

A. JAKUBOWSKI, R. OLSZANECKI, S. CHLOPICKI

INVOLVEMENT OF PLATELET ACTIVATING FACTOR IN IMMEDIATE HEART RESPONSE TO LIPOPOLYSACCHARIDE

Department of Experimental Pharmacology, Chair of Pharmacology Jagiellonian University
Medical College, Cracow, Poland

Although lipopolysaccharide (LPS) is recognized to induce a biphasic cardiovascular response its mechanism is not fully elucidated. In this study we analysed the involvement of PAF, TXA_2 and cysteinyl leukotrienes (cysLTs) in the acute cardiovascular effects of LPS in the isolated rat heart as well as in delayed phase of LPS response using a surrogate cellular model of the induction of NOS-2 by LPS in mouse macrophages. Perfusion of rat hearts with LPS resulted, in an immediate fall in heart contractility and coronary flow by $2.5 \pm 0.59 \text{ ml min}^{-1}$ and $560 \pm 81 \text{ mmHg sec}^{-1}$, respectively. This response was fully blocked by platelet activating factor (PAF) antagonist - WEB 2170 and partially inhibited, by inhibitor of cyclooxygenase (indomethacin) or by inhibitor of thromboxane synthase (camosagrel). The inhibition of leukotriene synthesis (BAY x1005) or cysLTs receptors (BAY x7195) was without effect. Administration of stable PAF analog (methylcarbanyl-PAF - MC-PAF) alone, mimicked heart response to LPS. In cultured mouse macrophages, MC-PAF did not induce NOS-2 expression and when given with LPS it slightly potentiated NOS-2 induction by LPS. However, in presence of WEB 2170 NOS-2 induction by LPS was inhibited in a dose-dependent manner. Inhibition of cyclooxygenase and leukotriene pathways had no effect on NOS-2 induced by LPS. These results indicate that PAF and TXA_2 , but not cysLTs mediate the instant heart response induced by LPS, while PAF alone mediates a delayed NOS-2 induction by LPS. Accordingly, PAF may constitute the mediator that links acute and delayed phases of LPS-induced cardiovascular response.

Key words: lipopolysaccharide, lipid mediators, platelet activating factor, TXA_2 , rat heart, mouse macrophages

INTRODUCTION

Administration of lipopolysaccharide (LPS) *in vivo* leads to the endotoxic shock which displays two distinct phases. The first occurs almost immediately

after LPS injection and involves transient pulmonary hypertension and systemic hypotension with concomitant fall in cardiac output (1). The delayed phase, which occurs a few hours after LPS injection involves gradual fall in blood pressure accompanied by vasoplegia, tissue hypoperfusion, microvessel injury, and multiple organ injury.

The biphasic pattern of heart response to LPS is also apparent. LPS decreases contractility of cardiomyocytes in isolated cardiomyocytes as well as in the isolated heart as soon as a few minutes after LPS administration (1,2) LPS also induces immediate coronary vasoconstriction (1). Moreover, LPS at a dose that does not induce vasoactive response itself profoundly modulates coronary artery response to angiotensin II, *via* mechanisms involving generation of superoxide anions in endothelial cells (3). On the other hand, 3 -12 h after LPS injection severe contractile dysfunction is observed (4) that is accompanied by release of troponin I (5), and progressive liberation of thromboxane A₂ and prostacyclin (6). It is postulated that NOS-2 induction *via* proinflammatory cytokines such as TNF- α is causally involved in late cardiac dysfunction in endotoxaemia (7, 8).

All these observations point out to heart as a target for immediate and delayed responses to LPS, however, the mechanisms of both phases of LPS-mediated cardiac dysfunction are still not clear. Both phases are regarded as distinct entities and the attention is focused on the delayed phase associated with pronounced alterations in heart function not on the immediate phase that is transient.

Noteworthy, there is evidence that suggests a possible role of PAF in immediate and delayed phases of heart response to LPS. Firstly, exogenous PAF mimics LPS effects as it exerts negative inotropic effect on myocardium (9) and induces coronary vasoconstriction in coronary arterioles (10, 11). Secondly, PAF may regulate induction of NOS-2, COX-2 and release of secretory PLA₂. Thirdly, LPS-induced release of PAF seems to be biphasic with an early, transient peak at 10 min and a delayed plateau at six hours after LPS (12). This is why we studied here involvement of PAF and other lipid mediators such as TXA₂ and cysteinyl leukotrienes in immediate cardiovascular response induced by LPS in the isolated rat heart. We also studied involvement of these mediators in a delayed phase of LPS response using a surrogate model of the induction of NOS-2 by LPS in mouse macrophage line.

MATERIAL AND METHODS

Reagents and drugs

LPS (*Escherichia coli* serotype 0127 : B8) indomethacin, MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide], BCIP [5-bromo-4-chloro-3-indolyl phosphate] and NBT [nitro blue tetrazolium] were purchased from Sigma Chemicals International; Germany; thiopental sodium (Tiopental) was from Biochemie GMBH, Germany; WEB 2170 (5-(2-chlorophenyl)-3,4-dihydro-10-methyl-3-[(4-morpholinyl)carbonyl]-2H,7H-cyclopenta[4,5]thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine) was a gift from Boehringer Ingelheim, Germany;

Camonagrel ([+]-5-[2-imidazole-1-ethyloxy]-1-indan-carboxylic acid hydrochloride) was a gift from Ferrer inc.; Spain. Methylcarbamy PAF C-16, an PAF acetylhydrolase resistant analog of PAF was from Cayman; USA. BAY x1005 ((R)-2-[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid) and BAY x7195 ((4S)-[4-carboxyphenylthio]-7-[4-(4-phenoxybutoxy)-phenyl]-hept-5-(z)-enoic acid) were a gift from Bayer AG; Germany. SDS-polyacrylamide gels and nitrocellulose membranes were purchased from Bio-Rad; USA; Rabbit polyclonal antibody to NOS-2 and alkaline phosphatase-conjugated secondary antibody were from Transduction Laboratories; USA; and Sigma; USA; respectively.

