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INCREASED PNEUMOTOXICITY OF LIPOPOLYSACCHARIDE FROM *E.COLI* IN NITRIC OXIDE DEFICIENT BLOOD-PERFUSED RAT LUNGS

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Isolated lungs of Wistar rats were ventilated by air that was enriched with 5% CO_2 and perfused with homologous blood in a closed circuit (9.5 ± 1 ml/min) using Hugo Sachs apparatus type 829. Lipopolysaccharide from *E.coli* (LPS, serotype 0127:B8) at a selected sub-toxic concentration of 300 μg/ml added to recirculating blood produced a biphasic response. Instant transient increase in pulmonary arterial and venous perfusion pressures, and a decrease in air tidal volume, and fifty minutes later slowly progressing decrease in air tidal volume without changes in pulmonary haemodynamics, were observed.

Inhibition of pulmonary nitric oxide synthase by instillation of N^G-nitro-L-arginine methyl ester (L-NAME) at a final concentration of 300μM to recirculating blood dramatically changed the responses to LPS. In nitric oxide deficient lungs LPS caused prompt increase in arterial and venous pressures and a fall in air tidal volume with accompanying rise in airway resistance. Within 6.3 ± 0.5 min a fulminant pulmonary oedema developed and all functions of the lung stopped abruptly.

We conclude that pulmonary nitric oxide plays a defensive role in protecting rat lungs against LPS-induced injury.

Key words: *isolated perfused lung; lipopolysaccharide; nitric oxide; N-nitro-L-arginine methyl ester*

INTRODUCTION

Sepsis due to gram-negative bacteria is a serious clinical condition associated with systemic hypotension and pulmonary hypertension, respiratory failure and multiorgan failure (1, 2) which may be fatal.

Intravenous administration of LPS from gram-negative bacteria to laboratory animals is used to produce experimental models of endotoxic shock.

During endotoxaemia, LPS directly or through released cytokines, such as tumour necrosis factor (TNF), interleukin-1 (IL-1), and interferon- (INF), induces nitric oxide (NO) synthase type II (NOS-II or iNOS) (3—5). NO and

peroxynitrite contribute to sepsis-induced systemic hypotension (6) and vasoplegia (7) which develops during the second phase of endotoxaemia (8).

Non-selective NOS-inhibitors such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) administered to animals with endotoxaemia may improve haemodynamic parameters (9) and prolong their survival time (10). However, in endotoxic shock NOS-inhibitors produce also detrimental effects such as an increase in pulmonary arterial pressure (11), drop in cardiac output (12), decreased organ perfusion (13), diminished oxygen delivery to tissues (14) and damage to visceral organs (15—17). Also in septic patients L-NMMA increases pulmonary and peripheral vascular resistance, reduces cardiac output and oxygen delivery to the tissues (9). The therapeutical role of non selective NOS-inhibitors in septic shock still remains to be clarified.

In our previous studies we demonstrated that pharmacological non-selective inhibition of NOS in rats enhanced their susceptibility to toxic action of LPS. In particular, in NO-deficient rats administration of LPS resulted in sudden death due to fulminant injury of the lung (18).

Here, we confirm the protective role of NO during endotoxin-induced lungs injury in the isolated blood-perfused lung.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS, *Escherichia coli* serotype o127:B8) and N^G-nitro-L-arginine methyl ester (L-NAME), HEPES were purchased from Sigma; bovine albumin (fraction V) from Serva; nadroparine (Fraxiparine) from Sanofi; thiopentone (Thiopental) from Biochemie GMBH

Animals

Lungs were isolated from female Wistar rats weighing from 200 to 230 g. Male Wistar rats of body weights from 300 to 400g were used as blood donors. Rats were kept at $22 \pm 1°C$, with a 12/12 hour-light/dark cycle, and were allowed water and standard rat chow *ad libitum*.

