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ENDOTOXAEMIA IN RATS: ROLE OF NO, PAF AND TXA₂ IN PULMONARY NEUTROPHIL SEQUESTRATION AND HYPERLACTATAEMIA

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Objective and Design: The involvement of PAF, TXA₂ and NO in LPS-induced pulmonary neutrophil sequestration and hyperlactataemia was studied in conscious rats. As pharmacological tools WEB 2170 (PAF receptor antagonist, 20 mg/kg), camonagrel (inhibitor of TXA₂ synthase, 30 mg/kg), N^G-nitro L-arginine methyl ester (L-NAME — non-selective nitric oxide synthase inhibitor, 30 mg/kg) were used.

Methods: Plasma lactate and NO₂⁻/NO₃⁻ levels as well as myeloperoxidase (MPO) activity in lung tissue were measured one and five hours after administration of LPS (4 mg/kg⁻¹).

Results: LPS induced a twofold increase in plasma lactate levels and nearly 10-fold increase in plasma NO₂⁻/NO₃⁻ levels five but not one hour after LPS administration. However, LPS-induced increase in pulmonary MPO activity was seen at both time intervals. Neither WEB 2170 nor camonagrel changed one or five hours responses to LPS (lactate, NO₂⁻/NO₃⁻, MPO). L-NAME potentiated LPS-induced rise in MPO activity in the lung and this potentiation was not affected by WEB 2170 or camonagrel. L-NAME suppressed plasma NO₂⁻/NO₃⁻ response and substantially potentiated plasma lactate response to LPS and both effects were partially reversed by WEB 2170 or camonagrel.

Conclusions: In summary, we demonstrated that PAF and TXA₂ play a role in overproduction of lactate during endotoxaemia in NO-deficient rats. However, these lipids do not mediate endotoxin-induced sequestration of neutrophils in the lung.

Key words: *lipopolysaccharide, pulmonary neutrophil sequestration, nitric oxide, platelet activating factor, thromboxane A₂*

INTRODUCTION

In various experimental models nitric oxide (NO) that is made by endothelial NO synthase (NOS-3) appears as a potent antileukocyte agent that inhibits activation (1, 2), adherence (3, 4) and migration (5, 6) of neutrophils. No wonder, that non-selective NOS inhibitors were reported to augment neutrophil-induced injury of liver or intestine (7, 8). It has been claimed that non-selective NOS inhibition may enhance endotoxin-induced damage of the lung (9, 10), however, it is not clear whether it is a neutrophil-dependent

phenomenon. Lately, we demonstrated that lipopolysaccharide (LPS) — induced acute injury of the lung in NO-deficient rats was mediated by the release of platelet activating factor (PAF) and thromboxane A₂(TXA₂) (11). Presently, we are looking for a link between this pneumotoxic effect of LPS and sequestration of neutrophils in the lung trying to spot the involvement of PAF and TXA₂ in this phenomenon, both in presence or in absence of endogenous NO. Additionally, we are attempting to elucidate the contribution of NO, PAF and TXA₂ in hyperlactatemia in rats exposed to LPS. As pharmacological tools were used: a non-selective NO synthase inhibitor L-NAME, a TXA₂ synthase inhibitor camonagrel and a PAF receptor antagonists WEB 2170.

MATERIALS AND METHODS

Animals

Male Wistar rats Lod: Wist BR from Animal Laboratory of Polish Mother's Memorial Research Institute Hospital in Łódź, Poland, weighting 250—350 g were used for experiments. Animal experiments conformed with the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Council of Europe No 123, Strasbourg 1985).

