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Short communication

Reversal of in vitro lipopolysaccharide-induced suppression of contraction in rat aorta by N^G-nitro-arginine, diphenyleneiodonium and di-2-thienyliodonium

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Abstract

The effects of N^G-nitro-L-arginine (L-NNA), D-NNA, diphenyleneiodonium and di-2-thienyliodonium on contraction were studied in endothelium-denuded rat aortic rings incubated for 4 h with lipopolysaccharide (10 μg ml^{-1}) or vehicle. Lipopolysaccharide reduced E_{max} and increased EC_{50} of the phenylephrine (10^{-9}–10^{-5} M) curve. Addition of D-NNA (4, 6×10^{-4} M), L-NNA (1, 10×10^{-6} M) and diphenyleneiodonium (1, 3×10^{-7} M), but not di-2-thienyliodonium (10^{-7} M), increased E_{max} and reduced EC_{50} of the phenylephrine curve of lipopolysaccharide-incubated but not control rings. Therefore, D-NNA, L-NNA and diphenyleneiodonium, but not di-2-thienyliodonium, inhibit inducible NO synthase in vascular smooth muscles.

Keywords: D-NNA (N^G-nitro-D-arginine); Diphenyleneiodonium; Di-2-thienyliodonium; Methylene blue; Nitric oxide (NO); Lipopolysaccharide; Contraction

1. Introduction

The exposure of vascular smooth muscle cells to bacterial lipopolysaccharide or cytokines is known to activate inducible nitric oxide (NO) synthase, generate large amounts of NO and diminish contractile activity (Schott et al., 1993; Shibano and Vanhoutte, 1993). Lipopolysaccharide and cytokines also decrease pressor response in vivo (Schott et al., 1993; Shibano and Vanhoutte, 1993). Lipopolysaccharide-induced vascular hyporeactivity in vivo and in vitro is inhibited by analogues of L-arginine (L-Arg), e.g., N^G-monomethyl-L-Arg (L-NMMA), N^G-nitro-L-Arg methyl ester (L-NAME) and N^G-nitro-L-Arg (L-NNA) (Gray et al., 1991; Schott et al., 1993; Joly et al., 1994).

N^G-substituted arginine analogues are widely believed to be enantiomerically specific such that the L- but not D-enantiomers inhibit endothelial NO biosynthesis, suppress endothelium-dependent relaxation of isolated blood vessels and raise blood pressure. L-NMMA but not D-NMMA restored contractile response to noradrenaline in endothelium-denuded aortic rings incubated with lipopolysaccharide in vitro (Fleming et al., 1990) and pressor response to noradrenaline in lipopolysaccharide-treated rats (Gray et al., 1991). Results from our laboratory, however, show that both L-NNA and D-NNA raise blood pressure (Wang et al., 1991) and inhibit endothelium-dependent relaxation in vivo, in vitro and ex vivo (Wang et al., 1993a) suggesting that D-NNA also inhibits the activity of endothelial NO synthase. In vivo studies show that D-NNA is as efficacious as L-NNA in raising blood pressure but is 2-fold less potent and has a slower (> 5-fold) onset of action (Wang et al., 1991). D-NNA is 40-fold less potent than L-NNA in inhibiting endothelium-dependent relaxation in vitro (Wang et al., 1993a). However, L-NNA but not D-NNA caused sustained contraction of endothelium-denuded aortic rings (Wang and Pang, 1994). It is unclear if D-NNA reverses lipopolysaccharide-induced vascular hyporesponsiveness.

A group of iodonium compounds, e.g., diphenyleneiodonium and di-2-thienyliodonium, have been shown

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to inhibit a flavoprotein coupled to NADPH-dependent enzymes (Stuehr et al., 1991), suppress activities of NO synthase in the macrophage, inhibit endothelium-dependent relaxation (Stuehr et al., 1991; Rand and Li, 1993; Wang et al., 1993b) and inhibit acetylcholine but not sodium nitroprusside-induced depressor response in conscious rats (Wang et al., 1993b). It needs to be verified if diphenyleneiodonium and di-2-thienyliodonium inhibit the activity of inducible NO synthase thereby reversing lipopolysaccharide-induced vascular hyporeactivity.

