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Platelet-activating factor increases inositol phosphate production and cytosolic free Ca^{2+} concentrations in cultured rat Kupffer cells

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Platelet-activating factor (PAF) stimulates glycogenolysis in perfused livers but not in isolated hepatocytes [(1984) J. Biol. Chem. 259, 8685–8688]. PAF-induced glycogenolysis in liver is associated closely with a pronounced constriction of the hepatic vasculature [(1986) J. Biol. Chem. 261, 644–649]. These and other observations suggest that PAF stimulates glycogenolysis in liver indirectly by interactions with cells other than hepatocytes. We have evaluated effects of PAF on hepatic Kupffer cells, which regulate flow through the hepatic sinusoids. Application of PAF to [^{3}H]inositol-labeled Kupffer cells produced dose-dependent increases in [^{3}H]inositol phosphates with an EC_{50} value of 4×10^{-10} M. Increases in inositol phosphate production in response to PAF were inhibited by a specific PAF receptor antagonist, SRI 63-675 (2×10^{-7} M), and stimulus of protein kinase C, phorbol 12-myristate 13-acetate (1×10^{-7} M). Measurements of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in single Kupffer cells loaded with Fura-2 demonstrated that application of PAF (2×10^{-9} M) resulted in significant increases in $[Ca^{2+}]_i$. These observations lead us to propose that interactions of PAF with Kupffer cells may result in the hemodynamic and metabolic responses to PAF in liver.

Platelet-activating factor; Phospholipase C; Inositol phosphate; Ca^{2+}; (Liver, Kupffer cell)

1. INTRODUCTION

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a unique phospholipid mediator which exerts potent glycogenolytic actions in perfused rat livers by mechanisms distinct from α-adrenergic agents or glucagon [1–6]. Most notable among the differences between PAF and these other glycogenolytic agents is the absence of glycogenolytic actions of PAF in isolated hepatocytes [2], the principle storage site for glycogen in liver. Interestingly, PAF stimulates degradation of phosphatidylinositol bisphosphate in hepatocytes without consequent production of inositol trisphosphate or Ca^{2+} mobilization [2,6,7]. This apparent enigma appears due to degradation of phosphoinositides by phospholipase A_2 following exposure of hepatocytes to PAF [8]. Associated with the glycogenolytic effect of PAF in perfused livers is a pronounced constriction of the hepatic vasculature resulting in several metabolic alterations consistent with ischemia within hepatic sinusoids [1,3,9]. It is well known that ischemia causes glycogenolysis in liver [10]. Thus, we have suggested that hepatocytes are not the primary site of action of PAF in the liver and that glycogenolysis in response to this lipid mediator is a result of interactions with the hepatic vasculature leading to vasoconstriction [3,4]. This concept is supported by the absence of glycogenolytic responses to pathophysiological concentrations of PAF in retrograde-perfused livers and liver slices as well as localization studies demonstrating that [^{3}H]PAF binds exclusively to periportal sinusoidal cells and not hepatocytes upon infusion into perfused livers [11].

It seemed entirely appropriate to investigate

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Abbreviations: PAF, platelet-activating factor; $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; PMA, 4β-phorbol 12β-myristate 13α-acetate; M199, medium 199; BSS, Earl's balanced salt solution

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agonist properties of PAF in hepatic Kupffer cells, which act as sphincters to regulate flow through the hepatic sinusoids [12]. Observations in the perfused liver indicate that biological responses to PAF may involve mobilization of Ca^{2+} from a small non-hepatocyte pool [13] and Ca^{2+} mobilization is an important signalling mechanism by which various hormones and mediators regulate cell function. Moreover, it is now well accepted that metabolism of inositol phospholipids can lead to the generation of second messengers which regulate $[Ca^{2+}]_i$ [14]. In the present communication we have performed experiments to explore agonist effects of PAF on inositol lipid hydrolysis and $[Ca^{2+}]_i$ in hepatic Kupffer cells. These studies provide the first evidence that PAF exerts agonist effects on Kupffer cells at concentrations comparable to those producing biological responses in liver.

2. MATERIALS AND METHODS

2.1. *Materials*

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) and lyso-PAF (1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine) were purchased from Bachem. *myo*-[2-^{3}H]Inositol was obtained from Amersham. SRI 63-675 was kindly provided by Dr Dean Handley, Sandoz Research Institute. All other chemicals were obtained from Sigma.

