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Pharmacology

Attenuation of the induction of nitric oxide synthase by endogenous glucocorticoids accounts for endotoxin tolerance *in vivo*

(endotoxin shock/vasodilatation/contraction/RU 486/tumor necrosis factor)

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ABSTRACT **An enhanced formation of nitric oxide (NO) due to induction of a calcium-independent (inducible) NO synthase (iNOS) contributes importantly to the cardiovascular failure caused by bacterial endotoxin. Repeated challenges with small doses of endotoxin result in tolerance to both peripheral vascular failure and death caused by subsequent injection of a higher dose of endotoxin. Here we investigate whether tolerance to endotoxin is associated with a lack of induction of iNOS *in vivo* and whether endogenous glucocorticoids play a role in the development of endotoxin tolerance. In anesthetized rats, i.v. administration of *Escherichia coli* endotoxin [lipopolysaccharide (LPS); 2 mg·kg^{-1}] resulted in a prolonged decrease in mean arterial blood pressure (MAP) and hyporeactivity to the contractile responses elicited by norepinephrine (NE; 10 nM) in aortic rings *ex vivo*. Hyporeactivity to NE was partially reversed by N^G-nitro-L-arginine methyl ester (0.3 mM) *in vitro*, suggesting that an enhanced formation of NO contributes to this hyporeactivity. There was a substantial increase in the activity of iNOS in the lung 3 h after i.v. injection of LPS (0.2 ± 0.1 to 6.6 ± 0.6 pmol·mg^{-1}·min^{-1}; $n = 5$; $P < 0.01$). Rats injected i.p. with LPS (0.5 mg·kg^{-1}) for 4 consecutive days became tolerant to an i.v. injection of LPS (2 mg·kg^{-1}) in that both hypotension and vascular hyporeactivity to NE were significantly attenuated. Moreover, in these endotoxin-tolerant rats, the induction of iNOS by LPS in the lung was attenuated by 63% ± 6%. Injection of LPS caused a 9-fold increase in plasma corticosterone (CCS) levels within 2 h and CCS levels remained significantly elevated 6 and 24 h after LPS. Animals rendered tolerant to endotoxin by administration of a low dose of LPS (0.5 mg·kg^{-1}, i.p.) for 4 days still had a 6-fold increase in plasma CCS levels 24 h after the last injection of LPS. When endotoxin-tolerant rats were treated with the glucocorticoid receptor antagonist RU 486 (50 mg·kg^{-1}, p.o. 3 h prior to LPS), there was a restoration of the effects of LPS (2 mg·kg^{-1}, i.v.) in causing hypotension, vascular hyporeactivity to NE, and iNOS induction in the lung. However, in control rats RU 486 enhanced neither the decrease in MAP nor the induction of iNOS in response to LPS (2 mg·kg^{-1}, i.v.). Thus, cardiovascular tolerance to endotoxin is accompanied and explained by reduced induction of iNOS *in vivo* due to the elevation of endogenous glucocorticoid levels.**

Endotoxin (or bacterial lipopolysaccharide; LPS), a component of the outer membrane of Gram-negative bacteria, is the prime initiator of septic shock. Despite the considerable progress made in understanding the pathophysiology of septic shock, therapy is still very limited and the mortality of patients with established shock remains high (1).

Tolerance to endotoxin develops after repeated administration of small doses to animals and is characterized by the reduced effect of subsequent challenge with a high-dose endotoxin (2). The mechanism underlying endotoxin tolerance is poorly understood, but tolerance to endotoxin occurs in two distinct phases. The early-phase tolerance begins within 24–96 h after endotoxin exposure and its mechanism is unclear. A late-phase tolerance develops several weeks after the initial exposure to endotoxin and is associated with the production of anti-endotoxin antibodies (2).