The aim of this study is to compare the effects of L-NNA with those of D-NNA, diphenyleneiodonium and di-2-thienyliodonium on lipopolysaccharide-induced loss of contractile responsiveness in endothelium-denuded rat aorta.

2. Materials and methods

2.1. Preparation of aortic rings

Male Sprague-Dawley rats (300–360 g) were killed by a blow on the head followed by exsanguination. The thoracic aorta was removed and cut into four 0.5 cm ring segments. The vascular endothelium was removed by gently rolling a blunt needle in the ring lumen. The rings were incubated for 4 h at 37°C in a cell culture incubator gassed with 95% air and 5% CO_2 in 3 ml of Dulbecco's modified Eagle's/Ham's F12 medium (D-MEM/F-12, Gibco, NY, USA) in the presence of lipopolysaccharide (10 μg ml^{-1}) or vehicle (30 μl of 0.9% saline, control). Some rings ($n = 6$ rings from different rats) were exposed to vehicle + polymyxin B (1 μg ml^{-1}) to assess if possible bacterial endotoxin contamination of the culture medium affects contraction. After incubation, the rings were suspended between two hooks at a passive force of 2 g, and placed in random order in separate baths containing Krebs' solution at pH 7.4 and 37°C as described in Wang et al. (1993a, b). After 60 min equilibration, the rings were pre-constricted with phenylephrine (EC_{90} at 10^{-6} M) followed by exposure to acetylcholine (2×10^{-6} M). All rings were considered to have undergone complete endothelial denudation as none relaxed to acetylcholine. The endothelium-denuded, control or lipopolysaccharide-treated rings were each separated into 11 groups ($n = 6$–8 rings from different rats).

After another 60 min, the rings were incubated for 20 min with vehicle (0.9% NaCl), L-NNA (10^{-6}, 10^{-5} M), D-NNA (3, 4 and 6×10^{-4} M), diphenyleneiodonium (1, 3 and 10×10^{-7} M) or di-2-thienyliodonium (10^{-7}, 10^{-6} M) followed by phenylephrine (10^{-9}–10^{-5} M). At the steady state response to phenylephrine (10^{-5} M), L-Arg (10^{-3} M) was added. Once the response to L-Arg stabilized (10–15 min), methylene blue (3×10^{-6} M) was added and the contraction was monitored for another 15 min. Afterwards, the rings were removed, dried overnight at 30°C and weighed.

2.2. Drugs

Phenylephrine, acetylcholine, methylene blue, L-Arg HCl, L-NNA, polymyxin B and lipopolysaccharide (*Escherichia coli* serotype 055:B5) were from Sigma Chemical Co. (MO, USA). D-NNA was from Bachem Bioscience (Philadelphia, PA, USA). Diphenyleneiodonium and di-2-thienyliodonium were from Colour Your Enzyme (Ontario, Canada). Diphenyleneiodonium and di-2-thienyliodonium were dissolved in 5% glucose so-

Table 1
Effects of 20-min treatment with D-NNA, L-NNA, diphenyleneiodonium (DPI), di-2-thienyliodonium (DTI) or vehicle (0.9% NaCl) on EC_{50} and E_{max} (mean ± S.E.M.) of the concentration-response curves to phenylephrine (PE) in endothelium-denuded rat aortic rings ($n = 6$–8 rats) incubated for 4 h in a medium containing lipopolysaccharide (LPS) (10 μg ml^{-1}) or vehicle (0.9% NaCl, control)

