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Effects of aminoguanidine administration on vascular hyporeactivity in thoracic aorta from endotoxaemic rats

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Abstract

Overproduction of nitric oxide has been implicated in the pathogenesis of the vascular hyporesponsiveness of endotoxic shock. In this study, we investigated the effects of aminoguanidine, an inducible nitric oxide synthase inhibitor, on the decreased vascular responsiveness in endotoxic shock. Male albino rats were administered intraperitoneally aminoguanidine (25, 50 or 75 mg kg^{-1}) 1 h after they received saline or lipolysaccharide (*Escherichia coli* serotype 055:B5). The thoracic aortas were removed 18 h after lipopolysaccharide administration and suspended in organ baths containing Krebs solution, and tested for vascular reactivity. Contractile responses to phenylephrine and potassium chloride, and relaxant responses to acetylcholine were reduced in endotoxaemic animals. Aminoguanidine was ineffective in improving the vascular hypocontractility at 25 and 75 mg kg^{-1} doses; but at 50 mg kg^{-1} dose, it restored the decreased contractile responses toward normal values. Diminished relaxant responses to acetylcholine were restored by aminoguanidine at all three different doses. There were no significant differences in sodium nitroprusside induced relaxant responses between all groups. Administration of aminoguanidine in control animals did not change vascular responses to any agent. These data suggest that aminoguanidine treatment improves the vascular hyporesponsiveness to contractile- and endothelium-dependent relaxant agents observed in endotoxic shock.

Keywords: Endotoxic shock; Aminoguanidine; Nitric oxide (NO); (Rat)

1. Introduction

Endotoxic shock is a syndrome characterized by hypotension, decreased systemic vascular resistance and hyporesponsiveness to vasoconstrictor agents; the mortality rate is high and despite advances in antimicrobial therapy and intensive care, treatment is often ineffective.

The initiating event in the pathophysiologic process of endotoxic shock is frequently the release of endotoxin. Animal studies suggest that the circulating endotoxin stimulates the release of various mediators such as tumour necrosis factor-α (TNF-α), interleukins (interleukin 1, 6, 8), platelet activating factor (PAF) and thromboxane A_2 (Johnston, 1988; Tabor et al., 1988; Boughton-Smith et al., 1989; Tracey et al., 1989; Beasley, 1990). These mediators and endotoxin itself induce the expression of inducible nitric oxide synthase (iNOS) in many cell types including macrophages, neutrophils, vascular endothelium and vascular smooth muscle cells (Nathan, 1992; Thiemermann, 1994). This induction results in overproduction of large amounts of nitric oxide (NO) (Moncada and Higgs, 1993). Enhanced formation of NO contributes to hypotension and vascular hyporeactivity to vasoconstrictor agents in endotoxic shock (Julou-Schaeffer et al., 1990; Thiemermann and Vane, 1990). It has been shown that non-selective NO synthase inhibitors may have some beneficial effects in endotoxic shock (Kilbourn et al., 1990; Petros et al., 1991). Other reports have suggested that inhibition of both endothelial NO synthase (eNOS) and iNOS may increase organ ischemia, microvascular thrombosis and mortality (Harbrecht et al., 1992; Hutcheson et al., 1990, Wright et al., 1992). According to these data, selective inhibition of iNOS may be a therapeutic intervention to improve the vascular hyporeactivity in endotoxic shock. It has been

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recently demonstrated that aminoguanidine is a mechanism-based inhibitor of NO synthase and is approximately 30 times more selective to iNOS enzyme than eNOS enzyme (Corbett et al., 1992; Griffiths et al., 1993). However, to date effects of in vivo aminoguanidine treatment on relaxant responses have not been evaluated in vitro. In this study, we examined the effects of in vivo administration of aminoguanidine on endotoxin-induced vascular hyporesponsiveness of thoracic aortas obtained from endotoxaemic rats.

2. Materials and methods

2.1. Experimental protocol

The study has been approved by the 'Animal Care Ethics Committee' of Faculty of Medicine, Ege University. Male albino rats (200–250 g) were used in the study. Rats were administered a single intraperitoneal injection of lipopolysaccharide (5 mg kg^{-1} of body weight in 1.5 ml of saline; lipopolysaccharide from *Escherichia coli* serotype 055:B5, L-2880, Sigma) or with the same volume of saline, 18 h prior to the beginning of functional studies. During this 18-h period, food and water were available ad libitum. The animals were placed in a quiet, temperature- and humidity-controlled room (21 ± 3°C and 60 ± 5%, respectively) in which a 12/12 h light–dark cycle was maintained (8:00 am–8:00 pm light).

