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Short communication

Inhibition of nuclear factor-κB prevents the loss of vascular tone in lipopolysaccharide-treated rats

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Abstract

We studied the role of nuclear factor-κB (NF-κB) on the tone and on the expression of inducible nitric oxide (NO) synthase, both evaluated in aortas from lipopolysaccharide-treated rats. Thoracic aorta rings from lipopolysaccharide-treated rats (4 mg/kg, i.p.), compared to those from naive animals, showed: (i) reduced contractility to phenylephrine, (ii) progressive loss in tone when contracted with phenylephrine, (iii) increased inducible NO synthase protein expression and NF-κB activation. Pyrrolidine dithiocarbamate (10, 30, 100 mg/kg, i.p.), an antioxidant inhibitor of NF-κB activation, dose dependently suppressed all these lipopolysaccharide-induced effects. These results demonstrate that in vivo inhibition of NF-κB activation prevented the lipopolysaccharide-induced loss of vascular tone, an effect which was correlated to reduced expression of inducible NO synthase protein.

Keywords: Aorta ring, rat; Lipopolysaccharide; Nitric oxide (NO); Nitric oxide (NO) synthase; Nuclear factor-κB (NF-κB); Vascular tone

1. Introduction

Septic shock is usually caused by Gram-negative bacteria, which release bacterial lipopolysaccharide into the blood stream. The main cardiovascular features of septic shock are a gradual fall in blood pressure and a lack of response to vasoconstrictor agents. Several studies have shown that sepsis-induced hypotension is mediated, at least in part, by overproduction of nitric oxide (NO) (Petros et al., 1991; Stoclet et al., 1993). NO is formed from L-arginine by both the constitutive NO synthase, present in the vascular endothelium (endothelial NO synthase), which has a role in the physiological control of blood pressure, and the inducible NO synthase (iNOS), which is expressed in the vessel wall in response to endotoxin or cytokines (for review see Nathan, 1992). Increased inducible NO synthase mRNA is usually correlated with activation of nuclear factor-κB (NF-κB), a transcription factor that plays a critical role in the transcriptional regulation of inducible NO synthase gene induction by lipopolysaccharide (Xie et al., 1994). Nuclear factor-κB is an ubiquitous transcription factor composed of a group of nuclear proteins: c-Rel, p50/p105 (NF-κB1), p65 (Rel A), p52/p100 (NF-κB2) and Rel-B. In the cytoplasm all NF-κB proteins occur as inactive dimers since they are bound to the inhibitory protein IκB-α. Signaling stimulated by lipopolysaccharide and cytokines triggers the phosphorylation and degradation of IκB-α, resulting in the dissociation of NF-κB from IκB-α. This process allows NF-κB to migrate to the nucleus where it binds to the promoter of NF-κB-regulated genes and initiates gene transcription (Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995). Recent studies demonstrated the in vivo function of NF-κB activation in the mediation of lipopolysaccharide-induced iNOS expression in the lung and the decrease in arterial blood pressure in response to lipopolysaccharide challenge (Liu et al., 1997; Ruetten and Thiemermann, 1997). In the present study we investigated the effect of pyrrolidine dithiocarbamate, an inhibitor of NF-κB activation (Xie et al., 1994), on the tone and sensitivity to phenylephrine of

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aortas from lipopolysaccharide-treated rats and correlated these effects to NF-κB activation and iNOS protein expression.

2. Materials and methods

2.1. Isolated aorta rings

Male Wistar rats (Mario Negri Sud, Milano, Italy), weighing 280–320 g, were used for all experiments. Control animals were treated with lipopolysaccharide of *Salmonella thyphosa* (4 mg/kg, i.p.). Pyrrolidine dithiocarbamate-treated animals received, 30 min before lipopolysaccharide injection, different doses of pyrrolidine dithiocarbamate (10, 30 and 100 mg/kg, i.p.) while control rats (lipopolysaccharide alone) received the carrier vehicle (1 ml/kg, i.p., of saline solution). Naive rats received neither lipopolysaccharide nor pyrrolidine dithiocarbamate. Thoracic aortas were removed 4 h after endotoxin administration and were cut into rings with endothelium of approximately 2 mm in length and placed under 1 g of tension in organ baths containing 10 ml of Krebs solution at 37°C and bubbled with 95% O_2 and 5% CO_2 as described by Zingarelli et al. (1994). All experiments were carried out in the presence of 10 μM indomethacin. After an equilibration period of 60 min, aorta rings were exposed to phenylephrine (300 nM) and the spontaneous loss of vascular tone was recorded for 4 h. In some experiments the aorta rings, after an equilibration period of 60 min, were contracted with cumulative concentrations of phenylephrine (1 nM–10 μM) in order to obtain concentration–response curves.