Perfusate

To obtain 45—50 ml of blood 3 donor rats were anaesthetised with thiopentone (Tiopental 120 mg/kg i.p.) and injected i.p. with nadroparine at a dose of 1000 I.U. The right carotid artery was cannulated and rats were exsanguinated.

For the initial perfusion of the lung during surgery the Krebs-Henseleit buffer of the following composition was used (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 12.5, with the addition of 4% albumin, 0.1% glucose and 0.3% HEPES.

Isolated lung preparation

The rat lungs were ventilated and perfused using the isolated lung apparatus type 829 from Hugo Sachs Elektronik (HSE).

Lungs were perfused through the pulmonary artery at constant flow of 9.5±1ml/min by a peristaltic pump (ISMATEC, HSE). Both arterial and venous pulmonary pressures were continuously monitored by ISOTEC pressure transducers (HSE).

For the ventilation of the lung Ventilation Control Module (VCM) module was used with optional either positive or negative pressure ventilation (HSE). The special valve allowed changing between the two types of ventilation. Positive pressure ventilation was used during surgery, negative pressure ventilation was used throughout the experiment. The inspired air was moistured by bubbling through water.

Air pressure within artificial thorax chamber during negative pressure ventilation was measured with a differential pressure transducer and adjusted to obtain required end-expiratory, end-inspiratory and deep breath end-inspiratory pressures (see below). Air flow velocity was measured with a pneumotachometer tube connected to a differential pressure transducer from which value of respiratory tidal volume was determined. The values of resistance, and compliance were calculated by Pulmodyn-pulmo software from the signals of air flow, tidal volume and chamber pressure according to the appropriate formulas (19).

The weight of lungs was continuously monitored by a transducer which allowed measurement of lung weight change during negative pressure ventilation (20).

The pH of perfusate was monitored and maintained at 7.35 by continuous addition of 5% CO_2 to the inspiratory air.

All data from the experiment were acquired by the PC transducer card and subsequently analysed by Pulmodyn-pulmo software (HSE).

Tidal volume, chamber pressure, pulmonary artery and venous pressures, as well as weight gain were continuously recorded on Graphtec linear recorder wr 3310.

Surgery

Rats anaesthetised with thiopentone (Thiopental 120 mg/kg, i.p.) were intubated intratracheally and ventilated with the positive pressure ventilation (80 breaths/min) using the VCM module of isolated lungs set-up (HSE). Rats were laparatomized, diaphragm removed and nabuparine at a dose of 750 I.U. was injected into the right ventricle to prevent microthrombi formation during surgery. Then animals were exsanguinated by the incision of left renal artery. The lungs were exposed via a medial sternotomy. The pulmonary artery was cannulated, and, at the same time, the ascending aorta was tied up. As soon as the second cannula was placed and secured in the left atrium the lungs started to be perfused through the pulmonary artery with warm (38°C) Kreb's buffer at a rate of 1—2 ml/min. Then the lung/heart block was dissected carefully — without touching of the lung — from the thorax and suspended by trachea in the specially designed water-jacked, and air-tight glass chamber, temperature of which was maintained at 38°C. After closing of the artificial thorax chamber the perfusion rate was gradually increased up to 6—10 ml/min to washout the blood, and negative pressure ventilation, was initiated with 80 breaths/minute and a breathing cycle 50%/50% of inspiratory/expiratory duration in each breath. The end-expiratory pressure in the chamber was set to -2 cm H_2O and inspiratory pressure was adjusted between -6 to -10 cm H_2O to give the initial tidal volume of about 2.0 ml. Every 5 min a deep breath of end-inspiratory pressure of -21 cm H_2O was automatically initiated by VCM module (HSE) to avoid atelectasis. 10 min after the lung was mounted in the set-up perfusion with 40-50 ml of blood obtained from donor rats, was initiated. The rate of perfusion was 9.5±1 ml/min, which resulted in the initial pulmonary arterial pressure of 18.5±2 cm H_2O. The venous pressure was set to 3-5 cm H_2O.

