Free Rad. Res. Comms., Vol. 17, No. 3, pp. 195–209
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PLATELET ACTIVATING FACTOR STIMULATES AND PRIMES THE LIVER, KUPFFER CELLS AND NEUTROPHILS TO RELEASE SUPEROXIDE ANION

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(*Received May 26, 1992; in revised form June 10, 1992*)

Platelet activating factor (PAF) is considered a key mediator in eliciting the immunologic and metabolic consequences of endotoxic shock and sepsis. Release of oxygen-derived radicals is one of the important and relevant actions of PAF. This study examines the direct and priming effects of PAF on superoxide anion release by perfused liver, isolated Kupffer cells and blood neutrophils. One hour after PAF infusion at a dose of 2.2 μg/kg body weight a significant amount of superoxide release (0.71 ± 0.1 nmol/min/g liver) was measured in the perfused liver compared with the control livers (0.2 ± 0.01). In the *in vitro* presence of either phorbol ester or opsonized zymosan, superoxide release following PAF treatment *in vivo* was significantly increased to 1.36 ± 0.2 and 4.29 ± 0.36, respectively. The administration of PAF receptor antagonist (SDZ 63-441) almost completely inhibited the release of this radical. Kupffer cells (KC1, KC2, KC3) and blood neutrophils isolated from PAF-treated rats were also primed for increased production when these cells were challenged *in vitro* by the activator of protein kinase C, opsonin-coated zymosan as well as the chemotactic factors, complement 5a and F-met-leu-phe. PAF added *in vitro* to the perfused livers, isolated Kupffer cells or neutrophils from normal animals stimulated the release of superoxide with or without the above agonists. The direct stimulatory effect of PAF on superoxide release was inhibited by the PAF receptor antagonist *in vitro*. The role of PAF in the LPS-induced superoxide release by the perfused liver was also examined by the administration of PAF antagonist in endotoxic rats. The antagonist inhibited the LPS-mediated superoxide release at 1 hr, but not at 3 hr post-treatment. These results indicate that PAF stimulates and primes the hepatic elements to release superoxide. PAF may be an important factor during the early phase of endotoxemia, while other bioactive substances may take over at a later phase. Therefore, PAF is a key mediator that can directly enhance the release of toxic oxygen-derived radicals which may contribute to organ failure during endotoxemia or sepsis.

KEY WORDS: Oxygen-derived radicals, macrophages, tissue injury, chemotactic factor, rat.

INTRODUCTION

Platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) has been implicated as one of the mediators of the immunologic and metabolic sequelae of endotoxic shock and sepsis. PAF is suspected to exert a multiplicity of actions among them enhancing the release of toxic oxygen-derived metabolites that can contribute to tissue injury.[1–3] Although PAF is also released by macrophages,[4] including the Kupffer cells, it may also have an autocrine function.[5–7] In addition, PAF targets other inflammatory cells such as neutrophils, eosinophils and platelets.

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As a result of such interaction between PAF and its target cells, oxygen-derived radicals are released which enhance the microbicidal activity of these cells. However, the release of reactive oxygen species may also cause injury to other tissues. The PAF-induced release of toxic oxygen-derived radicals is believed to result from increased influx of Ca^{++} into the cells, that leads to the activation of protein kinase C, and translocation of NADPH oxidase to the membrane.[8–10] This enzyme catalyzes single electron reduction of O_2 to O_2^-, leading to the formation of more toxic byproducts such as H_2O_2 and singlet oxygen. Hypochlorous metabolites (OCl^-) are also formed by the oxidation of H_2O_2 by myeloperoxidase in the presence of Cl^-. This may occur more commonly in neutrophils which contain myeloperoxidase in contrast to macrophages.[11]

PAF has been shown to participate in a wide spectrum of metabolic, immunologic and pathologic processes such as immune complex vasculitis[12] and microvascular immune injury.[2] The mechanism for such events is attributed to the stimulatory effect of PAF on neutrophil migration, activation and release of toxic oxygen-derived radicals at the site of inflammation.[2] Neutrophils are also primed by PAF to release large quantities of superoxide anion when challenged with preformed IgG-immune complexes.[12] The effect of PAF on other cells such as hepatocytes,[13] platelets,[14,15] monocytes,[16] eosinophils[17] and macrophages[5–7] have also been documented. For example, PAF has been shown to stimulate glucose consumption,[5] release of prostanoids[18] and oxygen-derived radicals[9,19] by leukocytes and peritoneal macrophages. However, very little is known about the effect of PAF on resident macrophages (Kupffer cells) in the liver which represent more than 80% of tissue macrophages. Kuppfer cells are also known to be the primary target of endotoxins (LPS). When LPS interact with Kupffer cells, a number of bioactive substances are released that may include PAF. Since PAF is considered to have an autocrine function in macrophages and leukocytes, a feedback loop may be activated during the early stage of endotoxemia or shock that can lead to tissue injury and organ failure.

In view of the above considerations, the present study is based on the hypothesis that PAF stimulates and primes the liver and hepatic phagocytes to release toxic oxygen-derived metabolites, and thus PAF is a contributory factor to the LPS-induced superoxide release during the early stage of endotoxemia. The formation of such radicals *in vivo* may therefore predispose the host to the deleterious effects of these substances leading to cytotoxicity, tissue injury and possibly organ failure. If PAF is a key mediator in the release of oxygen-derived radicals during endotoxemia, the LPS-induced release of reactive oxygen species may be reversed by the administration of PAF receptor antagonist SDZ 63-441. Therefore, this work examines the effect of PAF and the PAF receptor antagonist on superoxide anion release by the *in situ* perfused liver, isolated Kupffer cells and blood neutrophils, as well as the potential role of PAF in the LPS-induced superoxide anion production by the liver.

MATERIALS AND METHODS

Preparation of experimental animals

On the day before the experiments, male Sprague-Dawley rats (250–300 g, Charles River, Cambridge, MA) were cannulated with venous and arterial catheters using aseptic surgical techniques. Food was removed 18 hrs before the experiments, but

the animals were allowed free access to water. The following day, the rats received an intravenous infusion of PAF (99% pure, Sigma) at a dose of 2.20 μg/kg body weight. To determine the specificity of PAF, in another group of animals the receptor antagonist SDZ 63-441 (5 mg/kg, Sandoz Research Institute) was injected intravenously 5 min prior to PAF treatment followed by a continuous infusion for 1 hr (100 mg/kg/hr). To examine the possible contributions of arachidonic acid metabolites derived via the cyclooxygenase pathway, ibuprofen (10 mg/kg/hr, Upjohn Inc., Kalamazoo, MI) was infused together with PAF. Ibuprofen was also infused into some of the perfused livers as described below. In another experimental group, *Escherichia coli* lipopolysaccharide (LPS, Difco, MA) was injected intravenously at a dose of 1 mg/kg body weight 10 min after the injection of PAF receptor antagonist. The infusion of PAF receptor antagonist in the LPS-treated rats was continued for 1–3 hr. The control group received sterile saline.

