient heart to the effluent. This suggested that the coronary vasoconstrictor response of the recipient heart was not due to the presence of PAF in the effluent but to other vasoactive substances. Pretreatment of the recipient heart with the leukotriene receptor antagonist, L-649,923 (5 $\mu$M), partially reduced the vasoconstrictor effect of the effluent. Pretreatment of the first heart with indomethacin (2.8 $\mu$M) also partially reduced the vasoconstrictor effect of the effluent. The combination of indomethacin pretreatment of the first heart and L-649,923 pretreatment of the recipient heart completely abolished the vasoconstrictor effect of the effluent suggesting that both prostaglandins and leukotrienes were involved in the vasoconstrictor effect of the effluent. Pretreatment of both hearts with L-649,923 or the first heart with the leukotriene synthesis inhibitor (MK-886, 10 $\mu$M) completely abolished the vasoconstrictor effect of the effluent. This suggested that the indomethacin sensitive vasoconstrictor component of the effluent might be regulated by

leukotrienes in the first heart. However, infusion of leukotrienes ($LTB_4$, $LTC_4$ and $LTD_4$) to the first heart did not reproduce this vasoconstrictor component of the effluent in the recipient heart.

In conclusion, our study demonstrated through the use of a leukotriene receptor antagonist, a leukotriene synthesis inhibitor and a cyclooxygenase inhibitor that the vasoconstrictor effect of the effluent of the perfused rat heart after an injection of PAF was mediated by leukotrienes and prostaglandins. The ability of leukotriene receptor blockade and inhibition of leukotriene synthesis to mimick the effect of indomethacin indicates that the production and/or release of cyclooxygenase products in the effluent by PAF can be modulated by leukotrienes. The inability of exogenously applied leukotrienes to modulate the production and/or the release of cyclooxygenase products in the effluent suggests that the PAF-induced production of prostaglandins may be mediated by intracellular leukotrienes or at sites not accessible to exogenously applied leukotrienes.

## Introduction

Recently, platelet-activating factor (PAF) has been detected in the effluent blood from the coronary sinus in patients with coronary artery disease undergoing atrial pacing (Montrucchio *et al.*, 1986) and release of PAF from ischemic-reperfused rabbit heart has been demonstrated (Montrucchio *et al.*, 1989). The effects of PAF in the coronary circulation have been shown in a number of *in vivo* (Levi *et al.*, 1984; Piper & Stewart, 1986, 1987) and *in vitro* studies (Feuerstein *et al.*, 1984; Jackson *et al.*, 1986; Mehta *et al.*, 1986; Ezra *et al.*, 1987; Fielder *et al.*, 1987).

Regarding the mechanisms of the coronary vascular actions of PAF, it has been suggested that the effects of PAF are largely mediated by the release of other vasoactive substances. The importance of the lipoxygenase products, leukotriene $B_4$ ($LTB_4$), $C_4$ ($LTC_4$), and $D_4$ ($LTD_4$), and the cyclooxygenase products, prostaglandins and thromboxane $A_2$ in the coronary vascular effects of PAF has been demonstrated (Piper & Stewart, 1986, 1987). More recently, we showed that $LTC_4$ and $LTD_4$ may be responsible for the vasoconstrictor effect while $LTB_4$ may be responsible for the vasodilator effect of PAF in the perfused rat heart (see Part 3 of Result Section). Inasmuch as the precise mechanisms of actions of PAF on circulatory function have not been fully elucidated, the complex interaction between PAF and arachidonic acid metabolites in the coronary vascular effects of PAF is also not well defined. In the present study, a cascade perfusion model of isolated rat hearts was developed to investigate the properties of vasoactive substances released by PAF. The objectives of this study were (a) to clarify the role of arachidonic acid metabolites in the coronary vascular effects of PAF and (b) to gain additional insight into the characteristics of the vasoactive mediators released by PAF.

## Methods and Metarials

*Rat heart perfusion*

Following cervical dislocation, hearts from Sprague-Dawley rats (250 - 350 g) were rapidly excised and placed in cold Krebs-Henseleit solution oxygenated with 95% $O_2$ - 5% $CO_2$. The solution had a following composition (mM): NaCl 120, $NaH_2PO_4$ 1.18, $MgSO_4$ 1.18, KCl 4.76, $CaCl_2$ 1.25, $NaHCO_3$ 25.0, and glucose 11. The aorta was cannulated for coronary perfusion. The heart was allowed to beat spontaneously. The temperature of the perfusate was maintained at 37 $\pm$ 0.5°C and the coronary flow was controlled by a roller pump. The perfusion pressure was measured by a pressure transducer attached to a side arm of the aortic cannula. The perfusion pressure was recorded on a Gould chart recorder and monitored with a digital display.

*The cascade perfusion model*

In the cascade perfusion model, two hearts were first separately perfused with oxygenated Krebs-Henseleit solution. Each heart was equilibrated with Krebs-Henseleit solution for 20 min. During this period, the flow rate was adjusted to obtain a control perfusion pressure of 65 - 75 mm Hg and the flow rate was maintained constant for the rest of the experiment. Coronary vascular effects were measured as changes in the perfusion pressure. To enable drug pretreatments the perfusate for each heart was controlled by a multiple way stopcock. During cascade perfusion, the effluent from the first heart was collected by a funnel and immediately pumped to a second (recipient) heart. The effluent was oxygenated with 95% $O_2$ - 5% $CO_2$ to maintain the $PO_2$, and $PCO_2$ at the same level as the perfusate for the first heart. The pH, $PO_2$ and $PCO_2$ of the solutions were measured by a pH/blood gas analyzer. $Na^+$ and $K^+$ contents were

measured by flame photometry. The coronary flow rates of both hearts were controlled by the same roller pump motor so that the flow rate was kept identical. Solution accumulation in the funnel was kept to a minimum in order to reduce the dilution of vasoactive substances released from the first heart after challenge with PAF. One min prior to the injection of PAF to the first heart, the perfusion of the recipient heart was switched to the effluent from the first heart and continued to the end of experiment. Heart rates were monitored from the electrocardiographic recordings.