Experimental protocols

After being placed in the artificial thorax chamber, the lungs were allowed to equilibrate for 30 min until baselines of all measured parameters were stabilized. Then one of following protocols was followed.

(A) *Control group* ($n = 3$)

Isolated lungs were perfused with blood for 180 minutes without any interventions.

(B) *LPS treated group* ($n = 5$)

LPS dissolved in 1 ml of saline was administered as a bolus injection to the tubing leading to the pulmonary artery, in an amount that was necessary to achieve a final concentration of 300 μg/ml of LPS in recirculated blood.

(C) *L-NAME pretreated, LPS treated group* ($n = 5$)

L-NAME dissolved in 1 ml of saline was added to the blood reservoir in an amount required to reach a final concentration of 300 μM. Forty minutes later LPS was administered like in the group (B).

Statistics

The results were presented as arithmetic means ± standard errors (S.E.) for n determinations. Differences between means were evaluated by the unpaired Student's t-test inside the group and by paired Student's t-test between the group; $p > 0.05$ was considered as statistically significant.

RESULTS

In blood perfused lungs LPS at a sub-toxic concentration of 300 μg/ml of blood resulted in a transient (lasting no longer than 10—15 min) reduction of air tidal volume from 1.9±0.0 ml to 1.2±0.2 ml (*Fig. 1A*), while pulmonary resistance increased from 0.51±0.12 cm H_2O/ml/sec to 1.14±0.34 cm H_2O/ml/sec (*Fig.1B*), and pulmonary compliance decreased from 0.24±0.04 ml/cm H_2O to 0.1±0.01 ml/cm H_2O. On the haemodynamic site a transient rise in pulmonary arterial and venous perfusion pressures by 14.2±3.0 cm H_2O and by 12.8±7.8 cm H_2O, respectively were recorded (*Fig.1C, Fig.1D*). A transient oedema augmented the weight of LPS treated lungs by more than 150 mg.

The second phase of pneumotoxic action of LPS appeared fifty minutes later as a slowly creeping decrease in tidal volume. Hundred minutes later tidal volume was already 0.9±0.2 ml compared with 1.3±0.1 ml in control (A) group (*Fig.1A*). During second phase of LPS action pulmonary resistance increased from 0.51±0.12 cm H_2O/ml/sec to 1.2±0.42 cmH_2O/ml/sec (*Fig. 1B*) and pulmonary compliance decreased from 0.24±0.04 ml/cm H_2O to 0.10±0.02 ml/cmH_2O. However there was no accompanying changes in pulmonary arterial and venous pressures, and in weight gain as in the early phase of LPS action.

L-NAME given alone at a concentration of 300μM caused a small increase in pulmonary arterial and venous pressures (by 1.5±0.3 cm H_2O and 1.2±0.2 cm