Isolation of liver cells

Hepatocytes, endothelial and Kupffer cells were isolated from the liver by collagenase-pronase digestion followed by centrifugal elutriation as described previously.[20,21] Briefly, the liver was perfused for 5 minutes with 25 mg collagenase (Sigma) in 100 mL Hank's balanced salt solution supplemented with 0.001% bovine pancreatic deoxyribonuclease type I (Sigma) and 1 mM $CaCl_2$. The organ was surgically removed and cut into small pieces, resuspended in Gey's balanced salt solution and filtered through 4 layers of sterile gauze. The resulting cell suspension was centrifuged at 50 × g for 2 minutes to sediment the hepatocytes. The remaining tissues were further digested with 0.2% pronase E (Sigma), 0.001% deoxyribonuclease in Gey's balanced salt solution for 40 min at room temperature. Endothelial and Kupffer cells were collected by centrifugal elutriation at 875 × g at 23 mL/min (endothelial cells) and 29–45 mL/min (Kupffer cells) flow rates using a Beckman elutriator (J2-21 centrifuge, JE-6B elutriator rotor; Beckman Inc., Palo Alto, Ca). Subpopulations of Kupffer cells were separated at 29 mL/min flow rate at 875 × g (KCl), 45 mL/min at 875 × g (KC2) and at 45 mL/min at 1 × g (KC3) as described previously.[22] Using these methods, purity of hepatocytes, endothelial and Kupffer cells were above 95% as assessed by morphology (Giemsa-Wright stain) and peroxidase stain. Cell viability was above 97% as determined by Trypan blue exclusion.

Peroxidase staining

Peroxidase positive cells were identified using a modified peroxidase staining technique described previously.[23] Twenty μL of cell suspension (10×10^6 cells/mL) was added to 180 μl of Gey's balanced salt solution containing 1 mg/mL of diaminobenzidine (Sigma) and 1% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ). The cells were incubated for 10 min at 37°C. An aliquot (5 μL) was taken and examined under a microscope. Brown-stained cells were considered peroxidase positive. Kupffer cells were peroxidase positive while endothelial cells were negative.

Isolation of blood neutrophils

Heparinized blood (5–7 ml) was collected from the arterial catheter. Erythrocytes

were sedimented in the presence of 0.6% dextran 400 (Sigma) for 1 hr at 1 × g at room temperature. The leukocyte rich supernatant was collected centrifuged and resuspended in Hank's balanced salt solution, and layered onto a Ficoll-Hypaque density gradient of 1.077 and 1.119. Neutrophils were collected at the interface between these two gradients. This method was based on the procedure described by English and Andersen.[24] Using this method, neutrophil viability and purity was above 95%.

Preparation of opsonized zymosan

Zymosan particles were resuspended in sterile saline at a final density of 1 g/20 mL and boiled for 15 min. The boiled particles were cooled to room temperature and washed twice with sterile saline. Zymosan was resuspended in 10 ml of normal rat serum and incubated at 37°C for 30 min. The opsonin-coated zymosan particles were washed and finally resuspended in Hank's balanced salt solution at a final concentration of 1.98 mg/100 μL. Aliquots of 1–2 ml were kept at −20°C until used in the superoxide anion assay.

Preparation of endotoxin activated serum (C5a)

E. coli LPS was added to normal rat serum at a final concentration of 1 mg/mL. Serum was incubated at 37°C for 30 min to activate complement leading to the formation of C5a, and centrifuged at 100,000 × g at 4°C for 30 min. The supernatant was collected, divided in 1 mL aliquots and stored frozen at −20°C until used.

Measurement of superoxide anion release by in situ perfused liver

The portal vein was cannulated with a 20-gauge Teflon catheter (Delmed, Canton, MA). The liver was perfused with oxygen-saturated calcium-free Hank's balanced salt solution. The superior vena cava cannula served as the outflow tract, after ligation of the inferior vena cava. The blood was removed from the liver by continuous perfusion of 200 ml of the buffer at 2–3 mL/min/g wet liver at 37°C. Once the liver was cleared of blood, Hank's balanced salt solution containing 32 μmol ferricytochrome C (Sigma), 5 mmol glucose and 0.87 g/L bovine serum albumin (Sigma) was perfused through this organ. Phorbol myristate acetate (PMA; 10^{-7} M) or opsonized zymosan (1.98 mg/mL) was added at 4–5 min while Cu–Zn superoxide dismutase, derived from bovine liver (7500 Units/liver, Sigma) was introduced into the perfusion medium 8–10 minutes after the substrate had entered the liver. To determine the effect of cyclooxygenase inhibitors on superoxide anion release, ibuprofen (5 mg/mL/min) was infused into the liver without cytochrome c for 5 minutes followed by a 10 min perfusion in the presence of cytochrome c. Aliquots of (2.5 mL) of the perfusates were immediately placed in an ice bath, and when all the tubes were collected, they were centrifuged to remove contaminating cells and debris. The change in absorbance was measured at 550 nm. The concentration of detectable superoxide anion during liver perfusion was measured based on the slope of the delta absorbance of the reduced ferricytochrome C (minus SOD) which was converted to nmol using the molecular extinction coefficient of 21.1 M^{-1} cm^{-1}. The value is then expressed as nmol/min (of perfusion)/g wet weight. This procedure has been described in reference 25.

Superoxide anion assay on isolated cells

Superoxide anion assay on isolated cells was performed according to the procedure described previously.[26] Isolated hepatocytes, endothelial and Kupffer cells in Hank's balanced salt solution were each layered onto a 35 cm sterile petri dish (Costar) at a final cell density of 0.75–1.0 $\times$ 10^6 cells/plate. Ferricytochrome C (50 μM, Sigma) was added to the reaction mixture. Superoxide dismutase (SOD, 300 units/well) was added to the negative control. Phorbol myristate acetate (1 μmol), opsonized zymosan (1.98 mg/mL), complement activated serum, the source of C5a (10%), or formyl-methionyl-leucyl-phenylalanine (10^{-8} M) was added as stimulant. Superoxide anion was measured based on a change in absorbance (difference in absorbance with or without SOD) against a cell-free blank. Delta absorbance was converted into nmol using the molecular extinction coefficient of $21.1^{-M} \cdot cm^{-1}$. Superoxide anion is expressed in nmol/10^6 cells/hr.

Statistics

Data presented in this paper represent means $\pm$ SE of 5 to 10 independent experiments. Statistical significance was assessed by Student t test and non-parametric statistical analysis.

RESULTS

Effect of PAF infusion on superoxide anion produced by in situ perfused liver

Figure 1 shows that PAF infusion for 1 hr induced the reduction of ferricytochrome c by the *in situ* perfused liver. The rate of reduction was significantly greater than in the saline-treated control. The addition of superoxide dismutase towards the end of perfusion demonstrated that the reduction was due to superoxide anions, since the reduction of ferricytochrome was inhibited in the presence of this enzyme (Figure 1). The effect of superoxide dismutase on the inhibition of ferricytochrome c reduction in the control livers was not significant, indicating that this was not due to superoxide anion production. The estimated amount of superoxide released following PAF *in vivo* was 0.71 $\pm$ 0.1 nmol/min/g liver. The addition of PAF receptor antagonist *in vivo* inhibited the release of superoxide anion by the perfused liver following PAF. The antagonist had no effect on the superoxide release by the control livers (Figure 1).