Endotoxin induces the expression of a calcium-independent (inducible) isoform of nitric oxide (NO) synthase (iNOS) in macrophages, vascular smooth muscle cells, fibroblasts, hepatocytes, Kupffer cells, keratinocytes, and megakaryocytes *in vitro* and in a number of tissues including lung, spleen, liver, heart, kidney, as well as blood vessels *in vivo* (3, 4). An enhanced formation of NO in endotoxemia contributes importantly to several key features of the pathophysiology of septic shock (5, 6) including hypotension (7–10), vascular hyporeactivity to vasoconstrictor agents (10, 11), myocardial dysfunction (12–14), and, possibly, inhibition of cellular respiration (15, 16). Glucocorticoids are potent inhibitors of the induction of iNOS by LPS (17, 18). The protective cardiovascular effects of glucocorticoids in animal models of septic shock may be due in part to prevention of the induction of iNOS (10, 19).

Prolonged exposure of macrophages to LPS *in vitro* attenuates the expression of tumor necrosis factor (TNF) and interleukin 1 (IL-1) in response to a subsequent challenge with LPS (20, 21). Recent reports show that exposure to LPS reduces the subsequent ability of γ-interferon or LPS to induce iNOS in cultured macrophages (22, 23).

Here we investigate the relationship between the early-phase tolerance to LPS and the induction of iNOS *in vivo*. We demonstrate that the early-phase tolerance to endotoxin is associated with a reduced ability of endotoxin to induce iNOS because of increased circulating levels of endogenous glucocorticoids.

MATERIALS AND METHODS

Hemodynamic Measurements. Male Wistar rats (250–290 g; Glaxo) were anesthetized with thiopentone sodium (Intraval sodium, May 8 Baker, Dagenham, England; 120 mg·kg^{-1}, i.p.) and instrumented for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate, which were displayed on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA). Left or right femoral veins were cannulated for the administration of drugs.

Upon completion of the surgical procedures, cardiovascular parameters were allowed to stabilize for 15 min. After recording baseline hemodynamic parameters, animals re-

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Abbreviations: ADX, adrenalectomized; CCS, corticosterone; IL-1, interleukin 1; LPS, lipopolysaccharide; L-NAME, N^G-nitro-L-arginine methyl ester; MAP, mean arterial blood pressure; NE, norepinephrine; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor.

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ceived *Escherichia coli* LPS (2 mg·kg^{-1}, i.v.; in 0.3 ml of saline) as a slow injection over 2 min and hemodynamic parameters were monitored for 3 h.

Organ Bath Experiments. Thoracic aortae were rapidly removed, cleared of adhering periadventitial fat, and cut into rings 2 mm wide. Isometric contractions of endothelium-denuded rings to norepinephrine (NE; 10 nM) were measured (24, 25) before and after incubation with the NOS inhibitor (26, 27) N^G-nitro-L-arginine methyl ester (L-NAME; 20 min; 0.3 mM). Nine rings from four or five rats were used in each experimental group.

NOS Assay. Lungs were removed and frozen in liquid nitrogen. Frozen lungs were homogenized and conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline was measured in the homogenates (10, 28). Experiments performed in the absence of NADPH determined the extent of L-[^{3}H]citrulline formation independent of a specific NOS activity. Experiments carried out in the presence of NADPH, without calcium and with EGTA (5 mM), determined the calcium-independent NOS activity (i.e., iNOS).

Experimental Protocols. To induce tolerance to endotoxin, rats were injected i.p. with *E. coli* LPS (0.5 mg·kg^{-1}) daily for 4 consecutive days (Fig. 1). Six and 24 h after each of the four i.p. injections, some animals were killed by exsanguination under anesthesia, and lung samples were removed to measure iNOS activity. Six and 24 h after the first i.p. injection of LPS and 24 h after the last i.p. injection of LPS, rings of thoracic aortae were removed to study isometric contractions elicited by NE *ex vivo*.

Naive rats and endotoxin-tolerant rats (used 24 h after the last of four injections of LPS) were anesthetized and hemodymanic parameters were monitored for 3 h after giving LPS (2 mg·kg^{-1}, i.v.). Three hours after injection of LPS, lungs were removed for measurement of iNOS activity and thoracic aortae were removed for measurement of isometric contractions of vascular rings.

