Experimental paper

In vitro application of endotoxin enhances nitric oxide production in thoracic aortas from Mg-deficient rats

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Abstract. Since endotoxin-induced vascular hyporeactivity to phenylephrine is enhanced in Mg-deficient rats, this study was designed to determine whether endotoxin directly enhances nitric oxide (NO) production in thoracic aortas isolated from Mg-deficient rats in vitro. Thoracic aortas isolated from Mg-deficient and control rats were cultured for 6 h with or without endotoxin (LPS). LPS (0.01-1.0 µg) increased NO production in a concentration-dependent manner. NO production in the presence of 0.1 and 1.0 µg/mL LPS was significantly higher in Mg-deficient rat aortas compared to aortas from control rats. The enhanced NO production was not significantly affected by endothelium-denudation. LPS-stimulated NO production was fully inhibited by a selective iNOS inhibitor, 1400W (0.1, 1.0 µM), in control rat aortas, but in Mg-deficient rat aortas inhibition by 1400W was only partial. A similar inhibitory effect was observed with anti-CD14 and anti-TLR4 antibodies. These results suggest that endotoxin enhances NO production in Mg-deficient rat aortas directly, and that endotoxin receptors might, at least in part, contribute to this enhancement.

Keywords: endotoxin, magnesium, aorta, NO

It is well known that endotoxin induces vascular hyporeactivity to some vasoactive agents, such as phenylephrine, in vivo [1-3] and in vitro [4, 5]. It has been suggested that endotoxin-induced vascular hyporeactivity to phenylephrine might be dependent on endotoxin-induced nitric oxide (NO) production, which stimulates NO-cyclic GMP-potassium channel pathways [5-8]. Prevention of inducible nitric oxide synthase (iNOS) expression improves survival in rodent models of endotoxic shock [9, 10]. It has been reported that magnesium ions play an important role in many cell functions [11], and their deficiency or deficit induce multiple pathological consequences [12]. Recent reports show that, in Mg-deficient rats, endotoxin lethality is enhanced [13, 14]. Previous studies by our group and others showed that endotoxin-induced vascular hyporeactivity to phenylephrine is also enhanced in Mg-deficient rat thoracic aortas ex vivo [3] and in vitro [5], and that during Mg-deficiency in rats, cytokines [14, 15] and NO [16] increase in plasma. The purpose of the present study was therefore to assess whether in vitro application of endotoxin directly enhances NO production in thoracic aortas isolated from Mg-deficient rats, and also to assess the effects of a specific iNOS inhibitor and antibodies to endotoxin-receptors on this enhancement.

Materials and methods

Animals

Twenty adult male Wistar rats (8-10-weeks-old) were fed a Mg-deficient diet (Mg: 0.001%) for 20 days.
A control group (15 rats) received a normal diet (Mg: 0.07%). The composition of the purified diet has been described in detail in a previous report [17]. The rats were pair-fed and allowed free access to deionized water. Each rat was housed individually in a stainless steel cage at an ambient temperature of 22-25 °C under a 12 h light-dark cycle.

All experiments were performed according to the Guidelines for Animal Experiments of Kagoshima University.

**Reagents**

The reagents used in this study were as follows: penicillin G, streptomycin, Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen Co., USA), endotoxin (Escherichia coli 011: B4 lipopolysaccharides (LPS)) (Sigma Chemical Co., USA), 1400W and NO2⁻ assay kit F (Wako Pure Chemical Industries, Ltd., Japan), acetylcholine (Daiichi Pharmaceutical Co., Japan), mouse anti-human Toll-like receptor 4 (TLR4) monoclonal antibody, mouse anti-human CD14 monoclonal antibody, mouse IgG2a isotype control antibody (eBioscience, USA).

**Tissue preparation**

After feeding with the semi-synthetic diets for 20 days, the rats were anesthetized with pentobarbital solution (30 mg/kg body weight, i.p.), and the thoracic aortas were isolated from the rats under sterile conditions. The aortas were cleaned of all fat and connective tissue, and cut longitudinally into four segments. To remove the endothelium, the intimal surface was rubbed gently with a swab wetted with DMEM. The absence of endothelium was verified by a lack of relaxation when acetylcholine (1.0 M) was added at the end of the experiment. Each segment was preincubated for 1 h with DMEM supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL), and then cultured for 6 h with fresh DMEM in the presence or absence of LPS.