Experimental protocol

Animals were randomly divided into six experimental and two control groups:
Experimental groups:

- 1) LPS group — injection of LPS alone (4 mg/kg i.p.),
- 2) Camonagrel+LPS group — injection of camonagrel (30 mg/kg i.p.) 30 min later followed by injection LPS (4 mg/kg i.p.),
- 3) WEB 2170+LPS group — injection of WEB 2170 (20 mg/kg i.p.) 30 min later followed by injection of LPS (4 mg/kg i.p.),
- 4) L-NAME+LPS group — injection of L-NAME (30 mg/kg i.p.) 30 min later followed by injection of LPS (4 mg/kg i.p.),
- 5) L-NAME+Camomagrel+LPS group — injection of L-NAME (30 mg/kg i.p.) 15 min later followed by injection of camonagrel (30 mg/kg i.p.) 15 min later followed by injection of LPS (4 mg/kg i.p.),
- 6) L-NAME+WEB 2170+LPS group — injection of L-NAME (30 mg/kg i.p.) 15 min later followed by injection of WEB 2170 (120 mg/kg i.p.) 15 min later followed by injection of LPS (4 mg/kg i.p.),

Control group:

- 1) control group — injection of vehicle alone (1 ml of sterile saline i.p.),
- 2) L-NAME control group — injection of N^G-nitro-L-arginine methyl ester alone (30 mg/kg i.p.);

In groups each consisting of 12 rats blood and tissue samples were taken at two time intervals, i.e. 1 hour ($n = 6$) and 5 hours ($n = 6$) after LPS challenge. LPS, camonagrel or WEB 2170 were dissolved in sterile saline and injected intraperitoneally. In all experimental protocols injection volume was 1 ml. At the end of experiment rats were anesthetized with thiopentone sodium (120 mg/kg), trachea was cannulated, and catheter inserted into carotid artery to collect blood samples.

Directly after this procedure thorax was opened and 300 mg ± 50 mg of tissue was sampled from the right inferior lung lobe.

Assays of lactate and nitrate/nitrite concentrations in plasma

Immediately after collection blood samples were centrifuged for 4 min at 8000 g. Plasma samples were diluted 1:1 with sterile saline and ultrafiltrated using Milipore Ultrafree — MC centrifugal filters (Biomax 5000).

Lactate levels were measured enzymatically using commercially available lactate assay kit (MPR 3 Boehringer Mannheim) and expressed in mmol L⁻¹.

The total amount of nitrite and nitrate (NO₂⁻/NO₃⁻) in blood was quantified colorimetrically by Griess reaction using a modified method described by Verdon et al. (12). Briefly, after centrifugation, dilution and ultrafiltration (as above), 50 µl of filtrate was placed in duplicate in wells of the microssay plate. 10 µl of NADPH dissolved in 0.9% NaCl (final concentration 1 µM) was added followed by 40 µl of mixture consisting of nitrate reductase (final concentration 85 U/liter), glucose-6-phosphate dehydrogenase (final concentration 160 U/liter) and glucose-6-phosphate (final concentration 500 µM) dissolved in 20 mM sodium phosphate buffer. After 45 min of incubation at 20°C the Griess reagents were added: 100 µl of 1% (w/v) sulfanilamide dissolved in 5% phosphoric acid followed immediately by 100 µl of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride dissolved in 5% phosphoric acid. After 10 min of incubation the absorbance at 540 nm, was read using microplate reader (Bio-Tek Instruments Inc). Micromolar concentrations of NO₂⁻/NO₃⁻ were calculated from standard curve.

Assay of myeloperoxidase (MPO) activity in the lung

Immediately after excision lung tissue was washed with sterile saline, blotted to dry, weighted and homogenized using Omni Mixer Homogenizer (Camlab USA) in glass homogenizing tubes containing ice cold 0.5% hexadecyltrimethylammonium bromide (HTAB) dissolved in 50 mM phosphate buffer pH 6.0 (200 mg tissue/1 ml KH₂PO₄—Na₂HPO₄). The tissue homogenates were stored at -70°C. On the day of the assay they were thawed and centrifugated at 1200 g for 15 min at 4°C. The supernatants were harvested and assayed for myeloperoxidase activity using the technique described by Bradley et al. (13). Briefly, 0.1 ml aliquots of the supernatants were mixed with 2.9 ml 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured every 1 sec for 30 sec in 23°C using Beckman DU 640 BV diode array spectrophotometer equipped with kinetic software package. MPO activity was expressed as change in absorbance (absorbance unit) per 1 min per 1 gram of wet lung tissue (A/gram/min).

