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BIPHASIC RESPONSE TO LIPOPOLYSACCHARIDE FROM *E. COLI* IN THE ISOLATED VENTILATED BLOOD-PERFUSED RAT LUNG

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We characterised early circulatory and respiratory responses to lipopolysaccharide from *E. coli* (LPS, serotype O127:B8) in the isolated, ventilated and perfused rat lung preparation. Lungs were isolated from anaesthetised Wistar rats and perfused with full blood, platelet rich plasma (PRP), platelet poor plasma (PPP) or Krebs-Henseleit solution (KH). LPS (300 μg/ml) injected into the blood-perfused lung induced a characteristic biphasic response consisting of an immediate, transient decrease in respiratory tidal volume and an increase in pulmonary perfusion pressures followed by a delayed decrease in respiratory tidal volume. An immediate respiratory/circulatory response to LPS was of considerable magnitude only in full blood-perfused lung whereas the delayed response was fully expressed irrespective whether blood, PRP, PPP or KH was used for the lung perfusion. Immediate respiratory/circulatory response was inhibited by WEB 2170 (100 μM), a PAF receptor antagonist, and by camonagrel (300 μM), a TXA_2 synthase inhibitor, but not by MK 571 (100 μM), a cysteinyl leukotriene receptor antagonist. Delayed respiratory response was inhibited by camonagrel only. In summary, we demonstrated that the immediate coupled respiratory/circulatory response is mediated by blood cell-derived PAF and TXA_2 whereas the delayed uncoupled respiratory response is mediated by lung parenchyma-derived TXA_2.

Key words: *lipopolysaccharide, isolated lung, PAF, TXA_2, pulmonary vasoconstriction, bronchoconstriction*

INTRODUCTION

Despite many years of research, early circulatory and respiratory lung response to lipopolysaccharide (LPS) from *E. coli* is not as yet fully understood.

In pigs intravenous infusion of LPS induces an immediate, transient increase in pulmonary artery pressure and a parallel increase in airway resistance (1). Simultaneous occurrence of airway and vascular responses to LPS which occurs not only in pig but also in cat (2), and sheep (3), is indicative of common mechanisms involved in respiratory and circulatory facet of

immediate response to LPS. Indeed, PAF and/or TXA_2 were shown to be involved in both airway and vascular responses to LPS in some species (2—4), though the cellular source from which these mediators are released was not identified so far.

In rats *in vivo* an immediate vascular pulmonary response to LPS occurs within minutes after intravenous LPS injection and, similarly to the response in cat, pig and dog, is also mediated by the release of PAF and TXA_2 (5). However, it remains unknown whether immediate vascular response to LPS in rats *in vivo* is accompanied by prompt bronchoconstriction as it is in other species. On the other hand, in the isolated electrolite solution -perfused isolated rat lung LPS failed to induce an immediate response to LPS and elicited only a delayed solitary bronchoconstriction mediated by COX-2 derived TXA_2 (6). Several authors found that blood cells are mandatory for LPS to elicit vascular contraction (7—9), which may explain lack of vascular response to LPS in Krebs-perfused isolated rat lung. However, it remains intriguing that LPS-induced delayed bronchoconstriction shown in this preparation occurred only 60 —90 minutes after LPS injection (6) in contrast with an immediate pulmonary vascular response in rats *in vivo* observed by us (5). The above data may suggest that rat lung response to LPS is biphasic, and each phase is being mediated by bronchoconstrictor and vasoconstrictor lipids released from various cellular sources in blood or in the lung parenchyma. In this study we put this hypothesis to testing.

MATERIALS AND METHODS

Isolated lungs preparation

Lungs were isolated from Wistar rats weighing 200—250g (Lod:WIST BR from Animal laboratory of Polish Mother's Memorial Research Institute hospital in Lodz, Poland). In anaesthetised rats (thiopentone 120 mg/kg, i.p.), trachea was cannulated and lungs were ventilated with the positive pressures at a rate of 80 breaths/min (VCM module from Hugo Sachs Electronik-HSE). After laparotomy, diaphragm was cut and nadroparine, at a dose of 600 I.U. was injected into the right ventricle to prevent microthrombi formation during surgery. Then animals were exsanguinated by incision of left renal artery. Lungs were exposed via a medial sternotomy. The pulmonary artery and left atrium were cannulated via right and left atrium, respectively. Immediately after cannulation the lung/heart block was dissected from the thorax. Using the tracheal cannula the isolated lung was mounted in the water-jacketed (38°), air-tight glass chamber (HSE), and ventilated with negative pressures. The residual blood was washed out within first 10 minutes of the initial perfusion with prewarmed (38°C) Krebs-Hanseleit buffer. Then lungs were perfused with various fluids (see below) using a peristaltic pump (ISM 834, HSE) at a constant flow of about 16 ml/min. The venous pressure was set at a level of 2—5 cm H_2O.

The end-expiratory pressure in the chamber was set to be -2 cm H_2O and inspiratory pressure was adjusted between -6 to -10 cm H_2O to yield an initial tidal volume (TV) of about

2.0 ml. Breathing frequency was set to be 80 breaths/minute and a duration of inspiration versus expiration was 1:1 in each breath. Every 5 min throughout the experiment a deep breath of end-inspiratory pressure of -18 cm H_2O was automatically initiated by VCM module (HSE) to avoid atelectasis. The inspired air was moistured by bubbling through water. Airflow velocity was measured with a pneumotachometer tube connected to a differential pressure transducer (HSE) from which value of respiratory tidal volume was determined.