2.2. Study groups

Animals were divided into eight groups. Group 1 received only 1.5 ml of saline solution served as the control group. Group 2 received lipopolysaccharide as mentioned above. Groups 3, 4 and 5 received 25, 50 and 75 mg kg^{-1} of aminoguanidine intraperitoneally at 1 h after injection of 1.5 ml of saline solution, respectively. Groups 6, 7 and 8 received 25, 50 and 75 mg kg^{-1} of aminoguanidine intraperitoneally at 1 h after injection of lipopolysaccharide, respectively.

2.3. Vascular reactivity

After 18 h of lipopolysaccharide or saline administration, the animals were decapitated under deep ether anesthesia and the thoracic aortas were removed into 4°C Krebs–Henseleit solution. Adherent fatty and connective tissue was removed and the vessel was cut into rings 3 mm wide. In some of the rings, the endothelium was removed mechanically by inserting small forceps into lumen and gently rolling.

In each experiment, endothelium-intact and -denuded rings were suspended horizontally in 30-ml organ chambers containing Krebs–Henseleit solution of the following composition (mM); NaCl, 118.30; KCl, 4.70; $MgSO_4$, 1.20; KH_2PO_4, 1.22; $CaCl_2$, 2.50; $NaHCO_3$, 25.00; glucose, 11.10; pH, 7.4 when gassed with a 95% O_2, 5% CO_2 mixture and maintained at 37°C.

Each ring was connected to a force displacement transducer (MAY-COM FDT 10-A COMMAT Iletisim, Ankara, Turkey) for the measurement of isometric force, which was continuously displayed and recorded on-line on a personal computer via an 8 channel transducer data acquisition system (TDA 94, COMMAT Iletisim) using a software (Polywin 95 ver. 1.0 COMMAT Iletisim), which also had the capacity to analyse the data.

Tissues were equilibrated for 60–90 min under a resting tension of 2 g and washed with fresh oxygenated Krebs–Henseleit solution every 20 min. After the initial equilibration period, the endothelium-denuded rings were used to assess the contractile responses, and cumulative concentration–response curves were obtained by adding to the bath increasing concentrations of phenylephrine (0.003–30 μM) or potassium chloride (120 mM). The absence of endothelium in the rings was confirmed by the absence of relaxations to acetylcholine (1 μM) on preparations precontracted with a submaximal concentration of phenylephrine (0.3–1 μM). Relaxant responses were evaluated by using cumulative concentrations of either acetylcholine (0.003–30 μM) or sodium nitroprusside (0.001–1 μM) on endothelium-intact rings precontracted with phenylephrine