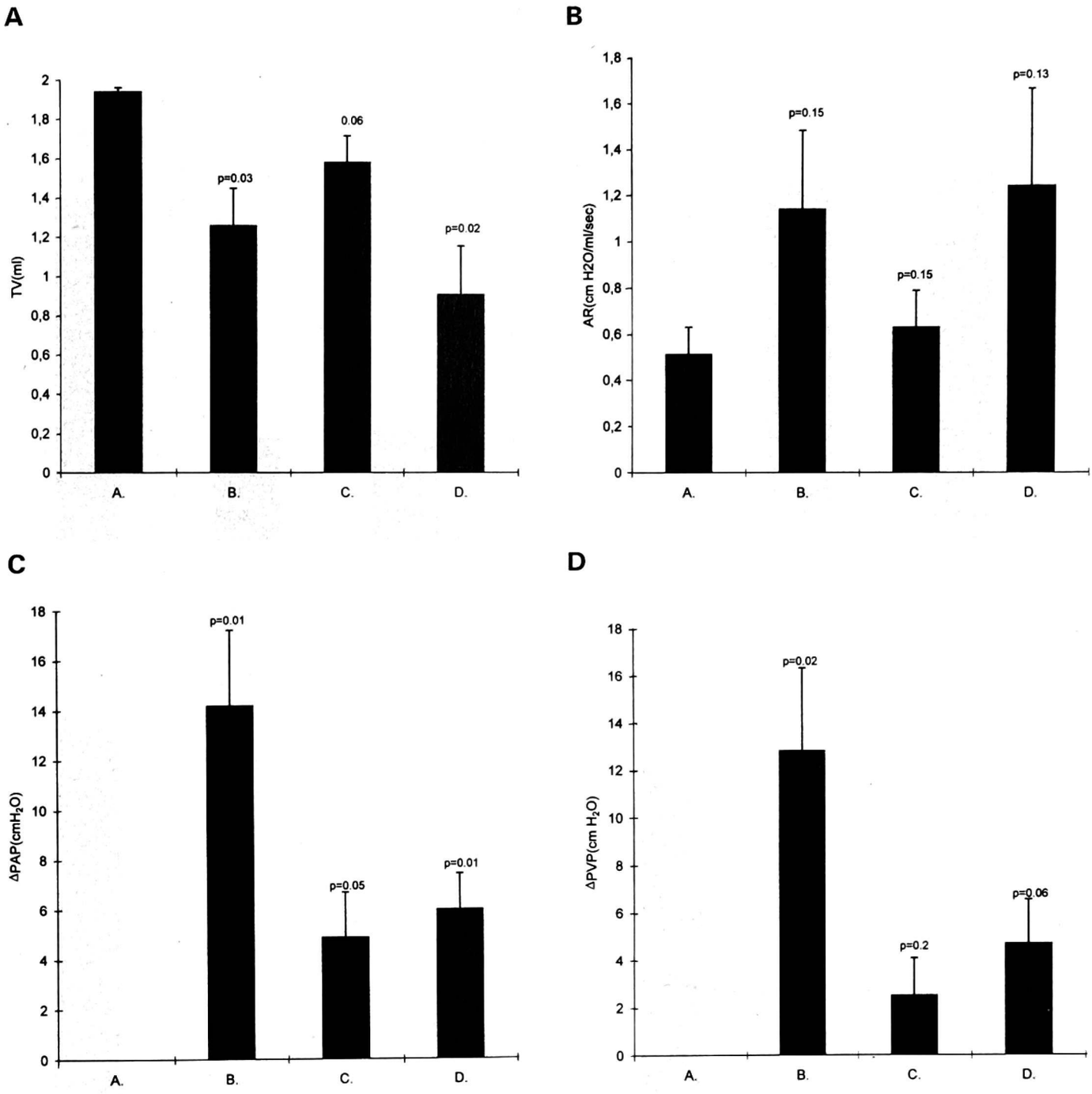

Fig. 1. Biphasic pattern of LPS (300 μg/ml) action on tial volume — TV (*Fig. 1A*), airway resistance — AR (*Fig. 1B*) and pulmonary arterial — PAP and venous — PVP pressures (*Fig. 1C* and *1D*, respectively). A/ Control, prior to LPS administration, B/ Peak of early phase of LPS action (10—15 min after LPS administration), C/ Partial recovery (40-50 min after LPS administration), D/ Late phase of LPS action (150 min after LPS administration). Columns represent arithmetic means from $n = 5$ experiments, and vertical bars represent S.E. of means.

H_2O, respectively) without an influence on the respiratory functions. In L-NAME-pre-treated lungs injection of LPS at a sub-toxic concentration of 300μg/ml induced a rapid arrest of all functions of the lungs within 6.3 ± 0.5 min, the pattern of which consisted of instant fall in tidal volume to zero (*Fig. 2A*), with accompanying increase in pulmonary resistance from 0.29 ± 0.01 cm H_2O/ml/sec to 0.90 ± 0.14 cm H_2O/ml/sec (*Fig. 2B*) and decrease in pulmonary compliance from 0.31 ± 0.02 cm H_2O/ml/sec to 0.11 ± 0.01 ml/cm

