Endotoxin triggers the expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in the rat aorta in vivo

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Abstract The free radicals nitric oxide (·NO) and superoxide (O_2^-) are known to react to form peroxynitrite ($ONOO^-$), a highly reactive species. Peroxynitrite has been suggested to play an important role in the cellular damage associated with the overproduction of ·NO, but there are very limited data regarding its in vivo formation. Here we demonstrate that injection of endotoxin into rats leads to the expression of an inducible isoform of ·NO synthase (iNOS) in the thoracic aorta at 6 h and an increase in the circulating levels of nitrite/nitrate. Moreover, at the same time point, there is a marked increase in the immunoreactivity of nitrotyrosine, a marker of peroxynitrite in the aorta. The formation of nitrotyrosine was prevented by inhibiting the activity of NOS by N^G-methyl-L-arginine in vivo. Our data suggest that during endotoxin shock, part of ·NO, produced following the induction of iNOS, is converted into peroxynitrite in the vicinity of large blood vessels. The demonstration of the in vivo formation of peroxynitrite at sites of ·NO overproduction may necessitate the development of novel and additional approaches for limiting or preventing ·NO-related cytotoxic or vasodilatory actions during circulatory shock.

Key words: Nitric oxide; Peroxynitrite; Superoxide; Septic shock; Nitrotyrosine; Immunohistochemistry; Contraction

1. Introduction

The production of large amounts of nitric oxide (·NO) by the inducible isoform of ·NO synthase (iNOS), in response to pro-inflammatory cytokines or endotoxin (bacterial lipopolysaccharide, LPS), has been implicated in the suppression of cellular respiration and in the pathophysiology of cell and organ damage associated with circulatory shock [1–3]. During circulatory shock, the vascular smooth muscle is a key site of ·NO overproduction, leading to reduced responsiveness to vasoconstrictor agents and a progressive dilatation of the vasculature (vascular decompensation) [1–3].

NO is known to react with superoxide anion (O_2^-) to form an even more toxic species, peroxynitrite ($ONOO^-$) [4,5]. The formation of peroxynitrite has been demonstrated from activated macrophages [6] endothelial cells [7] and human neutrophils [8]. Peroxynitrite is a highly reactive species capable of oxidizing many organic molecules [5]. We have previously shown that a major product from the spontaneous reaction of peroxynitrite with proteins is the formation of nitrotyrosine and that nitrotyrosine represents a specific protein modification mediated by peroxynitrite [9]. The formation of peroxynitrite has been proposed in a number of pathophysiological conditions that are associated with overproduction of NO and/or superoxide. Here we investigate the possibility that the induction of iNOS in the aorta during endotoxemia leads to the formation of peroxynitrite in the same tissue.

2. Materials and methods

2.1. Materials

Bacterial lipopolysaccharide (*E. coli*, serotype no. 0127:B8), calmodulin, NADPH, N^G-methyl-L-arginine monoacetate and Dowex 50W anion exchange resin were obtained from Sigma (St. Louis, MO). L-[2,3,4,5-^{3}H]arginine hydrochloride was obtained from Amersham (Arlington Heights, IL).

2.2. Animals

Male Wistar rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were injected with vehicle, vehicle and the NOS inhibitor N^G-methyl-L-arginine (15 mg/kg i.p. at 2 h after the injection of vehicle), with *E. coli* endotoxin (10 mg/kg i.p.) or with endotoxin and N^G-methyl-L-arginine (15 mg/kg i.p. at 2 h after the injection of endotoxin). At 6 h, animals were anesthetized with enflurane and blood was taken by cardiac puncture. After exsanguination, thoracic aortae were quickly removed and placed in tissue fixative for immunohistochemistry, or into liquid nitrogen for the measurement of nitric oxide synthase activity.

2.3. Nitric oxide synthase assay

Aortae were placed into a homogenation buffer composed of: 50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM phenylmethylsulfonyl fluoride (pH 7.4). Tissues were then homogenized in the buffer on ice using a Tissue Tearor 985-370 homogenizer (Biospec Products, Racine, WI). Conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was measured in the homogenates as described [10]. Briefly, cell homogenate (30 μl) was incubated in the presence of [^{3}H]L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and calcium (2 mM) for 30 min at 22°C. Reactions were stopped by dilution with 0.5 ml of ice-cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Experiments performed in the absence of NADPH determined the extent of [^{3}H]L-citrulline formation independent of a specific NOS activity. Experiments in the presence of NADPH, without calcium and with EGTA (5 mM), determined the calcium-independent (i.e. induced) NOS activity. Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]L-citrulline activity was measured by a Wallac scintillation counter (Wallac, Gaithersburg, MD).