Reagents and drugs

LPS (*Escherichia coli* serotype 0127:B8), L-NAME (N^G-nitro-L-arginine methyl ester), O-dianisidine, hexadecyltrimethylammonium bromide (HTAB), hydrogen peroxide, sodium nitrate, sodium nitrite, glucose-6-phosphate dehydrogenase, glucose-6-phosphate and NADPH were purchased from SIGMA Chemicals International; thiopentone sodium was from Biochemie GMBH, Germany; WEB 2170 15-(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-ethoxy]-1-indan-carboxylic acid hydrochloride) was a gift from Ferrer inc. Spain; LPS, camonagrel and WEB 2170 were dissolved in sterile saline immediately before injection.

Statistical evaluation

All values in the figures and text were expressed as mean \pm SEM of n experiments, where n represents the number of animals in the group. One way ANOVA followed by *post hoc* Duncan test was used for evaluation of differences between the groups. P value of less than 0.05 was considered statistically significant.

RESULTS

i) Effects of LPS on plasma lactate levels, NO_2^-/NO_3^- levels and MPO activity in the lung; modulation by WEB 2170, a PAF receptor antagonist, and camonagrel, a TXA₂ synthase inhibitor.

Five hours but not one hour after intraperitoneal injection of LPS at a dose of 4 mg/kg there was a twofold increase in plasma lactate levels (from 1.55 ± 0.36 to 2.97 ± 0.3 mM, p < 0.001) (Fig. 1) and nearly a 10-fold increase in