Both arterial and venous pulmonary pressures (PAP, PVP) were continuously monitored by ISOTEC pressure transducers (HSE).

The weight of lungs was monitored by a specially-designed transducer (HSE) (10).

TV, PAP, PVP and lung weight data were acquired by the PC transducer card and subsequently analysed by Pulmodyn-pulmo software (HSE), as well as continuously recorded on Graphtec linear recorder WR 3310.

The perfusion procedure and experimental protocols

The following fluids were used to perfuse the lung preparation: rat full blood (FB), rat platelet rich plasma (PRP), rat platelet poor plasma (PPP) or Krebs-Henseleit buffer (KH).

Blood was obtained from 2 Wistar rats (300—500 g body weight). Rats were anaesthetised (thiopentone 120 mg/kg i.p.), injected with nadroparine (600 I.U, i.p.) and exsanguinated through cannulated right carotid artery.

PRP or PPP were obtained by 10 min centrifugation of blood from donor rats at 200 g or 2000 g, respectively.

Krebs-Henseleit buffer (KH) was of the following composition (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 12.5, 4% albumin, 0.1% glucose and 0.3% HEPES.

The pH of perfusate was maintained at about 7.4 throughout the whole experiment by continuous addition of 5% CO_2 to the inspiratory air.

All lung preparations were allowed to equilibrate for the first 15 min until baseline PAP, PVP, TV and weight were stable. At this moment weight of the lung (value of which varied considerably between experiments) was set to zero.

LPS was injected 45 minutes after the beginning of the experiment. LPS was given as an injection to the perfusion line leading to the pulmonary artery at a dose necessary to achieve a final concentration required.

Camonagrel (300 μM), WEB 2170 (100 μM) or MK 571 (100 μM) were added to the reservoir with perfusate 30 min prior to the injection of LPS.

All experiments lasted 160 min. Control lungs perfused with blood, PRP, PPP or KH, subjected to no intervention were also included.

TV, PAP, PVP and weight were continuously monitored throughout the experiment, however, only values at intervals of -30, 0, 5, 10, 15, 30, 45, 60, 80, 100, 120 after LPS injection were analysed.

Data were expressed as means ± SEM of changes in TV (ΔTV), PAP (ΔPAP), PVP (ΔPVP), or weight (Δweight) from values before LPS injection (-30 min time interval). Significance of differences between groups was established by single factor analysis of variance (ANOVA) followed by t-test for multiple comparison.

Reagents and drugs

LPS (*Escherichia coli* serotype 0127 B8) was purchased from Sigma Chemicals International; bovine albumin fraction V was from Serva, Germany; thiopental sodium (Tiopental) was from Biochemie GMBH, Germany; MK 571 (3-(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)

((3-dimethylamino – 3-oxopropyl)thio)methyl)thio propanoic acid) was purchased from Biomol Research Lab. Inc., USA; 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 CAM, ([+] – 5-[2-imidazole – 1-ethyloxy]—1-indan-carboxylic acid hydrochloride) was a gift from Ferrer inc. Spain. LPS, CAM, WEB 2170 and MK 571were dissolved in 1 ml of saline immediately before administration.

RESULTS

Baseline TV, PAP, PVP values of isolated rat lung preparation

Baseline TV and PVP were similar in KH, PPP, PRP or blood-perfused lungs and in all 54 lungs used; TV was 2.0 ± 0.0 ml, and PVP was 2.8 ± 0.1 cm H_2O. However, baseline PAP was higher in blood-perfused lungs (15.8 ± 0.5 cm H_2O) than in lungs perfused with KH, PPP or PRP (10.9 ± 0.4, 9.3 ± 0.3, 9.4 ± 0.6 cm H_2O, respectively).

Isolated lung preparations perfused either with KH, PPP, PRP or with blood maintained their integrity for at least 160 min. In control lungs only a slight gradual increase in weight, in PAP and in PVP (less than 0.2 g/h, less than 2 cm H_2O/h and 1.2 cm H_2O/h, respectively) as well as a slight decrease in TV (less than 0.2 ml/h) were observed over time.

Characteristics of immediate and delayed response to LPS in blood-perfused rat lung

In preliminary experiments blood-perfused lung was treated with LPS at final concentrations ranging from 10 to 300 μg/ml. (*Fig. 1 A-C*). A concentration of 300 μg/ml of LPS was chosen for further experiments since only at that high concentration LPS produced immediate and delayed responses of sufficient magnitude to be studied.

An original tracings of a biphasic response of isolated blood-perfused lung to 300 μg/ml LPS is shown in *Fig. 2*. Immediate LPS response was coupled in circulatory and respiratory systems whereas in delayed response the respiratory changes were uncoupled from circulatory effects of LPS.

Immediate response

Immediate LPS-induced changes in ΔTV, ΔPAP, and ΔPVP started simultaneously 1.5 ± 0.1 min after LPS and reached its maximum ($\Delta TV_{max} = -0.5 \pm 0.2$ ml, $\Delta PAP_{max} = 10.9 \pm 2.3$ cm H_2O and $\Delta PVP_{max} = 9.3 \pm 2.4$ cm H_2O) as soon as 5.7 ± 0.3 min after LPS (*Fig. 1, 2*).