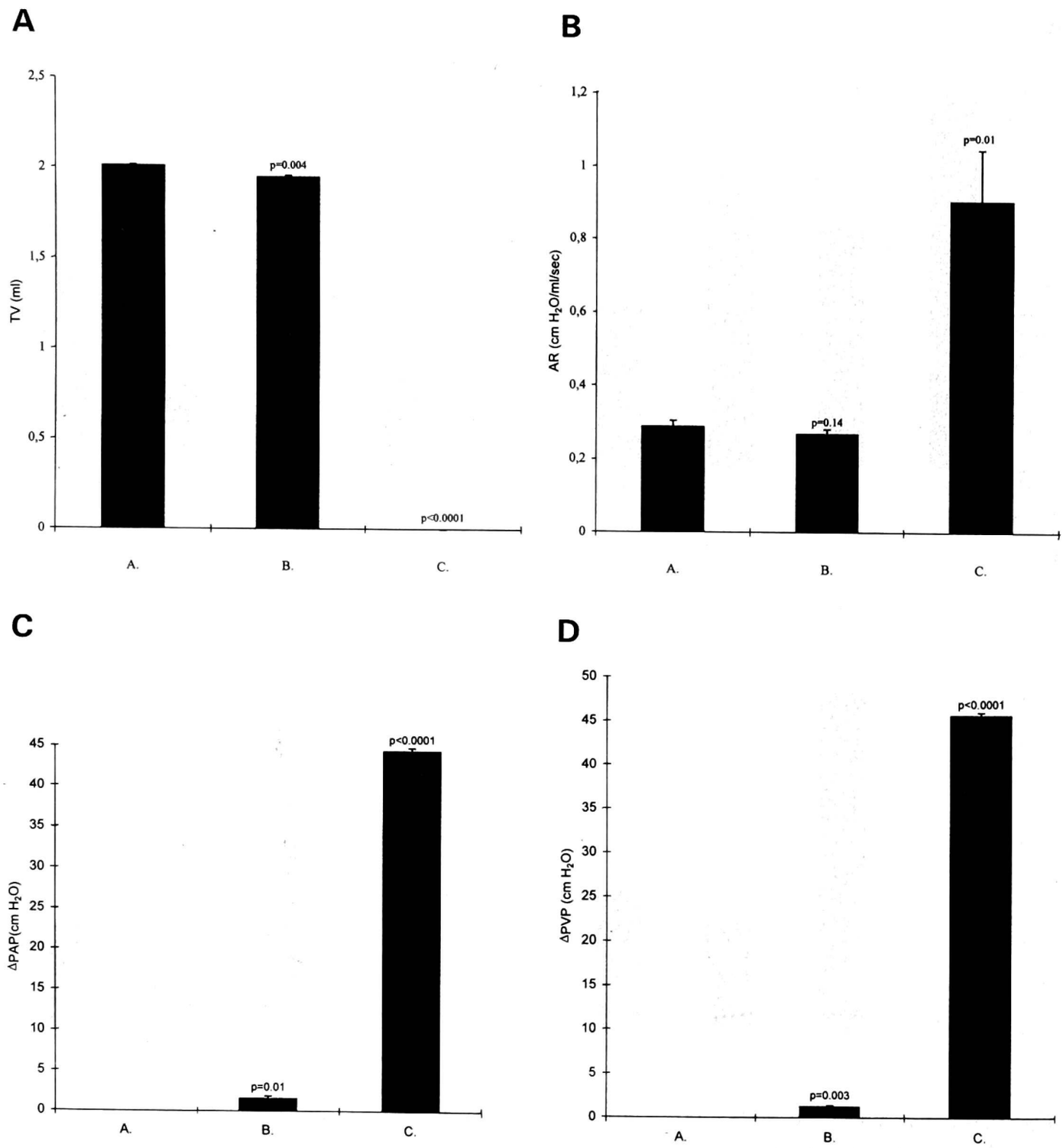

Fig. 2. Effect of LPS (300 μg/ml) in L-NAME (300 μM) pre-treated lungs on tidal volume — TV (*Fig. 2A*), airway resistance — AR (*Fig. 2B*) and pulmonary arterial — PAP and venous — PVP pressures (*Fig. 2C* and *2D*, respectively). A/ Control, prior to L-NAME administration, B/ 40 min after L-NAME pretreatment, C/ 5-8 min after LPS administration in the L-NAME pre-treated lung. Columns represent arithmetic means from $n = 5$ experiments, and vertical bars represent S.E. of means.

H_2O. In the same time prompt increase of pulmonary arterial and venous perfusion pressures by as much as 44.26 ± 0.4 cm H_2O and 45.44 ± 0.4 cm H_2O, respectively, was observed (*Fig. 2C, Fig. 2D*). This was accompanied by sudden development of the lung oedema (increase in the lung weight by more than 3000 mg).

DISCUSSION

Nitric oxide is an important mediator of endotoxaemia. Lipopolysaccharide (LPS) from gram-negative bacteria is used to study pathophysiology of endotoxic shock in experimental animals. LPS evokes endotoxic shock with clear-cut two phases. The first phase is characterised by an instant but transient fall of arterial blood pressure, while the second one develops during following hours and appears as aggravating arterial hypotension, vasoplegia, insufficient tissue perfusion, lactic acidosis and multiorgan failure leading to death. Previously we showed (18) that the first phase seemed to be associated with the LPS-induced local release of pneumotoxic lipids (e.g. PAF, TXA_2). The second phase is known to be associated with induction of NOS II and overproduction of toxic amounts of NO and peroxynitrite (6, 21—23).

The lung seems to be a prime target organ for toxic action of LPS. The main clinical complication of shock is the acute lung injury, defined as ARDS which is characterised by pulmonary artery hypertension, obturation of pulmonary blood vessels, increased shunt fraction, formation of atelectasis, arterial hypoxaemia, and a drop in cardiac output (24).