*Drug preparations and administrations*

In the experiments which required drug pretreatment, 10 min of equilibration with Krebs-Henseleit solution was followed by 10 min of pretreatment with a Krebs-Henseleit solution containing the leukotriene antagonist (L-649,923, Jones *et al.*, 1986) or the leukotriene synthesis inhibitor (MK-886, Gillard, *et al.*, 1989), and this was continued throughout the administration of PAF. The cyclooxygenase inhibitor, indomethacin, was added to the perfusion solution at the start of the stabilization period and the effect of PAF injection was tested after 20 min of perfusion in the presence of indomethacin. All stock solutions were made fresh daily and kept at 4°C between experiments. The leukotriene antagonist, L-649,923 (sodium (ß*S**, *R**)-4-(3-4(-acetyl-4-hydroxy-2-propylphenoxy)-propylthio)- -hydroxy-ß-methylbenzenebutanoate, Merck Frosst Canada Inc.) and the leukotriene synthesis inhibitor, MK-886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid, Merck Frosst Canada Inc.) were dissolved in distilled water and then diluted in Krebs-Henseleit solution to a concentration of 5 $\mu$M and 10 $\mu$M respectively. Indomethacin (Sigma Chemical Co.) was dissolved in 0.1 M $Na_2CO_3$ solution and diluted in Krebs-Henseleit solution to a final concentration of 2.8 $\mu$M. PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (prepared from bovine heart, Sigma Chemical Co.) was

prepared fresh daily in saline (0.9% NaCl) containing 0.25% bovine serum albumin (Sigma Chemical Co.). Bolus injections of PAF were given in a volume of 0.1 ml and over a one second period, into the perfusion line, 5-6 cm proximal to the aortic cannula. $LTB_4$, $LTC_4$ and $LTD_4$ (Merck Frosst Canada Inc.) were prepared fresh daily in saline (0.9% NaCl). The appropriate amount of $LTB_4$, $LTC_4$ or $LTD_4$ was continuously infused to the perfusion line 5-6 cm proximal to the aortic cannula for 5 minutes. Changes in perfusion pressure and the time at which maximum changes occurred were recorded.

*Statistical Analysis*

Data were analyzed by the Student's t-test and analysis of variance (ANOVA), followed by Duncan's test where appropriate. Values are expressed as means $\pm$ standard deviations (sd) and $P < 0.05$ was considered statistically significant.

## Results

*The effect of effluent from the first heart on the recipient heart in the cascade perfusion model*

After perfusion through the first heart, the $PO_2$ of the effluent was reduced. In order to maintain the pH, $PO_2$, and $PCO_2$ constant, the effluent from the first heart was oxygenated with 95% $O_2$ - 5% $CO_2$ before being perfused into the recipient heart. The pH, $PO_2$, and $PCO_2$ of the effluent were in the same range as normal oxygenated Krebs-Henseleit solution (Table 16). The $Na^+$ and $K^+$ concentrations of the effluent and normal Krebs-Henseleit solution were identical. Heart rate, contractility and the electrocardiogram were not altered by perfusion for up to 60 min with the effluent from the first heart (data not shown). There was also no significant alteration in the perfusion pressure of the recipient heart by perfusion with effluent (66.5 $\pm$ 1.3 and 68.8 $\pm$ 1.0 mm Hg at time 0 and after 30 min of perfusion with the effluent respectively, n=4).

*Assessment of PAF-released vasoactive substances in the effluent using the recipient heart of the cascade perfusion model*

When 100 pmol PAF was administered to the first heart, a brief vasodilator response followed by a vasoconstrictor response were observed as described previously. The effluent from this heart caused an increase in the perfusion pressure of the recipient heart (30.8 $\pm$ 8.7 mm Hg, n=6) at about 1.5 min after the injection of PAF to the first heart. However, no vasodilator response was observed in the recipient heart. The results of the effects of PAF on the perfusion pressure of the first and recipient hearts are summarized in Figure 9. To determine whether the coronary vascular effect of the

effluent was due to the presence of PAF per se or of released vasoactive substances, the recipient heart was pretreated with PAF (100 pmol) in order to eliminate the vasoconstrictor response to subsequent exposure to PAF (Piper & Stewart 1986, 1987; see also Part 2 of Results Section). The vasoconstrictor response of the recipient heart to the effluent was not affected by such a pretreatment (Figure 9). When both hearts were pretreated with PAF, no vasoconstrictor response to the effluent was observed in the recipient heart (Figure 9). The above results demonstrated that the vasoconstrictor response of the recipient heart to the effluent from the first heart was not caused by PAF itself, but by the presence of other PAF-released vasoactive substances. To eliminate the possible interference of the presence of PAF in the effluent in the determination of the properties of the PAF-released vasoactive substances in the effluent, 100 pmol PAF was administered to the recipient hearts in all subsequent experiments.

*The effects of L-649,923, indomethacin and MK-886 on the response to PAF in the cascade perfusion model*

To gain further insight into the characteristics of PAF-released vasoactive substances, pretreatments with a leukotriene receptor antagonist (L-649,923, 5 $\mu$M), a cyclooxygenase inhibitor (indomethacin, 2.8 $\mu$M) and a leukotriene synthesis inhibitor (MK-886, 10 $\mu$M) were used. The effects of pretreatment of the first heart with L-649,923, indomethacin and MK-886 on the coronary responses of these hearts to a bolus injection of 100 pmol PAF are shown in Figure 10. Pretreatment of heart with indomethacin did not significantly affect the vasodilator or the vasoconstrictor responses to PAF while pretreatment of heart with L-649,923 and MK-886 abolished both the vasodilator and vasoconstrictor responses to PAF.

The responses of the recipient hearts following the various pretreatments are summarized in Figure 11. Pretreating only the recipient heart with L-649,923 significantly attenuated ($P < 0.001$) but did not completely abolish the vasoconstrictor response of the recipient heart to the effluent from the first heart after an administration of 100 pmol PAF. The remaining vasoconstrictor component was abolished by pretreating the first heart with indomethacin and the recipient heart with L-649,923 ($P < 0.05$ when compared to the group pretreated with L-649,923 alone). Pretreatment of both hearts with L-649,923 or the first heart with MK-886 also abolished the vasoconstrictor effect of the effluent from the first heart on the recipient heart (Figure 11).

*The coronary vascular effects of the effluent from hearts given leukotriene infusions*

To explore the possible role of leukotrienes on the production of cyclooxygenase products as suggested by the results from the previous section, $LTB_4$, $LTC_4$ or $LTD_4$ infusion for 5 min was used. The effects of $LTC_4$ or $LTD_4$ infusion (300 pmol/min) on the perfusion pressure of the first and recipient hearts are summarized in Figure 12. With no pretreatment, $LTC_4$ and $LTD_4$ infusion resulted in a prominent increase in perfusion pressure in the first and recipient hearts and the increases were similar (Figure 12). With pretreatment of only the recipient heart with L-649,923 (5 $\mu$M), the effect of the effluent from hearts given $LTC_4$ or $LTD_4$ infusion on the perfusion pressure of the recipient heart was completely blocked ($P < 0.001$). Infusion of $LTB_4$ (300 or 600 pmol/min) resulted in vasodilatation in the first heart (-5.7 $\pm$ 1.2, n=12 and -10.7 $\pm$ 1.9 mm Hg, n=12 respectively). However, only a small vasodilatation was detected in the recipient heart when perfused with the effluent from hearts with these $LTB_4$ infusions (-2.2 $\pm$ 0.9, n=6 and -4.3 $\pm$ 2.4 mm Hg, n=6 for 300 and 600 pmol/min $LTB_4$ infusions respectively). Pretreatment of the recipient

heart with L-649,923 also did not result in any significant change in the response to effluent from hearts given $LTB_4$ infusions (-1.5 $\pm$ 0.8, n=6 and -1.2 $\pm$ 1.5 mm Hg, n=6 for 300 and 600 pmol $LTB_4$ infusions respectively).