Antagonism of Endogenous Glucocorticoids. Separate groups of control or endotoxin-tolerant rats received the glucocorticoid receptor antagonist RU 486 (50 mg·kg^{-1}) (29, 30) p.o. (in 1.5 ml of saline) 3 h prior to anesthesia and injection of LPS (2 mg·kg^{-1}, i.v.). Hemodynamic parameters were monitored for 3 h, after which time lungs were removed for measurement of iNOS activity and thoracic aortae were removed for measuring isometric contractions of vascular rings to NE. The above dose regimen for RU 486 prevents the antiinflammatory effects of dexamethasone in a carrageenin-induced pleurisy model in the rat (29).

Plasma CCS Assay. Plasma CCS levels were measured in blood samples of 5 ml taken from naive rats before and from separate groups of rats 2, 6, and 24 h after the first injection of LPS (0.5 mg·kg^{-1}, i.p.) by using a radioimmunoassay kit (ICN) (31). In addition, plasma CCS was measured 24 h after the last of 4 daily injections of LPS (0.5 mg·kg^{-1}, i.p.).

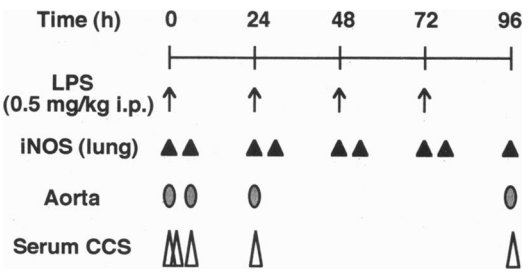

FIG. 1. Experimental protocol to induce endotoxin tolerance in anesthetized rats. Rats were injected with LPS (0.5 mg·kg^{-1}, i.p.) daily (arrows). At 0, 6, and 24 h after the first LPS injection, some animals were killed and lung samples were taken for measurement of iNOS activity (solid triangles). At 0, 6, and 24 h after the first LPS injection as well as at 96 h, thoracic aortae were removed from some rats to measure isometric contractions to NE (oval symbols). Serum corticosterone (CCS) was measured at 0, 2, 6, 24, and 96 h (open triangles).

Materials. Calmodulin, endotoxin (bacterial LPS; *E. coli* serotype 0127:B8), NADPH, L-NAME, NE bitartrate, and Dowex 50W anion-exchange resin were obtained from Sigma. RU 486 {(11β,17β)-11-[4-(dimethylamino)phenyl]-17-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one]; mifepristone} was a gift from Roussel-UCLAF. Solutions of RU 486 were made in saline and sonicated before use. L-[2,3,4,5-^{3}H]arginine hydrochloride was obtained from Amersham.

Statistical Evaluation. All values in the figures and text are expressed as means ± SEM of n observations, where n represents the number of animals or the number of blood vessels studied. Student's paired or unpaired t tests were used to compare means among or between groups, respectively. A P value of <0.05 was considered to be statistically significant.

RESULTS

Repeated Injections of LPS Abolish Its Ability to Induce iNOS. i.p. injection of LPS (0.5 mg·kg^{-1}) caused a significant increase in the activity of iNOS in the lung 6 h later. However, 24 h after injection of LPS, iNOS activity returned to control level. Subsequent challenges with LPS after 24, 48, and 72 h did not induce iNOS in the lung (Fig. 2*a*).

Contractions of aortic rings (without endothelium) to NE (10 nM) were reduced 6 h after the first injection of LPS ($P < 0.01$). Contractile responses to NE were significantly restored by *in vitro* incubation of aortic rings with L-NAME (Fig. 2*c*). In contrast, L-NAME did not affect the contractions of control vascular rings to NE (Fig. 2*c*). However, contractile responses to NE of rings obtained 24 h after the first injection of LPS were not different from controls (Fig. 2*c*).

