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Induced Nitric Oxide Impairs Relaxation but Not Contraction in Endotoxin-Exposed Rat Pulmonary Arteries

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Background. **Many patients with severe acute lung injury do not respond to nitric oxide (NO) inhalational therapy with alleviation of pulmonary arterial hypertension and hypoxemia, so this treatment remains controversial.**

Materials and methods. **We investigated in endotoxin-exposed Wistar rat pulmonary arteries whether endogenous NO alters contractile and relaxing responses, by electrochemical NO and isometric force measurements.**

Results. **Receptor-independent contraction was similar in control and endotoxin-exposed arteries, while thromboxane analogue (TxA)-dependent contraction was less in the latter. Neither non-selective NO synthase (NOS) inhibition by N^G-nitro-L-arginine (L-NA) or selective inducible-NOS2 inhibition by aminoguanidine (AG) improved TxA-induced contraction in endotoxin-exposed arteries. Acetylcholine-induced relaxation was impaired in endotoxin-exposed pulmonary arteries, despite a comparable acetylcholine-induced NO release in control arteries. Additionally, NO solution-induced relaxation of endotoxin-exposed arteries was impaired, but could be improved by L-NA or AG. Application of a phosphodiesterase-insensitive cyclic guanosine monophosphate analogue induced similar relaxation in both control and endotoxin-exposed arteries.**

Conclusions. **Endotoxin-associated NOS2-derived NO is thus associated with impaired NO-mediated relaxation, but does not underlie reduced receptor-mediated pulmonary contractile responses. An increased phosphodiesterase activity may underlie the former, so this route can be explored to replace or improve the effect of inhalational NO therapy in severe sepsis-induced acute lung injury in patients.**

Key Words: **sepsis; pulmonary artery; lung injury; nitric oxide; inducible nitric oxide synthase; cGMP; pulmonary hypertension.**

INTRODUCTION

Sepsis is the most frequent cause of severe acute lung injury, characterized by pulmonary arterial hypertension, permeability edema, atelectasis, and mismatching of regional perfusion to ventilation, resulting in hypoxemia. In many patients, nitric oxide (NO) inhalation, which has been advocated as an important adjunctive therapy during mechanical ventilatory support, does, however, not attenuate pulmonary hypertension and venous admixture, thereby rendering the therapy still controversial [1]. Endotoxin also induces NO inhalation hyporesponsiveness in rodent lungs [2–5].

A diminished vasoreactivity is likely to partially underlie NO inhalation hypo- or unresponsiveness, for instance associated with endogenously released vasoconstrictors such as endothelin and thromboxane [5–9] or vasodilator substances such as NO, the latter in part through activation of inducible NO synthase (NOS2) and smooth muscle cell (SMC) cyclic guanosine monophosphate (cGMP) [4, 10–15]. First, increased pulmonary arterial (SMC) NO production may compensate for predominating constrictor influences on the pulmonary arteries [5–9,13], as evoked by endothelial defects

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in NOS3-NO routes and other factors [10, 12, 14, 16–18]. However, the endotoxin-induced, NOS2-derived NO release may result in a fall in reactivity of pulmonary arteries to vasoconstrictors, depending on the animal species studied, the applied vasoconstrictor and endotoxin exposure duration [2, 3, 12–16, 19, 20]. Second, increased endogenous NO availability might also decrease vascular sensitivity and thus impair vasodilator responses to exogenous NO [2–5, 10, 12, 17, 18]. The question thus is whether alterations in both pulmonary arterial contractile and relaxation responses share a common pathogenic pathway, i.e., increased availability of NOS2-derived NO and thereby diminished sensitivity to exogenous NO.

Therefore, we investigated the role of endogenous and exogenous NO and cGMP in both contraction and relaxation of endotoxin-exposed isolated rat pulmonary arteries.

MATERIALS AND METHODS

Animals and Tissue Preparation

The Animal Care and Use Committee of the VU University Medical Center approved the experimental procedures. Wistar rats (males, 316.2 grams, $n = 54$; Harlan Zeist, The Netherlands) were anesthetized with sodium pentobarbital (Nembutal, Sanofi Sante BV Maassluis, The Netherlands; 60 mg/kg^{-1} i.p.) and lungs were removed. Lungs were kept in MOPS buffer (4°C, pH 7.4, equilibrated in room air) that consisted of (in mM): 145 NaCl, 4.7 KCl, 3 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 11.2 glucose, 2 pyruvate (Sigma-Aldrich, Zwijndrecht, The Netherlands), 0.02 EDTA, and 3 3-(N-morpholino) propanesulfonic acid (all components, except for pyruvate, were obtained from Merck, Darmstadt, Germany). Under sterile conditions, side branches of the main pulmonary artery were dissected, divided into two segments (length 1-2 mm) and used pair-wise [7].