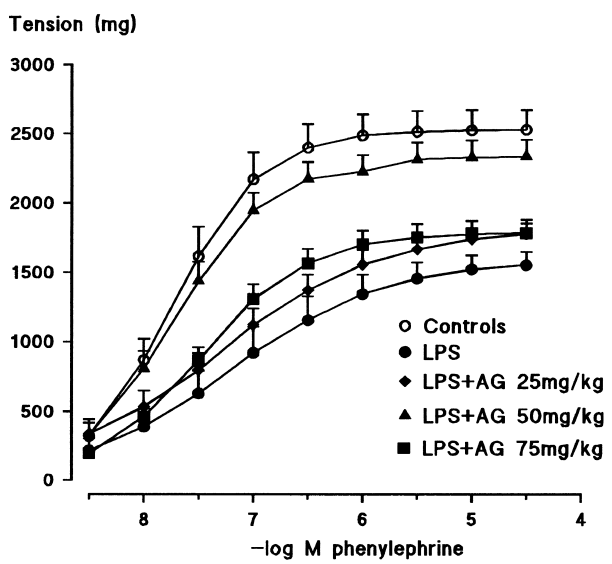

Fig. 1. Phenylephrine dose–response curves in endothelium denuded rat thoracic aortic rings (data are means ± S.E.M. of the groups; $n = 7–10$ in each group). Two-way ANOVA for repeated measures indicates significant difference between the curves of endotoxaemic rats (LPS) and saline-injected rats (controls) ($P < 0.05$). There was a statistically significant difference between the responses of endotoxaemic rats that received aminoguanidine at the dose of 50 mg kg^{-1} 1 h after endotoxin injection (LPS + AG 50 mg kg^{-1}) and endotoxaemic animals (LPS) ($P < 0.05$). No significant differences were observed between the responses of aminoguanidine administered endotoxaemic rats at the doses of 25 or 75 mg kg^{-1} (LPS + AG 25 mg kg^{-1}; LPS + AG 75 mg kg^{-1}) and endotoxaemic animals (LPS) ($P < 0.05$).

(0.3–1 μM) that was determined by using cumulative phenylephrine concentration–response curves for each ring.

2.4. Data analysis

Result were expressed as mean ± S.E.M. Concentration–response curves were fitted by nonlinear regression with simplex algorithm, and E_{max} and pD_2 ($-\log EC_{50}$) values were calculated by using software of a transducer system (Polywin 95 ver. 1.0 COMMAT Iletisim). Contractile responses were expressed as milligram tension developed, whereas relaxant responses were given as the percentages of phenylephrine precontraction. Comparisons of concentration–response curves were evaluated by two-way analysis of variance (ANOVA) for repeated measures. The differences between KCl, E_{max} and pD_2 were analysed by Kruskal–Wallis one-way ANOVA followed by Mann–Whitney *U*-test.

2.5. Reagents

Phenylephrine hydrochloride, acetylcholine hydrochloride, sodium nitroprusside and aminoguanidine bicarbonate were purchased from Sigma (USA); KCl was purchased from E. Merck (Darmstad, Germany). Stock solutions were prepared in distilled water. Aminoguanidine was prepared daily, dissolved in non-pyrogenic sterile saline before injection.

3. Results

3.1. Contractile responses

Phenylephrine (0.003–30 μM) induced contractile responses in endothelium-denuded rings in all groups are

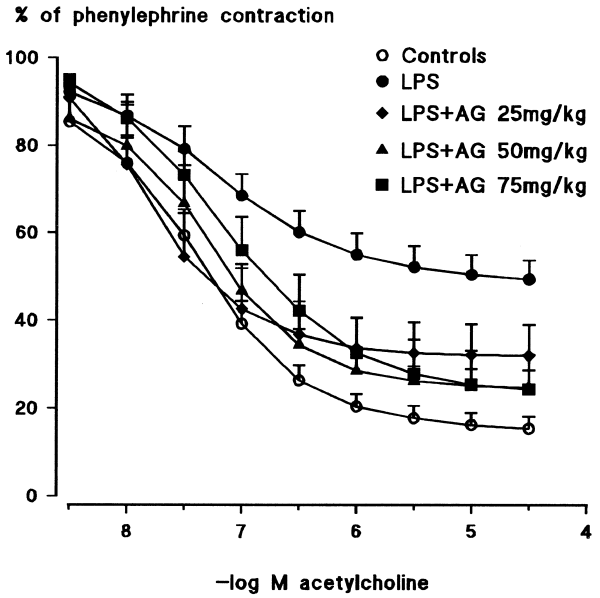

Fig. 2. Acetycholine dose–response curves in endothelium intact rat aortic rings precontracted with a submaximal concentration of phenylephrine (data are means ± S.E.M. of the groups; $n = 5–8$ in each group). Statistical analysis was performed by using two-way ANOVA for repeated measures between all groups. The differences between the responses of controls (control) and endotoxaemic animals (LPS) and between those of endotoxaemic rats which also received aminoguanidine (25, 50 or 75 mg kg^{-1}) 1 h after endotoxin injection (LPS + AG 25 mg kg^{-1}; LPS + AG 50 mg kg^{-1}; LPS + AG 75 mg kg^{-1}) and endotoxaemic group (LPS) were statistically significant ($P < 0.05$).