Studies in isolated rat heart

The method of isolated heart was described more in detail elsewhere (13, 14). Briefly, hearts were isolated from Wistar rats weighing 200-250g (Lod:WIST BR from Animal Laboratory of Polish Mother's Memorial Research Institute Hospital in Łódź, Poland). Anaesthetised rats (thiopentone 120 mg/kg, i.p.) were exsanguinated by the incision of left renal artery. Then hearts were removed and perfused at the constant pressure of 70 mm Hg retrogradely through the ascending aorta in the Langendorff apparatus (Hugo Sachs Elektronik - HSE) with the Krebs-Henseleit buffer of the following composition (mmole L⁻¹): NaCl 118.00, KCl 4.7, CaCl₂ 2.52, MgSO₄ 1.64, NaHCO₃ 24.88, KH₂PO₄ 1.18, glucose 10.00, sodium pyruvate 2.0, equilibrated with 95% O₂ + 5% CO₂ at 37°C. The hearts were paced at 330 impulses per minute through platinum electrodes inserted into the right atrium. Left ventricular pressure (LVP) was measured using the fluid-filled balloon inserted into the left ventricle and connected to a pressure transducer (Isotec, HSE). End diastolic pressure was set to 5-8 mmHg. The values of heart contractility were obtained from LVP signal by an analogue differentiation amplifier (DIF module, HSE). Coronary flow was monitored by Narcomatic Electronic Flowmeter (HSE).

The investigation conforms with the Guide for the Care and use of Laboratory Animals published by US National Institutes of Health and the experimental procedure used in present study was approved by the local Animal research Committee.

Experimental protocol

Hearts were perfused retrogradely through ascending aorta with Krebs buffer at a constant pressure according to the modified Langendorff technique. Isolated hearts were equilibrated at the perfusion pressure of 60 mm Hg for 15 min, then pressure was adjusted to 70 mm Hg and hearts were equilibrated again for 15 min before the beginning of experiment. After stabilization period, LPS (0.5 µg mL⁻¹) was added to the buffer perfusing isolated heart for 30 min. All hearts were randomised into seven experimental groups. Control hearts received only LPS (0.5 µg mL⁻¹) this final concentration of LPS was chosen on the basis of pilot experiments in which it was found to give the maximum coronary flow response (n=6). In other hearts an inhibitor of COX indomethacin (5 µmole L⁻¹), an inhibitor of TXA₂ synthase camonagrel (10 µmole L⁻¹), a PAF antagonist WEB 2170 (5 µmole L⁻¹), an inhibitor of five lipoxygenase activating protein - BAY x1005 (1 µmole L⁻¹), or antagonist of cysteinyl leukotrienes receptors BAY x7195 (1 µmole L⁻¹), each of them was perfused through the heart for 30 min before evoking response by LPS (n=6 in each group). These drugs were added to the perfusate reservoir in a dose necessary to achieve a required final concentration. In some hearts stable PAF analog - methylcarbamy PAF (1 mmole L⁻¹) was infused before and after NOS inhibition with L-NAME (100 µmole L⁻¹) (n=4). Coronary flow, left ventricular pressure, heart contractility were continuously measured and displayed throughout the experiment. For data analysis specially-designed software (PSCF-IGEL, Poland) was used. All experiments were completed in less than three hours.

Studies in macrophage Cell Line J774.2

The mouse macrophage cell line J774.2 was cultured in T75 flasks in DMEM that contained 10% foetal bovine serum supplemented with streptomycin (100 $\mu\text{g ml}^{-1}$), penicillin (100 U ml^{-1}), and fungizone (0,25 $\mu\text{g ml}^{-1}$). Flasks were kept at 37°C, in atmosphere of humidified air containing 5% CO_2 . Then, cells were seeded in 96-well plates and cultured in 200 μL of culture medium until reaching confluence (10^5 cells per well). Additionally, in order to obtain sufficient amount of material for immunoblotting, the cells were cultured in 6-well plates (10^6 cells per well) in 2 ml of culture medium.

In order to induce NOS-2 the cells were stimulated for 24 hrs with lipopolysaccharide from *E. coli* (LPS, 1 $\mu\text{g ml}^{-1}$) or stable analog of PAF - Methylcarbamylyl PAF C-16 (MC-PAF, 0.1 - 10 $\mu\text{mole L}^{-1}$). The effect of LPS was also tested after pre-treatment of macrophages with PAF receptor antagonist (WEB 2170, 1 - 100 $\mu\text{mole L}^{-1}$), indomethacin (1 - 10 $\mu\text{mole L}^{-1}$) and BAY x1005 (0.1 - 1 $\mu\text{mole L}^{-1}$). All compounds were given in fresh medium, 15 minutes prior to LPS challenge.

To exclude cytotoxic effect of used drugs, cell respiration viability assay was performed by mitochondrial-dependent reduction of MTT to formazan (15). Briefly, cells in 96-well plates were incubated with MTT (0,2 mg ml^{-1} for 60 minutes). Then, the culture medium was removed by aspiration and crystals of formazan were solubilized with DMSO (200 μL). The extent of reduction of MTT to formazan within cells was quantified by measurement of absorbance at 550 nm. None of pharmacological reagents used did modify MTT assay (data not shown).