Tissue Culture

For every experiment, two arterial segments from one animal were incubated in either control medium or medium containing 50 μg/ml^{-1} of *Escherichia coli* lipopolysaccharide (serotype 127:B8, Difco Laboratories, Detroit, MI) [7]. Briefly, culture plates were filled with 3 ml sterile Dulbecco's modified Eagle's medium containing 4 mM L-glutamine, 0.1 mM L-arginine, and 22.7 mM glucose and supplemented with 4% heat-inactivated fetal calf serum, 100 IU/ml^{-1} penicillin and 100 μg/ml^{-1} streptomycin (all Life Technologies, Inc., Breda, The Netherlands) for 20 h at 37°C (95% humidified air/5% CO_2) [7].

NO Measurements

Electrochemical NO measurements were performed on the endothelial layer as described earlier [21]. Briefly, an NO microsensor was produced by threading a single carbon fiber through the pulled end of a glass capillary according to the method previously described. The carbon fiber was coated with a polymeric layer of nickel (II)tetrakis(3-methoxy-4-hydroxyphenyl) porphyrin (Interchim Monluçon, France) and two 1.25% Nafion layers (Sigma-Aldrich Zwijndrecht, The Netherlands). The EMS-100 (Biological Claix, France) was used for amperometry at a potential difference of 680 mV. The porphyrinic electrode was free of interference from all reagents used. The sensitivity of each electrode was determined, in the absence of tissue, using aliquots of saturated NO solution as previously described [21]. The slope of the linear relation between the measured output current (pA) and the NO concentration (nM) determined the electrode sensitivity (1 nM/pA^{-1}).

Isometric Force Measurements

Isometric force measurements were performed in a double wire myograph [7]. Each vessel segment was mounted on two tungsten wires, which were connected to a micromanipulator or force transducer (Kistler Morse no. 46−1003−01), and suspended pair-wise in a water-jacketed organ chamber containing MOPS buffer (37°C). After stabilization, artery segments were stretched stepwise to a length that induced a passive force equivalent with a transmural pressure of about 13 mmHg, calculated according to a simplified Laplace equation [22]. This passive force value was used as baseline for active force measurements.

Protocol

Control and endotoxin-exposed arteries were constricted using either 60 mM KCl ($n = 5$), the Ca^{2+}-ionophore A23187 (10^{-9}-$3*10^{-6}$, $n = 6$), or the thromboxane (TxA) analogue U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$, 10^{-9}-10^{-6} M, $n = 6$). TxA is a mediator of endotoxin-induced lung injury in rats [2–5, 8, 19, 23]. Subsequently, we investigated whether NOS inhibition altered the endotoxin-induced changes in the contractile response to TxA. NOS2 activity was specifically inhibited by 20 h of co-incubation of the arteries with aminoguanidine (AG; 10^{-4} M; $n = 6$) [7]. Acute application of the non-selective inhibitor N^G-nitro-L-arginine (L-NA; 10^{-4} M) during force measurements was used to study the effects of overall NOS activity (Bachem, Bubendorf, Germany).

To study the effects of endotoxin on endothelial NOS, acetylcholine-induced NO release and relaxation were studied in KCl-preconstricted control and endotoxin-exposed arteries (10^{-6}-10^{-5} M, Sigma-Aldrich, Zwijndrecht, The Netherlands; $n = 5$). Subsequently, the response to exogenous NO was studied by application of increasing concentrations of diluted NO solution (10^{-8}-310^{-6} M; $n = 6$) [2]. KCl-preconstricted segments were acutely exposed to L-NA during the experiment (10^{-4} M). The effects of endothelium removal ($n = 6$), L-NA (10^{-4} M; $n = 5$), or aminoguanidine (10^{-4} M; $n = 6$) on NO-mediated relaxation were studied in different groups. To unravel whether endotoxin affects the response of pulmonary arteries to cyclic guanosine monophosphate (cGMP), KCl-preconstricted vessel segments were dilated with the membrane-permeable, phosphodiesterase-insensitive cGMP analogue 8-para-chlorophenylthioguanosine-3′,5′-cyclic guanosine monophosphate (8-pCPT-cGMP, 10^{-5} M, Biolog Life Science Institute, Bremen, Germany; $n = 6$).