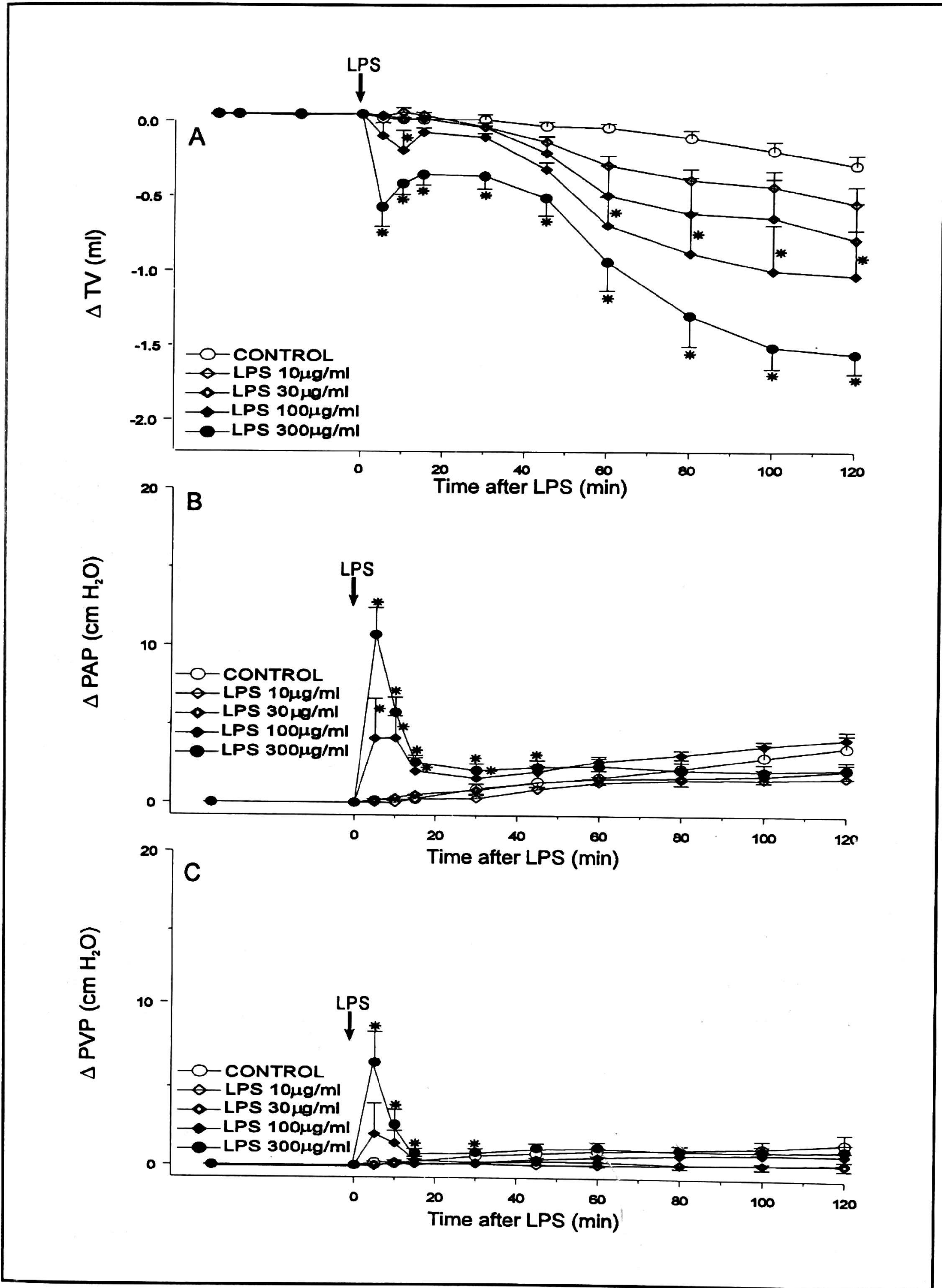

Fig. 1. Effects of LPS (at final concentrations ranging from 10 to 300 μg/ml) on tidal volume (ΔTV, *Fig. 1A*) and pulmonary pressures response (ΔPAP and ΔPVP *Fig. 1B, C*) in isolated blood-perfused rat lungs. Points represent means from n = 3÷7 experiments and vertical bars show S.E.M.*indicates $p<0.05$ vs blood-perfused lungs not injected with LPS