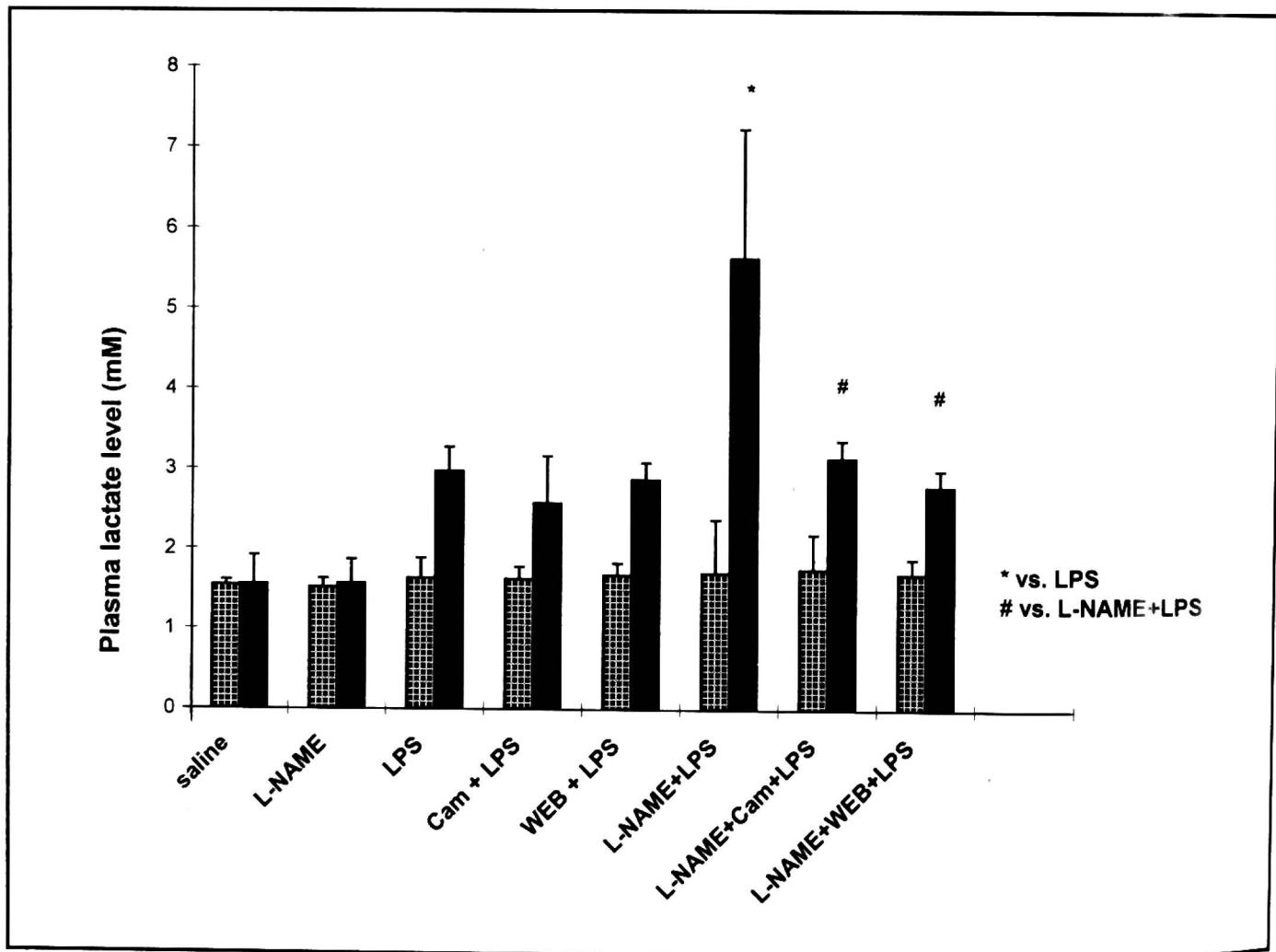


Fig. 1. Plasma lactate responses to LPS in control and L-NAME pretreated rats and their modulation by WEB 2170 (WEB), a PAF receptor antagonist., and camonagrel (Cam), a thromboxane A₂ synthase inhibitor. Measurements were done at 1 h (stippled columns) and 5 h (solid columns) after LPS injection.

* indicates significant difference (p < 0.05) with corresponding value in LPS group

indicates significant difference (p < 0.05) with corresponding value in L-NAME + LPS group