Statistics

All vasoconstrictor-induced arterial responses are expressed as force normalized to the axial segment length. Vasodilatory changes are expressed as the percentages of the pre-constriction response to 60 mM KCl. Data are given in means standard errors of the mean (SEM). Statistical analysis for concentration-response relations was performed using a repeated measurement analysis of variance (ANOVA). Other data were analyzed by paired or unpaired t-tests. Differences were considered statistically significant if $P < 0.05$. Statistical differences in one figure are indicated by *.

RESULTS

NO and Endotoxin-Mediated Pulmonary Contractile Dysfunction

Application of 60 mM KCl (Fig. 1A) or increasing concentrations of the Ca^{2+}-ionophore (Fig. 1B) induced

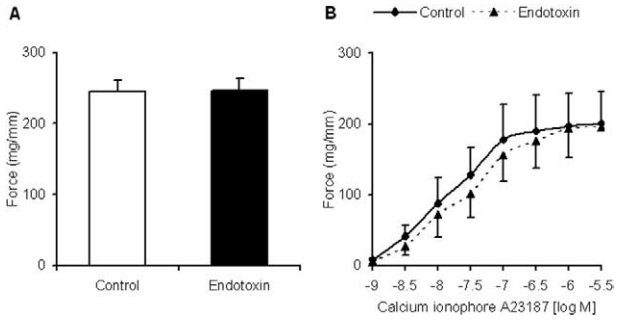

FIG. 1. Receptor-independent constriction by 60 mM KCl (panel A; $n = 5$) or a Ca^{2+}-ionophore (panel B; $n = 6$) similarly contracts control and endotoxin-exposed rat pulmonary arteries.

comparable contractions in control and endotoxin-exposed arteries. In contrast, endotoxin decreased the TxA-induced contraction (Fig. 2B) compared to control arteries (Fig. 2A; $P = 0.03$). In control arteries, L-NA induced contraction and enhanced the response to TxA (Fig. 2A), while L-NA did not contract endotoxin-exposed arteries (Fig. 2B). L-NA did also not improve the TxA-induced contraction of endotoxin-exposed arteries, and a difference between control and endotoxin-exposed arteries remained (Fig. 2B; $P < 0.05$). Moreover, co-incubation with the NOS2 inhibitor AG did not improve TxA-induced contraction of both control and endotoxin-exposed the arteries (Fig. 2A and 2B).

Acetylcholine-Induced NO Release and Relaxation

The effects of endotoxin on endothelial-dependent NO release and corresponding relaxation were studied by application of acetylcholine in KCl-preconstricted arteries (Fig. 3). Although acetylcholine-induced NO release was not different in control and endotoxin-exposed arteries (panel A), endotoxin-exposed arteries

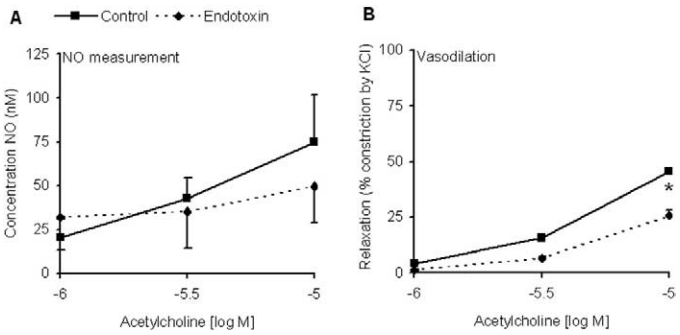

FIG. 3. Although acetylcholine-induced NO release is similar (panel A), the associated acetylcholine-induced relaxation response is higher in control than in endotoxin-exposed, KCl-preconstricted arteries (panel B; ANOVA $P = 0.001$; $n = 5$).

showed an impaired relaxation upon acetylcholine compared to controls (panel B; $P = 0.001$).

NOS2 Impairs NO-Induced Relaxation in Endotoxin-Exposed Pulmonary Arteries

The response to exogenous NO was studied by application of increasing concentrations of NO solution to both artery segments (Fig. 4A and 4B). Panel A shows that removal of the endothelium (-EC; $P = 0.02$ *versus* control) or co-incubation with L-NA ($P = 0.02$ *versus* control) or AG ($P = 0.008$ *versus* control) improved the response of control arteries to NO solution. Endotoxin-exposed arteries (Fig. 4B) showed a diminished response to NO solution ($P = 0.01$ *versus* control). This response was improved by removal of endothelium ($P = 0.01$ *versus* endotoxin) or co-incubation with L-NA ($P = 0.001$ *versus* endotoxin) or AG ($P = 0.01$ *versus* endotoxin). However, the response of control and endotoxin-exposed remained different after co-incubation with aminoguanidine ($P = 0.005$). These data suggest that NOS2-mediated NO