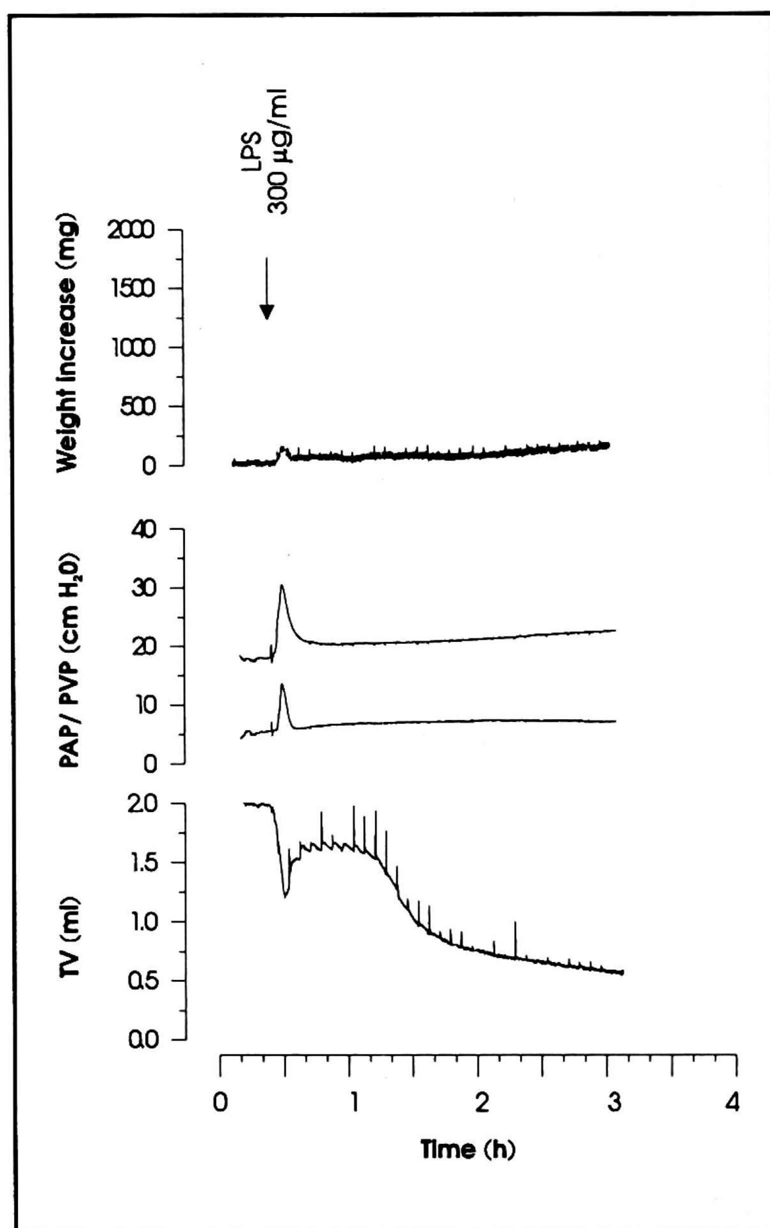

Fig. 2. Original tracing from an experiment showing a typical biphasic response to LPS (300 µg/ml) injected into the isolated blood — perfused rat lung. LPS induced an immediate transient response consisting of a decrease in tidal volume (ΔTV, *Fig. 2A*), increase of pulmonary arterial and venous pressures (ΔPAP, ΔPVP, *Fig. 2B, C*) followed by a delayed response consisting of a decrease in TV without accompanying changes in PAP, PVP and weight.

Secondary to LPS- induced hemodynamic response slight weight increase was noted ($\Delta weight_{max} = 157.6 \pm 32.8$ mg). Immediate response to LPS was transient and lasted 10—15 min (11.0 ± 1.2, 15.9 ± 1.4 and 15.0 ± 1.6 min for ΔTV, ΔPAP and ΔPVP responses, respectively) although 20 min after LPS slight ΔTV, ΔPAP and ΔPVP responses were still observed (-0.4 ± 0.1 ml, 3.3 ± 1.0 and 2.2 ± 1.1 cm H_2O, respectively).

Delayed response

Delayed response to LPS consisted of a gradual decline in TV without accompanying changes in TV, PAP PVP and weight. This response started at about 40 min after LPS and reached its plateau level at about 90—120 min after LPS injection. Delayed fall in TV was of approximately 2-fold greater magnitude then the immediate one (*Fig. 1, 2*).

Influence of composition of perfusing fluid on biphasic LPS response

The influence of perfusate composition on biphasic LPS response was studied using PRP, PPP or KH instead of blood for lung perfusion. When lungs were perfused with KH or PPP instead of blood, injection of LPS failed to induce an immediate ΔTV, ΔPAP ΔPVP and Δweight responses. On the contrary, when PRP was used as a perfusate, a mild immediate increase in PAP and negligible decrease in TV were noted (*Fig. 3A, B*), though there was no immediate response in PVP (*Fig. 3C*). Time course of ΔPAP response in PRP-perfused lung (*Fig. 3B*) was similar to that found in blood-perfused lungs but its maximum was substantially smaller (2.9 ± 0.4 versus 10.9 ± 2.3 cmH_2O in PRP and blood -perfused lungs, respectively). On the other hand, when KH, PPP or PRP were used for lung perfusion delayed airway constriction was observed, and its time course and magnitude was indistinguishable from that occurring in blood-perfused lung (*Fig. 3A*).

Pharmacological analysis of role of PAF, TXA_2 and cysLTs in immediate and delayed LPS responses

The role of platelet activating factor (PAF), thromboxane A_2 (TXA_2) and cysteinyl leukotrienes (cysLTs) in biphasic LPS response were assessed by using PAF receptor antagonist (WEB 2170), a TXA_2 synthase inhibitor (Camonagrel) and cysLTs receptor antagonist (MK 571), respectively. However, since the respiratory and vascular pulmonary responses to LPS were substantially different in blood-perfused and KH-perfused lung preparations, and pattern of LPS response in PPP- and PRP-perfused lungs was similar to that of KH-perfused preparations, (*Fig. 3*) our pharmacological analysis of LPS response was confined to a comparison of blood-perfused versus KH-perfused rat lungs.