2.2. *Kupffer cell culture*

Kupffer cells were isolated from livers of male Sprague-Dawley rats (200–250 g) employing previously described methods [15]. Briefly, livers were digested by collagenase/pronase perfusion and sinusoidal cells were purified by centrifugation on a metrizamide gradient. Cells were cultured in M199 containing 10% fetal bovine serum (4×10^6 cells/35 mm culture dish) at 37°C in a 5% CO_2 atmosphere. Culture medium was replaced after overnight incubation resulting in the removal of non-adherent endothelial cells [16] and cultures were used for experiments 1 to 5 days after plating. The purity of these Kupffer cell cultures was 90–95% as assessed by positive peroxidase staining, in agreement with previous histochemical as well as morphological and functional characterizations of these cultures [15,16]. For measurements of $[Ca^{2+}]_i$ in single Kupffer cells, Kupffer cells were cultured on 25 mm glass coverslips. As with plastic culture dishes, Kupffer cells but not endothelial cells attached to the glass surface [16].

2.3. *Incubations and analyses*

All studies of inositol phosphate production in Kupffer cells were performed at 37°C. For labeling inositol lipids, cultures were incubated 20–24 h in serum-free M199 containing *myo*-[2-^{3}H]inositol (1–2 μCi/ml). Cultures were rinsed and incubated in BSS for 60 min and then rinsed and incubated 15 min in BSS containing 5 mM LiCl prior to addition of PAF and other agents (time 0). Incubations were terminated 30 min following agonist addition by removing the medium and adding 1 ml of ice-cold methanol. Inositol phosphates were extracted by addition of 1.5 ml of chloroform:water (2:1) and samples of the aqueous phase were analyzed for total labeled inositol phosphates by batch elution from Dowex columns as described by Brown et al. [17].

$[Ca^{2+}]_i$ was measured in individual Kupffer cells using fluorescent videomicroscopy and digital image analysis of Fura-2-loaded cells as described by Sharma and Bhalla [18]. These experiments were performed with 3 day cultures following incubation in serum-free M199 for 20–24 h. Kupffer cells were loaded with Fura-2 by incubation with Fura-2/acetoxymethyl ester (5×10^{-6} M) for 45 min at 37°C and then incubated an additional 30 min in BSS at 37°C.

Results are expressed as means ± SE. Significance of differences between samples was assessed using analysis of variance and Sheffe's post hoc analysis.

3. RESULTS

Fig.1 illustrates the effect of PAF on inositol phosphate production in [^{3}H]inositol-labeled Kupffer cell cultures. Exposure of Kupffer cells to PAF (1×10^{-9} M) resulted in a significant increase ($p < 0.01$) in inositol phosphate production which was not observed in response to a 200-fold higher concentration of lyso-PAF. Incubation with vehicle (saline) alone did not increase [^{3}H]inositol phosphates in Kupffer cells above values observed at time 0.

Stimulation of Kupffer cell inositol phosphate production by PAF was dose-dependent with half-maximal responses occurring at approximately 4×10^{-10} M PAF (fig.2). The exquisite sensitivity of Kupffer cells to PAF can be appreciated by the small but significant ($p < 0.05$) increase in inositol phosphate production observed in response to 1×10^{-10} M PAF. Maximal responses to PAF were apparent at 3×10^{-9} M. At maximal concentrations, PAF increased [^{3}H]inositol phosphates in Kupffer cells over 700% compared to either saline or time 0 (not shown) values. Preliminary experiments employing HPLC separation of inositol phosphates have shown that increases in radioactivity in inositol 1,4,5-trisphosphate are apparent in Kupffer cells stimulated with PAF (2×10^{-9} M) for 15 s (not shown).

Activation of platelets by PAF can be inhibited by PAF receptor antagonists and activators of protein kinase C [19–21]. Activation of protein kinase C inhibits receptor-stimulated phospholipase C in a variety of cells and may represent an important feedback mechanism for regulating receptor re-

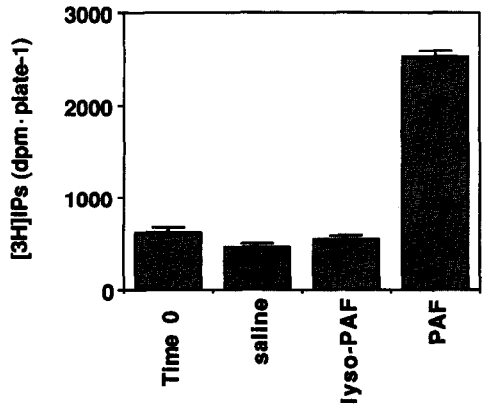

Fig.1. Effect of PAF on inositol phosphate production in cultured rat Kupffer cells. Kupffer cells were incubated with saline, lyso-PAF (2×10^{-7} M) or PAF (1×10^{-9} M) for 30 min before termination of incubation and measurement of inositol phosphates as described under section 2. Values shown represent means ± SE ($n = 3$).