2.4. Nitrotyrosine immunohistochemistry

Aortae were fixed for 3 h in 4% paraformaldehyde and cryoprotected by 1 h incubation in 10%, 20% and 30% sucrose in 0.1 M sodium cacodylate buffer. The tissues were placed on OTC-coated tissue-holders covered with a thin layer of OTC and frozen with distilled Freon 22 in liquid nitrogen. Eight μm thin sections were placed onto slides and stained with the anti-nitrotyrosine antibody. The blocking solution consisted of 4% fatty acid free BSA, 10% goat serum and 3% Triton X-100 in 0.1 M phosphate buffered saline (PBS), pH 7.2. Samples were incubated with either anti-nitrotyrosine polyclonal antibody at 2 μg/ml for 3 h, or antigen-competed primary antibody. The antigen-competed

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antibody was prepared by diluting the primary antibody at 2 μg/ml in 10 mM nitrotyrosine (Aldrich) in 0.1 M PBS, pH 7.4. The antibody binding on tissue sections was visualized by 1 h incubation with an anti-rabbit IgG conjugated to Texas red (1:100 dilution in PBS). Slides were examined under a Nikon Diaphot-TND epifluorescence inverted microscope. All pictures were obtained under the same magnification (25× lens), camera and printing settings.

2.5. Measurement of plasma nitrite/nitrate

Plasma was ultrafiltrated using 10,000 molecular weight cut-off ultrafiltration units (Fisher Scientific). The nitrate in the deprotinized filtrate was reduced to nitrite using *E. coli* as described previously [11] and nitrite was measured by the Griess reaction. The levels of nitrite/nitrate in control male Wistar rats was similar to the values reported previously for control male Fischer rats [12].

2.6. Statistical evaluation

All values in the figures and text are expressed as mean ± S.E.M. of n observations. Student's unpaired t-test was used to compare means between groups. A P-value less than 0.05 was considered to be statistically significant.

3. Results

In control rats, there was detectable calcium-dependent (constitutive, endothelial type) NOS activity, and no calcium-independent (inducible) NOS activity in the aorta. At 6 h after endotoxemia, there was a significant ($P < 0.01$), more than 4-fold increase in the total NOS activity in the aorta, approximately half of which was calcium-independent (inducible) (Fig. 1). The activity of NOS (both in the aortae of control and in endotoxemic rats) could largely be inhibited by N^G-methyl-L-arginine (3 mM) in vitro (Fig. 1). The increase in ·NO production in response to endotoxin was also reflected by a 20-fold increase in plasma levels of nitrite and nitrate, degradation products of ·NO (Fig. 2). This increase was significantly inhibited by N^G-methyl-L-arginine treatment in vivo (Fig. 2).