Administration of Camonagrel (300 μM), WEB 2170 (100 μM), or MK 571 (100 μM) into blood-or KH-perfused lung was without any influence on the baseline respiratory or circulatory parameters of the isolated lung (*Fig. 4A – C, Fig. 5*).

In the blood-perfused lung immediate ΔTV, ΔPAP and ΔPVP responses to LPS were abolished by pretreatment with Camonagrel, substantially inhibited by WEB 2170, and not affected by MK 571 (*Fig. 4 A* – C). Differently, delayed LPS-induced airway constriction was abolished by Camonagrel, whereas neither WEB 2170 nor MK 571 were effective (*Fig. 4 A* – C).

Similarly to the blood-perfused lung preparation, in KH-perfused lung, LPS-induced delayed airway constriction was abolished by Camonagrel but not influenced by WEB 2170 or by MK 571 (*Fig. 5*).

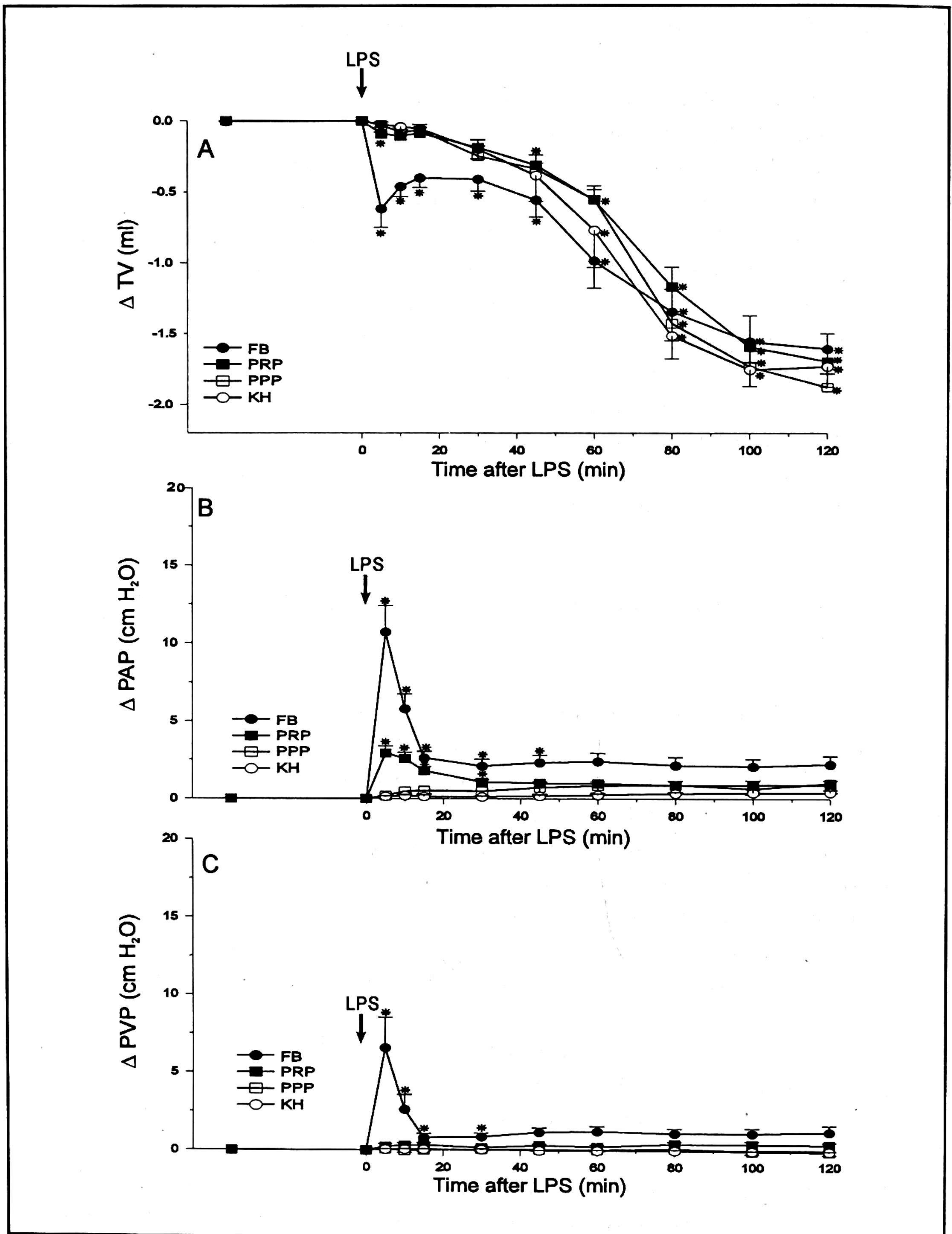

Fig.3. Influence of perfusate composition on immediate ΔTV, ΔPAP, ΔPVP and delayed ΔTV responses to LPS in isolated rat lung. LPS (300 μg/ml) was injected into isolated rat lung perfused with full blood (FB), platelet rich plasma (PRP), platelet poor plasma (PPP) or Krebs Henselait solution (KH). Characteristic biphasic response to LPS was only seen in full blood-perfused lungs whereas the delayed response was observed irrespective of the perfusing fluid. Points represent means from n = 4÷7 experiments and vertical bars show S.E.M.*indicates $p<0.05$ vs lungs not injected with LPS perfused with the same perfusing fluid i.e. full blood, PRP, PPP or KH.