2.2. Preparation of cytosolic fractions and nuclear extracts

In a separate set of experiments tissue extracts from intact thoracic aorta segments, removed 4 h after the treatments as described above, were prepared according to Iuvone et al. (1998) with some modifications. Briefly, the aorta segments were frozen in liquid nitrogen and then immediately suspended in 3 ml of ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulphonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and homogenized at the highest setting for 1 min in a Polytron PT 300 tissue homogenizer. The homogenates were divided into three aliquots of 1 ml, chilled on ice for 15 min and then vigorously shaken for 15 min in the presence of 25 μl of 10% Nonidet P-40. The nuclear fraction was precipitated by centrifugation at $1500 \times g$ for 5 min. The supernatant containing the cytosolic fraction was stored at −80°C until use. The nuclear pellet was resuspended in 600 μl of high salt extraction buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM phenylmethylsulphonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated under continuous shaking at 4°C for 30 min. The nuclear extract was then centrifuged for 15 min at $13{,}000 \times g$ and the supernatant was aliquoted and stored at −80°C. Protein concentration was determined by the Biorad protein assay kit.

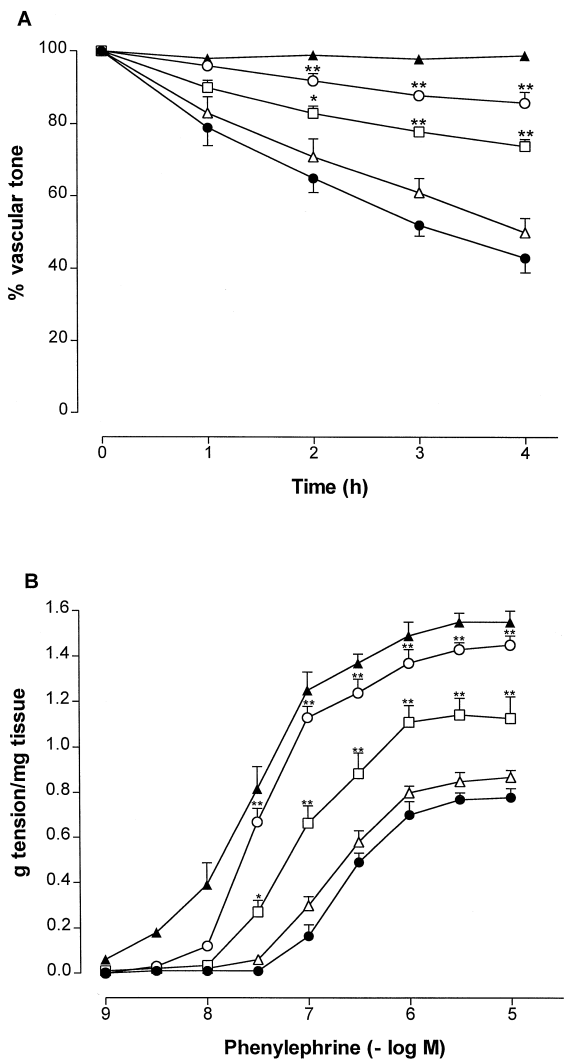

Fig. 1. (A) Time-dependent loss of tone in phenylephrine-contracted aorta rings from naive rats (▲) and lipopolysaccharide-injected rats treated with 10 (△), 30 (□) and 100 (○) mg/kg, i.p. pyrrolidine dithiocarbamate or saline (·). Pyrrolidine dithiocarbamate or saline (control group) was given 30 min before lipopolysaccharide injection. Each point represents the mean ± S.E.M. of 5–8 experiments. $*P < 0.05$; $**P < 0.01$ vs. control group. (B) Cumulative concentration–effect curves for phenylephrine in aortic rings from naive rats (▲) or lipopolysaccharide-injected rats treated with 10 (△), 30 (□) and 100 (○) mg/kg/i.p. pyrrolidine dithiocarbamate or saline (·). Pyrrolidine dithiocarbamate or saline was given 30 min before lipopolysacchariode injection. Each point represents the mean ± S.E.M. of 5–6 experiments. $*P < 0.05$; $**P < 0.01$ vs. control group.