Tolerance to Endotoxin Is Associated with Increased Basal Plasma CCS, Reduced Cardiovascular Response to LPS, and Reduced Induction of iNOS. Basal CCS levels in plasma from control rats were 40 ± 12 ng·ml^{-1} (n=16). The first injection of LPS caused an increase in plasma CCS levels to 373 ± 13 ng·ml^{-1} after 2 h, falling to 232 ± 48 ng·ml^{-1} within 24 h (n=5; $P<0.05$; Fig. 2*b*). However, 24 h after the final (fourth) injection of LPS, CCS plasma levels were still elevated (241 ± 21 ng·ml^{-1}; n=5; $P<0.01$; Fig. 2*b*).

LPS administration (2 mg·kg^{-1}, i.v.) to control rats resulted in a significant decrease in MAP from 118 ± 5 mmHg (control) to 88 ± 3 and 84 ± 2 mmHg 2 and 3 h after LPS ($P<0.01$; n=9; Fig. 3*a*). Endotoxemia for 3 h was also associated with a significant compensatory increase in heart rate (from 423 ± 8 to 456 ± 7 beats per min at 3 h; $P<0.01$; n=9; Fig. 3*b*). In endotoxin-tolerant rats, the fall in MAP elicited by a subsequent i.v. injection of LPS (2 mg·kg^{-1}) was substantially reduced when compared to control rats ($P<0.05$; n=11; Fig. 3*a*). In addition, injection of LPS into endotoxin-tolerant rats did not cause significant tachycardia (Fig. 3*b*).

The contractile effects of NE (10 nM) in endothelium-denuded aortic rings obtained from rats 3 h after i.v. challenge with LPS (2 mg·kg^{-1}) were significantly reduced ($P<0.01$). The contractile responses of these rings to NE were partially restored by *in vitro* incubation with L-NAME (Fig. 4). In contrast, rings obtained from endotoxin-tolerant rats 3 h after LPS injection (2 mg·kg^{-1}, i.v.) showed no reduction in the contractile effects of NE (10 nM) (Fig. 4). Moreover, L-NAME did not alter the contractile responses to NE in these vessels (Fig. 4).

In lungs obtained from naive rats 3 h after injection of LPS (2 mg·kg^{-1}, i.v.), there was a significant increase in the activity of iNOS from 0.2 ± 0.1 (control) to 6.6 ± 0.6

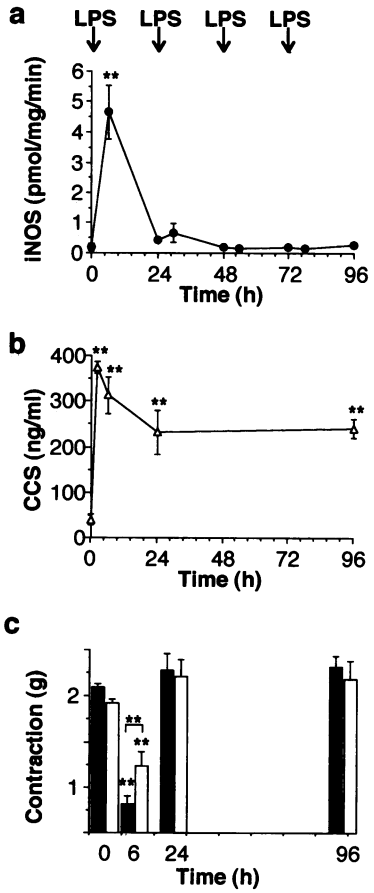

FIG. 2. Repeated challenge with LPS increases plasma CCS levels and produces tolerance to the induction of iNOS. Depicted are iNOS activities in homogenates of lungs (*a*; solid circles; n=4–6), plasma CCS levels (*b*; open triangles; n=5 or 6), and contractions to NE (10 nM) before (solid bars) and after (open bars) incubation with L-NAME (0.3 mM for 20 min; n=9) in rats before and after 4 daily consecutive injections of LPS (0.5 mg·kg^{-1}, i.p.) (*c*). Data are expressed as means ± SEM. Arrows represent daily injections of LPS (0.5 mg·kg^{-1}, i.p.). **, P<0.01; significant increases in CCS plasma levels or iNOS activity or significant decreases in contractions when compared to initial (baseline) values.