To examine the effect of a selective iNOS inhibitor, 1400W, after 1 h preincubation of each vascular segment, 1400W was added to DMEM 30 min prior to LPS application. To examine the effects of three kinds of antisera, IgG2a, TLR4 and CD14, after 1 h preincubation, each antiserum was added to fresh DMEM in the presence or absence of LPS.

To confirm the participation of iNOS in LPS-induced NO production, the effect of a highly selective iNOS inhibitor, 1400W [22], on LPS-induced NO production was studied.

**Determination of NO production**

The stable end product of NO, NO2⁻, was estimated in the culture medium using a fluorometric NO2⁻ assay kit. This assay was performed according to the manufacturer’s instructions using a Cytofluor Multi-Well Plate Reader Series 4000 (PE Applied Biosystems, USA), and the data were extrapolated from a standard curve prepared for each assay.

**Statistical analysis**

Results are expressed as the mean ± SEM of n observations, where n represents the number of animals studied. Statistical analyses were performed using the Student’s t test or Bonferroni test after one-way analysis of variance. Statistical significance was established when the probability level was equal to or less than 5%.

**Results**

**General**

As described in our early studies [18-21], the Mg-deficient rats used in this study showed a low plasma magnesium level (0.51 ± 0.05 mEq/L) after 20 days, however, plasma levels of calcium (4.95 ± 0.05 mEq/L), potassium (5.51 ± 0.30 mEq/L) and sodium (148.2 ± 0.71 mEq/L) were not significantly different from those of the controls. They also developed hyperemia of the ears after about 8 days on Mg-deficient diet.

**NO production from thoracic aortas**

To test NO production in vitro, thoracic aortas isolated from control and Mg-deficient rats were cultured for 6 h with or without LPS.

As shown in figure 1, LPS increased NO production in a concentration-dependent manner. The degree of this increment was significantly higher in Mg-deficient rat aortas than in control rat aortas in the presence of 0.1 and 1.0 μg/mL LPS. The enhanced NO production was not affected by endothelium denudation (figure 2).

1400W attenuates increased NO production by LPS

To confirm the participation of iNOS in LPS-induced NO production, the effect of a highly selective iNOS inhibitor, 1400W [22], on LPS-induced NO production was studied.
As shown in figure 3, treatment of thoracic aortas with 1400W significantly attenuated LPS-induced NO production. However, a significantly higher production of NO was observed in Mg-deficient rat aortas than in control aortas in the presence of 1400W.

CD14 and TLR4 antibodies attenuate increased NO production by LPS

It has been suggested that LPS-mediated signaling via CD14 and TLR4 is responsible for iNOS gene induction [23].

To determine the involvement of the above LPS receptors in LPS-induced NO production, the effects of anti-CD14- and anti-TLR4-antibodies were examined.

As shown in figure 4, LPS-induced NO production was significantly attenuated by anti-CD14- or anti-TLR4 antibodies. Treatment with a mouse isotype-matched control antibody (anti-IgG2a) did not affect LPS-induced NO production.

LPS-induced NO production in the presence of anti-CD14- or anti-TLR4 antibodies was higher in Mg-deficient rat aortas than in control aortas.

Discussion

Our previous ex vivo study showed that endotoxin-induced hyporeactivity to phenylephrine was enhanced in Mg-deficient rats, and suggested that some cytokines, such as interleukin-1β and tumor necrosis factor-α, might contribute to this enhancement [3].

The present results show that endotoxin directly stimulates NO production by the thoracic aorta. These data partly support those of previous studies, in which endotoxin was shown to stimulate iNOS expression and NO production in aortas and vascular smooth muscle cells [24-27].