## Discussion

In the cascade perfusion model, the recipient heart was pretreated by a bolus injection of PAF (100 pmol). After this treatment, the recipient heart loses the ability to respond to the direct effects of a further exposure to PAF (Piper & Stewart, 1986, 1987; see also Part 2 of Results Section). This enabled us to determine the properties of PAF-released vasoactive substances in the effluent of the first heart without the possible interference of the effect of PAF being present in the effluent.

Our results confirmed the existence of vasoactive substances in the effluent of the perfused rat heart after administration of PAF. This is consistent with the concept that PAF itself does not directly initiate the coronary vascular effects but that its effects are mediated through the release of other vasoactive substances (Piper & Stewart, 1986, 1987, see also Part 3 of Results Section). Our results showed that one PAF-induced vasoconstrictor component in the effluent was mediated by leukotrienes (blocked by the leukotriene receptor antagonist in the recipient heart or the leukotriene synthesis inhibitor in the first heart) while another component was mediated by cyclooxygenase products (blocked by the cyclooxygenase inhibitor pretreatment in the first heart). Use of the cascade model enabled us to study the interplay of the various vasoactive substances in mediating the coronary vascular effects of PAF.

There was an interaction between the two major arachidonic acid products, prostaglandins and leukotrienes in the perfused heart. This conclusion is based on the observation that the indomethacin-sensitive vasoactive component could also be blocked by pretreatment of the first heart with a leukotriene receptor antagonist or leukotriene synthesis inhibitor. These results demonstrate for the first time that cyclooxygenase products may be regulated by leukotrienes in the perfused rat heart and that this effect

is receptor mediated. Although it is more common to observe regulation of 5-lipoxygenase products by cyclooxygenase products, there have been a number of studies showing that leukotrienes directly regulate the synthesis and/or release of cyclooxygenase products in other systems. Busija & Leffler (1986) reported that leukotrienes were able to increase the levels of prostaglandins ($PGF_{2\alpha}$ and $PGE_2$) in the cerebral cortex of newborn pigs. A dose-dependent increase in the production of $PGI_2$ from human lymphatics by $LTC_4$ and $LTD_4$ has also been described, and each type of leukotriene tested ($LTC_4$, $LTD_4$, and $LTE_4$) has a similar effect on prostaglandin synthesis (Sinzinger *et al.*, 1986). In addition, the synthesis and release of 6-keto-$PGF_{1\alpha}$ and thromboxane $A_2$ in guinea pig lung by $LTD_4$ and $LTE_4$ was suggested to be receptor mediated (Mong *et al.*, 1986).

It has been reported in several studies that MK-886 is a potent inhibitor of leukotriene biosynthesis *in vivo* (Gillard *et al.*, 1989; Miller *et al.*, 1990; Rouzer *et al.*, 1990) and MK-886 has no direct effect on 5-lipoxygenase activity (Rouzer *et al.*, 1990) or cyclooxygenase activity (Gillard *et al.*, 1989). The ability of MK-886 to inhibit leukotriene biosynthesis is mediated via binding to the 5-lipoxygenase activating protein resulting in the prevention of translocation of 5-lipoxygenase from the cytosol to the active membrane location (Ford-Hutchinson, 1991). Our results have shown that pretreatment of the first heart with MK-886 completely abolished the PAF-induced vasoconstrictor effect of the effluent in the recipient heart. In contrast, L-649,923 pretreatment of the first heart would still require pretreatment of the recipient heart with L-649,923 since PAF-induced leukotriene production in the recipient heart would not be affected. Although a direct inhibition of cyclooxygenase by L-649,923 cannot be ruled out in the present study, the evidence from using both L-649,923 and MK-886 strongly supports the concept that the synthesis and/or release of cyclooxygenase

products are leukotriene receptor-mediated in the coronary circulation of the perfused rat heart as concluded in the previous section.

Piper & Stewart (1986, 1987) showed that indomethacin attenuated the increase in perfusion pressure in the isolated rat and guinea pig hearts following bolus injections of PAF. It had also been reported that indomethacin blocked the coronary vasoconstriction produced by intracoronary injection of PAF in the pig heart *in vivo* (Feuerstein *et al.*, 1984). However, our previous study (Part 3 of Results Section) and current data showed that pretreatment of the heart with indomethacin did not affect the coronary vascular effect of PAF in the same heart. Thus cyclooxygenase products are unlikely to participate in the coronary vascular response under our experimental conditions. But results obtained from the cascade perfusion model indicate the presence of cyclooxygenase products based on the response of recipient hearts to effluents from hearts with or without pretreatment with indomethacin. One possible explanation is that the cyclooxygenase products are released from the venous side of the coronary circulation and therefore cause vasoconstriction only in the recipient heart.

Leukotrienes and prostaglandins are extensively metabolized. However, it is clear that a sufficient amount of these vasoactive arachidonic acid products was present in the coronary effluent after an administration of PAF to initiate a vasoconstrictor response in the recipient heart comparable to that in the first heart. In contrast, in spite of the presence of a vasodilator response in the first heart, no noticeable vasodilatation was observed in the recipient heart. This suggests that $LTB_4$, proposed to be the mediator of the vasodilator response in the first heart (Part 3 of Results Section), is unstable in coronary effluent and was therefore present in insufficient quantity to produce vasodilatation in the recipient heart.

