ELSEVIER

European Journal of Pharmacology 281 (1995) 251–254

ejp

# Hyporesponsiveness to nitrovasodilators in rat aorta incubated with endotoxin and L-arginine

Shinya Tsuchida *, Masahiro Hiraoka, Masakatsu Sudo, Ikunobu Muramatsu

*Departments of Pediatrics and Pharmacology, Fukui Medical School, Matsuoka, Fukui 910-11, Japan*

Received 21 November 1994; revised 19 April 1995; accepted 20 April 1995

**Abstract**

We studied the effect of L-arginine on relaxation responses to sodium nitroprusside or SIN-1 (3-morpholinosydonimine-*N*-ethyl-carbamine) in the rat thoracic aorta incubated with endotoxin. Sodium nitroprusside or SIN-1 produced a reproducible relaxation in the aorta incubated for 12 h with endotoxin. However, the response to both nitrovasodilators was remarkably attenuated when the aorta was preincubated for 12 h with endotoxin and L-arginine. D-Arginine could not substitute for L-arginine. The attenuated response to sodium nitroprusside or SIN-1 was partially restored by the inhibition of nitric oxide (NO) production with $N^{\omega}$-nitro-L-arginine. Cycloheximide prevented the inhibitory effect of preincubation with L-arginine. These results suggest that the prolonged exposure to muscle-derived NO induces hyporesponsiveness to nitrovasodilators.

*Keywords:* Nitric oxide (NO); Muscle-derived nitric oxide (NO); Endotoxin; L-Arginine; Nitrovasodilator; Tolerance

## 1. Introduction

Sodium nitroprusside and other nitrovasodilators exert their pharmacological functions by releasing nitric oxide (NO) (Murad et al., 1978; Braughler et al., 1979; Ignarro et al., 1981). NO is released from not only exogenous nitrovasodilators but also from endogenous sources such as vascular endothelial cells, macrophages, neutrophils and hepatic Kupffer cells (for review: Moncada et al., 1991a). Moncada et al. (1991b) demonstrated that the continuous production of endothelium-derived NO could result in a reduced sensitivity of smooth muscle to nitrovasodilators, suggesting an endogenous-exogenous NO interaction. However, vascular smooth muscle cells themselves were shown to produce NO (muscle-derived NO) following the induction of NO synthase with endotoxin or cytokines (Busse and Mülsch, 1990; Nunokawa et al., 1993), which may be responsible for the hypotension during endotoxin shock (Wakabayashi et al., 1987; Takakura et al., 1994). In view of these observations, we speculated that the muscle-derived NO, like endothelium-derived NO, might induce subsensitivity to nitrovasodilators.

To verify this possibility, aortic strips were incubated with endotoxin in bath medium containing L-arginine (NO precursor) in order to enhance the production of muscle-derived NO, and then the effect of L-arginine on relaxation responses to sodium nitroprusside and SIN-1 (3-morpholinosydonimine-*N*-ethyl-carbamine) was examined.

## 2. Materials and methods

### 2.1. Chemicals

Phenylephrine hydrochloride, sodium nitroprusside, acetylcholine, 8-bromo-cyclic GMP (8-Br-cyclic GMP) and $N^{\omega}$-nitro-L-arginine were purchased from Sigma Chemical Co. (St. Louis, MO), L-arginine, cycloheximide and papaverine hydrochloride were from Nacalai tesque (Kyoto, Japan). SIN-1 (3-morpholinosydonimine-*N*-ethyl-carbamine) was from Funakoshi (Tokyo, Japan) and Escherichia coli lipopolysaccharide serotype 055:B5 was from Difco Laboratories (Detroit, MI).

* Corresponding author. Department of Pediatrics, Fukui Medical School, Matsuoka, Fukui 910-11, Japan. Tel. 81-0776-61-3111, fax 81-0776-61-8129.