As shown in figures 1 and 2, endotoxin-induced enhancement of NO production was observed in
Mg-deficient rat aortas, and this enhancement was not affected by removing endothelial cells. These results suggest that the enhancement of endotoxin-induced NO production does not depend on endothelial nitric oxide synthase (eNOS). The following results strongly support the non-dependency on eNOS in endotoxin-induced NO production, which show a selective iNOS inhibitor, 1400W, suppressed endotoxin-induced NO production to basal levels in control rat thoracic aortas. In Mg-deficient rat thoracic aortas, however, the suppression with 1400W was significantly weaker than in control rats, and endotoxin-induced NO production was not suppressed to basal levels (figure 3). These results suggest that endotoxin induces much more iNOS activity in thoracic aortas from Mg-deficient rats than in those from control rats. The result was similar to previous data in which NOS activity in lungs was not significantly different between control and Mg-deficient rats, but its induction by endotoxin was significantly higher in Mg-deficient rats than control rats [16]. In the absence of endotoxin, NO production from Mg-deficient rat thoracic aortas was not significantly different from control aortas. The data suggest that the amount of iNOS in thoracic aortas might not be affected by Mg-deficiency. Our previous data showed that iNOS mRNA level in thoracic aorta was higher in Mg-deficient rats than control ones [5]. In this experiment, however, the NO production from Mg-deficient rat’s aorta without stimulation of endotoxin was not higher than that from control rats. The precise reason is unclear, but it might be dependent on the higher sensitivity of the assay method of mRNA than that of NO. Endotoxin enhanced vascular hyporeactivity to phenylephrine much more in Mg-deficient rats’ thoracic aortas than control ones, and its effect was observed at 0.01 μg/mL endotoxin [5]. However, in this experiment enhanced production of NO was not observed at this dose of endotoxin (figure 1). This difference might be dependent on the higher sensitivity to NO in Mg-deficient rat aorta [20, 21].

A recent study using cDNA array technology showed that most genes (78%) in rat neutrophils were expressed at a level more than two-fold higher in Mg-deficient rats compared to control rats [28]. These data suggest the possibility that endotoxin receptors on the thoracic aorta might increase during Mg-deficiency. Some studies have indicated that TLR4 and CD14 might mediate a response to endot-

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**Figure 2.** Effect of endothelium-denudation on NO production from thoracic aortas of Mg-deficient rats (n = 5).
oxin [29-32]. Recent reports have shown that both CD14 and TLR4 are required for endotoxin-induced iNOS induction in chicken macrophages [32].

Therefore, we speculated that during Mg-deficiency the expression of endotoxin receptors might be upregulated. To confirm this speculation, the effect of specific antibodies to TLR4 and CD14 on NO production from the thoracic aorta was examined. As shown in figure 4, both antibodies significantly suppressed endotoxin-induced NO production. However, the degree of suppression was significantly weaker in Mg-deficient rat aortas than in control aortas. These data strongly suggest that a greater number of endotoxin receptors leads to stronger signal transduction and ultimately higher iNOS expression and activity in Mg-deficient rat aortas than in control aortas. Data reported recently, which showed the presence of CD14 on vascular smooth muscle cells [33], support, at least in part, the above suggestion.

It has been suggested that Mg deficiency induces hyperlipidaemia, which plays an important role in the pathogenesis of vascular injury [39]. Mg deficiency was shown to enhance vascular endothelial injury, thus promoting the development and progression of atherosclerosis [40, 41]. As shown in figure 2, however, endotoxin-induced NO production of Mg-deficient rat aortas was not affected by removing endothelial cells. From the present results, the endothelial cells might be injured and endothelial dysfunction for spontaneous NO production might be occurring. However, the relation between NO production and atherosclerosis during Mg deficiency is unclear.

Further studies should focus on the complex relationship between NO, vascular hyporeactivity and atherosclerosis for a better understanding of the
pathophysiology of endotoxin shock during Mg deficiency.

**Conclusion**

The present data demonstrate that endotoxin directly stimulates NO production by the thoracic aorta, and that its production is enhanced in Mg-deficient rats. The data also suggest that endotoxin receptors might contribute to this enhancement.

**References**


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