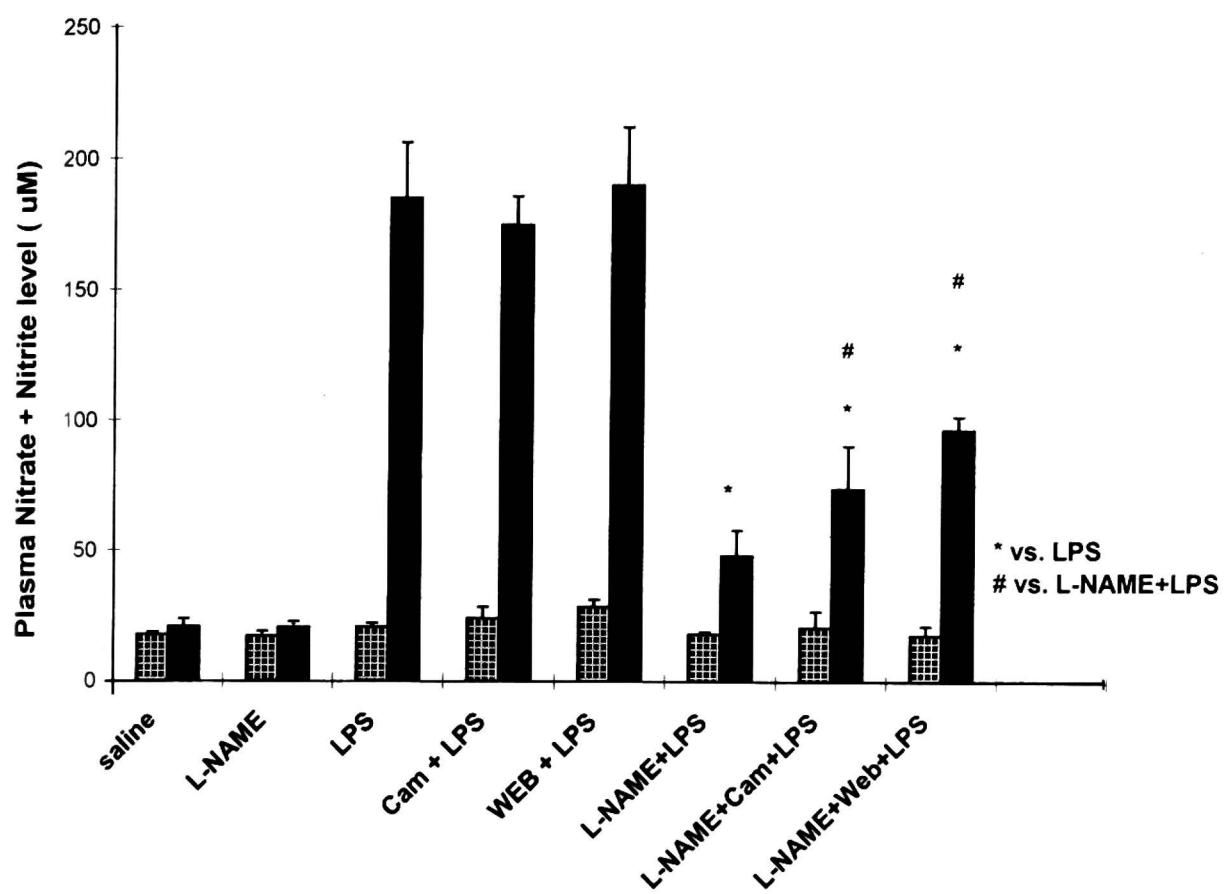


Fig. 2. Plasma nitrite and nitrate responses to LPS in control and L-NAME pretreated rats and their modulation by WEB 2170 (WEB), a PAF receptor antagonist., and camonagrel (Cam), a thromboxane A₂ synthase inhibitor. Measurements were done at 1 h (stippled columns) and 5 h (solid columns) after LPS injection.

* indicates significant difference ($p < 0.05$) with corresponding value in LPS group

indicates significant difference ($p < 0.05$) with corresponding value in L-NAME + LPS group

plasma $\text{NO}_2^-/\text{NO}_3^-$ levels (from 20.68 ± 2.98 to 185 ± 21.08 μM , $p < 0.001$) (Fig. 2). In contrast, LPS-induced increase in MPO activity in lung tissue was seen at both time intervals at 1 and at 5 hours after LPS injection (from 1.36 ± 0.094 to 13.4 ± 1.46 , $p < 0.001$, or from 3.3 ± 0.09 to 13.7 ± 1.48 A/g/min, $p < 0.001$), respectively, (Fig. 3). The pretreatment with camonagrel or WEB 2170 did not change the 1 or 5 hour response to LPS (lactate, $\text{NO}_2^-/\text{NO}_3^-$ or MPO) with the notable exception of a modest increase in the pulmonary MPO levels ($p < 0.05$) in the groups pretreated with WEB 2170 (Fig. 3).

ii) Effects of L-NAME on LPS response.

L-NAME by itself at a dose of 30 mg/kg i.p. changed neither lactate plasma levels (Fig. 1) nor $\text{NO}_2^-/\text{NO}_3^-$ plasma levels (Fig. 2) nor MPO activity in the lung (Fig. 3). However, L-NAME did change response to LPS. L-NAME augmented lactate plasma response (from 2.97 ± 0.3 to 5.63 ± 1.6 mM

$p < 0.005$) and suppressed $\text{NO}_2^-/\text{NO}_3^-$ plasma responses to LPS (from 185 ± 21 to $48.3 \pm 9.8 \mu\text{M}$, $p < 0.001$) at 5 hours but not at 1 hour after LPS. On the other hand, LPS-induced rise in the pulmonary MPO was potentiated by L-NAME at both time intervals (18.90 ± 1.68 vs. 13.40 ± 1.46 , $p < 0.001$ and 20.90 ± 1.64 vs. $13.70 \pm 1.48 \text{ A/min/g}$, $p < 0.001$, at 1 and 5 hours, respectively).