*SSDI* 0014-2999(95)00245-6

### 2.2. Functional studies

Male Wistar rats aged 6–8 weeks (180–260 g) were killed and the thoracic aorta was rapidly removed. The aorta was cleaned of adherent connective tissue and cut helically under a dissecting microscope. The strips were mounted under 1 g of resting tension in 20 ml organ baths containing a modified Krebs-Henseleit solution of the following composition (mM): NaCl 112, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25.0, $NaH_2PO_4$ 1.2 and glucose 11.5, at 37°C and bubbled with 95% $O_2$:5% $CO_2$ (pH 7.4). Developed tension was recorded isometrically with force-displacement transducers (Nihon Kohden TB-651T). The endothelial cells were removed by rubbing them with filter paper, and their removal was confirmed by the loss of the relaxant response to acetylcholine. 60 and 90 min after setting up, the responses to sodium nitroprusside, SIN-1, L-arginine, 8-Br-cyclic GMP or papaverine were recorded on strips precontracted with the concentration of phenylephrine (1 μM) which produced nearly maximal contraction (approximately 90%) throughout the experiments. The second response to each drug served as control and this step was considered the beginning (0 h incubation) of the experimental protocol. After recording of the second response (control), the strips were incubated with 0.1 μg/ml endotoxin (lipopolysaccharide) and/or L-arginine. At an appropriate incubation time (9 h and 12 h incubation), the bath medium was exchanged to L-arginine-free medium and then 1 μM phenylephrine was added to test the relaxation response to sodium nitroprusside, SIN-1 or L-arginine. In some experiments, after the final responses were recorded at 12 h, the aortic strips were washed and then treated with 100 μM $N^{\omega}$-nitro-L-arginine for 30 min before concentration-response curves for sodium nitroprusside or SIN-1 were measured. To study the effects of cycloheximide, aortic strips were immersed in the medium containing 10 μM cycloheximide immediately after isolation and throughout the experiments.

### 2.3. Statistical analysis

Experimental values are given as the means ± S.E. of six to ten experiments. Results were analyzed by using Bonferroni/Dunnett's test or Student's *t*-test. Probability of less than 0.05 was considered significant.

## 3. Results

### 3.1. Effects of preincubation with lipopolysaccharide and L-arginine

Sodium nitroprusside (0.001–10 μM) and SIN-1 (0.01–100 μM) produced concentration-dependent relaxations in the rat endothelium-denuded aorta. The responses changed little up to 12 h in the aorta preincubated with 0.1 μg/ml lipopolysaccharide only (Fig. 1A,C). However, when the aortic strips were preincubated with 0.1 μg/ml lipopolysaccharide and 1000 μM L-arginine for appropriate periods and then washed with L-arginine-free medium, the subsequent responses to sodium nitroprusside and SIN-1 were attenuated; at 12 h preincubation the maximum relaxation was 60 ± 5% and 71 ± 3%, respectively (Fig. 1B,D, open triangles). Such an inhibitory effect of L-arginine-preincubation on the responses to sodium nitroprusside or SIN-1 was concentration-dependent (10–1000 μM), while D-arginine (100 and 1000 μM) had no effect on the responses to sodium nitroprusside and SIN-1 (Fig. 2). In contrast, no attenuation of sodium nitroprusside or SIN-1 responses was produced in the aorta coincubated with cycloheximide (10 μM). Furthermore, after