pmol·mg^{-1}·min^{-1} (n=5; P<0.01). In contrast, in endotoxin-tolerant rats, the increase in the activity of iNOS elicited by LPS after 3 h (2 mg·kg^{-1}, i.v.) was significantly smaller. Thus, iNOS activity in the lungs of endotoxin-tolerant rats was only 37% ± 6% of that measured in LPS-treated naive animals (Fig. 5).

In Endotoxin-Tolerant Rats, the Glucocorticoid Antagonist RU 486 Prevents Blunting of the Cardiovascular Response and Restores iNOS Induction. Pretreatment of endotoxin-tolerant rats with the glucocorticoid receptor antagonist RU 486 (50 mg·kg^{-1}, p.o.) significantly enhanced the hypotensive response elicited by a subsequent injection of LPS (2 mg·kg^{-1}, i.v.) (Fig. 3*a*). Importantly, pretreatment with RU 486 completely restored the capacity of LPS to induce iNOS activity in the lungs of endotoxin-tolerant rats (Fig. 5). Endotoxin-tolerant rats pretreated with RU 486 also developed a significant vascular hyporeactivity to NE 3 h after injection of LPS (2 mg·kg^{-1}, i.v.), an effect that was overcome by L-NAME (Fig. 4). However, the degree of vascular hyporeactivity in these vessels was significantly less (P<0.01) than that found in naive rats after LPS injection.

RU 486 did not significantly influence the hypotension and tachycardia (data not shown) or the induction of iNOS in the lung (Fig. 5) caused by LPS (2 mg·kg^{-1}, i.v.) in naive animals.

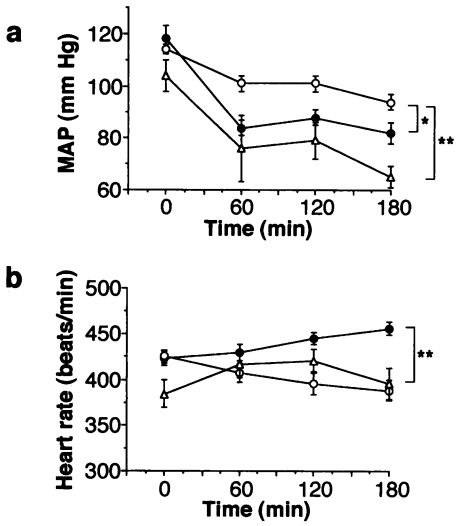

FIG. 3. Repeated challenge with LPS inhibits the cardiovascular response to LPS. Depicted are changes in MAP (*a*) and heart rate (*b*) caused by LPS (2 mg·kg^{-1}, i.v.) in naive control rats (solid circles; n=9), endotoxin-tolerant rats (see *Materials and Methods*; open circles; n=11), or endotoxin-tolerant rats pretreated with RU 486 (50 mg·kg^{-1}, p.o., 3 h prior to instrumentation for hemodynamic measurements; open triangles; n=5). LPS was administered at time 0. Data are expressed as means ± SEM. *, P<0.05; **, P<0.01; significant differences between groups.

DISCUSSION

It has been proposed that several different mechanisms contribute to the development of endotoxin tolerance. The early-phase tolerance to endotoxin is associated with a reduced release of TNF, colony-stimulating factor, and γ-interferon (32–35). These cytokines are mediators of the cardiovascular dysfunction elicited by endotoxin (36). Activation of the pituitary–adrenocortical axis (34, 37), inhibition of neutrophil activation (38), and alterations in guanine nucleotide binding protein activity (39) have also been demonstrated in endotoxin-tolerant animals.