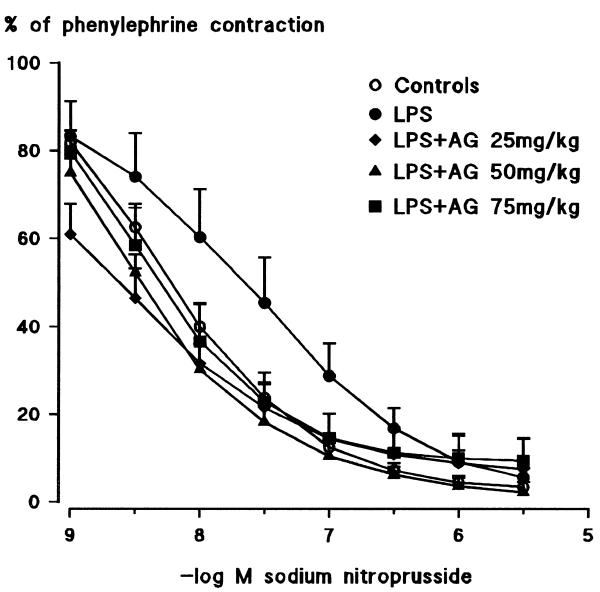

Fig. 3. Sodium nitroprusside dose–response curves in endothelium intact rat aortic rings precontracted with a submaximal concentration of phenylephrine (data are means ± S.E.M. of the groups; $n = 5–8$ in each group). Statistical analysis was performed by using two-way ANOVA for repeated measures between all groups. No statistically significant differences were observed between the responses of control group (controls) and endotoxaemic group (LPS) and between those of aminoguanidine administered endotoxaemic groups (LPS + AG 25 mg kg^{-1}; LPS + AG 50 mg kg^{-1}; LPS + AG 75 mg kg^{-1}) and endotoxaemic group (LPS).

Table 1
The pD_2 and E_{max} values of concentration–response curves of phenylephrine and tension developed to a single concentration of potassium chloride (120 mM). Data are expressed as mean ± S.E.M. of the groups ($n = 7–10$). LPS, lipopolysaccharide; AG, aminoguanidine; KCl, potassium chloride

	Phenylephrine		KCl (tension mg)
	pD_2	E_{max} (tension mg)	
Controls	7.76 ± 0.11	2529 ± 144	1576 ± 85
LPS	7.20 ± 0.24[a]	1579 ± 91[a]	1188 ± 111[a]
LPS + AG (25 mg. kg^{-1})	7.37 ± 0.27	1860 ± 67	1106 ± 94
LPS + AG (50 mg. kg^{-1})	7.76 ± 0.10	2338 ± 123[b]	1555 ± 105[b]
LPS + AG (75 mg. kg^{-1})	7.53 ± 0.07	1786 ± 95	1366 ± 79

[a] $P < 0.05$ compared to controls.
[b] $P < 0.05$ compared to lipopolysaccharide group.

Table 2
Maximum relaxations (expressed as percentage of phenylephrine precontraction) and pD_2 values for acetylcholine and sodium nitroprusside. Data are expressed as mean ± S.E.M. of the groups ($n = 5–8$). LPS, lipopolysaccharide; AG, aminoguanidine

	Acetylcholine		Sodium nitroprusside	
	pD_2	Maximal relaxation (%)	pD_2	Maximal relaxation (%)
Controls	7.68 ± 0.24	84.63 ± 2.79	8.25 ± 0.11	96.59 ± 1.20
LPS	7.25 ± 0.28	50.63 ± 4.33[a]	7.85 ± 0.34	94.37 ± 2.12
LPS + AG (25 mg kg^{-1})	7.79 ± 0.21	68.00 ± 7.01[b]	8.64 ± 0.13	92.48 ± 6.90
LPS + AG (50 mg kg^{-1})	7.84 ± 0.54	75.29 ± 4.01[b]	8.44 ± 0.10	97.74 ± 2.38
LPS + AG (75 mg kg^{-1})	7.17 ± 0.15	75.56 ± 7.79[b]	8.44 ± 0.18	90.53 ± 5.05

[a] $P < 0.05$ compared to controls.
[b] $P < 0.05$ compared to lipopolysaccharide group.

shown in Fig. 1. The pD_2 and E_{max} values elicited by phenylephrine and the tension developed to 120 mM KCl are shown in Table 1. The contractile responses to phenylephrine and KCl were significantly decreased in the endotoxaemia group when compared with the control group ($P < 0.05$). The pD_2 and E_{max} values elicited by phenylephrine were significantly diminished ($P < 0.05$).