In summary, the present study used a cascade perfusion model to study the characteristics and the interaction of PAF-released vasoactive substances. Our results suggest the following. Upon the administration of PAF in the perfused rat heart, leukotrienes ($LTB_4$, $LTC_4$, and $LTD_4$) are released. While $LTB_4$ may be responsible for the observed vasodilator effect of PAF, $LTC_4$ and $LTD_4$ are responsible for the vasoconstrictor effect of PAF. In addition to directly mediating part of the coronary vascular effects of PAF, leukotrienes also modulate the synthesis and release of cyclooxygenase products via a receptor mediated mechanism. Both lipoxygenase and cyclooxygenase products are present in the effluent after the administration of PAF in the perfused rat heart and contribute to the coronary vascular effects of the effluent observed in the recipient heart. However, exogenously applied leukotrienes cannot mimick the endogenously produced leukotrienes released by PAF in regulating the synthesis of cyclooxygenase products. This suggests that the PAF-induced production of prostaglandins may be mediated by intracellular leukotrienes or at sites not accessible to exogenous leukotrienes. Indeed it has been demonstrated that leukotrienes, in particular $LTC_4$, may serve as intracellular mediator of somatostatin-induced increase of neuronal M-current (Schweitzer *et al.*, 1990) and regulation of prostaglandin synthesis by leukotrienes. Hence, further work regarding the cellular mechanism(s) of the interaction between these PAF-released vasoactive substances and the elucidation of the site where this interaction occurs in the coronary vasculature seems warranted.

**Table 16**

**$PO_2$, $PCO_2$, pH, $Na^+$ and $K^+$ concentrations of the solutions used in the cascade perfusion model**

| | A | B | C | D | n |
|---|---|---|---|---|---|
| $PO_2$ (mm Hg) | 462.4 ± 34.0 | 199.3 ± 32.3 | 434.0 ± 14.0 | 196.2 ± 20.9 | 7 |
| $PCO_2$ (mm Hg) | 43.6 ± 9.3 | 40.4 ± 5.7 | 42.6 ± 8.7 | 40.7 ± 4.9 | 7 |
| pH | 7.37 ± 0.07 | 7.37 ± 0.05 | 7.36 ± 0.07 | 7.36 ± 0.04 | 6 |
| $Na^+$ (meq/l) | 149 ± 2 | 150 ± 2 | 150 ± 2 | 151 ± 2 | 6 |
| $K^+$ (meq/l) | 5.5 ± 0.5 | 5.5 ± 0.5 | 5.5 ± 0.5 | 5.5 ± 0.5 | 6 |

(A) Solution before perfusion into the first heart, (B) effluent collected from the first heart, (C) solution before perfusion into the second heart, and (D) effluent collected from the second heart.

Values represent mean ± sd, n = number of experiments. $Na^+$ concentration was determined to 1 meq/l accuracy and $K^+$ concentration to 0.1 meq/l accuracy.

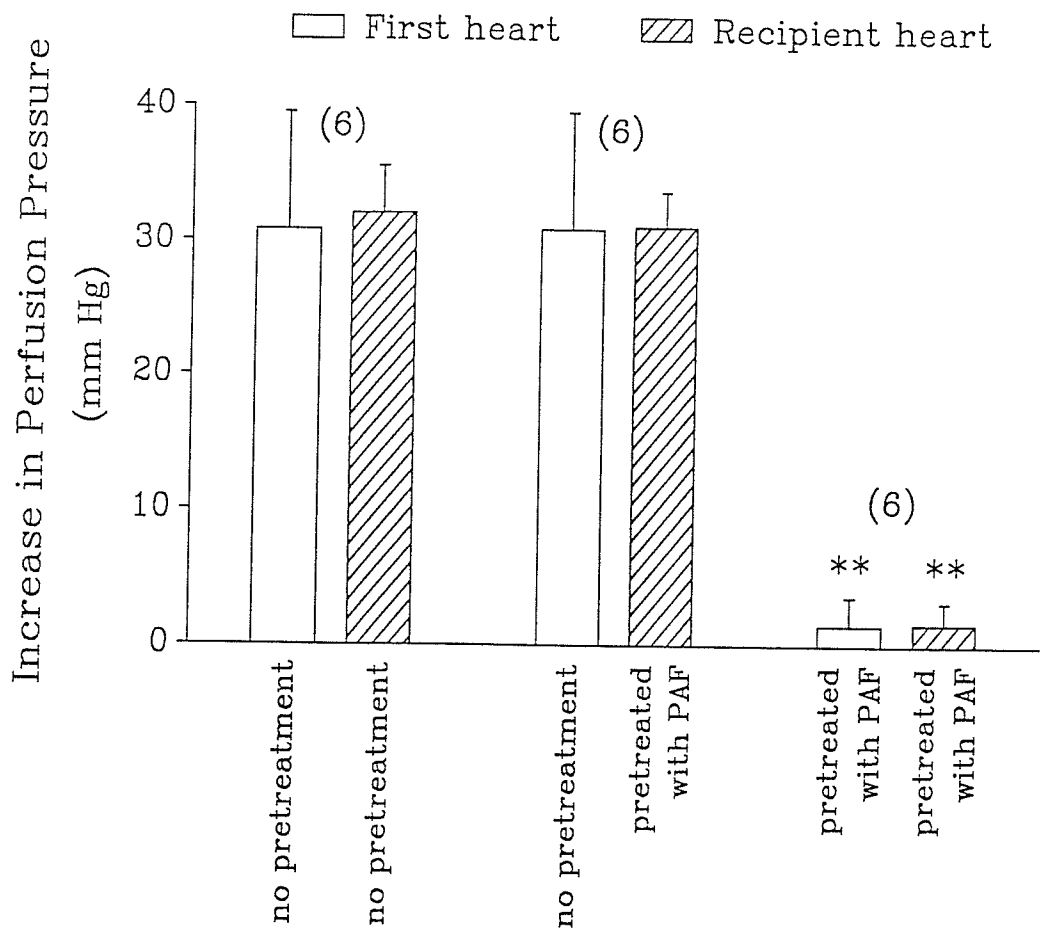



**Figure 9.** The effects of 100 pmol PAF on the perfusion pressure of the first and recipient hearts with and without pretreatment with PAF. Values represent mean $\pm$ sd. Numbers in parenthesis indicate the number of experiments. ** $P < 0.01$ when compared to the response of the corresponding group with no pretreatment.