2.3. Electrophoretic mobility shift assay (EMSA) and Western blot analysis

EMSA study and Western blot analysis were performed on nuclear and cytosolic extracts respectively as previously described (Iuvone et al., 1998).

2.4. Materials

Phosphate-buffered saline was from Celbio. DL-dithiothreitol, phenylmethylsulfonilfluoride, soybean trypsin inhibitor, pepstatin A, leupeptin and benzamidine were from Calbiochem. [^{32}P]γATP was from ICN Biomedicals. Poly dI–dC was from Boehringer Mannheim. Nonfat dry milk was from Biorad. All the other reagents were from Sigma.

2.5. Statistics

Results (mean ± S.E.M.) are expressed as grams of tension per milligram of tissue for n experiments. Statistical significance was calculated by analysis of variance (ANOVA) and Bonferroni's corrected P value for multiple comparisons. The level of statistically significant difference was defined as $P < 0.05$.

3. Results

3.1. Vascular tone and reactivity

In preliminary experiments we established that in aorta rings from animals treated with pyrrolidine dithiocarbamate alone (10, 30 and 100 mg/kg, i.p.) neither the vascular tone nor the sensitivity to phenylephrine were different from those observed in rings from naive rats. Addition of 300 nM phenylephrine to the organ bath contracted the aorta rings (80%–90% of the maximum response). In all rings the contractions reached a plateau after 30 min followed by a gradual decrease of tone over the next 4 h, except for aorta rings from naive rats in which tone was stable over this period. The loss of tone of aorta rings from control rats ($n = 8$) decreased by 21 ± 5%, 35 ± 4%, 48 ± 3% and 57 ± 4% respectively after 1, 2, 3 and 4 h. In aorta rings from pyrrolidine dithiocarbamate-treated rats (10, 30 and 100 mg/kg, i.p. 30 min before lipopolysaccharide injection), the loss of tone after 4 h was reduced in a dose-dependent fashion by 13 ± 4% ($n = 5$), 55 ± 2% ($n = 8$; $P < 0.01$) and 86 ± 3% ($n = 8$; $P < 0.01$) respectively (Fig. 1A). Furthermore, lipopolysaccharide treatment reduced the contractile response to phenylephrine (1 nM–10 μM) of aorta rings (Fig. 1B). The maximum force of contraction (g tension/mg tissue) induced by 10 μM phenylephrine in aorta rings from naive rats was 1.55 ± 0.05 ($n = 6$), whereas it was 0.78 ± 0.04 ($n = 6$; $P < 0.01$) in rings from control rats. The maximum force of contraction induced by 10 μM phenylephrine in aorta rings from pyrrolidine dithiocarbamate-treated rats (10, 30 and 100 mg/kg, i.p., 30 min before lipopolysaccharide injection) was increased in comparison to that of rings from control rats; in fact, it was 0.87 ± 0.03 ($n = 5$), 1.13 ± 0.09 ($n = 5$; $P < 0.01$) and 1.45 ± 0.04 ($n = 5$; $P < 0.01$) respectively.