Nitrite concentration in culture medium

Nitrite accumulation in J774.2 cell culture supernatant, a measure of NOS-2 activity, was measured using the Griess method (16). Briefly, 100 μL of 1% sulphanilamide in 5% phosphoric acid, followed by 100 μL of 0,1% N-(1-naphtyl)-ethylene diamine in 5% phosphoric acid were added to 100 μL of culture medium. After 10 minutes of incubation at 23°C the absorbance at 550 nm was read. The final concentration of nitrite in a sample was calculated from the standard curve constructed using sodium nitrite as a reference compound.

NOS-2 immunoblotting

24 hrs after LPS stimulation the cells were lysed in lysis buffer (1% Triton X-100, 0,1% SDS in PBS containing 1 mmole L^{-1} PMSF, 10 $\mu\text{mole L}^{-1}$ leupeptin, 50 $\mu\text{mole L}^{-1}$ pepstatin A). Protein concentrations in lysates were determined using Bradford method. Samples, containing equal amounts of total protein were mixed with gel loading buffer [50 $\mu\text{mole L}^{-1}$ Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml bromophenol blue] in a ratio 1:1 (v/v) and boiled (4 min). Then, samples (30 μg of total protein) were separated on 7,5% SDS-polyacrylamide gels (Mini-Protean II) using Laemmli buffer system and proteins were semi-dry transferred to nitrocellulose membranes. Non-specific binding sites were blocked overnight in 4°C with 5% non-fat dried milk and the membranes were then incubated 2 hrs in room temperature (RT) with rabbit polyclonal antibody to NOS-2 (1:1000). Bands were detected with alkaline phosphatase-conjugated secondary antibody (1 hrs in RT, 1:5000) and developed with BCIP and NBT. Rainbow markers were used for molecular weight determinations. Protein bands were scanned and analyzed with Scion image software (Scion Corporation, USA).

Statistics

Significance of differences between groups was established by single factor analysis of variance (ANOVA) followed by t-test for multiple comparison. All data represent mean \pm S.E.M. A value of $P < 0.05$ was considered to be significant.

RESULTS

Involvement of PAF and TXA₂ but not of cysteinyl leukotrienes in coronary vasoconstriction induced by LPS in isolated rat heart

The average basal coronary flow in isolated rat heart was 6.76 ± 0.34 ml min⁻¹ (n=40). Administration of LPS ($0.5 \mu\text{g mL}^{-1}$) resulted in an immediate fall in coronary flow by 2.5 ± 0.3 ml min⁻¹ ($P < 0.005$ vs control group). Addition of indomethacin ($5 \mu\text{mole L}^{-1}$), camonagrel ($10 \mu\text{mole L}^{-1}$), BAY x1005 ($1 \mu\text{mole L}^{-1}$), or BAY x7195 ($1 \mu\text{mole L}^{-1}$), had no effect on basal coronary flow while administration of WEB 2170 produced a slight and non significant increase in coronary flow (by 0.4 ± 0.25 ml min⁻¹).

As shown in *Fig. 1* in presence of WEB 2170, indomethacin or camonagrel change in coronary flow induced by LPS amounted to $+0.3 \pm 0.1$ ml min⁻¹, -1.5 ± 0.12 ml min⁻¹ ($P < 0.05$ vs LPS group) and -0.8 ± 0.11 ml min⁻¹ ($P < 0.01$ vs LPS group), respectively. On the other hand, in the presence of BAY x1005 or BAY x7195 LPS-induced fall in coronary flow did not differ from that in induced by LPS alone (by 2.1 ± 0.27 ml min⁻¹ and by 2.3 ± 0.27 ml min⁻¹, respectively). MC-PAF ($1 \mu\text{mole L}^{-1}$) produced similar vascular effect to LPS as it decreased coronary flow by 1.98 ± 0.31 ml min⁻¹. This response was not affected by NOS inhibition with L-NAME (1.82 ± 0.22 ml min⁻¹).

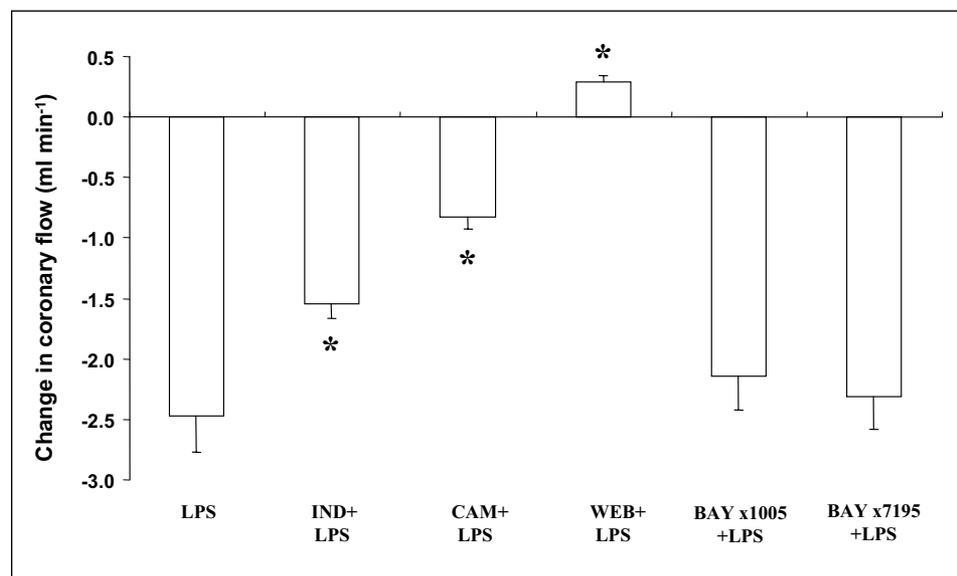


Figure 1. Indomethacin (IND - $5 \mu\text{mole L}^{-1}$), Camonagrel (CAM - $10 \mu\text{mole L}^{-1}$) and WEB 2170 ($10 \mu\text{mole L}^{-1}$) decrease LPS-induced ($0.5 \mu\text{g mL}^{-1}$) change in coronary flow in isolated rat heart while BAY x1005 ($1 \mu\text{mole L}^{-1}$) and BAY x7195 ($1 \mu\text{mole L}^{-1}$) stay without effect. Data represent mean \pm SEM for n = 6 -36 experiments (* $P < 0.05$ vs LPS group).