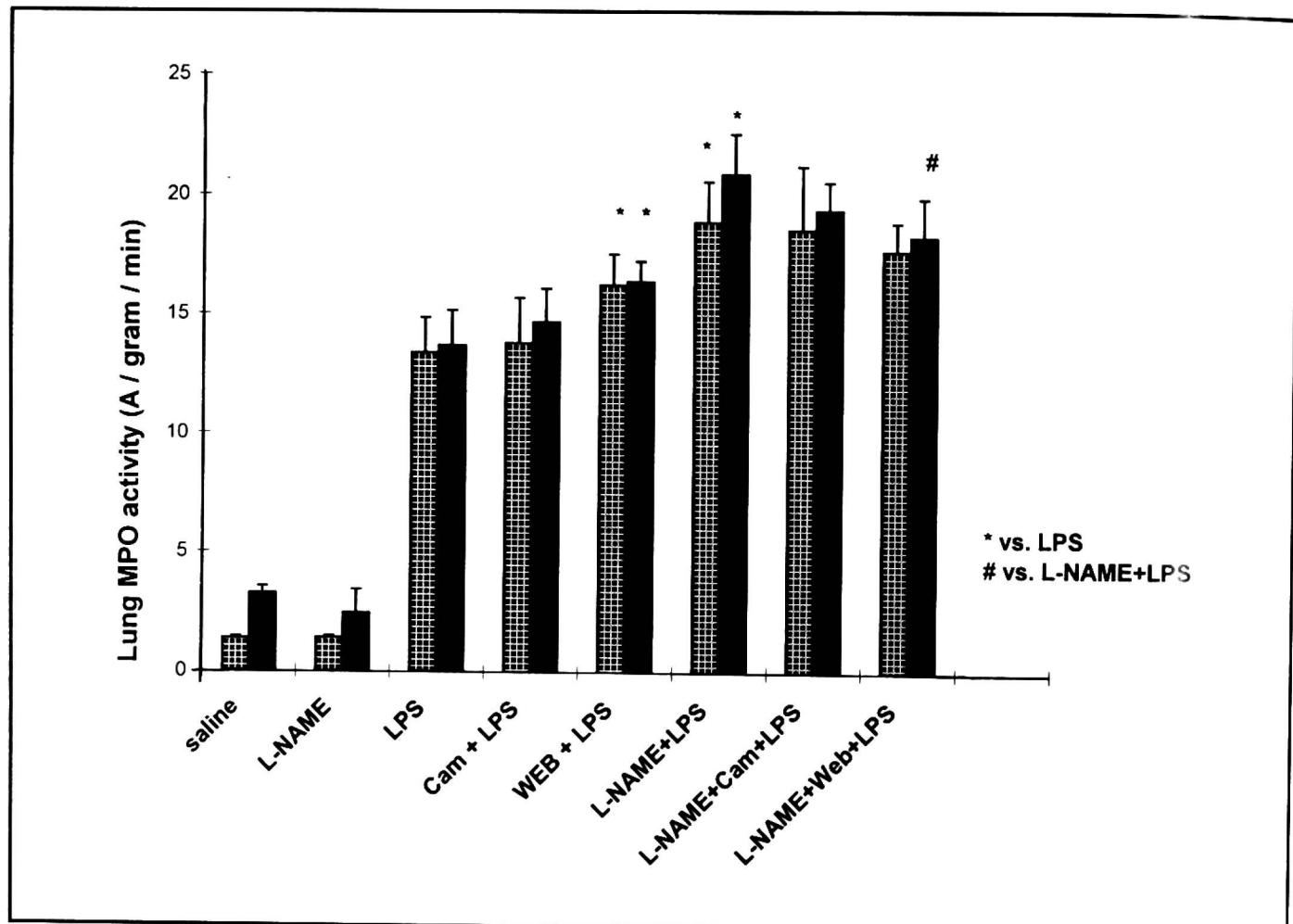


Fig. 3. Lung MPO activity responses to LPS in control and L-NAME pretreated rats and their modulation by WEB 2170 (WEB), a PAF receptor antagonist., and camonagrel (Cam), a thromboxane A₂ synthase inhibitor. Measurements were done at 1 h (stippled columns) and 5 h (solid columns) after LPS injection.