The present study shows that the reduced cardiovascular response to LPS, which occurs after repeated administration of small doses of LPS, is associated with a reduced capacity

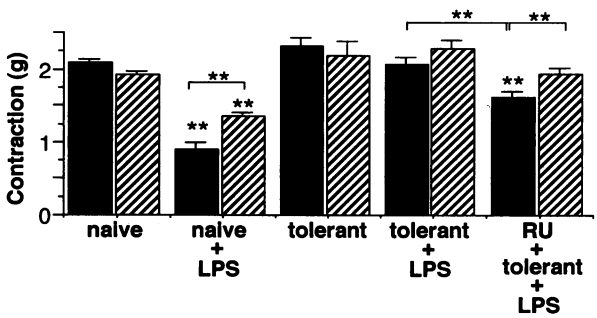

FIG. 4. Repeated challenge of rats with LPS prevents development of vascular hyporeactivity to NE in thoracic aortic rings *ex vivo*. Depicted are contractions to NE (10 nM) in aortae isolated from naive rats (naive; n=9), from naive rats 3 h after LPS injection (2 mg·kg^{-1}, i.v.; naive + LPS; n=9), from endotoxin-tolerant rats (tolerant; n=9), from endotoxin-tolerant rats 3 h after LPS injection (2 mg·kg^{-1}; i.v.; tolerant + LPS; n=9), and from RU 486-pretreated endotoxin-tolerant rats 3 h after LPS injection (2 mg·kg^{-1}, i.v.; RU + tolerant + LPS; n=9) before (control; solid bars) and after incubation with L-NAME (0.3 mM for 20 min; hatched bars). Data are expressed as means ± SEM. **, P<0.01; significant differences from control, or between groups, as indicated.

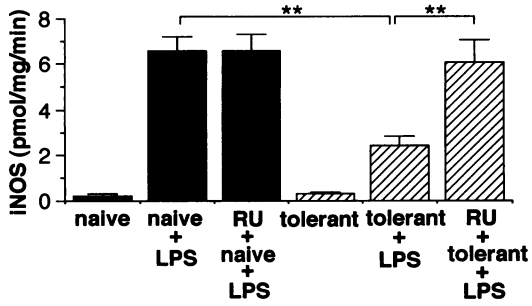

FIG. 5. Repeated challenge with endotoxin reduces the ability of endotoxin to induce iNOS. This can be reversed by the glucocorticoid receptor antagonist RU 486. Depicted are iNOS activities in lung homogenates obtained from naive rats (naive; $n=4$), from naive rats 3 h after LPS injection (2 mg·kg^{-1}; i.v.; naive + LPS; $n=9$), from naive rats pretreated with RU 486 3 h prior to LPS (RU + naive + LPS; $n=4$), from endotoxin-tolerant rats (tolerant; $n=4$), from endotoxin-tolerant rats 3 h after LPS (tolerant + LPS; $n=11$), and from endotoxin-tolerant rats pretreated with RU 486 3 h prior to LPS (RU + tolerant + LPS; $n=5$). Data are expressed as means ± SEM. There was a significant ($P<0.01$) increase in iNOS activity 3 h after LPS in naive and endotoxin-tolerant rats when compared to the respective baseline levels (prior to LPS). **, $P<0.01$; significant differences between groups.

of LPS (*i*) to induce iNOS in the lung *in vivo* and (*ii*) to cause NO-mediated vascular hyporeactivity *ex vivo*.

Pretreatment of rats (25) or mice (40) with a monoclonal antibody to TNF, or of rats with the endogenous IL-1 receptor antagonist (41), attenuates the induction of iNOS by LPS. These agents also prevent the LPS-induced decrease in MAP and the development of hyporeactivity to NE in the thoracic aorta *ex vivo* (25, 41). Thus, induction of iNOS by LPS *in vivo* is, at least in part, mediated by TNF and IL-1. Glucocorticoids reduce the formation of TNF and IL-1 in response to LPS (35, 42, 43), so that a reduction in the release of these cytokines is the most likely mechanism by which glucocorticoids suppress iNOS induction *in vivo*.