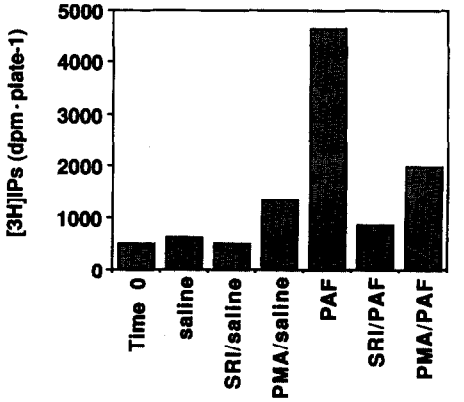

Fig.3. Effects of SRI 63-675 and PMA on PAF-stimulated inositol phosphate production in cultured rat Kupffer cells. SRI 63-675 (2×10^{-7} M) and PMA (1×10^{-7} M) were added to Kupffer cells 15 min prior to addition of saline or PAF (2×10^{-9} M) for 30 min. Incubations and inositol phosphate measurements were performed as described under section 2. Values shown represent means of duplicate experiments.

sponses [14]. To characterize PAF receptor responses in Kupffer cells further, PAF-stimulated inositol phosphate production was evaluated in the presence of a PAF receptor antagonist (SRI 63-675) [22] and activator of protein kinase C (PMA). Fig.3 shows that PAF-stimulated inositol phosphate production in Kupffer cells was reduced to approximately 10% and 35% of control values by SRI 63-675 (2×10^{-7} M) and PMA (1×10^{-7} M), respectively. Interestingly, incubation of Kupffer cells with PMA produced increases in [^{3}H]inositol phosphates of approximately 100%. Similar results have been reported in MDCK cells where PMA increased basal levels of inositol monophosphate but inhibited receptor-mediated production of inositol phosphates [23].

In view of our findings that PAF receptors are coupled to activation of phosphoinositide phospholipase C in Kupffer cells, preliminary experiments were performed to evaluate effects of PAF on $[Ca^{2+}]_i$ in Kupffer cells. $[Ca^{2+}]_i$ in un-

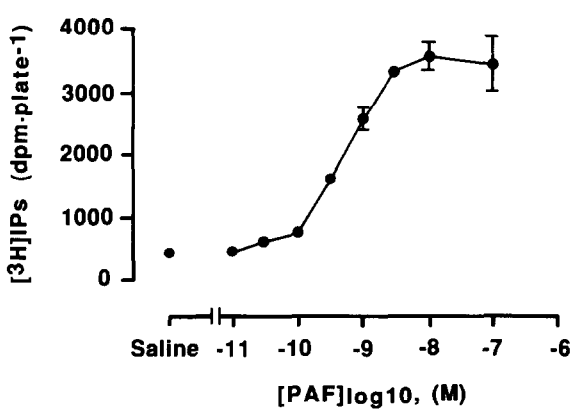

Fig.2. Dose response of PAF on inositol phosphate production in cultured rat Kupffer cells. Kupffer cells were incubated with saline or various concentrations of PAF for 30 min before termination of incubation and measurement of inositol phosphates as described under section 2. Values shown represent means ± SE ($n = 3$).

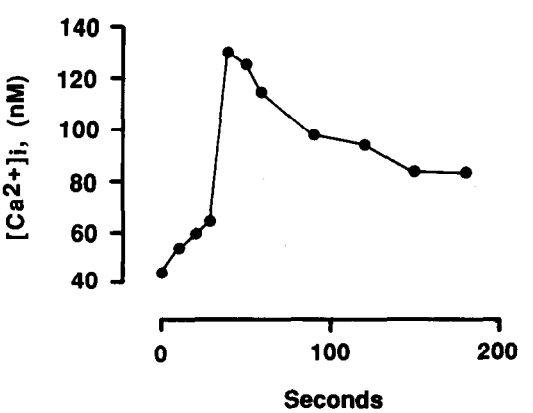

Fig.4. Effect of PAF on $[Ca^{2+}]_i$ in an individual rat Kupffer cell. PAF was added to the microscope chamber at time 0 as an equal volume addition to provide a final PAF concentration of 2×10^{-9} M. $[Ca^{2+}]_i$ was measured as described under section 2.

stimulated Kupffer cells averaged 62 ± 8 nM ($n = 12$). Fig.4 illustrates a representative effect of PAF (2×10^{-9} M) on $[Ca^{2+}]_i$ in a single Kupffer cell. Although maximal increases in $[Ca^{2+}]_i$ in response to PAF exposure were apparent at the first sampling period (10 s) in some cells, typically there was a delay as shown in fig.4. These variations were observed even with different cells on the same coverslip suggesting some cell-cell variability in responses to PAF. After the peak increase in $[Ca^{2+}]_i$ was attained, $[Ca^{2+}]_i$ decreased to a level which remained elevated above basal values for the duration of our experiments (3 min). In 7 individual cells, exposure to PAF increased $[Ca^{2+}]_i$ to 118 ± 23 nM ($p < 0.01$).