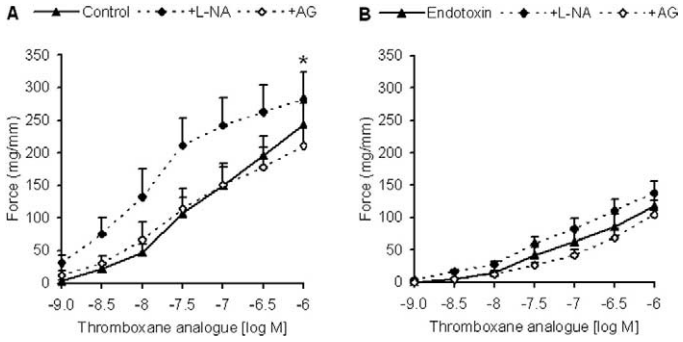

FIG. 2. TxA-induced contraction is impaired in endotoxin-exposed compared to control arteries (panel B and A, respectively; ANOVA $P = 0.03$; $n = 6$). L-NA enhanced the TxA-induced contractile response in control (panel A; * $P < 0.05$), but not in endotoxin-exposed arteries (panel B). Furthermore, co-incubation with aminoguanidine (AG) did not improve TxA-induced contractility in control or endotoxin-exposed arteries (panel A and B).

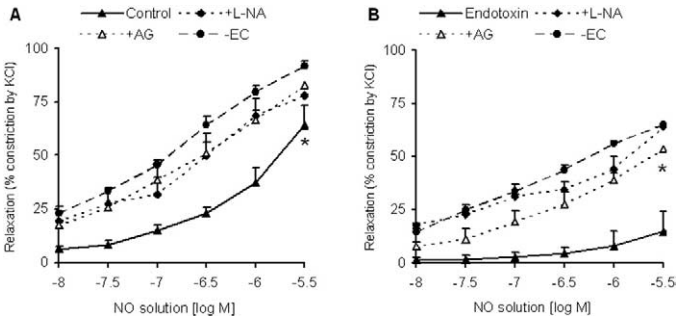

FIG. 4. Endotoxin-exposed pulmonary arteries show an impaired response to NO (panel D) compared to control arteries (panel C; ANOVA $P = 0.01$; $n = 6$). Removal of the endothelium, inhibition of NOS by L-NA or NOS2 by aminoguanidine improved the endotoxin-exposed pulmonary artery response. However, after aminoguanidine exposure, a difference remained between control and endotoxin-exposed arteries (ANOVA, $P = 0.005$).

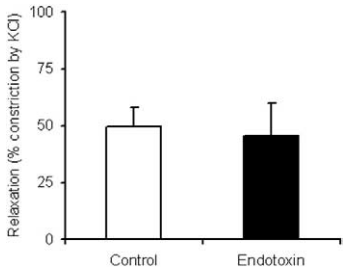

FIG. 5. KCl-preconstricted arteries exposed to 8-pCPT-cGMP, a phosphodiesterase-insensitive cGMP analogue: both control and endotoxin-exposed arteries respond similarly.

partly contributes to the impaired response of endotoxin-exposed pulmonary arteries to exogenous NO.

Vasorelaxation by a cGMP Analogue

Arteries were pre-constricted with 60 mM KCl, and subsequently the phosphodiesterase-insensitive cGMP analogue was applied. cGMP induced a similar reduction in force in control arteries (50.8%) and endotoxin-exposed arteries (46.2%; Fig. 5), implying that both control and endotoxin-exposed SMCs equally relaxed to an increase in cGMP.

DISCUSSION

Endotoxin impaired receptor-mediated but not receptor-independent vasoconstriction in isolated pulmonary arteries. Furthermore, our data suggest that NOS2-derived NO reduced the response of SMCs to NO, but did not ameliorate the impaired contraction of endotoxin-exposed pulmonary arteries. The diminished response to NO together with a normal response to a phosphodiesterase-insensitive cGMP analogue argues in favor of accelerated cGMP breakdown. This implies that the pulmonary vascular dysfunction is located in the SMCs, as supported by unaltered endothelium-dependent, acetylcholine-induced endothelial NO release after exposure to endotoxin.