The elevated plasma levels of CCS that we found in endotoxin-tolerant rats support the view that these steroids play a central role in the development of endotoxin tolerance. In addition, we show here that the reduced ability of LPS to cause hypotension and iNOS induction in endotoxin-tolerant rats is reversed by the glucocorticoid receptor antagonist RU 486. Thus, elevated levels of endogenous glucocorticoids in endotoxin-tolerant rats can account for the protection against cardiovascular failure and the attenuation of the induction of iNOS by LPS. The notion that an increase in plasma levels of corticosteroids plays a key role in the development of endotoxin tolerance is also supported by the finding that adrenalectomized (ADX) rats, which lack endogenous glucocorticoids, do not become tolerant to LPS (34).

Inhibition of glucocorticoid receptors with RU 486 enhances the cardiovascular response and the induction of iNOS elicited by LPS in endotoxin-tolerant, but not in naive, rats. Clearly, agents that inhibit induction of iNOS by LPS, such as glucocorticoids, exhibit their effects only when administered prior to LPS (5). In naive rats, a significant increase in plasma CCS occurs only after injection of LPS (this study; see also ref. 31). This delayed increase in CCS does not inhibit the LPS-induced release of TNF or IL-1 (31), explaining why RU 486 does not affect the release of the above cytokines (31), the subsequent induction of iNOS, or the associated hemodynamic response in naive rats injected with LPS. However, successive administrations of LPS resulted in a prolonged increase in glucocorticoid levels. Indeed, in the present study CCS levels in plasma of endotoxin-tolerant rats (24 h after the last of four i.p. injections of LPS) were ≈6-fold higher than basal levels. Thus, elevated plasma glucocorticoids attenuate the induction of iNOS by LPS in endotoxin-tolerant rats.

The hypothesis that basal endogenous glucocorticoids also regulate the degree of NOS induction caused by LPS in normal (nontolerant) rats is supported by the finding that ADX rats become exquisitely sensitive to LPS and show supersensitivity to iNOS induction (44). However, in contrast to ADX rats, treatment of naive rats with the glucocorticoid antagonist RU 486 did not lead to an enhanced cardiovascular response to LPS or increased iNOS induction. This suggests that other factors may also be involved in the enhanced sensitivity to LPS found in ADX rats. It is noteworthy that ADX rats, but not normal rats pretreated with RU 486, respond to LPS with increased production of TNF and IL-1 (31).

Injection of LPS to naive rats resulted in a marked impairment of the contractions of aortic rings to NE *ex vivo*. This was due in part to an enhanced formation of NO, as it was partially reversed by treatment with L-NAME *in vitro* (see also refs. 11, 25, and 41). In contrast, there was no vascular hyporeactivity to NE in endotoxin-tolerant rats. Interestingly, pretreatment of these rats with RU 486 partially restored the ability of LPS to reduce the contractile responses of aortic rings to NE (Fig. 4). Thus, another factor(s), apart from NO, contributes to this hyporeactivity, and RU 486 restores only that part of the hyporeactivity that is due to induction of iNOS. It is noteworthy that RU 486 treatment fully restored the ability of LPS to induce iNOS in the lungs of endotoxin-tolerant rats (Fig. 5). The nature of the additional factor(s) causing vascular hyporeactivity is not clear, but it does not appear to be regulated by endogenous glucocorticoids.

In conclusion, the present study shows that the development of cardiovascular tolerance to endotoxin in the rat is associated with a reduced ability of LPS to induce iNOS. In addition, endotoxin tolerance is associated with a marked increase in plasma glucocorticoid levels. Thus, we propose that the attenuation of the induction of iNOS by endogenous glucocorticoids accounts for endotoxin-induced cardiovascular tolerance. Elevated glucocorticoids play a key role in the development of endotoxin tolerance and may serve to protect individuals with chronic inflammatory conditions from deleterious consequences of excessive NO production.

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