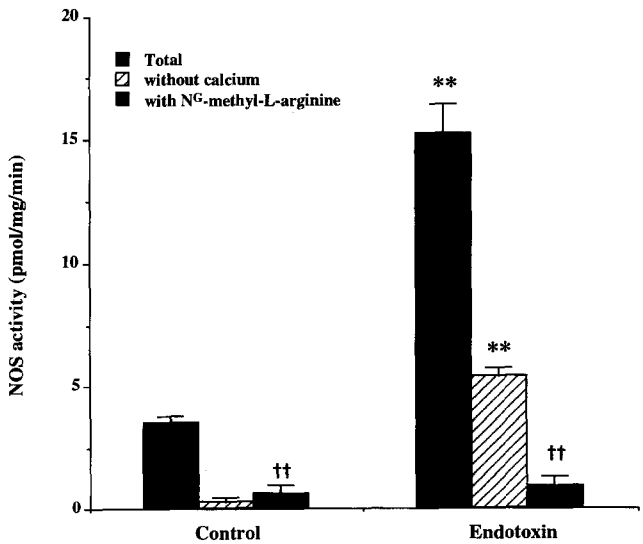

Fig. 1. Total (black columns) and calcium-independent (inducible, hatched columns) nitric oxide synthase activity in the aorta of vehicle-treated control rats and in the aorta of rats at 6 h after *E. coli* lipopolysaccharide (10 mg/kg i.p.) ($n = 3$–4). Data are expressed as means ± S.E.M. of n observations. Grey columns represent total NOS activities in the presence of the NOS inhibitor N^G-methyl-arginine (3 mM). **Represents significant difference between the respective NOS activities in control animals vs. in rats treated with endotoxin ($P < 0.01$). ††Represents significant inhibition of NOS activity by N^G-methyl-arginine in vitro ($P < 0.01$).

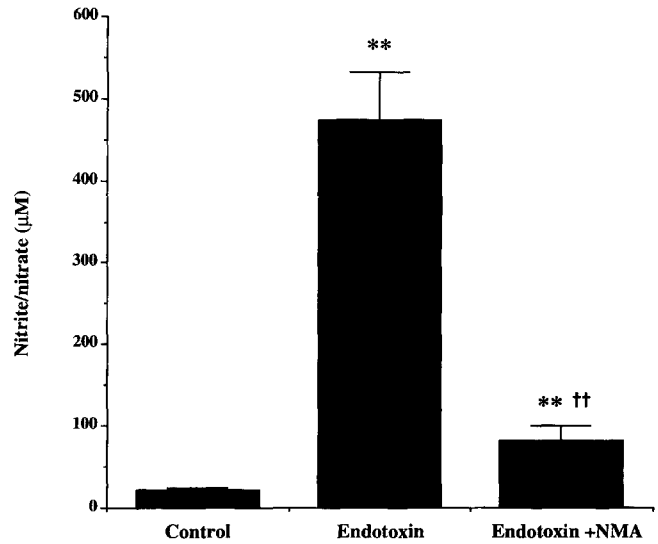

Fig. 2. Plasma nitrite/nitrate levels in control animals, in animals at 6 h after *E. coli* lipopolysaccharide (10 mg/kg i.p.) treatment and in animals treated with endotoxin and the NOS inhibitor N^G-methyl- arginine (15 mg/kg i.p. at 2 h after the injection of endotoxin). **Represents significant increases in nitrite/nitrate plasma levels in rats treated with endotoxin (with or without N^G-methyl-arginine treatment) when compared to control animals ($P < 0.01$). †Represents significant reduction by N^G-methyl-arginine in plasma nitrite/nitrate levels in rats treated with endotoxin ($P < 0.05$).

In the aortae of control rats, very low nitrotyrosine immunoreactivity could be observed (Fig. 3a). Similarly, low nitrotyrosine immunoreactivity could be observed in the aortae of N^G-methyl-L-arginine control rats. In contrast, there was a marked increase in the nitrotyrosine immunoreactivity in the aortic tissues obtained from rats injected with endotoxin, (Fig. 3b), which was almost completely absent in the aortae of septic animals that received treatment with N^G-methyl-L-arginine (Fig. 3c). The development of nitrotyrosine immunoreactivity in the aortae of septic rats could also be inhibited by competition by excess nitrotyrosine during the incubation with the nitrotyrosine antibody (Fig. 3d).

4. Discussion

Using the measurement of tyrosine nitration, a specific marker of the presence of peroxynitrite [9,13–16], here we have demonstrated that endotoxemia leads to a substantial increase in the levels of biologically active peroxynitrite in the vicinity of large blood vessels, such as the thoracic aorta. Considering that there was a diffuse immunoreactivity throughout the whole tissue and that in the vascular smooth muscle, a substantial increase in the calcium-independent (inducible) NOS activity could be demonstrated, we assume that mainly iNOS, induced in the vascular tissue, is the source of ·NO. The source of superoxide may be the vasculature itself and/or activated neutrophil granulocytes. Using N^G-methyl-L-arginine, we have also demonstrated that the nitrotyrosine immunoreactivity (i.e. the formation of peroxynitrite), can be prevented by inhibition of ·NO biosynthesis.