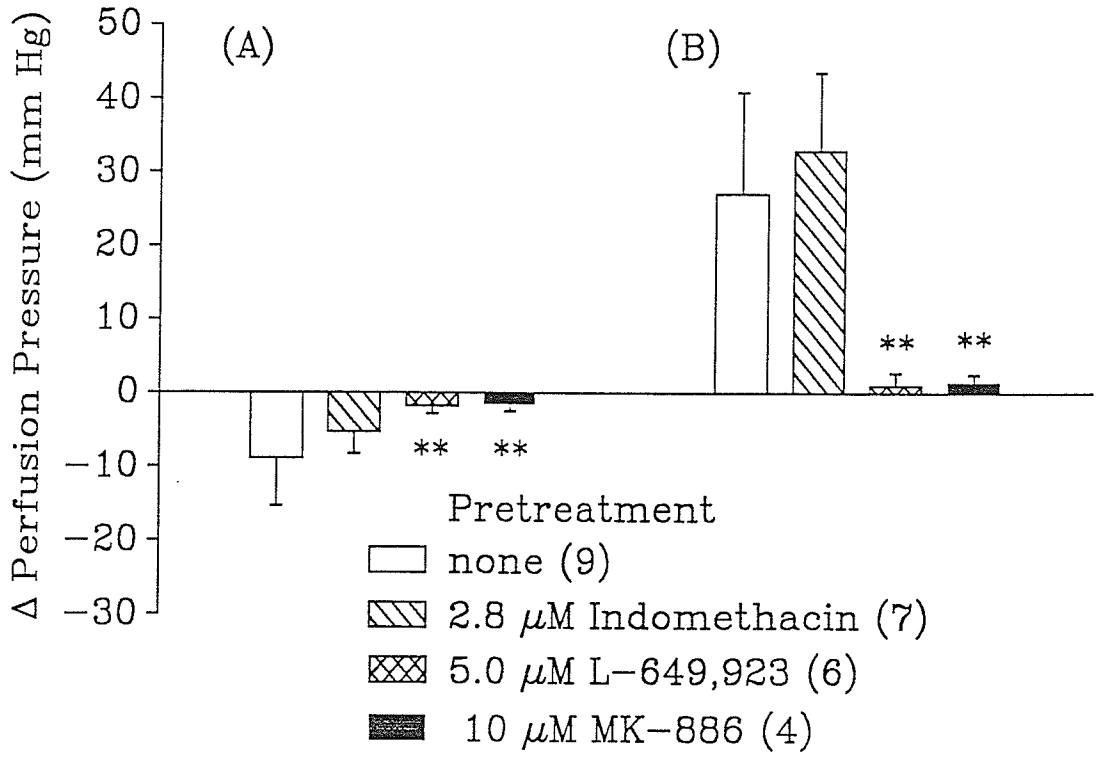



**Figure 10.** The effects of 100 pmol PAF on the perfusion pressure of the first heart after various pretreatments. Panel A shows the initial vasodilator response to PAF and panel B shows the subsequent vasoconstrictor response to PAF. The concentrations of L-649,923, indomethacin and MK-886 were 5.0, 2.8 and 10 $\mu$M. Values represent mean $\pm$ sd. Numbers in parenthesis indicate the number of experiments. ** $P < 0.01$ when compared to the response of hearts with no pretreatment.

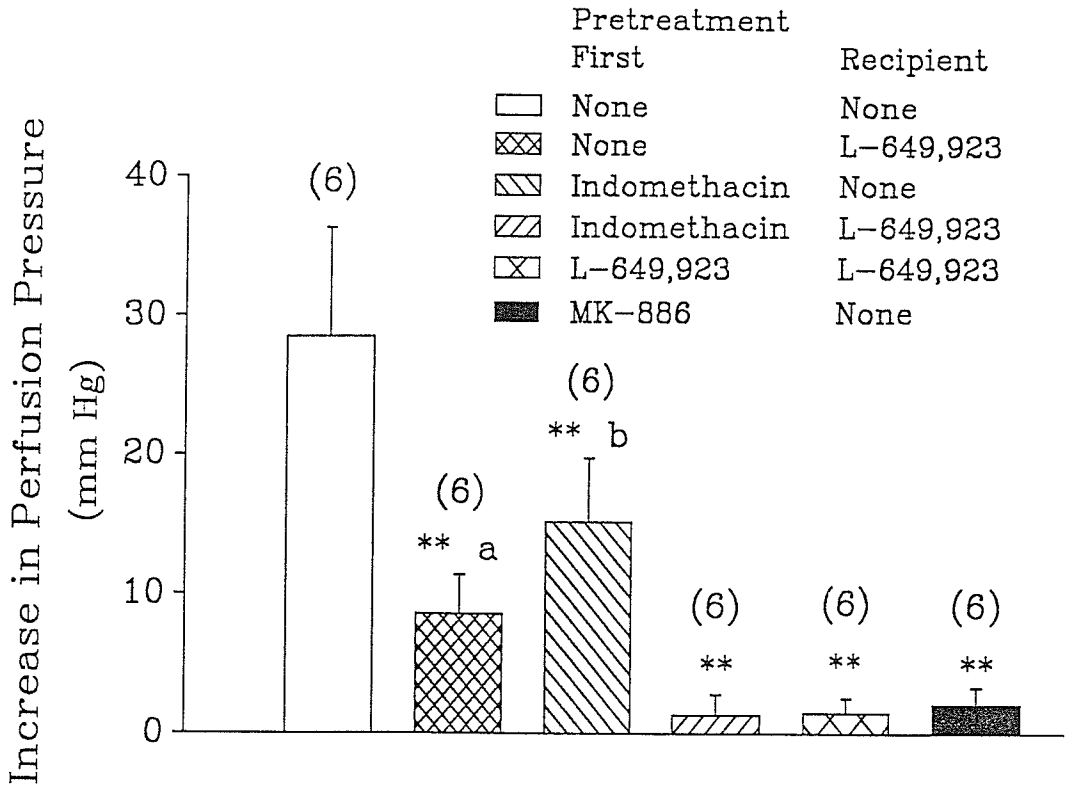



**Figure 11.** The response of the recipient heart to the effluent from the first heart - the effect of various pretreatments to the first and recipient hearts. The concentrations of L-649,923, indomethacin and MK-886 were 5.0, 2.8 and 10 $\mu$M. Values represent mean $\pm$ sd. Numbers in parenthesis indicate the number of experiments. ** $P < 0.01$ and *** $P < 0.001$ when compared to the response of recipient hearts with no pretreatment. [a] $P < 0.05$ when compared to the responses of the last four groups. [b] $P < 0.01$ when compared to the responses of the last three groups.