Treatment		PE concentration-response curve			
		EC_{50} ($\times 10^{-9}$ M)		E_{max} (g per mg dried tissue)	
		Control	LPS	Control	LPS
Vehicle (0.9% NaCl)		22 ± 3	123 ± 15 [a]	1.41 ± 0.06	1.00 ± 0.04 [a]
D-NNA:	3×10^{-4} M	22 ± 4	101 ± 19 [a]	1.33 ± 0.17	1.25 ± 0.09
	4×10^{-4} M	22 ± 2	42 ± 8 [b]	1.40 ± 0.12	1.47 ± 0.15 [b]
	6×10^{-4} M	38 ± 1	52 ± 16 [b]	1.45 ± 0.16	1.53 ± 0.15 [b]
L-NNA:	1×10^{-6} M	24 ± 6	78 ± 18 [a]	1.33 ± 0.13	1.36 ± 0.13 [b]
	1×10^{-5} M	23 ± 8	44 ± 6 [b]	1.42 ± 0.08	1.43 ± 0.07 [b]
DPI:	1×10^{-7} M	24 ± 6	80 ± 18 [a]	1.36 ± 0.10	1.38 ± 0.07 [b]
	3×10^{-7} M	22 ± 2	43 ± 9 [b]	1.29 ± 0.17	1.47 ± 0.10 [b]
	1×10^{-6} M	102 ± 16 [b]	101 ± 17	1.18 ± 0.08 [b]	1.11 ± 0.08
DTI:	1×10^{-7} M	36 ± 6	121 ± 21 [a]	1.41 ± 0.08	1.19 ± 0.10
	1×10^{-6} M	152 ± 61 [b]	192 ± 55	1.22 ± 0.06 [b]	1.35 ± 0.08 [b]

[a] Significantly different ($P < 0.05$) from the respective control group. [b] Significantly different ($P < 0.05$) from the corresponding values of EC_{50} or E_{max} of vehicle-treated LPS-incubated aorta or control aorta.

lution but all other drugs were dissolved in normal saline (0.9% NaCl).

2.3. Calculations and statistical analysis

The results are expressed as g of tension generated per mg dried tissue (mean ± S.E.M.) and analyzed by analysis of variance/co-variance followed by Duncan's multiple range test, with $P < 0.05$ as the criterion for statistical significance. EC_{50} and E_{max} were calculated from individual phenylephrine curves by non-linear regression (Wang and Pang, 1993).

3. Results

3.1. Effect of lipopolysaccharide on phenylephrine curve

Incubation of endothelium-denuded aortic rings with vehicle + polymyxin for 4 h did not affect EC_{50} ($20 \pm 3 \times 10^{-9}$ M) or E_{max} (1.38 ± 0.10 g per mg dried tissue) of the concentration-response curve to phenylephrine, relative to values in rings incubated with the vehicle (Table 1). Thus, there was negligible contamination of the vehicle-incubated rings with bacterial lipopolysaccharide. Therefore, polymyxin was not added to the vehicle groups in the remaining studies. Incubation with lipopolysaccharide caused a rightward shift of the phenylephrine curve causing > 5-fold increase in EC_{50} and a decrease in E_{max} (Table 1).

3.2. Effects of L-NNA, D-NNA, diphenyleneiodonium and di-2-thienyliodonium on phenylephrine curve

Neither L-NNA, D-NNA, diphenyleneiodonium nor di-2-thienyliodonium affected baseline tension of vehicle- or lipopolysaccharide-treated aortic rings during the 20-min incubation. Neither D-NNA nor L-NNA affected the phenylephrine curve of control rings incubated with the vehicle (Table 1). In rings incubated with lipopolysaccharide, D-NNA and L-NNA caused a left and upward shift of the phenylephrine curve resulting in concentration-related reductions in EC_{50} and increases in E_{max} (Fig. 1A; Table 1).