Involvement of PAF and TXA₂ but not of cysteinyl leukotrienes in negative inotropic response induced by LPS in isolated rat heart

The basal heart contractility (dp/dt_{max}) in isolated rat heart was 1239 ± 42 mmHg sec⁻¹ (n=40). Again all used drugs with the exception of WEB 2170 did not influence dp/dt_{max} (data not shown). PAF receptor antagonist produced an increase in dp/dt_{max} by 77 ± 9 mmHg that was not, however, statistically significant. Simultaneously with a decrease in coronary flow, LPS caused a fall in dp/dt_{max} by almost 50% (by 560 ± 81 mmHg sec⁻¹; P<0.001 vs control group). Fall in heart contractility was observed as soon as 5 min after endotoxin administration with maximum effect approximately 10 min later. Kinetics of a decrease in heart contractility and coronary vasoconstriction induced by LPS were matched.

In hearts pre-treated with WEB 2170 a drop of dp/dt_{max} induced by LPS was profoundly inhibited (55 ± 23 mmHg sec⁻¹; P<0.005 vs LPS group). In the presence of indomethacin or camonagrel LPS-induced fall in dp/dt_{max} was partially inhibited (271 ± 32 mmHg sec⁻¹; P<0.05 vs LPS group and by 291 ± 42 mmHg sec⁻¹; P<0.05 vs LPS group), respectively. Again the drugs that affect leukotriene pathway (1 μ mole L⁻¹), BAY x1005 and BAY x7195 (1 μ mole L⁻¹), did not prevent LPS-induced depression of dp/dt_{max} (fall by 437 ± 76 mmHg sec⁻¹ and 514 ± 75 mmHg

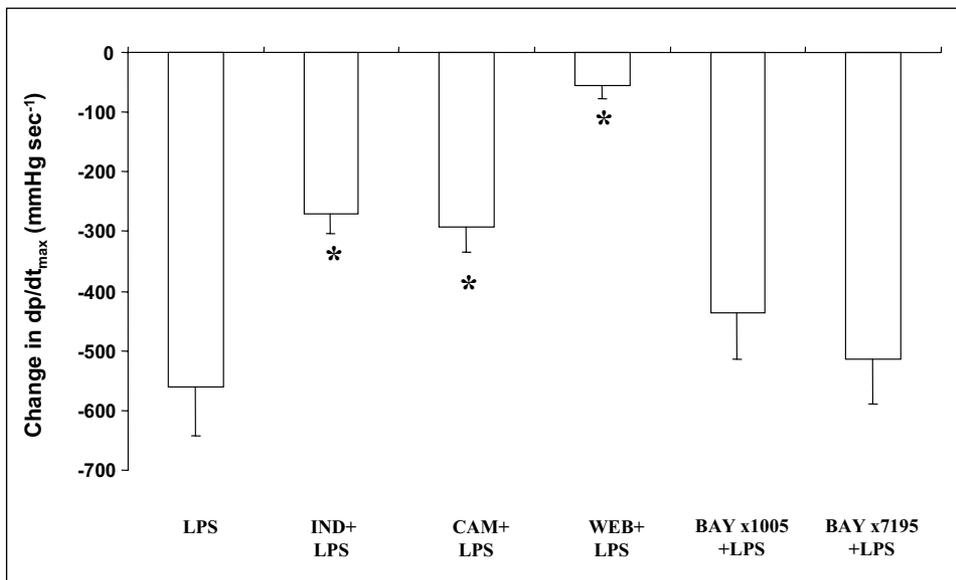


Figure 2. Indomethacin (IND - 5 μ mole L⁻¹), Camonagrel (CAM - 10 μ mole L⁻¹) and WEB 2170 (10 μ mole L⁻¹) but not BAY x1005 (1 μ mole L⁻¹) and BAY x7195 (1 μ mole L⁻¹) inhibit LPS-induced (0.5 μ g mL⁻¹) decrease in heart contractility in isolated rat heart. Data represent mean \pm SEM for n = 6-36 experiments (*P<0.05 vs LPS group).