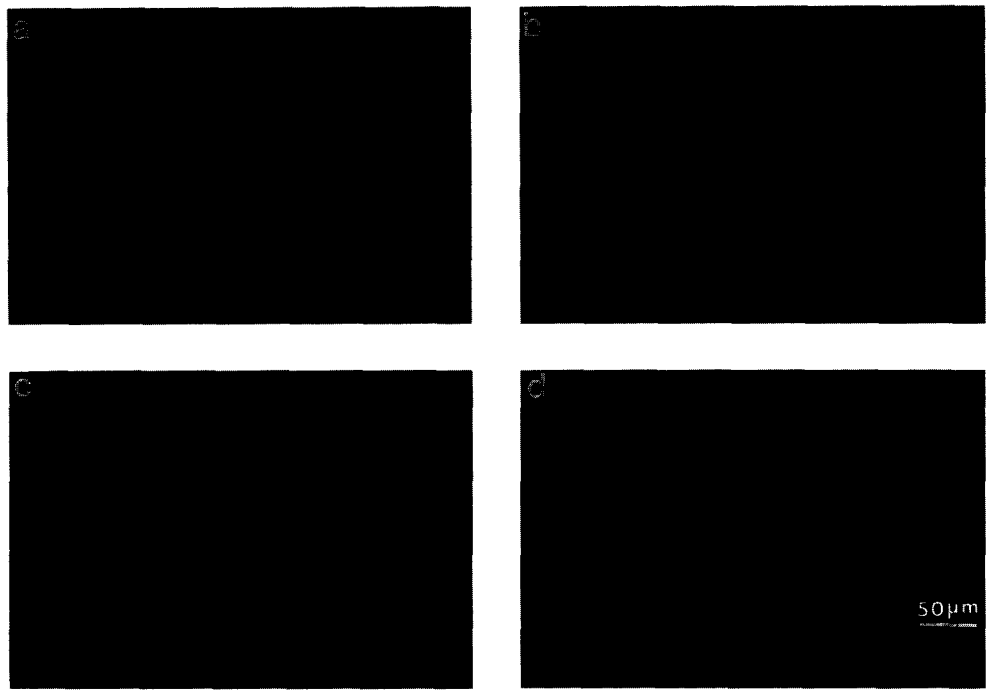

Fig. 3. Representative nitrotyrosine immunofluorescence photomicrographs of rat thoracic aortae. (a) Control rats; (b) rats injected with endotoxin; (c) rats injected with endotoxin combined with N^G-methyl-L-arginine; (d) immunoreactivity in the aortae of septic rats where antibody binding was inhibited by excess nitrotyrosine during the incubation period. Similar results were observed in four different aortae in each experimental group.

Increased nitrotyrosine levels have been detected in human diseases associated with of oxidative stress; in atherosclerotic plaques of human coronary vessels [13], in the lungs of infants with acute lung injury and ARDS [14,15], and in synovial fluid and plasma from patients with rheumatoid arthritis [16]. Endotoxin-mediated induction of hepatic ·NO increased peroxynitrite formation and injury during reperfusion of ischemic rat liver [17]. Moreover, an increase in nitrotyrosine immunoreactivity was recently found in the lung of rats injected with bacterial endotoxin [18]. It is noteworthy in this contest that among many organs studied, the lung expresses the highest specific activity of iNOS in response to endotoxin injection in the rat [10]. Thus, it appears that endotoxin shock results in induction of iNOS, with subsequent formation of peroxynitrite.

Based on the protective effect of inhibitors of NOS in various animal models of shock, it is generally assumed that ·NO, per se, is the active vasodilator and cytotoxic molecule in endotoxemia [1–3]. The present data, however, suggest that endotoxemia is associated with the formation of large amounts of peroxynitrite in the vicinity of the vasculature. If peroxynitrite is formed from iNOS-derived ·NO in vivo, this would necessitate the introduction of different therapeutic strategies in order to ameliorate cytotoxic actions. It is noteworthy that peroxynitrite also has marked vasodilator properties, and its vascular actions are substantially different from that of ·NO [19,20].

In conclusion, we have provided indirect evidence for the in vivo formation of peroxynitrite in the vascular smooth muscle, a pertinent site of ·NO overproduction in endotoxaemia. The realization of the formation of peroxynitrite during sepsis at sites of ·NO overproduction may open ways of developing novel, additional approaches for limiting or preventing ·NO-related cytotoxic or vasodilatory actions during circulatory shock and other pathophysiological states.

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