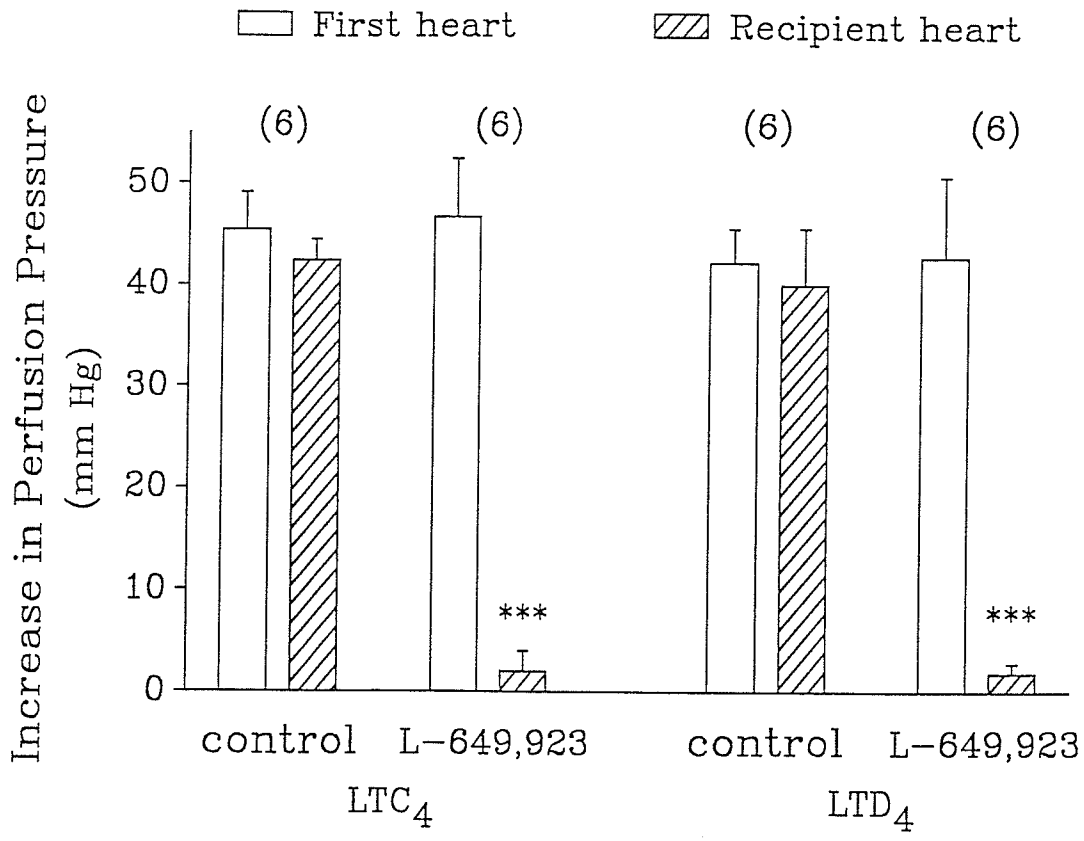



**Figure 12.** The effects of $LTC_4$ and $LTD_4$ infusion on the perfusion pressure of the first and recipient hearts. $LTC_4$ and $LTD_4$ infusion rates were 300 pmol/min and the amount of L-649,923 used for pretreatment of the recipient heart was 5.0 $\mu$M. Values represent mean $\pm$ sd. Numbers in parenthesis indicate the number of experiments. *** $P < 0.001$ when compared to the response of the corresponding values with no L-649,923 pretreatment.

# General discussion

In comparing of the hypotensive effects of PAF with that of palmitoyl carnitine and lysophosphatidylcholine, our data showed that PAF is the most potent hypotensive compound. The hypotensive effect of palmitoyl carnitine was about 1000 times less potent than that of PAF and was not mediated through the same site or mechanism as PAF. Lysophosphatidylcholine had no significant hypotensive action. Regarding the mechanisms of the powerful and long-lasting hypotension produced by PAF, Shigenobu *et al.* (1987) reported that this action of PAF was endothelium-dependent. Relatively low concentration of PAF ($10^{-9}$-$10^{-7}$ M) produced endothelium-dependent relaxation of the rat aorta. PAF-induced vasodilatation of resistance vessels was thought to be through the release of endothelium-derived relaxing factor. Lysophosphatidylcholine is a normal component of biological membranes and a small amount is presented in most membranes. It alters the general properties of the membrane such as fluidity and permeability (Shier *et al.*, 1976) and may function as a membrane transducer by diffusing though the lipid portion of the membranes to modify the activity of membrane associated enzymes. Lysophosphatidylcholine stimulates guanylate cyclase production due to its perturbation effect of membrane architecture (Fink and Gross, 1984). It has also been demonstrated that lysophosphatidylcholine or palmitoyl carnitine were quantitatively incorporated into the sarcolemma, resulting in alterations of membrane molecular dynamics. Bing *et al.* (1988) reported that lysophosphatidylcholine was an effective relaxant of vascular smooth muscle *in vitro* and might play a role in the regulation of vascular activity *in vivo*. Menon *et al.* (1989) demonstrated that lysophosphatidylcholine was an endothelium-dependent and cyclic GMP-producing vascular relaxant *in vivo* and in rabbit aortic strips. The degree of relaxation was related to the activation of guanylate cyclase. The relaxing effect of lysophosphatidylcholine on vascular smooth muscle was not related to endothelium-

derived relaxing factor. Both palmitoyl carnitine and lysophosphatidylcholine accumulate in the ischemic myocardium. Bing *et al.* (1988) showed that lysophosphatidylcholine which was injected as micelles into the left atrium of rabbits caused a decline in coronary vascular resistance and an increase in coronary blood flow. Coronary vascular vasodilatation induced by lysophosphatidylcholine and palmitoyl carnitine in the ischemic myocardium could have beneficial effects, but the exact mechanisms remain to be further studied. It can be concluded that although PAF, palmitoyl carnitine and lysophosphatidylcholine are structurally similar, their properties are significantly different.

Ischemia potently stimulates the generation and release of several lipid and nonlipid mediators that, by complex interactions, may upset normal tissue function. This is particularly true in the heart in which the consequences of an ischemic event, resulting in a marked myocardial disturbance, are generally ascribed to release of catecholamines, eicosanoids (leukotrienes, prostaglandins and thromboxane $A_2$), glycerophospholipids (lysophosphatidylcholine and PAF) and other cytotoxic compounds. Among these mediators, PAF is considered to be involved in cardiac dysfunction and tissue damage associated with ischemia-reperfusion. PAF has been identified in the effluent blood from the coronary sinus of patients with ischemic heart disease undergoing atrial pacing (Montrucchio *et al.*, 1986).

The diverse cardiovascular effects of PAF in coronary circulation have been shown in a number of studies *in vivo* and *in vitro*. In isolated perfused heart, the coronary vascular responses to PAF have been described as vasoconstriction (Levi *et al.*, 1984, Piper and Stewart, 1986, 1987). When PAF was administered by intracoronary injection in intact anesthetized animals, such as pigs, dogs and sheep, vasodilatation (Jackson *et al.*, 1986), vasoconstriction (Feuerstein *et al.*, 1984) and

biphasic responses characterized by an initial dilatation followed by long-lasting constriction were reported. The results obtained from these studies varied depending on different experimental methods and conditions, animal species, amount of PAF and route of administration. The results are therefore not comparable. In the study outlined in this thesis, all coronary vascular responses, i.e. vasodilatation, vasoconstriction and a biphasic response, could be produced in the same experimental model of isolated perfused heart with a bolus injection of PAF. The baseline perfusion pressure, the amount of PAF and the inclusion of serum albumin in the preparation of PAF solution are the determining factors for the coronary vascular effects of PAF. Moreover, the amount of PAF ranging from extremely low (1.0 fmol) to 1,000 pmol produced only a vasoconstrictor response in the isolated perfused guinea-pig heart. These results also suggest that animal species is a contributing factor to the diversity of coronary vascular responses to PAF.