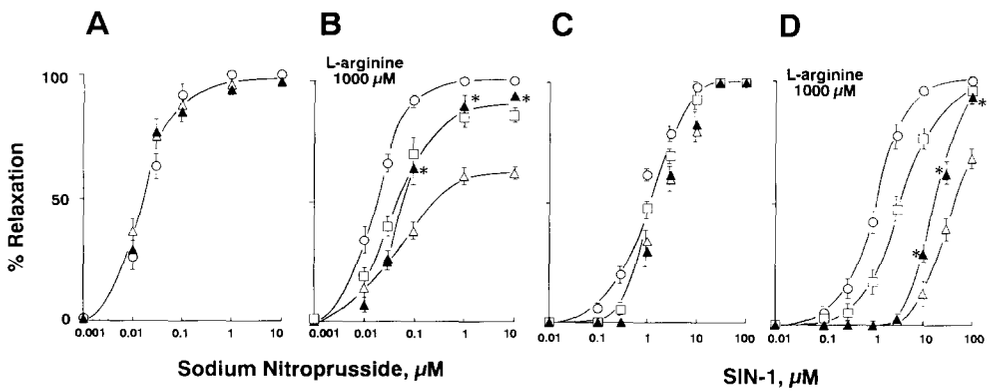


Fig. 1. Concentration-relaxation curves for sodium nitroprusside or SIN-1 in the rat thoracic aorta incubated for 0 h (○), 9 h (□), 12 h (△) with 0.1 μg/ml lipopolysaccharide. In B and D, L-arginine (1000 μM) was present during the preincubation period, but the response to sodium nitroprusside or SIN-1 was recorded in L-arginine-free medium after the preincubation. Closed triangles (▲) indicate the response to sodium nitroprusside or SIN-1 in the presence of $N^{\omega}$-nitro-L-arginine (100 μM), which was obtained after recording the responses at 12 h. Data shown are the means ± S.E. of six to ten experiments. * Significant difference between (△) and (▲) ($P < 0.05$, Student's *t*-test).

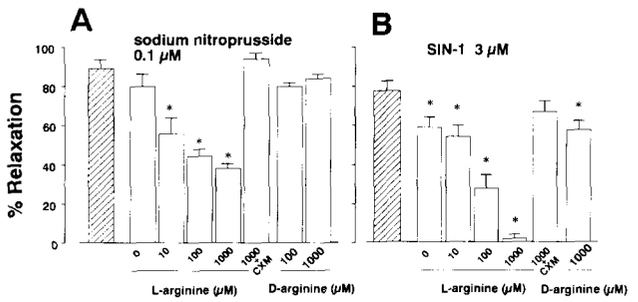


Fig. 2. Effects of preincubation of lipopolysaccharide and L-arginine, D-arginine or cycloheximide (CXM) on the relaxation response to sodium nitroprusside (A) or SIN-1 (B) in the rat thoracic aorta. The aorta was preincubated for 12 h with lipopolysaccharide (0.1 μg/ml) and the drugs indicated under each column. Then, the response to 0.1 μM sodium nitroprusside or 3 μM SIN-1 was recorded in L- or D-arginine-free medium. Shaded column: the response at 0 h incubation. Data shown are the means ± S.E. of six to eight experiments. * Significantly different from the 0-h response ($P < 0.05$, Bonferroni/Dunnett's test).

preincubation with lipopolysaccharide and L-arginine for 12 h, the attenuated response to sodium nitroprusside or SIN-1 was significantly restored by additional treatment with 100 μM $N^{\omega}$-nitro-L-arginine ($P < 0.05$) (Fig. 1B,D, closed triangles). The contractile responses to 1 μM phenylephrine were gradually attenuated with an increase in the period of preincubation with lipopolysaccharide only (0.92 ± 0.04 and 0.60 ± 0.05 g/mg wet weight at 0 and 12 h preincubation, respectively, $n = 6$). Similarly, the preincubation with lipopolysaccharide and L-arginine for 12 h followed by the removal of L-arginine attenuated the contractile response to 1 μM phenylephrine (0.52 ± 0.06 g/mg wet weight), which was not significantly different from the response of the aorta preincubated with lipopolysaccharide only for 12 h. The relaxation responses to papaverine or 8-Br-cyclic GMP were not significantly different between the aortic strips preincubated with and without L-arginine for 12 h (data not shown).

### 3.2. Relaxation response to L-arginine

L-Arginine (1–1000 μM) itself evoked a concentration-dependent relaxation in the aorta preincubated with 0.1 μg/ml lipopolysaccharide. This relaxation began to be observed after 3 h incubation and gradually developed with an increase in the incubation time, reaching a plateau (92 ± 3% relaxation, $n = 6$) at 9–12 h. However, L-arginine failed to produce a relaxation in the aortic strips preincubated with lipopolysaccharide and cycloheximide (10 μM).