In our model of blood-perfused isolated rat lungs we studied the pneumotoxicity of LPS. Rat lungs are highly resistant to toxic action of LPS. Given alone at a concentration as high as 300 μg/ml resulted only in minute changes in circulatory and respiratory functions of lungs. To study the regulatory role of endogenous NO a non-selective NOS inhibitor L-NAME was used. In the NO-deficient lungs LPS at the same sub-toxic concentration (300 μg/ml) produced a sudden arrest of all functions of lungs, their instant oedema and death, within 6.3 ± 0.5 min.

We showed previously that in rats *in vivo* pharmacological inhibition of NOS prior to administration of LPS caused a dramatic increase in mortality of animals. Their survival time was shortened 15-fold as compared to rats which were treated with LPS only (18). Our present data from isolated blood perfused preparation of the lung from the rat seem to support our original assumption (18), that NO generated within lungs keeps at bay dangerous and potentially pneumotoxic mediators released by LPS and saves the lung against direct or more likely indirect lethal effects of LPS. The mechanisms of interaction between pneumotoxic mediators released by LPS (TXA_2, PAF, LTs) and pneumoprotective NO taking place in the lung as well as the mechanisms by which NO exerts its protective role remains to be elucidated in future studies.

There is extensive evidence that large amounts of NO made by LPS-induced NOS II may contribute to mortality in the late phase of endotoxic shock. The novel finding of previous (18) and present studies of ours is that NO released by LPS instantly after its administration — which most

likely depends on the activation of NOS III (18) — has a potent pneumoprotective action.

If any practical suggestions may come out of our studies, they are as follows. Firstly, NOS II selective inhibitors (25—28) have a better chance in therapy of endotoxic shock than non-selective NOS inhibitors. Secondly, attempts to apply inhalations of gaseous NO (29—31) or NO-donors (e.g. S-nitroso-N-acetyl-penicyllamine) in septic shock, gain from our study an additional support.

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