In vehicle-incubated control rings, diphenyleneiodonium (1 or 3×10^{-7} M) did not affect either the E_{max} or EC_{50} of the phenylephrine curve (Table 1), but the high concentration (10^{-6} M) increased the EC_{50} and reduced E_{max}. In lipopolysaccharide-incubated rings, the two low concentrations of diphenyleneiodonium caused a left and upward shift of the phenylephrine curve (Fig. 1B), resulting in an increase in E_{max} and a decrease in EC_{50}, relative to the those of lipopolysaccharide-incubated rings not treated with diphenyleneiodonium (Table 1). The highest concentration of diphenyleneiodonium, however, shifted the phenylephrine curve back towards that of the vehicle-treated, lipopolysaccharide-incubated rings (Fig. 1B; Table 1).

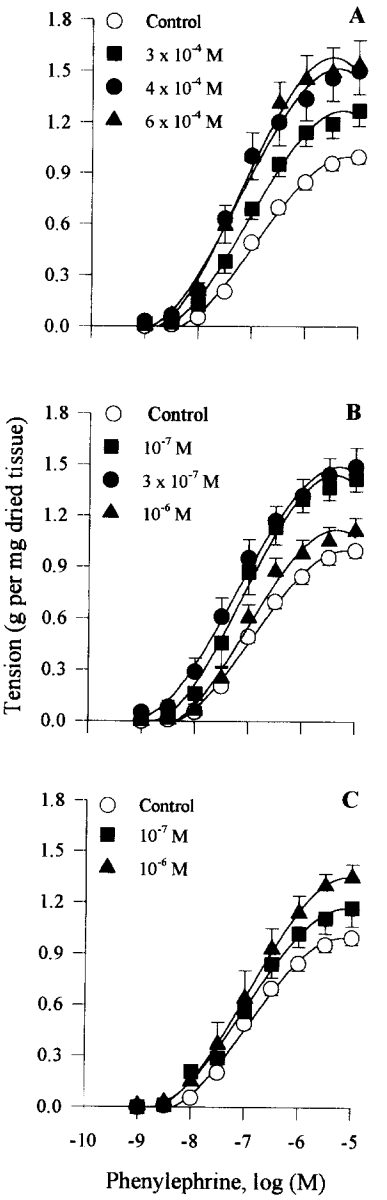

Fig. 1. Effects (mean ± S.E.M.) of 20-min exposure to (A) D-NNA, (B) diphenyleneiodonium, (C) di-2-thienyliodonium or vehicle (0.9% NaCl, open circles) on concentration-response curve to phenylephrine in endothelium-denuded rat aortic rings ($n = 6$–8 per group) incubated for 4 h in a culture medium containing *E. coli* lipopolysaccharide (10 $\mu g\ ml^{-1}$).

Di-2-thienyliodonium (10^{-7} M) had no effect on the phenylephrine curve of lipopolysaccharide-treated or control rings. Di-2-thienyliodonium (10^{-6} M) increased EC_{50} of both rings but increased only the E_{max} of lipopolysaccharide-treated rings (Fig. 1C; Table 1).

3.3. Effect of L-Arg and methylene blue

At steady state response to phenylephrine (10^{-5} M) in the presence of the vehicle, D-NNA (6×10^{-4} M) or

L-NNA (10^{-5} M), L-Arg relaxed the rings that were incubated with lipopolysaccharide by 73, 80 and 87%, respectively, but not the control rings incubated with the vehicle. Methylene blue subsequently restored the response to phenylephrine in lipopolysaccharide-treated rings, but did not affect the response of vehicle-incubated control rings.

In the presence of 10^{-6} M of diphenyleneiodonium or di-2-thienyliodonium, neither L-Arg nor methylene blue affected tension developed to phenylephrine in rings incubated with either lipopolysaccharide or vehicle (data not shown).