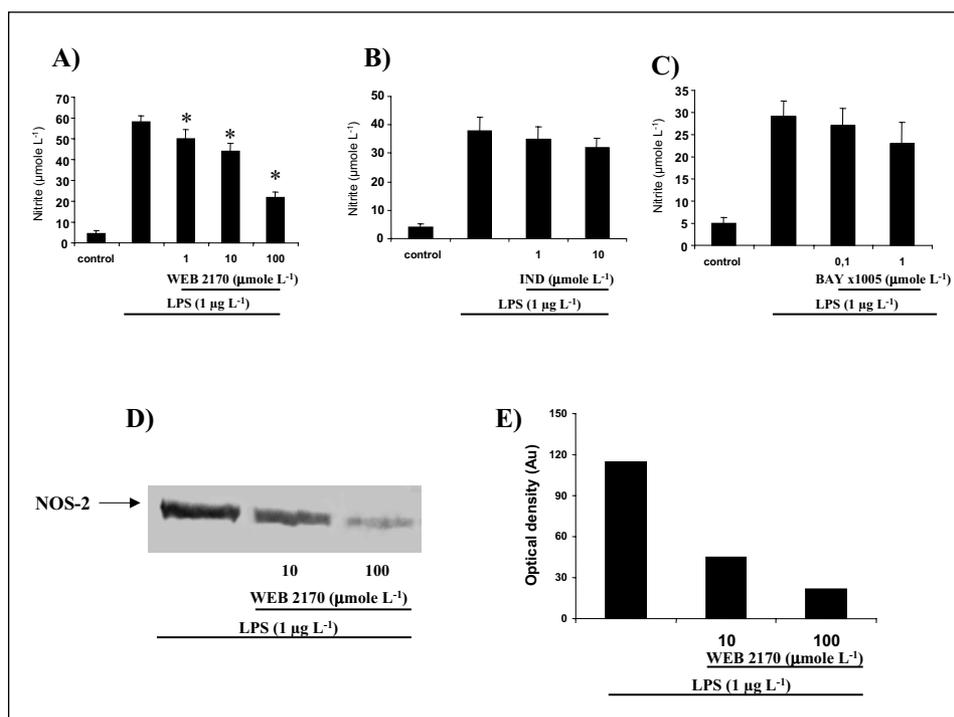


Figure 3. PAF receptor antagonist, WEB 2170 (1 - 100 $\mu\text{mole L}^{-1}$), given 15 minutes prior to LPS (1 $\mu\text{g mL}^{-1}$) dose-dependently inhibits 24-hrs nitrite accumulation in culture medium of J774.2 cells (A). Neither indomethacin (IND, 1 - 10 $\mu\text{mole L}^{-1}$) (B) nor BAY x1005 (0.1 - 1 $\mu\text{mole L}^{-1}$) (C), influence 24-hrs nitrite accumulation in culture medium of LPS-stimulated J774.2 cells. Nitrite data represent mean \pm SEM for n = 6 -12 experiments ($*P < 0.05$ vs LPS group). (D) - Immunoblotting of NOS-2 protein: WEB 2170 (10 $\mu\text{mole L}^{-1}$ - 100 $\mu\text{mole L}^{-1}$) dose-dependently inhibits NOS-2 protein induction in LPS-treated J774.2 cells. (E) - densitometry of bands from panel A (Au - arbitrary units).

sec^{-1} , respectively) (Fig. 2). PAF analog mimicked effect of LPS on heart contractility as it decreased $\text{dp}/\text{dt}_{\text{max}}$ (by $481 \pm 119 \text{ mmHg sec}^{-1}$). L-NAME did not affect MC-PAF-induced fall in $\text{dp}/\text{dt}_{\text{max}}$ (fall by $445 \pm 92 \text{ mmHg sec}^{-1}$).

Role of PAF in induction of NOS-2 protein by LPS in cultured macrophages

As shown in Fig. 3, 24-hrs stimulation by LPS (1 $\mu\text{g mL}^{-1}$) strongly induced NOS-2 in mouse macrophage cell line J774.2 as evidenced by an increased level of nitrite in cell culture supernatant ($35 \pm 3 \mu\text{mole L}^{-1}$ vs $3.5 \pm 1 \mu\text{mole L}^{-1}$, $P < 0.001$) and a presence of NOS-2 protein in stimulated cells (in control cells NOS-2 protein was not detectable - data not shown). Pre-treatment with PAF receptor antagonist, WEB 2170 dose-dependently decreased nitrite accumulation in culture medium of LPS-treated macrophages (Fig. 3A). This effect was due to inhibition of NOS-2

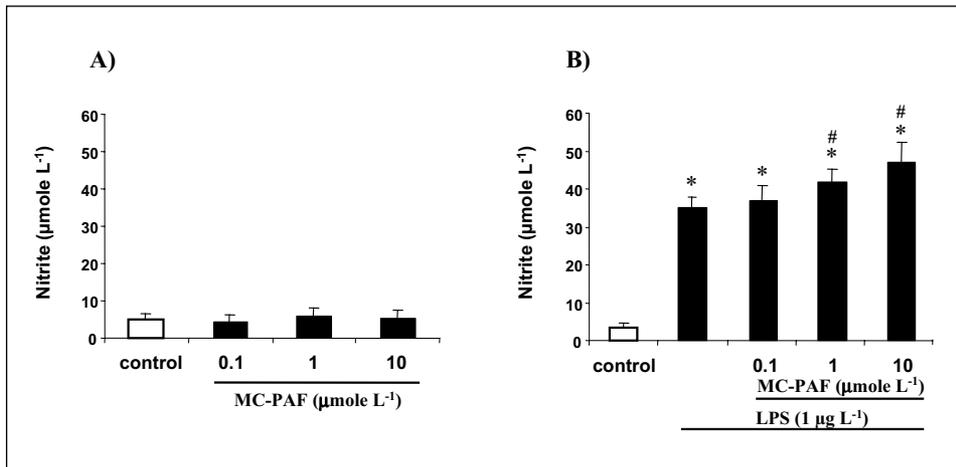


Figure 4. Methylcarbamy PAF - (MC-PAF; 0.01 $\mu\text{mole L}^{-1}$ - 10 $\mu\text{mole L}^{-1}$) does not increase 24-hrs nitrite accumulation in J774.2 cell culture (A) however, it potentiates 24-hrs nitrite accumulation in LPS-stimulated (1 $\mu\text{g ml}^{-1}$) J774.2 cell culture (B). Nitrite data represent mean \pm SEM for n = 6-12 experiments (* $P < 0.05$ vs control group; # $P < 0.05$ vs LPS group).

induction by LPS (Fig. 3D and 3E). On the contrary, neither pre-treatment with indomethacin nor with an inhibitor of FLAP (BAY x1005), influenced 24-hrs nitrite accumulation in LPS-treated J774.2 cells (Fig. 3B and 3C).