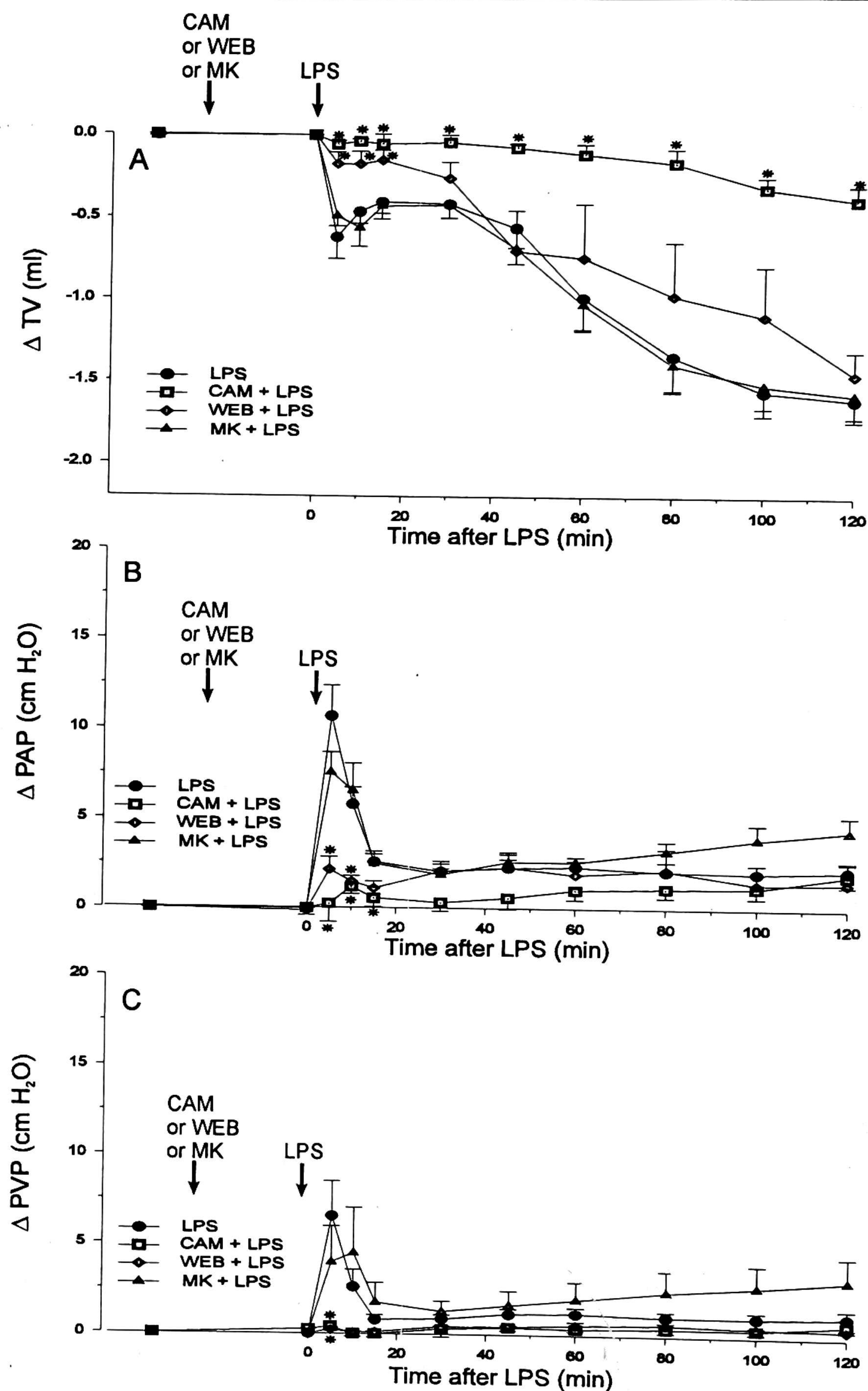

Fig. 4. Involvement of TXA_2 and PAF in immediate ΔTV, (*Fig. 4A*), ΔPAP, ΔPVP, (*Fig. 4B-C*) and in delayed ΔTV responses to LPS (300 μg/ml) in the isolated blood-perfused rat lung. Both camonagrel (300 μM), a thromboxane A_2 synthase inhibitor (CAM+LPS); and WEB 2170 (100 μM), a PAF receptor antagonist (WEB+LPS) inhibited an immediate response whereas delayed response was inhibited only by camonagrel. MK 571 (100 μM), an cysteinyl leukotriene receptor antagonist did not influence either immediate nor delayed response. Points represent means from n = 4÷7 experiments and vertical bars represent S.E.M.*indicates $p<0.05$ vs lungs injected with LPS (300 μg/ml) alone.