* indicates significant difference ($p < 0.05$) with corresponding value in LPS group
indicates significant difference ($p < 0.05$) with corresponding value in L-NAME + LPS group

iii) Modulation by WEB 2170 and camonagrel of LPS response in L-NAME pretreated rats

The eminent potentiation of plasma lactate response to LPS by L-NAME at 5 hours after the LPS challenge (see results ii) was abrogated both by WEB 2170 ($p < 0.005$) or by camonagrel ($p < 0.01$) (Fig. 1). Interestingly, each one of these drugs blunted the inhibitory effect of L-NAME on LPS-induced $\text{NO}_2^-/\text{NO}_3^-$ plasma response 5 hours after LPS administration ($p < 0.05$) (Fig. 2). At both time intervals in NO-deficient rats neither WEB 2170 nor

cambonagrel affected the augmented MPO response to LPS with one exception only. Five hours after LPS challenge in WEB 2170 pretreated rats a slight but significant ($p = 0.042$) decrease in MPO activity occurred (Fig. 3).

DISCUSSION

Elevated plasma levels of PAF (14, 15) and of metabolites of TXA₂ (16, 17) were demonstrated in septic patients. PAF (18, 19) and TXA₂ (20, 21) were shown to be released to circulation by lipopolysaccharide (LPS) in various models of endotoxic shock (22, 23). We studied the involvement of PAF and TXA₂ in the LPS-induced pulmonary neutrophil sequestration as evidenced by lung myeloperoxidase (MPO) activity both in presence and in absence of endogenous NO. Endogenous NO production was monitored by NO₂⁻/NO₃⁻ levels in plasma. In addition, the involvement of PAF and TXA₂ in LPS-induced hyperlactataemia was investigated in analogous conditions.

Lactate and NO₂⁻/NO₃⁻ in blood as well as MPO activity in lung homogenates were assayed at two time intervals corresponding to the early and late phases of endotoxic shock in which activation of NOS-3 (11) and induction of NOS-2 would contribute to the haemodynamic state, respectively (24).

Several studies demonstrated that blood lactate levels correlate well with the incidence and severity of organ failure during sepsis (25, 26) and lactate blood test was proven to be a reliable predictor of outcome in patients with septic shock (27, 28). Plasma levels of NO₂⁻/NO₃⁻ have not such a predictive value but they offer a reliable estimation of the degree of NOS-2 induction not only in experimental animals but also in patients with septic shock (29, 30).

We showed that one hour after administration of LPS, plasma levels of lactate or NO₂⁻/NO₃⁻ had not differed from control levels, irrespectively of pharmacological procedures applied. Meaningful changes were observed five hours after LPS challenge and, accordingly our analysis is restricted to that time interval only. Late but not instant increase in plasma lactate levels in endotoxaemia, as reported previously (31, 32), indicates that endotoxin-induced hyperlactataemia is not mediated by direct influence on glycolytic enzymes but it may rather reflect impairment of tissue perfusion. Although previously we measured by porphyrinic electrode an immediate of (10—15 min duration) rise in pulmonary NO generation (11), obviously this rise was not big enough to be detected one hour after administration of LPS by an assay of NO₂⁻/NO₃⁻ in plasma, and only NOS-2 derived rise in NO₂⁻/NO₃⁻ was detected 5 hours after LPS.

We showed that neither PAF nor TXA₂ is involved in LPS-induced plasma lactate and NO₂⁻/NO₃⁻ response. Obviously, pretreatment with L-NAME

inhibited LPS-induced rise in plasma $\text{NO}_2^-/\text{NO}_3^-$ levels, but surprisingly it potentiated LPS-induced hyperlactataemia. Interestingly, the above effects of L-NAME pretreatment on LPS induced lactate and $\text{NO}_2^-/\text{NO}_3^-$ response were partially reversed by WEB 2170 and camonagrel. Accordingly, the role of PAF and TXA₂ became apparent only after pharmacological removal of endogenous NO. There are several possible explanations for these observations.

Pharmacological removal of endogenous NO by L-NAME was previously shown to potentiate hypoperfusion of various organs during endotoxic shock (7, 33) and this effect was shown to be reversed by exogenous NO donor (34). Many authors point out to blood flow maldistribution and non-vital organs hypoperfusion as a major cause of hyperlactatemia during sepsis (35, 36). Consequently, our data prompt us to speculate that if NO production is preserved, effects of PAF and TXA₂ released by LPS sufficiently counteracted by vasodilator actions of NO. However, if endogenous NO production is inhibited, actions of these lipid mediators unopposed by endogenous NO lead to non-vital organs underperfusion and to substantial impairment of their oxygenation as evidenced by the increased blood lactate level.