It has been demonstrated that leukotrienes and thromboxane $A_2$, which are released by the heart upon ischemia-reperfusion, have harmful effects on the myocardium by causing coronary constriction, impairment of ventricular force, generation of arrhythmia and increase in microvascular permeability (Allan and Levi, 1980 and Piper, 1984). These effects are in contrast to the beneficial effect of prostacyclin ($PGI_2$) formed by blood vessel walls in preservation of ischemic myocardial cells. Piper and Stewart (1986, 1987) identified the presence of 6-keto-$PGF_{1\alpha}$, thromboxane $B_2$ and leukotriene-like active compounds in the effluent of the perfused heart after PAF administration. Pretreatment of hearts with indomethacin attenuated the vasoconstrictor effect of PAF in isolated rat hearts following bolus injections of PAF. Also, indomethacin blocked the coronary constriction produced by intracoronary injection of PAF in the pig heart (Feuerstein *et al.*, 1984). However, only vasoconstriction was observed in these studies. Another aim of our study is to

elucidate the mechanisms of coronary vasodilator and vasoconstrictor effects of PAF. We propose that the coronary vascular effects of PAF are indirectly mediated by other vasodilator and vasoconstrictor compounds which are released upon the administration of PAF. The most likely candidates are the cyclooxygenase and lipoxygenase products. However, under our experimental conditions, pretreating the rat hearts with indomethacin had no significant effect on the coronary vascular responses following a bolus injection of PAF in perfused hearts. The data presented in Part 3 therefore suggest that cyclooxygenase products are not playing an important role in mediating the coronary vascular effects of PAF. Pretreatment of the isolated rat hearts with leukotriene antagonists and a leukotriene synthesis inhibitor completely blocked both the coronary vasodilator and vasoconstrictor responses to a bolus injection of PAF. Following the bolus injection of PAF, the release of $LTB_4$, $LTC_4$, and $LTD_4$ were significantly increased in the effluent of perfused heart and were detected by radioimmunoassays. Bolus injection of $LTB_4$ produced a decrease in perfusion pressure, while a bolus injection of $LTC_4$ or $LTD_4$ resulted in increases in perfusion pressure. Therefore, both components of the coronary vascular responses could also be mimicked by bolus injections of leukotrienes. These data suggested that the coronary vascular responses to PAF in isolated rat heart are mainly mediated by lipoxygenase products, leukotrienes.

The development of the cascade perfusion model allowed the effluent from the first heart to act on the recipient heart. In this system, the properties of the vasoactive compounds released from the isolated heart can be pharmacologically characterized. Moreover, the use of the cascade perfusion model enables us to study the interplay of various vasoactive compounds in mediating the coronary vascular effects of PAF. The results in Part 4 confirmed the existence of vasoactive compounds in the effluent of the perfused heart after the administration of PAF. PAF-induced vasoconstriction in the

recipient heart consists of two components. One component could be blocked by leukotriene antagonists and the second component could be blocked by indomethacin. Both components could also be blocked by the leukotriene synthesis inhibitor. Our data therefore demonstrate that cyclooxygenase products could be regulated by endogenous leukotrienes in the perfused rat heart and this effect is receptor mediated. However, exogenously applied leukotrienes could not mimic the endogenously produced leukotrienes induced by PAF in regulating the synthesis of cyclooxygenase products. The mechanisms of the interaction between these vasoactive substances should be elucidated by future research.

It has been well documented that PAF acts via receptors on the membranes of responsive cells and the binding of PAF could be blocked by PAF antagonists (Braquet *et al.*, 1987). The $K_d$ values usually are between $10^{-10}$ and $10^{-9}$ M. The receptors are stereospecific and binding correlates well with the responses by whole cells. Receptors have been described in many cells and tissues. The rank order of potency for several antagonists was different in human neutrophils and platelets and the ionic requirement for PAF receptor binding was also different in the platelets and leucocytes. Then the presence of multiple PAF receptors was suggested by Hwang (1988). The functionally opposite vasodilator and vasoconstrictor effects of PAF could be explained by the existence of such PAF receptor subtypes in the heart. The study presented in this thesis has provided additional evidence to support this hypothesis. The vasodilator effect is mediated by a type of PAF receptor which has a higher affinity for PAF. The receptor is activated by very small amounts of PAF (1.0 fmol to 1.0 pmol). The vasoconstrictor effect of PAF is mediated by another type of PAF receptor which has a slightly lower affinity for PAF and the activation of the receptor requires a higher concentration of PAF ($>10$ pmol). The different time course of the vasodilator (peak effect 20-30 s) and vasoconstrictor (peak effect 60-90 s) responses to PAF is compatible with this

hypothesis. The desensitization properties of the vasodilator and vasoconstrictor responses are also different. In compariing the effects of a first and second injection of PAF, the second injection of 100 pmol PAF did not produce the vasoconstrictor response. However, the vasodilator effect of PAF was still observed following the second injection. Thus, it appears that the PAF receptor involved in vasoconstriction is easily desensitized while the PAF receptor involved in vasodilatation tends to desensitize more slowly. The functionally opposite vasodilator and vasoconstrictor effects of PAF could be selectively abolished by different PAF antagonists. CV-6209, one of PAF structural analog antagonists, showed selectivity for the vasodilator response and a higher concentration (10 fold) was required to block the vasoconstrictor response. The PAF antagonists, FR-900452, WEB 2086 and BN-50739, showed selectivity for the vasoconstrictor response (10, 20 and 1,000 fold, respectively).