4. Discussion

Our results show that both D-NNA and L-NNA concentration-dependently inhibited lipopolysaccharide-induced depression of contractile response to phenylephrine; the response of D-NNA (4 or 6×10^{-4} M) was similar to that of L-NNA (10^{-5} M) (Table 1). Since a higher concentration of L-NNA (3×10^{-5} M) did not cause greater restoration of phenylephrine response in the lipopolysaccharide-incubated rings (results not shown), D-NNA appears to be as efficacious as, but less potent than, L-NNA in suppressing the activities of inducible NO synthase. Thus, the L-enantiomeric configuration of NNA is favored but not essential in inhibiting activities of inducible NO synthase.

The concentration of L-NNA that causes 50% inhibition of the response to lipopolysaccharide appears to be in the same range as the IC_{50} of L-NNA that inhibits relaxation response to acetylcholine in intact rat aortic rings in vitro (Wang et al., 1993a). L-NNA was reported to be a more potent inhibitor of the activity of endothelial NO synthase than that of inducible NO synthase in macrophages and endothelium-denuded arteries (Lambert et al., 1991). Differences in experimental conditions are likely to be the cause of these discrepancies.

Diphenyleneiodonium ($1–3 \times 10^{-7}$ M) restored response to phenylephrine in lipopolysaccharide-incubated rings but did not affect phenylephrine response in control rings. The approximated IC_{50} of diphenyleneiodonium (1×10^{-7} M) is similar to the IC_{50} for inhibiting relaxation to acetylcholine in aortic rings (Wang et al., 1993b). These inhibitors, therefore, have the same rank of potency (diphenyleneiodonium > L-NNA > D-NNA) in suppressing activities of endothelial (Wang et al., 1993a, b) and inducible NO synthase of vascular smooth muscles.

Diphenyleneiodonium (10^{-6} M) increased the EC_{50} and decreased E_{max} of the phenylephrine curve in the control rings. Rand and Li (1993) reported that a high (10^{-5} M), but not a low (10^{-7} M), concentration of diphenyleneiodonium suppressed contractile response of smooth muscles. Suppression of contraction by diphenyleneiodonium might have been due to impairment of ATP production as diphenyleneiodonium was shown to inhibit the activities of mitochondrial NADH-dependent enzymes (Ragan and Bloxham, 1977). In line with this interpretation, the concentration of diphenyleneiodonium (IC_{50} at 5×10^{-8} M) required to inactivate NO synthase in macrophages (Stuehr et al., 1991) was lower than that (IC_{50} at 1.3×10^{-5} M) to inhibit macrophage mitochondrial respiration (Hancock and Jones, 1987).

Di-2-thienyliodonium, in contrast to diphenyleneiodonium, did not completely restore responses to phenylephrine in lipopolysaccharide-treated rings. Di-2-thienyliodonium was less potent than, but as efficacious as, diphenyleneiodonium in inhibiting the activity of inducible NO synthase in macrophages (Stuehr et al., 1991). Similar to diphenyleneiodonium, the high dose of di-2-thienyliodonium also suppressed contractile activity in control rings.

Our observation of L-Arg causing relaxation of phenylephrine-preconstricted rings incubated with lipopolysaccharide but not those of vehicle-incubated control rings is in agreement with the results of Fleming et al. (1990) and Schott et al. (1993). This suggests that lipopolysaccharide-induced vascular hyporeactivity is due to activation of the L-Arg/NO/soluble guanylyl cyclase pathway and that extracellular L-Arg is rate-limiting for the activity of inducible NO synthase in vitro. L-Arg also reduced response to phenylephrine in lipopolysaccharide-incubated rings pretreated with D-NNA or L-NNA, but not those treated with diphenyleneiodonium or di-2-thienyliodonium. The effects of L-Arg were inhibited by methylene blue. These results suggest that Arg analogues are reversible, competitive inhibitors of inducible NO synthase in vascular smooth muscle cells.

To summarize, D-NNA, L-NNA and diphenyleneiodonium, but not di-2-thienyliodonium, are efficacious inhibitors of the activity of inducible NO synthase in aortic smooth muscle. The inhibitory effects of D-NNA and L-NNA are reversed by L-Arg suggesting involvement of the L-Arg/NO pathway.

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