The *in vivo* priming effect of PAF on superoxide release by the perfused liver when it was challenged by either PMA or zymosan was also observed (Figure 1). The amounts of superoxide released in response to these agonists were 1.36 $\pm$ 0.2 and 4.29 $\pm$ 0.36 nmol/min/g liver, respectively. Control livers released less than 0.5 nmol in the presence of these agonists.

Effect of PAF in vitro on superoxide anion release by the perfused liver

Immediately after the removal of blood during perfusion of control liver, PAF at a final dose of 100 μg/liver was infused for 10 minutes followed by another 10 minutes in the presence of ferricytochrome c. Figure 2 shows that *in vitro* PAF induced the release of superoxide anion by the perfused liver. The estimated amount of superoxide

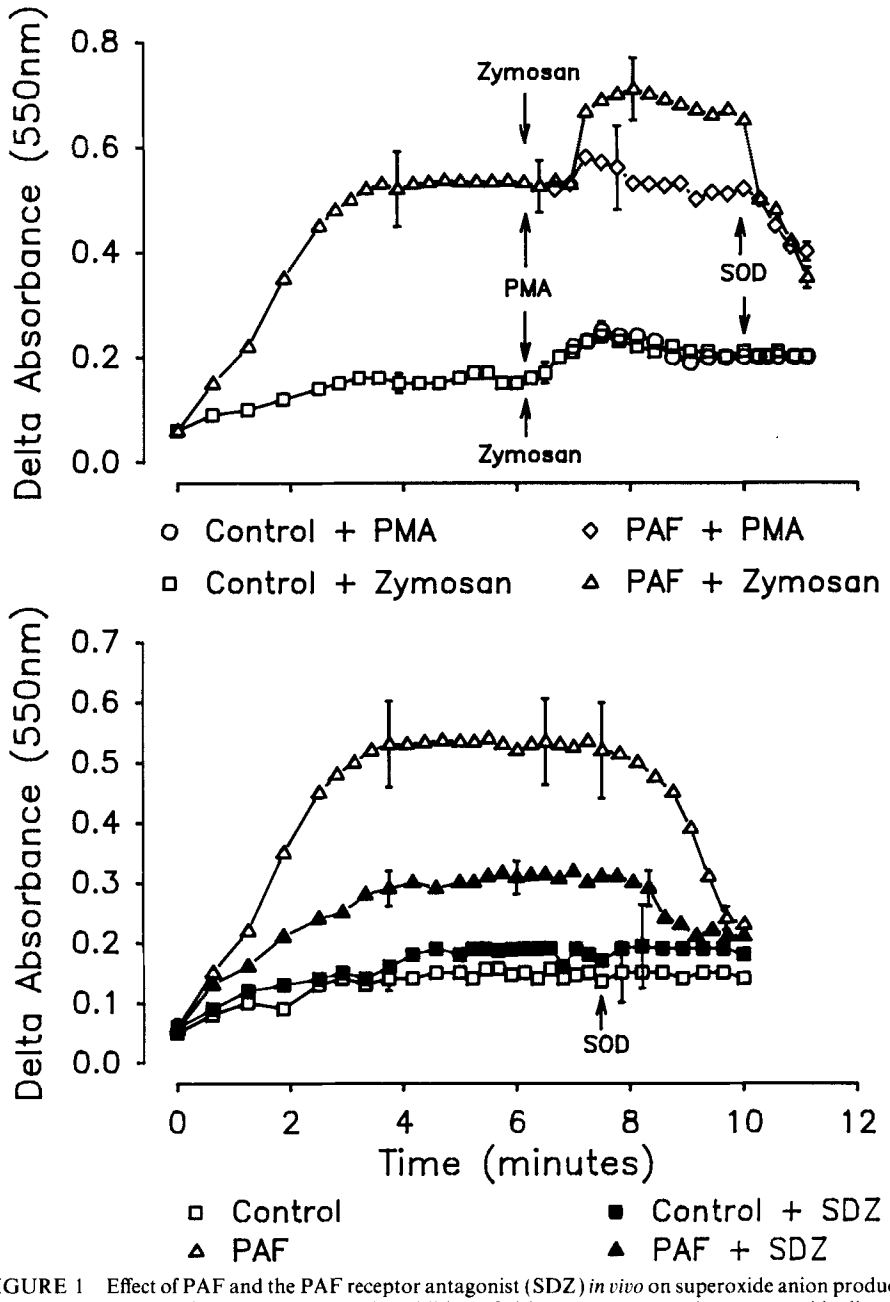

FIGURE 1 Effect of PAF and the PAF receptor antagonist (SDZ) *in vivo* on superoxide anion production by the perfused liver. The arrows indicate the addition of either zymosan, PMA or superoxide dismutase (SOD). The upper panel refers to the effect of PAF on agonist-stimulated superoxide release as indicated by the arrows, while the lower panel refers to the effect of PAF receptor antagonist on PAF-induced superoxide release. The concentration of cytochrome c used in this experiment was 32 μM. For the determination of superoxide release by isolated cells, it was necessary to increase the concentration of cytochrome c to 50 μM, because neutrophils generate large amount of superoxide *in vitro*.