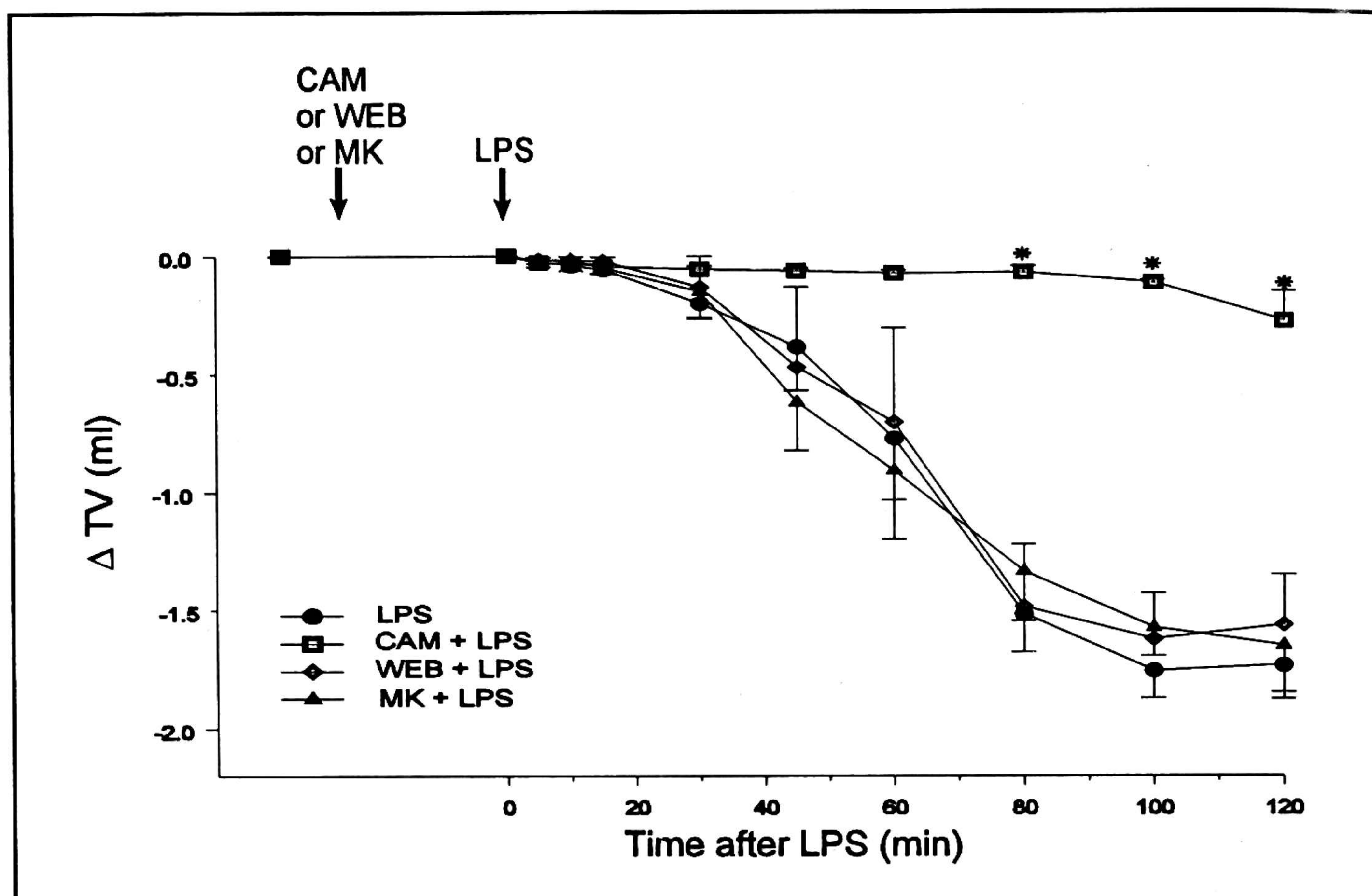

Fig. 5. Involvement of TXA_2, in delayed bronchoconstriction induced by LPS (300 μg/ml) in the isolated KH-perfused rat lung. Camonagrel (300 μM) (CAM+LPS); but not WEB 2170 (100 μM), (WEB+LPS) and not MK 571 (100 μM), (MK+LPS) abolished delayed LPS-induced bronchoconstriction. Points represent means from $n = 4 \div 7$ experiments and vertical bars represent S.E.M.*indicates $p<0.05$ vs lungs treated with LPS (300 μg/ml) alone.

DISCUSSION

Whole blood-perfused and ventilated lung mimics closely *in vivo* conditions and allows for simultaneous assessment of circulatory and respiratory functions of the lung, in an easier fashion than *in vivo* (11). Indeed, in most studies *in vivo* rather the effects of LPS on vascular tone but not on the lung mechanics were measured (1, 5). Here, we characterized for first time both circulatory and respiratory facets of rat lung response to LPS.

Wistar rat is known to be much less sensitive to LPS than other species (12, 13). We had to use LPS at a concentration of 300 μg/ml in blood-perfused rat lung to mimic the response of 10 mg/kg of LPS from our previous studies *in vivo* (5, 14). However, that anticoagulation of blood with heparin may also contribute to the low responsiveness of isolated blood-perfused lung to LPS, since heparin is known to bind LPS (15—17).