In summary, the coronary vasodilator and vasoconstrictor effects of PAF are mediated by different PAF receptor subtypes (Figure 13). The first subtype of PAF receptor, which mediates the vasodilator response, could be activated by lower concentrations of PAF and induces the production and release of $LTB_4$. Then, $LTB_4$ activates the leukotriene receptor and causes coronary vasodilatation. When the second type of PAF receptor, which mediates the vasoconstrictor response, is activated by higher concentrations of PAF, the production and release of $LTC_4$ and $LTD_4$ are increased and a coronary vasoconstrictor response occurs. CV-6209 selectively blocks the PAF-induced vasodilator response at a lower concentration. The PAF antagonists, FR-900452, BN-50739 and WEB 2086, are selective in blocking the PAF-induced vasoconstrictor response at lower concentrations. Both leukotriene synthesis inhibitor, MK-886 and leukotriene receptor antagonists, e.g. L-649,923 can abolish the PAF-induced coronary vascular responses. Using the cascade perfusion heart model, it was also shown that there are complicated interactions between the PAF-induced vasoactive

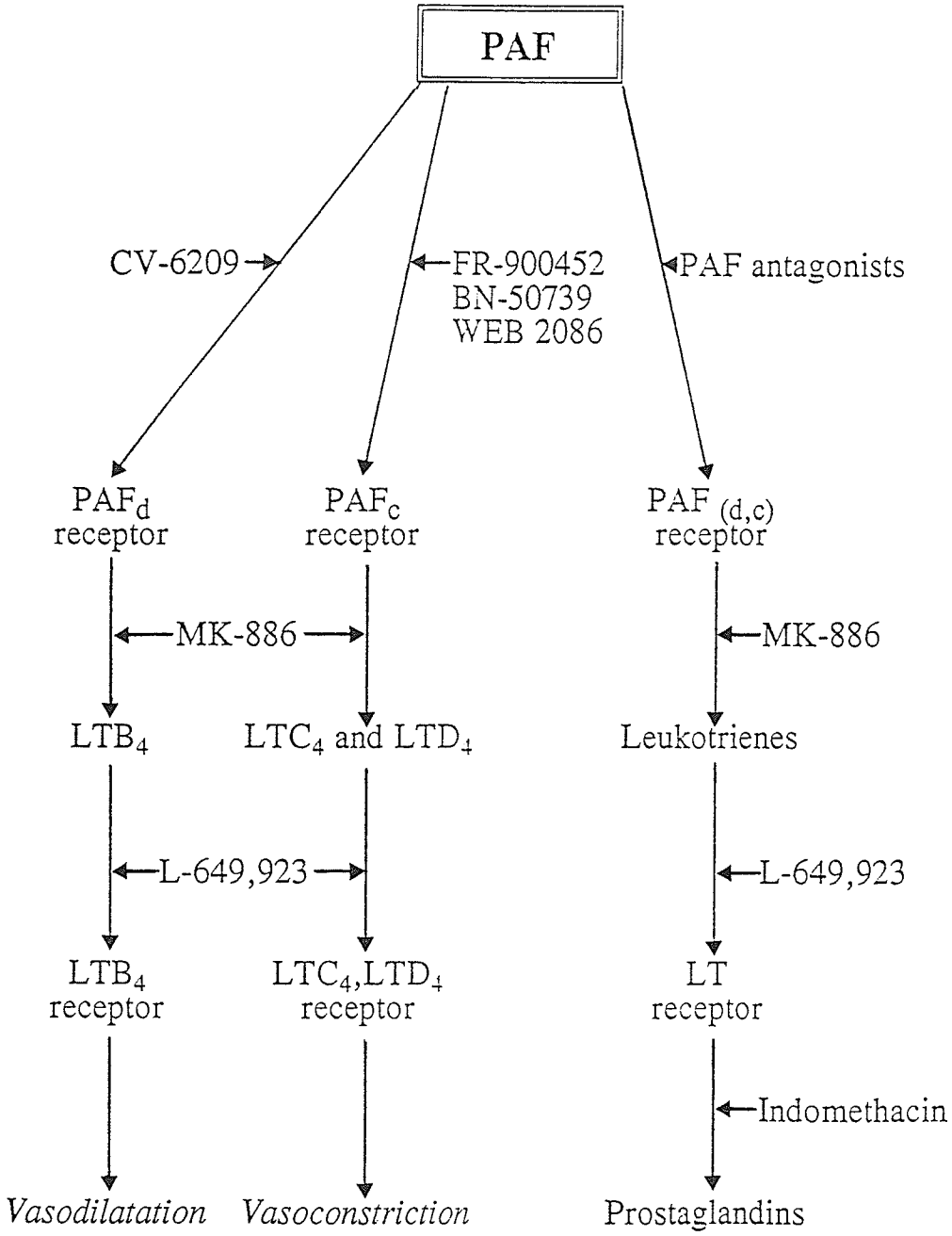



**Figure 13.** Schematic diagram illustrating the proposed PAF receptor subtypes mediating the vasodilator and vasoconstrictor effects of PAF and the interaction of PAF released vasoactive substances in perfused rat heart. $PAF_d$ receptor indicates the receptor mediating the vasodilator effect of PAF and $PAF_c$ indicates the receptor mediating the vasoconstrictor effect of PAF. LT receptor represents the leukotriene receptor. Compounds that are known to affect the receptor or synthesis of other vasoactive mediators in this study are listed.

compounds, such as, leukotrienes and prostaglandins. PAF-induced production and release of prostaglandins are mediated by leukotrienes and can be blocked by either leukotriene synthesis inhibitor or leukotriene receptor antagonists. Both leukotrienes and prostaglandins can be detected in the effluent of a perfused heart. We propose that endogenously produced leukotrienes are responsible for this regulation and that this is an intracellular event. But at this stage, it is still unknown which kind of PAF receptors and leukotrienes are involved.

As outlined in the introduction of the thesis, the receptors of PAF have not been purified or characterized at a molecular level. This is firstly because PAF is amphiphilic and results in high levels of nonspecific binding to membranes in the receptor binding studies. Since PAF is readily incorporated into mixed micelles, attempts to solubilize the receptor with detergent have been severely limited. The second problem is that there are only several hundred to several thousand receptors on most types of responsive cells. The study of PAF receptor at the present stage is focused on using radiolabeled receptor binding assays with selective PAF receptor antagonists which have less nonspecific binding. Future studies should concentrated on purification of the PAF receptors and the use molecular biology methods to clone the PAF receptor genes. This may facilitate the investigation into the different structures and sequences of the various proposed PAF receptor subtypes and their regulation at a molecular level.

Considerable interdisciplinary cooperation has led to major achievements in our understanding of PAF and related lipids with respect to chemical synthesis, their chemical and physical properties, structural features required for biological activities, factors that influence cellular metabolism and secretion, biological activities, receptors, interactions with other cell mediators and metabolic pathways and their regulation.

Continued efforts in these areas are expected to extend this current base of information as well as develop new concepts about the cellular role of PAF. Nevertheless, the challenge of future PAF research will be to elucidate the molecular mechanism(s) of its action in relation to the various biological responses it triggers. The ultimate goal is that continued advances in new basic knowledge and developments in the PAF field will ultimately lead to applications that provide a basis for understanding, preventing and treating disease processes as varied as hypertension, coronary vascular diseases, inflammation and allergic disorders where PAF has been implicated.

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