4. DISCUSSION

The data presented in this report provide the first demonstration that Kupffer cells respond to biologically-relevant concentrations of PAF and that this response involves activation of phosphoinositide phospholipase C and increases in $[Ca^{2+}]_i$. Phospholipase C responses to PAF in Kupffer cells occurred at low molar concentrations of PAF but not the biologically inactive form of PAF (lyso-PAF) and were prevented by a specific PAF receptor antagonist. These observations suggest that PAF interacts with specific receptors in Kupffer cells to regulate inositol phosphate production.

It is important to consider the relationship between the present results and the biological actions of PAF in liver. We have suggested that the potent glycogenolytic actions of PAF in perfused livers are secondary to PAF-induced constriction of the hepatic sinusoids [3,4]. Implicit in this hypothesis are the requirements that PAF does not produce direct glycogenolytic effects in hepatocytes and that primary interactions of PAF occur with cells regulating flow through the hepatic sinusoids. In keeping with these criteria, PAF has no effects on glycogenolysis in freshly isolated hepatocytes, cultured hepatocytes or liver slices [2,6,11]. Moreover, this study has demonstrated primary effects of PAF on Kupffer cells, cells which restrict blood flow by acting as 'sphincters' in the hepatic sinusoids [12]. The effects of PAF on Kupffer cells described in this study occurred at PAF concentrations comparable to those producing glycogenolysis and vasoconstriction in perfused rat livers, i.e., 2×10^{-11} to 2×10^{-9} M [3,4]. EC_{50} values for these effects of PAF in perfused livers ($\sim 2 \times 10^{-10}$ M) [4] are very similar to values observed in this study for effects of PAF on Kupffer cell inositol phosphate production. These observations support the concept that Kupffer cells are a primary site of PAF action in liver.

Recently, Kuiper et al. [24] reported that PAF stimulates production of prostaglandin D_2 from Kupffer cells. These authors demonstrated that prostaglandin D_2 increases glycogenolysis in hepatocytes and perfused livers and suggested that PAF stimulates glycogenolysis in liver indirectly by stimulation of Kupffer cell prostaglandin D_2 production. However, PAF stimulated prostaglandin D_2 production in Kupffer cells only at concentrations greater than 1×10^{-8} M. Because PAF stimulates glycogenolysis and vasoconstriction in perfused livers at much lower concentrations their results suggest, to the contrary, that Kupffer cell-derived prostaglandin D_2 is not involved in hepatic responses to pathophysiological concentrations of PAF. Recently, Lapointe and Olson (personal communication and [25]) demonstrated that low concentrations of indomethacin or ibuprofen could be used to completely block prostaglandin D_2 and thromboxane A_2 production in perfused livers without inhibiting glycogenolytic and hemodynamic responses to PAF. In view of these observations and the absence of glycogenolytic responses to PAF (1×10^{-9} M) in liver slices and retrograde-perfused livers [11], it seems unlikely that hepatic responses to PAF are secondary to the production of Kupffer cell mediators. However, a possible autocrine role of PAF in regulating Kupffer cells is suggested by the recent demonstration that Kupffer cells produce PAF in response to the calcium ionophore A23187 [26].

Although we have provided insight into actions of PAF on Kupffer cells, we have not demonstrated cause and effect relationships between Kupffer cell responses in vitro and responses to PAF in perfused livers. We also acknowledge that it is difficult to demonstrate that such relationships are valid, particularly when responses in vivo are not mimicked by individual cells. However, it is well recognized that changes in $[Ca^{2+}]_i$ are an important mechanism by which hormones and mediators regulate cell function including alterations in cell shape. Moreover, $^{45}Ca^{2+}$ washout

studies in perfused livers have suggested the possible involvement of increases in Kupffer cell $[Ca^{2+}]_i$ in the biologic actions of PAF [13].

In view of the foregoing evidence, it seems appropriate to suggest that Kupffer cells play a primary role in hemodynamic and metabolic responses to PAF in liver. We propose that PAF binding to Kupffer cells increases $[Ca^{2+}]_i$ resulting in hepatic vasoconstriction by alterations in Kupffer cell shape or 'sphincter cell' activity. Hydrolysis of inositol lipids by receptor-mediated phospholipase C activation leads to production of second messengers which can increase $[Ca^{2+}]_i$ by effects on Ca^{2+} mobilization and/or Ca^{2+} influx [14]. While we have not defined the mechanisms underlying PAF-induced increases in $[Ca^{2+}]_i$ in Kupffer cells, our results are consistent with a role for inositol lipid biosignalling in this process.

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