In the present work we show that in blood-perfused rat lung the immediate, transient response to LPS consists of coupled circulatory and respiratory

reactions, i.e. an increase in pulmonary perfusion pressures and decrease in respiratory tidal volume. On the contrary, the delayed response to LPS consists of a sustained decrease in respiratory function that is uncoupled from circulatory changes which simply do not occur.

Both respiratory and circulatory immediate responses were inhibited by a PAF receptor antagonist, by a TXA_2 synthase inhibitor, but not by a cysteinyl leukotriene receptor antagonist, whereas the delayed respiratory response was inhibited only by a TXA_2 synthase inhibitor. Immediate responses to LPS were expressed only in blood-perfused lung and rudimentarily in PRP-perfused lung, whereas a delayed response was of the same magnitude and time course irrespective whether full blood, PRP, PPP or KH solution were used for the lung perfusion.

Accordingly, PAF and TXA_2 released from blood cells seem to participate in immediate vascular and airway responses to LPS, whereas the delayed airway response is likely to depend on the TXA_2 synthesis that is completed within lung tissue.

PAF was originally reported to be released from basophils in response to IgE stimulation (18) and, later, it was found to be released by numerous types of activated cells including platelets, neutrophils, macrophages (19) and epithelial cells (20). Platelets and neutrophils were shown to be directly activated by LPS within minutes after LPS stimulation (21, 22) so PAF released from these cells could contribute to the immediate LPS-induced broncho- and vaso- constriction. LPS may also activate endothelial cells (19), however, PAF is then not released but it remains associated with plasma membrane, and in a juxtacrine fashion along with selectin P, is involved in neutrophil recruitment (23). Accordingly, blood cells seem to be more feasible candidates as a source of TXA_2 and PAF in the immediate LPS response, then vascular endothelium.

Exogenous PAF was shown to release TXA_2. Indeed, exogenous PAF-induced bronchoconstriction and vasoconstriction were largely prevented by COX inhibitors (24, 25) and some authors proposed that TXA_2 as a final mediator of PAF induced response (26, 27), however, both PAF (28, 29) and LPS (21) are known to activate platelets. In our blood-perfused lung TXA_2 released from platelets being activated either by PAF or directly by LPS seem to act as a final broncho- and vaso- constrictor in the immediate LPS response.

We show here that LPS injected into PRP-perfused lung elicits a response substantially smaller than that in blood-perfused lung. It seems therefore that platelet activation alone could not generate enough PAF and TXA_2 to reproduce the immediate airway and vascular response to LPS observed in full blood. On the other hand, removal of either platelets (30) or leukocytes (31)

from blood was shown to diminish the immediate response to LPS *in vivo* in other species. This may suggest that participation of both types of cells is required for the immediate LPS effects in the lung.

Activated isolated neutrophils or platelets release PAF only in modest amounts (32). On the contrary, coincubated platelets and neutrophils generate PAF abundantly (33). Neutrophils cannot synthetize TXA_2 on their own since these cells lack TXA_2 synthase (34), but owing to transcellular arachidonic acid transfer, coincubation of activated neutrophils and platelets augments production of TXA_2 by platelets (35). Our recent data (unpublished) showing that LPS is a potent inducer of platelet-neutrophil adhesion *in vitro* may speak for a possible involvement of platelet-neutrophil interactions in the immediate LPS response.

In contrast with the immediate phase of LPS response, delayed airway constriction did not require blood cells and could be evoked in KH-perfused lung as found earlier (6). We also showed that platelets would not contribute to this response as evidenced by the similar response in KH and PRP-perfused lung. The response was still of approximately the same magnitude when plasma alone was used to perfuse the lung instead of KH. These data suggest that TXA_2 responsible for delayed airway constriction is released from lung parenchyma directly by LPS without participation of blood-cell derived lipids, LPS binding protein or soluble CD14 (36—38).

Among over forty types of cells present in the lung only a few of them are capable to produce TXA_2 *in vitro* e.g fibroblasts, mononuclear cells (34) or endothelial cells (39). Immunostaining of TXA_2 synthase in the lung points out to alveolar and bronchial macrophages as the cells most likely to produce TXA_2 in the lung tissue (34). Moreover, alveolar macrophages were shown to produce TXA_2 upon LPS stimulation *in vitro* (40). Delayed airway constriction which was recently shown to depend on COX-2 (6) was in our hands abolished by camonagrel, a thromboxane synthase inhibitor. These data taken together suggest that TXA_2 responsible for the LPS-induced delayed airway constriction is produced most likely in lung macrophages by consitutive TXA_2 synthase fed with cyclic prostaglandin endoperoxides made by LPS-induced COX-2 isoform of the enzyme.

In summary, analysis of circulatory and respiratory responses to LPS in isolated rat lung perfused either with blood or plasma or platelets rich plasma or Krebs buffer leads us to the conclusion that the immediate, coupled respiratory/circulatory response to LPS occurs only in blood-perfused lung and it is mediated by blood cells-derived TXA_2 and PAF. On the other hand the delayed uncoupled respiratory response to LPS is mediated

also by TXA_2, although the generation of this mediator is confined to the intrapulmonary sources.

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