Stable analog of PAF (methylcarbamy PAF C-16) given alone at a concentration ranging from 0.1 to 10 $\mu\text{mole L}^{-1}$ did not induce NOS-2 in macrophages as evidenced by lack of increase of 24-hrs nitrite accumulation in cell culture supernatant (Fig. 4A). However, methylcarbamy PAF C-16, slightly and dose-dependently augmented LPS-dependent 24-hrs nitrite formation by J774.2 cells (Fig. 4B)

DISCUSSION

On one hand, we studied here the involvement of lipid mediators (PAF, TXA_2 , cysteinyl leukotrienes) in immediate cardiovascular response induced by bacterial endotoxin from *E. Coli* (LPS) in the isolated rat heart assessing coronary flow and contractility changes. On the other, we studied involvement of these mediators in a delayed phase of LPS response using as an end-point the surrogate model of NOS-2 induction in mouse macrophage line.

We demonstrated that LPS had induced an immediate coronary vasoconstriction and depression of myocardial contractility in isolated rat heart. This response was mediated by PAF and TXA_2 but not by cysteinyl leukotrienes. On the other hand, we demonstrated that PAF but not TXA_2 and cysteinyl leukotrienes was involved in NOS-2 induction induced by LPS in cultured

macrophages. Although our data come from two distinct experiment systems such as isolated heart and cultured macrophages they give us a reason to speculate that PAF constitutes an important mediator that links early and delayed cardiovascular effects of bacterial endotoxin.

The biphasic nature of cardiovascular response to LPS *in vivo* is well known (17, 18,19). Importantly, an immediate transient response to LPS and a delayed sustained phase of LPS response are often regarded as separate entities. Some authors even consider the immediate phase of LPS response as unimportant epiphenomenon (20). The main reason for that may be that immediate hypotension and pulmonary vasoconstriction with accompanying bradycardia, extrasystoles (21) and decreased cardiac output (1) induced by LPS are transient and observed only with high LPS doses, while a delayed phase consisting of a gradual fall in blood pressure accompanied by microvessel injury, disturbances in blood coagulation and tissue hypoperfusion is present even with low doses of LPS and may lead to multiorgan failure and death. Importantly, hypotension and vasoplegia of endotoxic shock are mediated by induction of nitric oxide synthase (NOS-2) and subsequent nitric oxide (NO) overproduction. Here we provide evidence that PAF may be a mediator that links both phases of cardiovascular response to LPS.

We showed that immediate coronary vasoconstriction and negative inotropic response induced by LPS were mimicked by stable PAF analog and fully blocked by PAF receptor antagonist WEB 2170. These results points to PAF as a main mediator of the heart response to LPS. Additionally, LPS-induced cardiac response was partially inhibited by non-specific COX inhibitor - indomethacin or by inhibitor of thromboxane A₂ synthesis - camonagrel. In contrast, neither inhibition of five lipooxygenase activating protein with BAY x1005 (22) nor blockade of cysteinyl leukotriene receptors with BAY x7195 (23,24) suppressed heart response to LPS. Accordingly, PAF with TXA₂, but not cysteinyl leukotrienes, are involved in immediate heart response to LPS.

These results seem to stay in agreement with known ability of exogenous PAF to induce negative inotropic response and coronary vasoconstriction (9,11,25). Importantly, PAF-induced heart response is accompanied by a profound rise in coronary venous thromboxane B₂ levels (25). In our study LPS-induced response was abolished by PAF receptor antagonist while high concentration of COX inhibitor or thromboxane A₂ synthase inhibitor were only partially effective. This would suggest that heart response to LPS is mediated by PAF subsequently involving its direct and indirect action, the latter *via* TXA₂ release. This is in line with the ability of PAF to mimic the response to LPS (26,27) that was also observed in our experiments with PAF analog.

Some authors postulate that LPS may provoke immediate vasodilation by immediate activation of NOS-3 (28). In our experiments L-NAME, the non-selective NOS inhibitor did not show any effect on heart response induced by LPS (data now shown) or by PAF analog. The reason of this discrepancy might be that

in experiments of Cannon et al (28) coronary arteries were strongly precontracted with thromboxane A₂ mimetic and PAF may act as vasodilator or vasoconstrictor (29) depending on the tone of the vessel. Indeed, Cannon et al (28) demonstrated that LPS - induced vasodilation in precontracted coronary vessels was inhibited by PAF receptor antagonist, further supporting the major role of PAF in immediate heart response to LPS. Of course, we do not exclude a possible role of other mediators such as histamine (30), catecholamines (31) or TNF α (1,6) in LPS-induced heart response. Their interaction with PAF remained to be determined.

Although PAF was originally reported to be released from neutrophils, basophils, macrophages and platelets (32) obviously these cells cannot constitute the source of PAF in Krebs buffer-perfused isolated heart. Endothelial cells (33,34) and/or cardiomyocytes (35) remain the most likely cellular source of LPS-released PAF in the heart. TXA₂ most likely derives from cardiomyocytes but not from endothelial cells and; is released in response to LPS and/or to PAF.

We showed previously that in the isolated blood-perfused rat lung immediate lung response to LPS was also mediated by PAF and TXA₂, but they were released from blood cells not from lung parenchyma (19). However, within the heart, endothelial cells or, possibly, cardiomyocytes represent the source for these mediators implicating role of these cells in inflammatory response.

Our data suggest that increased amount of PAF but not of TXA₂ and cysteinyl leukotrienes may also contribute to the late phase of endotoxic shock. Indeed, WEB 2170 but not indomethacin or BAY x1005 inhibited induction of NOS-2 protein in response to LPS in mouse macrophage line. Previously PAF was suggested to be involved in NOS-2 induction in astrocytes and Kupffer cells (36,37). It was also shown that PAF antagonist diminished induction of COX-2 by LPS in hippocampal neurons (38) and is involved in the induction of secretory PLA₂ genes in astrocytes (39). On the other hand, PAF by itself did not stimulate NO release (assumed as nitrite level) from macrophages, however, it potentiated in a dose-dependent manner NO production by these cells when they were stimulated with LPS. These observations suggest that exogenous PAF is not able to induce NOS-2 and it may only slightly facilitate LPS response. However, the endogenous PAF may be causally involved in LPS-induced NOS-2 induction. Indeed, it was shown that pre-treatment with WEB 2170 prevented late hemodynamic consequences of LPS injection *in vivo* in rats (40).