Endotoxin impaired receptor-mediated, but not receptor-independent vasoconstriction, support the previously reported impairment of TxA/norepinephrine-induced contraction after 20 h of endotoxin exposure [2, 14, 19]. In contrast, other authors suggested an unaltered TxA/phenylephrine-induced contractile response after similar exposure times [14, 16, 19]. We hypothesized that NO is central in the impaired receptor-dependent contractile response of endotoxin-exposed arteries, as suggested by studies showing that impaired contraction during endotoxemia can be improved by NOS inhibition [3, 12–15, 19, 21]. However, we were unable to improve TxA-induced vasoconstriction by NOS inhibition, suggesting that NO did not mediate impaired contractility after endotoxin exposure in our experiments. These differences, as compared to other studies, might be a result of the used animal species (porcine, sheep), the duration of exposure to endotoxin (<15 h or >15 h) or the applied vasoconstrictor (norepinephrine, endothelin). Additionally, receptor-independent Ca^{2+}-influx was not affected by endotoxin, suggesting that the mechanism of impaired pulmonary arterial contractility depends on alterations in receptor expression or intracellular signaling. Endotoxin may suppress vasopressin, angiotensin II, and endothelin receptor gene expression, which might account for the reduced response to these vasoconstrictors [24]. We did not study nor are we aware of TxA receptor depression in endotoxin-induced lung injury. Because of the limited number of studies investigating altered pulmonary SMC signaling during endotoxemia, we can only speculate on the role of mechanisms downstream of receptor expression.

Our data suggest intact endothelial NO release in endotoxin-exposed pulmonary arteries, while the NO-induced diameter response of these vessel segments was impaired. Indeed, the impaired endothelium-dependent acetylcholine-induced vasodilator response agrees with the literature [10, 16–18]. Interestingly, NOS inhibition during force measurements only induced vasoconstriction in control arteries, implying that, despite functional endothelial NOS3, endotoxin affects the responsiveness of pulmonary SMCs to NO, in line with other studies showing impaired response to the endothelium-independent vasodilator sodium nitroprusside [10, 17, 18]. Additionally, endotoxin-associated enhanced vasoconstrictor availability may increase vascular tone [6–9] and blunt L-NA-induced contraction, as reported before [25]. Endotoxin-exposed pulmonary arteries showed impaired relaxation to NO solution in our study, which could be improved by unspecific NOS and specific NOS2 inhibition. In combination with the equal endothelial NO release in control and endotoxin-exposed arteries, this suggests that NOS2-mediated NO release induces hyporesponsiveness of pulmonary SMCs to other sources of NO. Because of the *in vitro* incubation setup, interference of adjacent pulmonary tissue and blood was avoided during the experiments. Therefore, the mechanism of endotoxin-induced hyporesponsiveness to NO must be localized in the vessel wall. Extending observations by other studies, showing that endotoxin decreases the response to inhaled NO in isolated perfused lung preparations [2–5]. In contrast, some studies suggest absence of NOS2-induction and resultant impairment of relaxation in endotoxin-exposed pulmonary arteries, which is probably due, at least in part, to the short duration of endotoxin exposure applied in these studies [17, 20]. However, we show that inhibition of NOS, and in particular NOS2, improved the relaxation capacity of endotoxin-exposed pulmonary arteries.

NO-mediated relaxation acts through activation of the enzyme guanylate cyclase (GC), resulting in the transition of guanosine triphosphate (GTP) into cGMP. It has been suggested that NO-induced cGMP release might decrease in endotoxic pulmonary SMCs because of down-regulation of GC activity or a NO-mediated inhibition of cGMP-dependent protein kinase (PKG) expression [10, 26–28]. Besides, an increased cGMP-specific phosphodiesterase activity during endotoxemia might exaggerate the degradation of cGMP [2, 3, 17]. We used a phosphodiesterase-insensitive cGMP analogue, bypassing the cGMP degradation pathway by phosphodiesterases, to verify whether NO/cGMP underlies the mechanism of impaired SMC relaxation [2, 3, 17]. Endotoxin-exposed and control arteries similarly relaxed to the cGMP analogue, in contrast with their response to NO. This suggests that an increased phosphodiesterase activity may underlie impaired NO-mediated relaxation in our isolated endotoxin-exposed pulmonary arteries, as in sheep with pulmonary hypertension or lungs isolated from endotoxin-challenged rats, opening the potential for therapy with nebulized cGMP-specific phosphodiesterase inhibitors or phosphodiesterase-insensitive cGMP analogs [2, 3, 5, 23].

In conclusion, endotoxin-associated NOS2-derived NO is associated with impaired NO-mediated relaxation, but does not underlie reduced receptor-mediated pulmonary contractile responses in isolated endotoxin-exposed rat pulmonary arteries. Because an increased phosphodiesterase activity may underlie the former, our *in vitro* data support exploration of this route to replace or improve the effect of inhalational NO therapy in severe sepsis-induced acute lung injury.

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