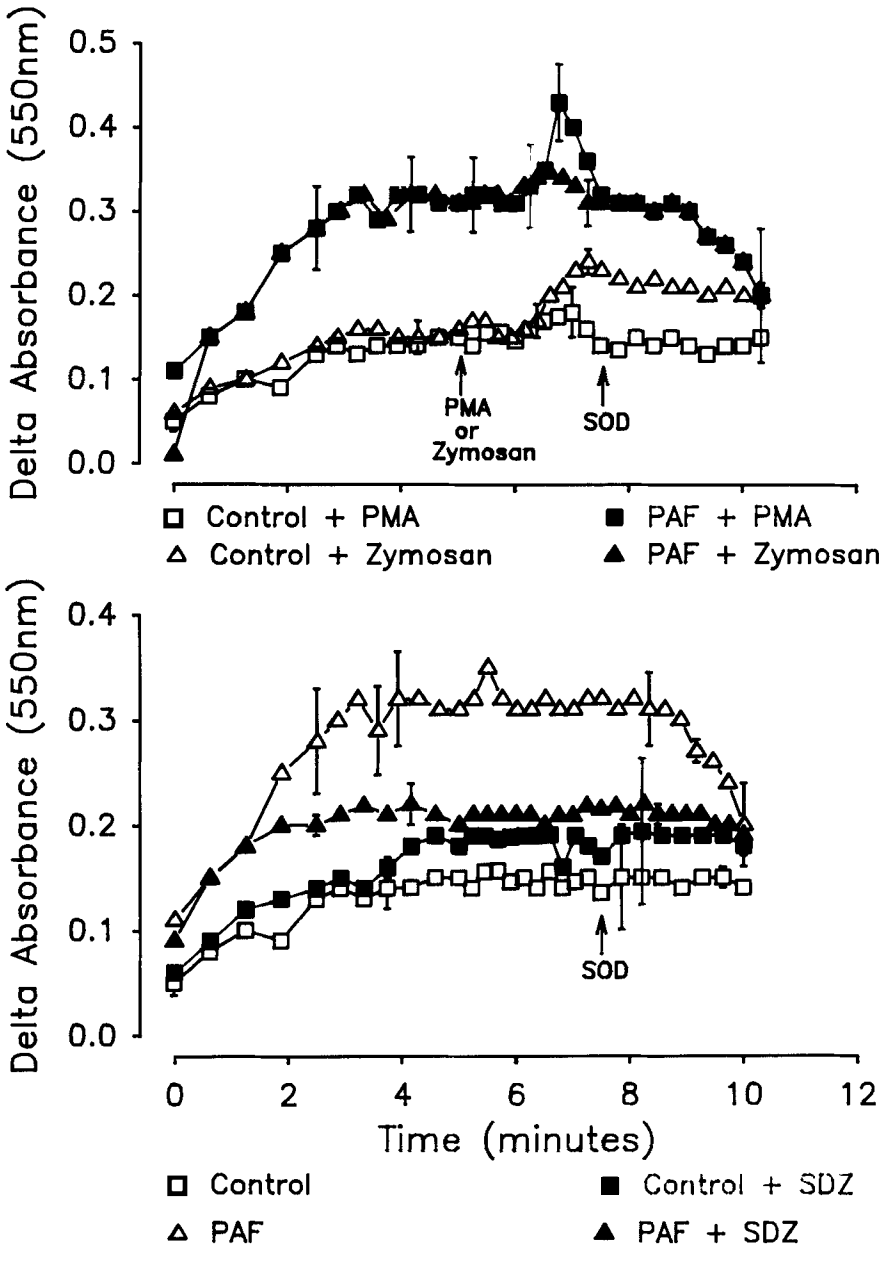

FIGURE 2 Effect of PAF and the PAF receptor antagonist *in vitro* on superoxide anion release by the perfused liver. Arrows indicate the addition of zymosan, PMA or superoxide dismutase (SOD). The upper panel refers to the agonist-stimulated superoxide release as indicated by the arrows, while the lower panel refers to the effect of PAF receptor antagonist on PAF-induced superoxide release. The concentration of cytochrome c used in this study was 32 μM. For the determination of superoxide by isolated cells, it was necessary to increase the concentration of cytochrome c to 50 μM, because neutrophils generate large amount of superoxide anion *in vitro*.

released by liver after *in vitro* PAF was 0.3 ± 0.04 nmol, and was elevated slightly to 0.6 and 1.0 after the addition of zymosan or PMA, respectively. The addition of PAF receptor antagonist inhibited the superoxide release induced by *in vitro* PAF. The addition of either PMA or zymosan slightly increased superoxide formation, but these values did not reach statistical significance compared to the parallel controls (Figure 2).

Effect of ibuprofen on PAF-induced superoxide release by the perfused liver

Ibuprofen infusion *in vivo* and *in vitro* inhibited the PAF-mediated superoxide production by the perfused liver by almost 80%. The amount of PAF-induced superoxide produced in the presence of ibuprofen was 0.12 ± 0.02 nmol/min/g liver, compared with PAF alone (0.71 ± 0.1). Ibuprofen alone did not have any significant effect on superoxide release by control livers.

Effect of PAF in vivo and in vitro on superoxide anion release by isolated Kupffer cells and neutrophils in vitro

Table I shows that 1 hr after PAF infusion *in vivo* all Kupffer cells subpopulations (KC1, KC2 and KC3) generated significant amounts of superoxide anion spontaneously. In the presence of the chemotactic factors, C5a and f-met-leu-phe, opsonin-coated zymosan or PMA, the amount of superoxide released was further increased. The values of superoxide released among the 3 subpopulations of Kupffer cells were not statistically different. Neutrophils taken from PAF treated rats were also primed for increased superoxide release when these cells were challenged *in vitro* with the same agonists (Table I).

The direct *in vitro* effect of PAF was also determined in isolated Kupffer cells and blood neutrophils. Kupffer cells isolated following collagenase-pronase digestion was

TABLE I
Superoxide anion release by Kupffer cells and neutrophils following PAF treatment *in vivo*

Superoxide anion (nmol/10^6 cells/hr)

Agonist	Kupffer cell 1	Kupffer cell 2	Kupffer cell 3	Neutrophils
None	1.2 ± 0.9†	2.0 ± 0.2	3.0 ± 0.4	0†
	(0)†	(0.25 ± 0.1)	(0.9 ± 0.13)	(0)†
C5a	6.4 ± 1.1	8.3 ± 1.2	9.6 ± 0.8	14.0 ± 3.0
	(1.4 ± 0.1)	(2.5 ± 0.1)	(3.2 ± 0.6)	(4.0 ± 0.3)
F-met-leu-phe	3.7 ± 1.0	4.4 ± 1.0	6.2 ± 1.0	6.0 ± 0.3
	(0)	(0.25 ± 0.1)	(0.9 ± 0.1)	(3.0 ± 1.0)
Opsonized zymosan	7.3 ± 0.8	7.4 ± 0.9	8.0 ± 1.6	21.3 ± 5.0
	(1 ± 0.1)	(0.8 ± 0.2)	(2.1 ± 0.2)	(6.0 ± 0.7)
PMA	3.0 ± 1.0†	7.4 ± 0.9*	7.5 ± 1.3*	23.0 ± 2.0
	(1.4 ± 0.1)†	(0.9 ± 0.2)	(2.4 ± 0.4)	(7.5 ± 0.5)

Values in parentheses refer to the saline-treated group.
*P < 0.001 vs KCl.
†Not significant.
All values between control and PAF-treated groups were statistically different.
N = 5.

cultured *in vitro* for 24 to regenerate the PAF receptors that may have been lost during the isolation procedures. Figure 3 shows that PAF stimulated the release of superoxide anion by the isolated Kupffer cells and neutrophils from naive untreated rats in a dose-dependent manner. In the presence of PAF receptor antagonist, the amount of superoxide released in the presence of PAF was markedly diminished in the Kupffer cells. The PAF receptor antagonist completely inhibited the PAF-induced superoxide anion release by neutrophils (Table II).

Superoxide production by either the endothelial cells or isolated hepatocytes taken from control and PAF-treated rats were negligible. The addition of PAF *in vitro* to these cells did not stimulate superoxide anion release (data not shown).