## 4. Discussion

NO synthase is induced after treatment with lipopolysaccharide and/or cytokines in various cell types including rat vascular smooth muscle cells (Busse and Mülsch, 1990; Nunokawa et al., 1993). L-arginine serves as a precursor for muscle-derived NO and provokes a continuous production of NO (Moritoki et al., 1991). In the present study, L-arginine elicited a relaxation after preincubation with lipopolysaccharide for more than 3 h, whereas in the aorta preincubated with lipopolysaccharide and cycloheximide, which prevents the induction of endotoxin-inducible NO synthase (Rees et al., 1990; Moritoki et al., 1992), L-arginine failed to cause relaxation. Therefore, it is likely that NO synthase is induced after incubation for more than 3 h with lipopolysaccharide, resulting in a sustained production of muscle-derived NO after preincubation with L-arginine.

The aorta preincubated with lipopolysaccharide only produced reproducible relaxations in response to sodium nitroprusside or SIN-1 for 12 h. However, when the aorta was preincubated with lipopolysaccharide and L-arginine, a remarkable attenuation of the sodium nitroprusside or SIN-1 response was observed. This attenuation does not appear to reflect non-specific hyporesponsiveness of the aorta to relaxant drugs, because the relaxation responses to 8-Br-cyclic GMP and papaverine were not inhibited even when the aortic strips had been preincubated with or without L-arginine. The attenuation of the response to sodium nitroprusside or SIN-1 was specifically produced by L-arginine, and D-arginine (an enantiomer of L-arginine) was without effect. Such an inhibitory effect of L-arginine was evoked in a concentration-dependent manner. Therefore, the inhibitory effect of L-arginine seems to result from the prolonged exposure to muscle-derived NO, which is generated by the lipopolysaccharide-inducible NO synthase. The findings that co-incubation with cycloheximide completely blocked the induction of the inhibitory effect of L-arginine and that $N^{\omega}$-nitro-L-arginine (a NO synthase inhibitor) partially restored the attenuated response to sodium nitroprusside or SIN-1 further support the above possibility.

A long preincubation with lipopolysaccharide and L-arginine for 12 h was necessary for the development of the attenuation of sodium nitroprusside or SIN-1 responses, suggesting the requirement of a prolonged exposure to muscle-derived NO. Further, the hyporesponsiveness to nitrovasodilators was maintained even after washout of L-arginine from the medium. It is likely that the exposure to muscle-derived NO could be sustained by using endogenous L-arginine. By contrast, the inhibition of NO synthesis by $N^{\omega}$-nitro-L-arginine for 30 min partially restored the hyporesponsiveness to nitrovasodilators, indicating that part of the hyporesponsiveness is acutely reversible after removal of NO.

The mechanisms for the attenuation of the sodium nitroprusside or SIN-1 response by the prolonged exposure to muscle-derived NO remain to be explored.

Since the response to 8-Br-cyclic GMP was not affected, it is likely that muscle-derived NO induces the attenuation of sodium nitroprusside or SIN-1 responses as a result of a decrease in soluble guanylate cyclase activation, an effect that has been described for exogenous NO as tolerance to nitrovasodilators (Keith et al., 1982). In addition, the muscle-derived NO might cause an impairment of NO-formation from sodium nitroprusside or SIN-1, as has previously been reported for the tolerance to glyceryl trinitrate (Chung and Fung, 1990; Kojda et al., 1994).

Such hyporesponsiveness to nitrovasodilators might be also observed in vivo as a result of an endogenous-exogenous NO interaction. Actually, Moncada et al. (1991b) demonstrated that the removal of endothelium-derived NO by the administration of a NO synthase inhibitor leads to a specific supersensitivity to nitrovasodilators in vivo. However, Salvemini and coworkers (Salvemini et al., 1992) observed no change in the hypotensive response to SIN-1 in rats treated with lipopolysaccharide for 18 h. Reasons for this discrepancy are now unknown, but it is interesting to note that NO synthase induction is transient in lipopolysaccharide-treated rats (Szabo et al., 1994).