In summary, in isolated rat heart, in the absence of blood cells and plasma mediator systems, LPS exerts immediate coronary vasoconstriction and depression of myocardial contractility, which are mediated by PAF. Additionally, PAF is involved in the induction of NOS-2 by LPS. It is therefore tempting to speculate that PAF may constitute the link between early and late phases of the cardiovascular response to bacterial endotoxin.

REFERENCES

1. Stamm C, Cowan DB, Friehs I, Noria S, del Nido PJ, McGowan FX Jr. Rapid endotoxin-induced alterations in myocardial calcium handling: obligatory role of cardiac TNF-alpha. *Anesthesiology*, 2001; 95: 1396-1405.
2. Panaro J, Amati L, Sisto M et al. Embryonal cardiotoxicity of the Helicobacter pylori lipopolysaccharide. *Immune Endocrine&Metabolic Disorders*, 2001; 1: 31-44.
3. Bachschmid M, Thurau S, Zou MH, Urlich V. Endothelial cell activation by endotoxin involves superoxide/NO-mediated nitration of prostacyclin synthase and thromboxane receptor stimulation. *FASEB J.*, 2003; 17: 914-916.
4. Hocherl K, Dreher F, Kurtz A, Bucher M. Cyclooxygenase-2 inhibition attenuates lipopolysaccharide-induced cardiovascular failure. *Hypertension*, 2002; 40: 947-953.
5. Tavernier B, Li JM, El-Omar MM et al. Cardiac contractile impairment associated with increased phosphorylation of troponin I in endotoxemic rats. *FASEB J.*, 2001; 15: 294-296.
6. Grandel U, Fink L, Blum A et al. Endotoxin-induced myocardial tumour necrosis factor-alpha synthesis depresses contractility of isolated rat hearts: evidence for a role of sphingosine and cyclooxygenase-2-derived thromboxane production. *Circulation*, 2000; 102: 2758-2764.
7. Muller-Werdan U, Engelmann H, Werdan K. Cardiodepression by tumour necrosis factor-alpha. *Eur Cytokine Network*, 1998; 9: 689-691.
8. Pinsky DJ, Cai B, Yang X, Rodriguez C, Sciacca RR, Cannon PJ. The lethal effects of cytokine-induced nitric oxide on cardiac myocytes are blocked by nitric oxide synthase antagonism or transforming growth factor beta. *J Clin Invest*, 1995; 95: 677-685.
9. Massey CV, Kohout TA, Gaa ST, Lederer WJ, Rogers TB. Molecular and cellular actions of platelet-activating factor in rat heart cells. *J Clin Invest*, 1991; 88: 2106-2116.
10. Huang Q, Wu M, Menger C, Kelly K, Yuan Y. Neutrophil-dependent augmentation of PAF-induced vasoconstriction and albumin flux in coronary arterioles. *Am J Physiol*, 1998; 275(4 Pt 2):H1 138-147.
11. Giessler C, Ponicke K, Steinborn C, Brodde OE. Effects of PAF on cardiac function and eicosanoid release in the isolated perfused rat heart: comparison between normotensive and spontaneously hypertensive rats. *Basic Res Cardiol*, 1995; 90: 337-347.
12. Camussi G, Biancone L, Iorio EL et al. Porins and lipopolysaccharide stimulate platelet activating factor synthesis by human mesangial cells. *Kidney International*, 1992; 42: 1309-1318.
13. Olszanecki R, Kozlovski VI, Chlopicki S, Gryglewski RJ. Paradoxical augmentation of bradykinin-induced vasodilatation by xanthine/xanthine oxidase-derived free radicals in isolated guinea pig heart. *J Physiol Pharmacol* 2002;53:689-699.
14. Chlopicki S, Kozlovski VI, Gryglewski RJ. No-dependent vasodilation induced by nebivolol in coronary circulation is not mediated by beta-adrenoceptors or by 5 HT1A-receptors. *J Physiol Pharmacol* 2002;53:615-624.
15. Olszanecki R, Gębska A, Kozlovsky V, Gryglewski R J. Flavonoids and nitric oxide synthase. *J Physiol Pharmacol*, 2002; 53(4 Pt 1): 571-584.
16. Wu CC, Thiemermann C, Vane JR. Glibenclamide-induced inhibition of the expression of inducible nitric oxide synthase in cultured macrophages and in the anaesthetized rat. *Br J Pharmacol*. 1995;114:1273-1281.
17. Thiemermann Ch. Nitric oxide and circulatory shock: an update. In *Nitric Oxide - Basic Research and Clinical Applications*. Gryglewski RJ, Minuz P (eds). IOS Press, 2001; 317(A): 117-125.
18. Gryglewski R J, Wolkow PP, Uracz W et al. Protective role of pulmonary nitric oxide in the acute phase of endotoxemia in rats. *Circ Res*, 1998; 82: 819-827.