Effect of PAF receptor antagonist on the LPS-induced superoxide release by the perfused liver

We have previously shown that the LPS-induced superoxide release by the perfused liver was detected at 1 hr post-treatment. The superoxide release peaked at 3 hr and returned to normal by 6 hr. Figure 4 shows that the infusion of PAF receptor antagonist into LPS-treated rats inhibited the release of superoxide-induced by endotoxin by almost 70% 1 hr post-treatment. The receptor antagonist-induced inhibition of LPS-induced superoxide release was not detected at 3 hr post-treatment.

DISCUSSION

These data indicate that PAF directly stimulates the liver, isolated Kupffer cells and blood neutrophils to release superoxide anion. PAF also primed the hepatic phagocytes for increased superoxide production when these cells were challenged *in vitro* with chemotactic factors, activator of protein kinase C and opsonin coated zymosan particles. The PAF-induced stimulation of superoxide release was markedly diminished by the addition of PAF receptor antagonist suggesting the involvement of PAF receptors in these processes. This study also demonstrates that *in vivo* treatment of endotoxic rats with the PAF-receptor antagonist downregulated superoxide production during the early phase of endotoxemia, but not in the later phase, implying that other mediators such as TNF may be involved during this latter stage.

PAF is a potent autocoid mediator produced by neutrophils, eosinophils, monocytes, macrophages, platelets and endothelial cells. As a result of the interaction of PAF with these cells, toxic reactive oxygen species are produced.[9,19] Neutrophils and eosinophils are also primed for increased aggregation and chemotaxis, diapedesis and endothelial cell infiltration.[2,17,19] These events lead to plasma extravasation, interstitial edema, arterial hypotension and shock.[1–3] In the liver, PAF has also been suggested to stimulate hepatic glycogenolysis and vasoconstriction during ovalbumin-induced anaphylaxis.[27] This finding indicates that PAF not only acts on vascular endothelial cells and leukocytes but also on hepatocytes. It has been suggested that PAF-mediated increase in glycogenolysis in the perfused liver occurs through an indirect mechanism via the vasoconstriction induced alterations in perfusate flow or hypoxia.[27] The same mechanism may play a role in the PAF-induced superoxide anion release by the perfused liver as shown in this study. Hepatic ischemia followed by reperfusion primes the Kupffer cells to generate superoxide anion.[28] This is also

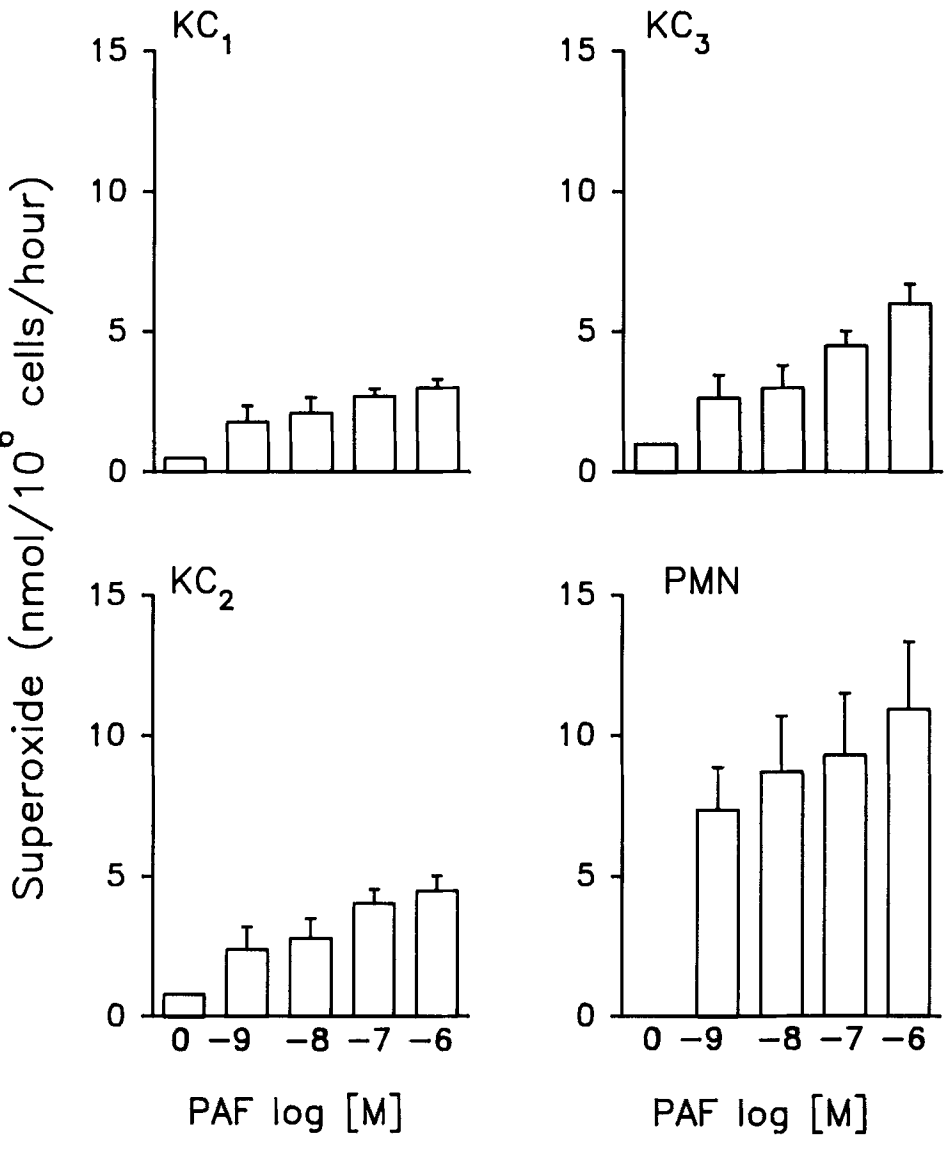

FIGURE 3 Effect of increasing concentration of PAF on superoxide release by Kupffer cells and blood neutrophils. Kupffer cells from normal rats were isolated as described in the Materials and Methods section. Isolated Kupffer cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (Hyclone), 50 μg/ml gentamicin and 100 units/ml penicillin and streptomycin (Sigma) for 24 hr to regenerate the PAF receptors that may have been lost during the isolation procedures. On the day of the experiment, RPMI was removed and the Kupffer cell monolayer was washed with pre-warmed Hank's balanced salt solution. Blood neutrophils were freshly isolated and assayed for superoxide release on the day they were collected. A final volume of 1.0 mL Hank's buffer was layered onto the monolayers of either Kupffer cells or neutrophils in a 6 well-plate (Costar). Different concentration of PAF was added to each well. Ferricytochrome c (50 μM final concentration) was also added to each well. Appropriate blank with or without 300 units/well of superoxide dismutase was set up. The cells were incubated for 60 min at 37°C. Superoxide anion was determined as described in the Materials and Methods section.