Aminoguanidine treatment significantly enhanced the diminished phenylephrine and KCl responses in the endotoxaemia group when compared to nontreated-endotoxaemia group, only at the dose of 50 mg kg^{-1} ($P < 0.05$; Fig. 1, Table 1). No statistically significant differences were found between pD_2 values of aminoguanidine treated-endotoxaemia groups and nontreated-endotoxaemia group. Treatment of control group with aminoguanidine did not alter the contractile responses as compared with the nontreated-control group (data not shown).

3.2. Relaxant responses

On endothelium-intact rings precontracted with phenylephrine, acetylcholine (0.003–30 μM) and sodium nitroprusside (0.001–30 μM) induced relaxations in all groups (Figs. 2 and 3, respectively). The pD_2 values and the maximum relaxations expressed as a percentage of their corresponding phebylephrine precontraction are given in Table 2. Relaxation responses to acetylcholine in endotoxaemic aortas were decreased as compared to the control group ($P < 0.05$), whereas the responses to sodium nitroprusside did not show any significant changes. There were no significant differences between the pD_2 values of both acetylcholine and sodium nitroprusside in the control group and the endotoxaemia group.

Aminoguanidine treatment at three doses augmented the diminished relaxations to acetylcholine significantly in endotoxaemic rats ($P < 0.05$) without altering the pD_2 values. There were no statistically significant differences in the relaxant responses to sodium nitroprusside between all groups. The pD_2 values elicited by sodium nitroprusside were not different. Aminoguanidine administration to control rats did not show any significant changes in relaxant responses to both acetylcholine and sodium nitroprusside when compared to the nontreated control rats (data not shown).

4. Discussion

In the present study, endotoxin injection to rats produced decreased contractile responses to phenylephrine and KCl compared to the control animals. These results are in agreement with other studies, which have reported decreased vascular responsiveness to various agents in similar endotoxic shock models (Wakabayashi et al., 1987; Julou-Schaeffer et al., 1990; Parker and Adams, 1993; Umans et al., 1993). Treatment with aminoguanidine after endotoxin challenge restored the vascular responsiveness in endotoxaemic animals.

Evidence in the literature suggests that induction of iNOS activity by lipopolysaccharide causes prolonged production of large amounts of NO that contributes significantly to the cardiac and vascular hyporesponsiveness characteristic of endotoxic shock (Julou-Schaeffer et al., 1990; Thiemermann and Vane, 1990; Szabo et al., 1993). In this regard, studies examining the role of NO in the decreased vascular contractility in endotoxic shock have used L-arginine analogues, such as N^G-nitro-L-arginine-methyl ester and N^G-methyl-L-arginine, nonselective NOS inhibitors and shown some beneficial effects (Kilbourn et al., 1990; Fleming et al., 1991). However, recent studies have suggested that inhibition of both eNOS- and iNOS-mediated production might have deleterious effects such as enhanced liver injury, reduced survival and decreased cardiac output in septic shock (Klaubunde and Ritger, 1991; Harbrecht et al., 1992; Minnard et al., 1994). These deleterious effects may be associated with the physiological functions of eNOS-mediated NO production such as maintenance of vascular tone, platelet aggregation, neutrophil adhesion and modulation of the inotropic response of normal myocardium (Moncada et al., 1991).

On the basis of these data, we used aminoguanidine to inhibit lipopolysaccharide-induced production of NO with-

out compromising tonic production of NO by eNOS in determining whether in vivo administration of this agent will improve the vascular hyporesponsiveness to contractile and relaxant agents in endotoxic shock.

The impairment of contractile responses to depolarization or α-adrenoceptor agonists caused by endotoxin as seen in our results has been previously demonstrated in the aortas from endotoxaemic animals (Wakabayashi et al., 1987; McKenna, 1988; Beasley et al., 1990; Julou-Schaeffer et al., 1990; Can et al., 1998). This generalized contractile defect can be explained by basic alterations in calcium mobilization or changes in the contractile apparatus within vascular smooth muscle (Wakabayashi et al., 1987; Bigaud et al., 1990). In this study, aminoguanidine improved the decreased phenylephrine and KCl responses in endothelium-denuded aortic rings only at the dose of 50 mg kg^{-1} 1 h after endotoxin injection. It is well established that aminoguanidine has effects unrelated to inhibition of iNOS such as inhibition of diamine oxidase and nonenzymatic glycosylation (Edelstein and Brownlee, 1992; Griffiths et al., 1993). Failure of aminoguanidine to restore the decreased contractile responses at 25 or 75 mg kg^{-1} doses may be due to these effects, their significance in our experimental model of endotoxaemia is unknown.