19. Chlopicki S, Bartus JB, Gryglewski RJ. Biphasic response to lipopolysaccharide from *E. Coli* in the isolated ventilated blood-perfused rat lung. *J Physiol Pharmacol*, 1999; 50: 551-565.
20. Parratt JR. Nitric oxide and cardiovascular dysfunction in sepsis and endotoxemia: an introduction and an overview. In *Shock, sepsis and organ failure-nitric oxide*. Berlin: Springer, Verlag (eds) 1995; 1-20.
21. Koulchitsky SV, Subbotin OV, Azev OA, Kulchitsky VA. NO-ergic mechanisms are implicated in a disturbed cardiac rhythm after systemic application of lipopolysaccharide *E. Coli* to rats. *Autonomic Neurosciences-Basic&Clinical*, 2002; 98: 99-101.
22. Hatzelmann A, Fruchtmann R, Mohrs KH, Raddatz S, Muller-Peddinghaus R. Mode of action of the new selective leukotriene synthesis inhibitor BAY x 1005 {[R]-2-[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid} and structurally related compounds. *Biochem Pharmacol*, 1993; 45: 101-111.
23. Gorenne I, Ortoz JL, Labat C et al. Antagonism of leukotriene responses in human airways by BAY x7195. *Eur J Pharmacol*, 1995; 275:207-212.
24. Francis HP, Patel UP, Thompson AM, Carpenter TG, Gardiner PJ, Abram TS. The effects of a novel sulphidopeptide leukotriene antagonist, BAY x7195, against elicited bronchoconstriction in the anaesthetized guinea-pig. *Br J Pharmacol*, 1998; 123: 39-44.
25. Ezra D, Laurindo FR, Czaja JF, Synder F, Goldstein RE, Feuerstein G. cardiac and coronary consequences of intracoronary platelet activating factor infusion in the domestic pig. *Prostaglandin*, 1987; 34: 41-57.
26. Bessin P, Bonnet J, Apffel D et al. Acute circulatory collapse caused by platelet-activating factor (PAF-acether) in dogs. *Eur J Pharmacol*, 1983; 86: 403-413.
27. Mulder MF, van Lambalgen AA, van Kraats AA et al. Systemic and regional hemodynamic changes during endotoxin or platelet activating factor (PAF)-induced shock in rats. *Circ Shock*, 1993; 41: 221-229.
28. Cannon TR, Mann GE, Baydoun AR. mechanisms of acute vasodilator response to bacterial lipopolysaccharide in the rat coronary microcirculation. *Br J Pharmacol*, 1998; 123: 637-644.
29. Man RYK, Hu W, Kinnaird AAA. Coronary vascular response to platelet activating factor in the perfused rat heart. *J Lipid Mediat*, 1990; 2: 75-83.
30. Matsuda N, Hattori Y, Zhang XH, Fukui H, Kemmotsu O, Gando S. Contractions to histamine in pulmonary and mesenteric arteries from endotoxemic rabbits: modulation by vascular expressions of inducible nitric-oxide synthase and histamine H1-receptors. *J Pharmacol.&Experim. Therap.* 2003; 307:175-81
31. Pleiner J, Heere-Ress E, Langenberger H, Sieder AE, Bayerle-Eder M, Mittermayer F, Fuchsjäger-Mayrl G, Böhm J, Jansen B, Wolzt M. Adrenoceptor hyporeactivity is responsible for *Escherichia coli* endotoxin-induced acute vascular dysfunction in humans. *Arteriosclerosis, Thrombosis&Vascular Biology*. 2002; 22:95-100
32. Camussi G, Bussolino F, Salvidio G, Baglioni C. Tumour necrosis factor/cachectin stimulates peritoneal macrophages, polymorphonuclear neutrophils, and vascular endothelial cells to synthesize and release platelet-activating factor. *J Exp Med*, 1987; 166: 1390-1404.
33. Camussi G, Pawlowski I, Bussolino F, Caldwell PR, Brentjens J, Andres G. Release of platelet activating factor in rabbits with antibody-mediated injury of lung: the role of leukocytes and of pulmonary endothelial cells. *J Immunol*, 1983; 131: 1802-1807.
34. Camussi G, Aglietta M, Malavasi F et al. Release of platelet-activating factor from human endothelial cells in culture. *J Immunol*, 1983; 131: 2397-2403.
35. Janero DR, Burghardt C. Production and release of platelet-activating factor by the injured heart-muscle cell (cardiomyocyte). *Res Comm Chem Pathol&Pharmacol*, 1990; 67: 201-218.
36. Wang JH, Sun GY. Platelet activating factor (PAF) antagonists on cytokine induction of iNOS and sPLA2 in immortalized astrocytes (DITNC). *Neurochem Res*, 2000; 25: 613-619.

37. Mustafa SB, Flickinger BD, Olson MS. Suppression of lipopolysaccharide-induced nitric oxide synthase expression by platelet activating factor receptor antagonists in the rat liver and cultured rat Kupffer cells. *Hepatology*, 1999; 30 1206-1214.
38. Serou MJ, DeCoster MA, Bazan NG. Interleukin-1 beta activates expression of cyclooxygenase-2 and inducible nitric oxide synthase in primary hippocampal neuronal culture: platelet-activating factor as a preferential mediator of cyclooxygenase-2 expression. *J Neurosci Res*, 1999; 58: 5930598.
39. Li W, Xia J, Sun GY. Cytokine induction of iNOS and sPLA2 in immortalized astrocytes (DITNC): response to genistein and pyrrolidine dithiocarbamate. *J Interferon&Cytokine Res*, 1999; 19: 121-127.
40. Bochenski J, Chlopicki S, Gryglewski RJ. Role of thromboxane A2 and platelet activating factor in early haemodynamic response to lipopolysaccharide in rats. *J Physiol Pharmacol*, 1999; 50: 287-297.

Received: July 31, 2003

Accepted: March 18, 2004

Authors address: Stefan Chlopicki, Department of Experimental Pharmacology Chair of Pharmacology Jagiellonian University Medical College, Grzegórzecka 16, Kraków 31-531, Poland, Phone: (012) 211 168, Fax: (012) 217 217
E-mail: mfschlop@cyf-kr.edu.pl