TABLE II
Effect of PAF receptor antagonist (SDZ 63-441) on the PAF-induced superoxide release by Kupffer cells and neutrophils from normal rats

Superoxide anion (nmol/10^6 cells/hr)

Cell type	PAF*	PAF + Antagonist
Kupffer cell 1	4.0 ± 0.8	0.75 ± 0.5
Kupffer cell 2	4.5 ± 0.7	1.2 ± 0.8
Kupffer cell 3	5.0 ± 0.4	0.3 ± 0.4
Neutrophils	10.0 ± 1.1	0

*$P < 0.001$ vs PAF + Antagonist.

Cells from naive rats were isolated as described in the Materials and Methods sections. Isolated Kupffer cells were cultured for 24 hr to generate the PAF receptors that may have been lost during the isolation procedures. Neutrophils were freshly isolated and assayed for superoxide release on the day they were collected. Cells were treated with 1 μM PAF in the presence or absence of PAF receptor antagonist (10 μg/l × 10^6 cells) for 15 minutes. Ferricytochrome c (50 μM) with or without 300 Units superoxide dismutase was added to the culture. The cells were incubated at 37°C for 60 min. Superoxide anion was determined as described in the Method section.

accompanied by increased neutrophil infiltration into the liver and priming of these cells for increased respiratory burst.[28] Tissue injury during hepatic ischemia as assessed by elevated plasma glutathione has been attributed to reactive oxygen species released by phagocytic cells.[29] It is unlikely that hepatocytes and hepatic endothelial cells are the predominant sources of these radicals, because these cells did not release significant amounts of superoxide anion *in vitro* as shown in this study and in our previous publication.[30] The most likely sources of superoxide released by the perfused liver following PAF treatment *in vitro* or *in vivo* are the Kupffer cells. Neutrophils in the liver probably did not contribute significantly as their number was very small ($< 2.0 \times 10^6$/liver). PAF infusion for 1 hr did not significantly increase the number of neutrophils sequestered in the liver, in contrast to previous observations during endotoxemia,[30] TNF infusion,[25] and *in vivo* activation of the reticuloendothelial system.[31] However, blood neutrophils were primed for increased superoxide release following *in vivo* infusion as shown in the Results section.

The mechanism for increased respiratory burst by phagocytic cells remains to be fully elucidated. However, it has been shown that PAF directly enhances the influx of Ca^{++} into the cells.[8,9] Ca^{++} bind to the membranes and increase the activity of protein kinase C.[32] As a result, PAF can directly stimulate superoxide release by this mechanism. The influx of Ca^{++}, and intracellular Ca^{++} mobilization can activate protein kinase C to induce the translocation of NADPH oxidase to the plasma membrane, leading to the formation of O_2^- from O_2. Therefore, the priming of increased superoxide release through ligand (chemotactic factors, C5a and F-met-leu-phe, or opsonized zymosan)- mediated activation of phagocytic cells may be achieved through this pathway. Increased intracellular Ca^{++} triggered by PAF increases the activity of calpain to cleave protein kinase C to a form which is independent of Ca^{++} and phospholipid.[33] This may be responsible for the priming effect of PAF on phorbol ester induced superoxide production by isolated Kupffer cells and neutrophils. Another aspect of the mechanism of action of PAF on its target cells is through the modulation of inositol phospholipids via deacylation and

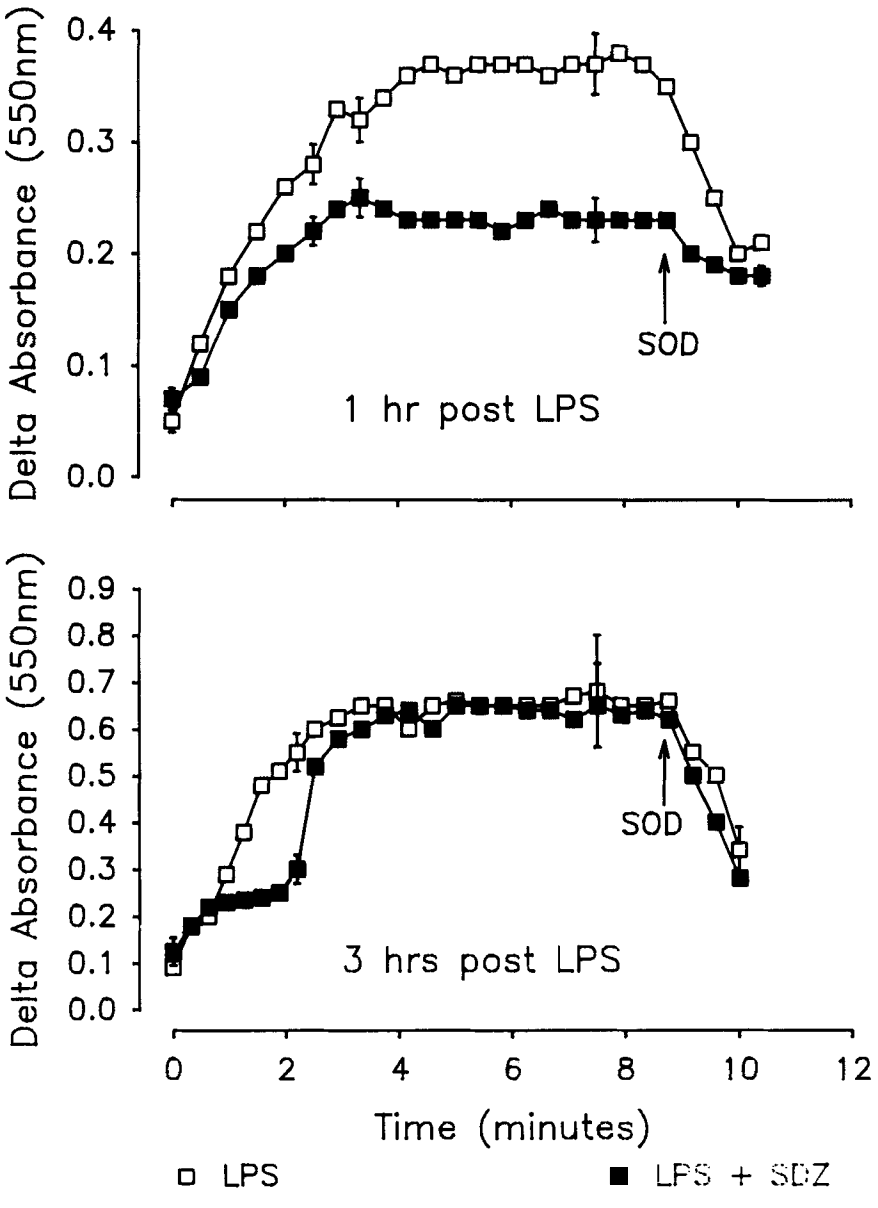

FIGURE 4 Effect of PAF receptor antagonist on the LPS-induced superoxide anion release by the perfused liver. Upper panel refers to 1 hr after LPS treatment, while the lower panel refers to 3 hr post-LPS.

phospholipase C activation leading to the upregulation of arachidonic acid metabolism as has been observed in Kupffer cells.[8] Stimulation of eicosanoid biosynthesis has been demonstrated to be involved in activation of the respiratory burst in neutrophils[34] and Kupffer cells following latex phagocytosis *in vivo* and *in vitro*.[31] The present study also demonstrates that in the perfused rat liver

oxygen-derived radical production induced by PAF may also be mediated at least in part by arachidonic acid metabolism. This assumption was based on the experiments which showed that the addition of ibuprofen, an inhibitor of the cyclooxygenase, attenuated the PAF-stimulated production of superoxide anion by the perfused liver.