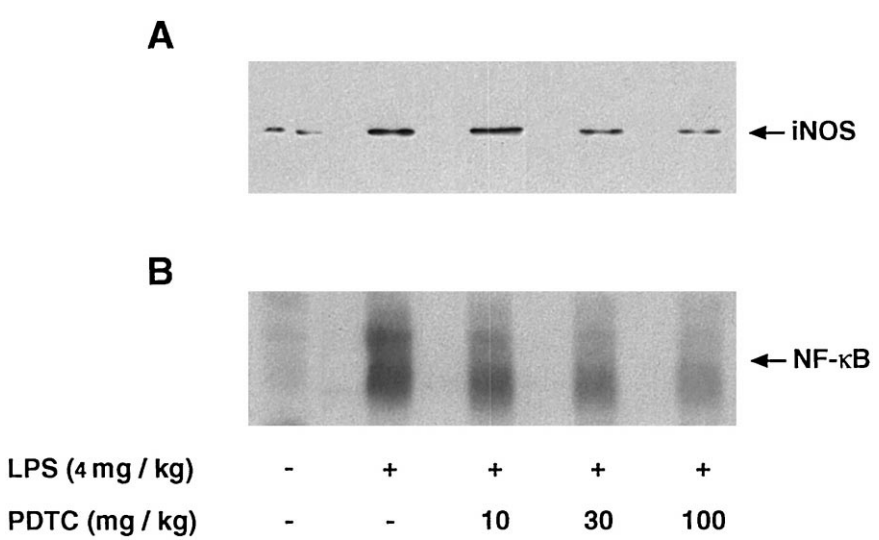

Fig. 2. (A) Western blot analysis shows the effect of pyrrolidine dithiocarbamate on inducible NO synthase protein expression in cytoplasmic extracts from rat aorta rings. The results illustrated are from a single experiment and are representative of a total of three separate experiments. (B) The EMSA shows the effect of pyrrolidine dithiocarbamate on lipopolysaccharide-induced NF-κB/DNA binding in nuclear extracts from rat aorta rings. The results illustrated are from a single experiment and are representative of a total of three separate experiments. Lipopolysaccharide (LPS); nuclear factor-κB (NF-κB); inducible NO synthase (iNOS); pyrrolidine dithiocarbamate (PDTC).

3.2. Effect of pyrrolidine dithiocarbamate on lipopolysaccharide-induced inducible NO synthase protein expression in rat aorta

Low levels of inducible NO synthase protein expression were detectable in aorta segments from naive rats. In contrast, an increase in inducible NO synthase protein expression was observed in aortas from control animals, and this increase was dose dependently prevented in aortas from pyrrolidine dithiocarbamate-treated rats (10, 30, 100 mg/kg, i.p.) (Fig. 2A).

3.3. Effect of pyrrolidine dithiocarbamate on NF-κB activation in rat aorta

A low level of NF-κB/DNA binding activity was detected in nuclear protein extracts from aortas of naive rats. Conversely, a retarded band was clearly detected in aortas from control rats. NF-κB activation was inhibited in a dose-dependent fashion by pyrrolidine dithiocarbamate treatment (10, 30 and 100 mg/kg, i.p.) (Fig. 2B). The specificity of the NF-κB/DNA binding complex was evident by the complete displacement of NF-κB/DNA binding in the presence of a 50-fold molar excess of unlabelled NF-κB probe in the competition reaction. In contrast, a 50-fold molar excess of unlabelled mutated NF-κB probe or transcription factor Sp-1 consensus oligonucleotide had no effect on DNA binding activity (data not show).

4. Discussion

The results of the present study show that in vivo inhibition of NF-κB activation by pyrrolidine dithiocarbamate dose dependently attenuates either the loss of vascular tone or the sensitivity to phenylephrine of aortas from lipopolysaccharide-treated rats. Furthermore, we have shown that the effects of pyrrolidine dithiocarbamate are closely correlated to the inhibition of NF-κB activation and reduced inducible NO synthase expression, both evaluated in aorta rings from lipopolysaccharide-treated rats. This suggests that the reduction of NF-κB activation by pyrrolidine dithiocarbamate may account for the attenuation of the decrease in tone and the reduced sensitivity to phenylephrine produced by this agent. It has previously been reported that exposure of rat aortas in vitro and in vivo to endotoxin results in a decreased responsiveness to vasoconstrictors and a progressive loss of vascular tone that occurs as a consequence of the induction of iNOS and the increased generation of NO (Knowles et al., 1990; Rees et al., 1990). Our results are in agreement with these reports since immunoblotting experiments with anti-inducible NO synthase antibody demonstrated that rat aortas obtained from lipopolysaccharide-challenged rats expressed increased levels of inducible NO synthase protein compared to those observed of naive rats. It has recently been shown that pyrrolidine dithiocarbamate and calpain inhibitor I, two structurally unrelated inhibitors of NF-κB activation, are able to attenuate the induction of inducible NO synthase protein and activity in the lung and liver of lipopolysaccharide-treated rats and consequently attenuate the circulatory failure as well as the multiple organ dysfunction (Liu et al., 1997; Ruetten and Thiemermann, 1997). Furthermore, it has been shown that pyrrolidine dithiocarbamate prolongs survival time in rats with endotoxemia and improves the septic shock syndromes both in vivo and ex vivo (Hong et al., 1998). However there is no direct evidence that correlates the systemic hypotensive response to lipopolysaccharide in vivo with NF-κB activation and inducible NO synthase protein expression in vascular tissue. In the present study we demonstrated that lipopolysaccharide-induced inducible NO synthase protein expression in rat aorta was accompanied by activation of NF-κB. In conclusion, in this study the modulation of NF-κB activation in vascular tissue may represent a critical in vivo regulatory mechanism mediating lipopolysaccharide-induced inducible NO synthase expression and the resultant regulation of vascular tone. Moreover, our results suggest that NF-κB may represent a novel molecular target for the treatment of circulatory shock or for the management of vasodilatation associated with local or systemic inflammation.

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