In summary, the present study demonstrates that the responsiveness to nitrovasodilators is remarkably attenuated by exposure to muscle-derived NO of the rat aorta preincubated with lipopolysaccharide and L-arginine.

## Acknowledgements

We thank N. Aoki for secretarial assistance. This study was supported in part by Grants from the Ministry of Education, Science and Culture, Japan and from the Smoking Research Foundation of Japan.

## References

Braughler, J.M., C.K. Mittal and F. Murad, 1979, Effects of thiol, sugars, and protein on nitric oxide activation of guanylate cyclase, J. Biol. Chem. 254, 12450.

Busse, R. and A. Mülsch, 1990, Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells, FEBS Lett. 275, 87.

Chung, S.J. and H.L. Fung, 1990, Identification of the subcellular site for nitroglycerin metabolism to nitric oxide in bovine coronary smooth muscle cells, J. Pharmacol. Exp. Ther. 253, 614.

Ignarro, L.J., H. Lippton, J. Edwards and W. Baricos, 1981, Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of *S*-nitrosothiols as active intermedeates, J. Pharmcol. Exp. Ther. 218, 739.

Keith, R.A., A.M. Burkman, T.D. Sokoloski and R.H. Fertel, 1982, Vascular tolerance to nitroglycerin and cyclic GMP generation in rat aortic smooth muscle, J. Pharmacol. Exp. Ther. 221, 525.

Kojda, G., J.K. Beck, W. Meyer and E. Noack, 1994, Nitrovasodilator-induced relaxation and tolerance development in porcine vena cordis magna: dependence on intact endothelium, Br. J. Pharmacol. 112, 533.

Moncada, S., R.M. Palmer and E.A. Higgs, 1991a, Nitric oxide: physiology, pathophysiology, and pharmacology, Pharmacol. Rev. 43, 109.

Moncada, S., D.D. Rees, R. Schulz and R.M. Palmer, 1991b, Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis in vivo, Proc. Natl. Acad. Sci. USA 88, 2166.

Moritoki, H., H. Ueda, T. Yamamoto, T. Hisayama and S. Takeuchi, 1991, L-Arginine induces relaxation of rat aorta possibly through non-endothelial nitric oxide formation, Br. J. Pharmacol. 102, 841.

Moritoki, H., S. Takeuchi, T. Hisayama and W. Kondoh, 1992, Nitric oxide synthase responsible for L-arginine-induced relaxation of rat aortic rings in vitro may be an inducible type, Br. J. Pharmacol. 107, 361.

Murad, F., M. CK, A. WP, K. S and K. H, 1978, Guanylate cyclase: activation by azide, nitro compounds, nitric oxide, and hydroxy radical and inhibition by hemoglobin and myoglobin, Adv. Cycl. Nucl. Res. 9, 145.

Nunokawa, Y., N. Ishida and S. Tanaka, 1993, Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 191, 89.

Rees, D.D., S. Cellek, R.M. Palmer and S. Moncada, 1990, Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock, Biochem. Biophys. Res. Commun. 173, 541.

Szabo, C., C. Thiemermann, C.C. Wu, M. Perretti and J.R. Vane, 1994, Attenuation of the induction of nitric oxide synthase by endogenous glucocorticoids accounts for endotoxin tolerance in vivo. Proc. Natl. Acad. Sci. USA 91, 271.

Salvemini, D., V. Mollace, A. Pistelli, E. Änggård and J. Vane, 1992, Metabolism of glyceryl trinitrate to nitric oxide by endothelial cells and smooth muscle cells and its induction by *Escherichia coli* lipopolysaccharide, Proc. Natl. Acad. Sci. USA 89, 982.

Takakura, K., Y. Goto, S. Kigoshi and I. Muramatsu, 1994, Comparison between the effects of treatment in vitro and in vivo with lipopolysaccharide on responsiveness of rat thoracic aorta, Circ. Shock 42, 141.

Wakabayashi, I., K. Hatake, E. Kasashita and K. Nagai, 1987, Diminition of contractile response of the aorta from endotoxin-injected rats., Eur. J. Pharmacol. 141, 117.