The ability of PAF to stimulate phagocytic cells to release superoxide anions and other toxic oxygen metabolites is beneficial for maintenance of the microbicidal function of these cells. PAF also primes phagocytes to be highly sensitized to chemotactic factors such as C5a and the bacterial synthetic product, f-met-leu-phe. In the present study, Kupffer cells and neutrophils taken from PAF-treated rats released large amounts of superoxide anion when these cells were challenged with C5a or f-met-leu-phe *in vitro*, compared to those cells taken from saline treated rats. Breviario *et al.*[35] have shown that PAF enhanced the adhesion of neutrophils to vascular endothelial cells pretreated with either IL-1 or TNF which was reversed by the addition of PAG antagonists. Neutrophils pretreated with PAF were also primed to release superoxide when challenged with TNF or GM-CSF[2]. These findings indicate that PAF alone or in combination with other cytokines has a significant role in host-defense mechanisms during infection. However, the release of oxygen-derived radicals by phagocytes may also cause cytotoxicity to the surrounding cells such as the hepatocytes and endothelial cells in the liver that could contribute to tissue injury and organ failure during endotoxic shock and sepsis.

The pharmacological actions of PAF have been shown to mimic the pathophysiology of shock.[1] In the liver, PAF induces vascular constriction, glycogenolysis[13,36] and inositol turnover.[13] Endotoxemia is known to induce a sequence of events that includes the release of humoral and cellular mediators leading to the clinical manifestations of endotoxic shock.[1] The wide array of mediators secreted during endotoxemia includes cytokines, vasoactive amines, complement components and bioactive lipids. Among the lipid mediators, PAF is considered to be a potent autocoid known to regulate leukocyte function, hepatic metabolism, bronchopulmonary system, gastrointestinal function and the immune system.[1] Following endotoxemia, Kupffer cells which are the primary targets of LPS are activated, resulting in the release of bioactive substances including PAF. Therefore, PAF can interact with Kupffer cells and other cells adjacent to this cell type such as hepatocytes and endothelial cells, resulting in vasoconstriction, glycogenolysis and hyperglycemia.[13,36,37] This may be followed by systemic activation of leukocytes for chemotaxis and sequestration in the liver. The presence of activated Kupffer cells and the accumulation of neutrophils in the liver during the acute phase of endotoxemia may compromise this organ, because of tissue injury mediated by toxic oxygen-derived radicals that are released by these cells. PAF was shown to have a significant contribution to the LPS-induced superoxide release during the early stage of endotoxemia. This seems to be specific for PAF, since the administration of PAF receptor antagonist *in vivo* inhibited the LPS-mediated superoxide release by the liver. However, after 3 hr endotoxin treatment, attenuation of the LPS-associated superoxide formation in the liver by the PAF antagonist was not evident, suggesting that PAF is involved during the early phase of endotoxemia. During the latter phase other mediators are probably taking over. The most likely mediator is TNF which peaks at 90 min after LPS treatment.[38]

In conclusion, these studies demonstrate the PAF-induced stimulation and priming of the liver, Kupffer cells and neutrophils to release superoxide anion may be mediated

by PAF receptors since the PAF receptor antagonist significantly inhibited superoxide release. The increased sensitivity of neutrophils to chemotactic factors following *in vivo* PAF may be a factor required for increased sequestration of neutrophils to the liver during the early phase of endotoxemia. The priming of Kupffer cells by PAF to release large amounts of oxygen-derived radicals in response to receptor-mediated phagocytosis, phorbol ester and chemotactic factors could predispose the hepatocytes and endothelial cells to the deleterious effects of these radicals. PAF is probably involved in the induction of metabolic and immunologic sequelae during the early phase of endotoxemia while other bioactive mediators may be responsible for the latter phase. Therefore, PAF is a key mediator that can stimulate oxidative burst leading to the formation of toxic oxygen metabolites that may contribute to organ failure during endotoxic shock.

Acknowledgement

This work was supported by the National Institutes of Health grant GM 32654. The excellent technical assistance of Jean Carnal, Lisa Luke, Kirk Elliott and Howard Blakesley is greatly appreciated.

References

1. M. Santiago-Crespo and S.F. Gallardo (1991) Pharmacological modulation of PAF: A therapeutic approach to endotoxic shock. *Journal of Lipid Mediators*, **4**, 127–144.
2. P. Braquet, M. Paubert-Braquet, R.H. Bourgain, F. Bussolino and D. Hosford (1989) PAF/cytokine auto-generated feedback networks in microvascular immune injury: Consequences in shock, ischemia and graft rejection. *Journal of Lipid Mediators*, **1**, 75–112.
3. T. Mozes, J.P.C. Heiligers, J.A.M. Crine, F.J. Zijlstra, S. Ben-Efrain, P.R. Saxena and I.L. Bonta (1991) Platelet activating factor is one of the mediators involved in endotoxic shock in pigs. *Journal of Lipid Mediators*, **4**, 309–326.
4. G. Camussi, F. Bussolino, G. Salvidio and C. Baglioni (1987) Tumor necrosis factor/cachectin stimulates peritoneal macrophages, polymorphonuclear neutrophils, and vascular endothelial cells to synthesize and release platelet-activating factor. *Journal of Experimental Medicine*, **166**, 1390–1404.
5. H. Hayashi, I. Kudo, S. Nojima and K. Inoue (1991) Biological response of guinea pig peritoneal macrophages to platelet activating factor. *Lipids*, **26**, 1193–1199.
6. W. Chao, H. Liu, M. Debysere, D.J. Hanahan and M.S. Olson (1989) Identification of receptors for platelet activating factor in rat Kupffer cells. *Journal of Biological Chemistry*, **264**, 13591–13598.
7. F.H. Valone (1988) Identification of platelet activating factor receptors in P388D1 murine macrophages. *Immunology*, **140**, 2389–2394.
8. C.R. Gandhi and M.S. Olson (1991) PAF effects on transmembrane signalling pathways in rat Kupffer cells. *Lipids*, **26**, 1038–1043.
9. H. Sasaki, K. Maeyama and T. Watanabe (1991) Intracellular Ca^{++} concentration and H_2O_2 production in mouse peritoneal macrophages are stimulated by platelet activating factor. *Lipids*, **26**, 1209–1213.
10. B. Dewald and M. Baggliolini (1985) Activation of NADP oxidase in human neutrophils. Synergism between FMLP and the neutrophil products PAF and LTB4. *Biochemical Biophysical Research Communications*, **128**, 297–304.
11. P.P. Bradley, D.A. Priebat, R.D. Christensen and G. Rothstein (1982) Measurement of cutaneous inflammation: Estimation of neutrophil content with an enzyme marker. *Journal of Investigative Dermatology*, **78**, 206–209.
12. J.S. Warren, D.M. Mandel, K.J. Johnson and P.A. Ward (1989) Evidence for the role of platelet activating factor in immune complex vasculitis in the rat. *Journal of Clinical Investigation*, **83**, 669–678.
13. S.D. Shukla, B.D. Buxton, M.A. Olson and D.J. Hanahan (1983) Acetylglycerol ether phosphocholine. A potent activator of hepatic phosphoinositide metabolism and glycogenolysis. *Journal of Biological Chemistry*, **258**, 10212–10214.
14. A.J. Marcus, L.B. Safier, H.L. Ullman, K.T.H. Wong, M.J. Brockman, B.B. Weksler and K.L. Kaplan (1981) Effects of acetyl glycerol ether phosphocholine on human platelet function *in vitro*. *Blood*, **58**, 1027–1031.
15. P.M. Henson (1976) Activation and desensitization of platelets by platelet activating factor (PAF) derived from IgE-sensitized basophils. *Journal of Experimental Medicine*, **143**, 937–952.
16. T. Yasaka, L.A. Boxer and R.L. Baehner (1982) Monocyte aggregation and superoxide anion release in response to formyl-methionyl-leucyl-phenylalanine (FMLP) and platelet activating factor (PAF).