Endotoxin treatment selectively impaired acetylcholine-induced endothelium-dependent relaxation of rat aorta, but did not produce any change in sodium nitroprusside-induced endothelium-independent vasorelaxation, in accordance with previous studies (Wylam et al., 1990; Umans et al., 1993; Parker and Adams, 1993). It is well known that sodium nitroprusside releases nitric oxide spontaneously. The mechanism of this NO release has not yet been clarified (Feelisch, 1991). The molecule itself does not possess a direct enzyme activating potency. The released NO directly activates the soluble guanylate cyclase in smooth muscle, resulting in intracellular increase of endogenous vasodilator cGMP. Previous studies have shown that lipopolysaccharide can affect cGMP concentrations in vascular smooth muscle (Beasley, 1990). However, our results are consistent with other studies suggesting that it spares the vascular smooth muscle's responsiveness to agents that involve the guanylyl cyclase-cGMP pathway (Parker et al., 1991; Umans et al., 1993; Wylam et al., 1990). Aminoguanidine restored acetylcholine responses in lipopolysaccharide-treated group toward normal values. Diminished responses to acetylcholine have been attributed to endothelial damage in previous studies (Reidy and Schwart, 1983; Young et al., 1991), whereas others have reported that lipopolysaccharide has a direct inhibitory effect on endothelial NO biosynthesis, independent of the effects of lipopolysaccharide on vascular smooth muscle (Beasley et al., 1990; Myers et al., 1992; Umans et al., 1993). On the other hand, recent studies suggest that negative feedback regulation in NO synthesis takes place. It has been demonstrated that NO and NO donors inhibit the neuronal nitric oxide synthase (nNOS) and iNOS (Rogers and Ignarro, 1992; Assreuy et al., 1993). Similar results have been reported for eNOS that NO and NO-donor agents markedly inhibit endothelium-dependent relaxation and NO generation without changing the sensitivity of vascular smooth muscle cells to direct relaxant effect of NO (Buga et al., 1993). Furthermore, another recent study have found decreased activity of the constitutive enzyme in lipopolysaccharide-incubated tissues when there was significantly increased activity of iNOS (Scott et al., 1996). It seems plausible that selective inhibition of iNOS-mediated NO production by aminoguanidine abolishes the inhibition of eNOS-mediated NO production, which is required for vasodilator mechanism of acetylcholine.

Aminoguanidine had no demonstrable effect on acetylcholine-induced relaxation in endothelium-intact rings from control rats. These findings may be explained by absence of inhibition of the constitutive enzyme in rat thoracic aorta, consistent with studies reporting that aminoguanidine is a selective and potent iNOS inhibitor (Corbett et al., 1992; Griffiths et al., 1993; Wolff and Lubeskie, 1995).

Our results further support other reports suggesting that selectively inhibition of iNOS may have beneficial effects in the treatment of endotoxic shock. For instance, aminoguanidine has been shown to attenuate the vascular hyporeactivity to phenylephrine in rat aortic rings in vitro (Joly et al., 1994; Scott et al., 1996). In another study, treatment with aminoguanidine after endotoxin challenge has significantly prevented the increase in both lung iNOS activity and serum nitrite/nitrate level suggesting that it may be useful in preventing the development of acute respiratory failure in endotoxic shock (Arkovitz et al., 1996). Furthermore, it has been demonstrated that aminoguanidine attenuates hypotension, liver injury and mesenteric blood flow alterations and improves survival caused by endotoxic shock (Wu et al., 1995; Hock et al., 1997; Iskit et al., 1999).

In conclusion, present findings demonstrated for the first time in vitro that in vivo administration of aminoguanidine restored the endotoxin induced vascular hyporeactivity to acetylcholine, an endothelium-dependent relaxant agent. Thus, selective inhibition of iNOS resulted in maintaining physiological levels of NO generated by eNOS while blocking the overproduction of NO generated by iNOS. However, aminoguanidine has effects unrelated to inhibition of NOS. The potential significance of these effects in endotoxaemia remains to be determined by further investigations.

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