It was lately shown, that in endotoxaemia NOS could not efficiently oxidate arginine and might produce superoxide anions rather than NO (37). We are tempted to speculate that pharmacological suppression of NO synthesis during endotoxaemia triggers tissue hypoperfusion and subsequent hypoxia develops. Then, the remaining activity of NOS generates O₂⁻ rather than NO. PAF receptor blockade or TXA₂ synthesis inhibition reverses tissue hypoperfusion and shifts the balance between NO and O₂⁻ back to the prevalence of NO as evidenced by increasing $\text{NO}_2^-/\text{NO}_3^-$ levels and accompanying decrease in lactate plasma levels (*Fig. 2* and *Fig. 1*).

Although many authors reported NOS-3 as a source of "protective" NO in endotoxaemia (38, 39), our data point out to a possible beneficial role of NO derived from NOS-2 that abides vascular wall during endotoxaemia. NOS-2-derived NO is likely to minimize tissue hypoperfusion. Indeed, other authors recently suggested that NOS-2-derived NO, being a culprit of hypotension and vasoplegia (40, 41), may also attenuate some of the deleterious consequences of endotoxaemia (42).

The direct influence of NOS-2 derived NO on cell metabolism should be also considered. It was demonstrated that NO inhibited Krebs cycle enzymes (43) or mitochondrial respiratory chain reactions (44). However, in the present study inhibition of NOS potentiates LPS-induced plasma lactate response, which leaves this line of explanation unlikely.

Another possible explanation of our data refers to the direct stimulatory effect of PAF on NOS-2 induction (45). However, our data show that PAF

receptor antagonist loosened the inhibitory effect of L-NAME on NO synthesis as it PAF was to contribute to inhibiton rather than to stimulation of NOS-2 induction.

In contrast with lactate and $\text{NO}_2^-/\text{NO}_3^-$ response LPS-induced pulmonary sequestration of neutrophils in the lung was observed both one and five hours after administration of endotoxin. This was evidenced by myeloperoxidase (MPO) activity assay in tissues which is thought to be reliable index of tissue neutrophil content (13, 46).

We confirmed that LPS was responsible for sequestration of neutrophils in the rat lung, which occured rapidly after LPS and persisted, as many authors reported previously (47—49). Interestingly, pharmacological inhibition of NO synthases potentiated LPS-induced elevation of MPO activity in the lung. It may well be that in endotoxic lung NO prevents LPS-induced pulmonary sequestration of neutrophils (50) because NO inhibits their activation (2) and expression of adhesion molecules (4). Activation of neutrophils seems to be mandatory for their atrappment in the lung of LPS-treated animals (49—51), however, as we showed here, the PAF or TXA_2 -trigerred mechanisms are not likely to be involved. An important difference is that although PAF and TXA_2 do not contribute to pulmonary trapping of neutrophils in LPS-treated rats, both of these lipids may contribute to the activation of neutrophils (52), especially when neutrophils are already sequestrated in the lung. Indeed, we previously demonstrated that PAF receptor blockade or TXA_2 synthase inhibition both would substantially prolong survival time and prevent lethal lung injury in NO-deficient LPS-treated rats (11). The pathways leading to sequestration of neutrophils and to their activation are obviously different.

In summary, we showed that inhibition of NO synthase before administration of LPS result in potentiation of plasma lactate response. This exaggerated lactate response to LPS was abrogated by either blockade of PAF receptors or by inhibition of thromboxane A₂ synthesis. However, neither PAF nor TXA_2 seem to contribute to augmented sequestration of neutrophils after LPS when production of endogenous NO is inhibited. We speculate that in rats with endotoxic shock, effects of non-selective NO synthase inhibition on lactate production may be mediated by an increased generation of PAF and TXA_2 , leading to excessive vasoconstriction and tissue hypoperfusion. The relative contribution of NOS-2-derived NO to excessive hyperlactatemia seems likely but remains to be proven.

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