Journal of Immunology, **128**, 1939–1944.
17. C. Elliot Sigal, F.H. Valone, M. Holtzman and E.J. Goetzl (1987) Preferential human eosinophil chemotactic activity of the platelet activating factor (PAF) 1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphocholine (AGEPC). *Journal of Clinical Immunology*, **7**, 179–184.
18. F.H. Chilton, J.T. O'Flaherty, M.J. Thomas, R.L. Wukle, L.R. de Chatelet and M.L. Waite (1982) Platelet activating factor: Stimulation of the lipoxygenase pathway in polymorphonuclear leukocytes by 1-*O*-hexadecyl-2-*sn*-glyceryl-3-phosphocholine. *Journal of Biological Chemistry*, **257**, 5402–5407.
19. G.M. Vercelloti, H.Q. Yin, K.S. Gustafson, P.O. Nelson and H.S. Jacob (1988) Platelet activating factor primes neutrophil responses to agonists: Role in promoting neutrophil-mediated endothelial cell damage. *Blood*, **71**, 1100–1107.
20. C.G. Figdon, J.M.M. Leemans, W.S. Bont and J.E. Vries (1983) Theory and practice of centrifugal elutriation (CE). Factor influencing the separation of human blood cells. *Cell Biophysiology*, **5**, 105–112.
21. R.J. Sanderson and K.E. Bird (1977) Cell separation by counterflow centrifuge. In: *Methods in Cell Biology*, vol. 15, pp. 1–4 (D.M. Prescott, ed.), Academic Press, New York.
22. A.P. Bautista, N.B. D'Souza, C.H. Lang, J. Bagwell and J.J. Spitzer (1991) Alcohol downregulates superoxide anion release by hepatic phagocytes in endotoxemic rats. *American Journal of Physiology*, **260**, R969–R976.
23. G.D. Johnson and J. Dorling (1981) Immunofluorescence and immunoperoxidase techniques. In: *Techniques in Clinical Immunology*, p. 131 (R.A. Thompson, ed.), Blackwell Scientific Publications, Oxford.
24. D. English and B.R. Andersen (1974) Single-step separation of red blood cells: Granulocytes and mononuclear leukocytes on discontinuous density gradient of Ficoll-Hypaque. *Journal of Immunological Methods*, **5**, 249–252.
25. A.P. Bautista, A. Schuler, Z. Spolarics and J.J. Spitzer (1992) Tumor necrosis factor alpha stimulates superoxide anion generation by perfused rat liver and Kupffer cells. *American Journal of Physiology*, **261**, G891–G895.
26. R.B. Johnson, C.A. Godzik and Z. Cohn (1978) Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *Journal of Experimental Medicine*, **148**, 115–127.
27. K.L. Jines and R.A. Fisher (1992) Regulation of hepatic glycogenolysis and vasoconstriction during antigen-induced anaphylaxis. *American Journal of Physiology*, **262** (in press).
28. H. Jaeschke, A.P. Bautista, Z. Spolarics and J.J. Spitzer (1991) Superoxide generation by Kupffer cells and priming of neutrophils during reperfusion after hepatic ischemia. *Free Radical Research Communications*, **15**, 277–284.
29. H. Jaeschke and A. Farhood (1991) Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *American Journal of Physiology*, **260**, G355–G362.
30. A.P. Bautista, K. Meszaros, J. Bojta and J.J. Spitzer (1990) Superoxide anion release in the liver during the early stage of endotoxemia in rats. *Journal of Leukocyte Biology*, **48**, 123–128.
31. A.P. Bautista, A. Schuler, Z. Spolarics and J.J. Spitzer (1992) In vivo latex phagocytosis primes the Kupffer cells and hepatic neutrophils to generate superoxide anion. *Journal of Leukocyte Biology*, **51**, 39–45.
32. J.C. Gay (1990) Priming of neutrophil oxidative responses by platelet activating factor. *Journal of Lipid Mediators*, **2**, S161–S175.
33. A. Kishimoto, N. Kajikawa, M. Shiota and Y. Nishizuka (1983) Proteolytic activation of calcium-activated phospholipid-dependent protein kinase by calcium-dependent neutral protease. *Journal of Biological Chemistry*, **258**, 1156–1164.
34. S.L. Reinhold, S.M. Prescott, G.A. Zimmerman and T.M. McIntyre (1990) Activation of human neutrophil phospholipidase D by three separable mechanisms. *FASEB Journal*, **4**, 208–214.
35. F. Breviario, F. Bertocchi, E. Dejana and F. Bussolino (1988) Interleukin-1 induced adhesion of polymorphonuclear leukocytes to cultured human endothelial cells. Role of platelet activating factor. *Journal of Immunology*, **141**, 3391–3397.
36. K.L. Hines, A. Braillon and R.A. Fisher (1991) Platelet activating factor increases hepatic vascular resistance and glycogenolysis *in vivo*. *American Journal of Physiology*, **260**, G471–G480.
37. C.H. Lang, C. Dobrescu, D.M. Hargrove, G.J. Bagby and J.J. Spitzer (1988) Platelet activating factor-induced increases in glucose kinetics. *American Journal of Physiology*, **254**, E193–E200.
38. N.B. D'Souza, G.J. Bagby, S. Nelson and J.J. Spitzer (1989) Acute ethanol infusion suppresses endotoxin-induced serum tumor necrosis factor. *Alcoholism: Clinical and Experimental Research*, **13**, 295–298.

Accepted by Prof. B. Halliwell