Effect of methylguanidine on rat blood pressure: role of endothelial nitric oxide synthase

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1 The effect of acute i.v. administration of methylguanidine (MG) on mean arterial blood pressure (MABP) was investigated in anaesthetized male Wistar rats.

2 MG (1–30 mg kg⁻¹ i.v.) produced an increase in MABP in a dose-dependent manner both in normal and in hexamethonium (5 mg kg⁻¹, i.v.)-treated rats.

3 L-Arginine (30 or 150 mg kg⁻¹, i.v.), but not its enantiomer D-arginine (30 or 150 mg kg⁻¹, i.v.), reversed the effect of MG on MABP in both normal and hexamethonium-treated rats.

4 L-Arginine (150 mg kg⁻¹, i.v.) administered 2 min before MG (30 mg kg⁻¹, i.v.) prevented the increase in MABP caused by MG in either normal or hexamethonium-treated rats. This effect was not observed with D-arginine (150 mg kg⁻¹, i.v.).

5 Thus, the rise in MABP caused by MG in the anaesthetized rat is due to inhibition of endothelial NO-synthase activity. We speculate that the rise in the plasma concentration of endogenous MG associated with uraemia may contribute to the hypertension seen in patients with chronic renal failure.

Keywords: Methylguanidine; nitric oxide; L-arginine; hexamethonium; rat blood pressure; hypertension; uraemia

Introduction

Nitric oxide (NO) produced by endothelial cells is a mediator involved in the regulation of vascular tone (Rees *et al.*, 1989; Amezcua *et al.*, 1989). NO is synthesized from the guanidino group of the amino acid L-arginine, and an impairment in its synthesis has been implicated in several diseases (Moncada *et al.*, 1991). In fact, hypertension of different aetiologies is associated with a decrease in either synthesis or release of NO (Linder *et al.*, 1990; Panza *et al.*, 1990; Pinto *et al.*, 1992). The reasons of these alterations are as yet unclear. In principle, a reduced formation of NO can be caused either by direct endothelial cell damage (Forstermann *et al.*, 1986; Jacobs *et al.*, 1990) or by accumulation of endogenous NO-synthase inhibitors in the plasma (Iyengar *et al.*, 1987; Vallance *et al.*, 1992; Sorrentino *et al.*, 1993).

Studies regarding the physiological and pathological role of NO have been conducted with specific inhibitors of NO-synthase, such as N^G-methyl-L-arginine (Palmer *et al.*, 1988) or N^G-nitro-L-arginine methyl ester (Rees *et al.*, 1990). These and several other inhibitors of NO-synthase have modifications of the guanidino group of L-arginine (Moncada *et al.*, 1991). Vallance and coworkers (1992) have shown that asymmetrical dimethylarginine, an analogue of L-arginine, is an endogenous inhibitor of NO synthesis which accumulates in the plasma of patients with end-stage chronic renal failure and might contribute to the hypertension and immune dysfunction associated with this pathology.

Patients with chronic renal failure show a retention of several nitrogen-containing products derived from protein catabolism. It has been hypothesized that the high blood concentration of these products may cause uraemic symptoms, i.e. hypertension, immune dysfunction and neurological disorders (Jones & Burnett, 1975). In fact, a dietary protein restriction or haemodialysis reduces these symptoms (Lowrie *et al.*, 1981). Methylguanidine (MG) is a product of protein catabolism that increases and accumulates within cells in chronic renal failure (Matsumoto *et al.*, 1976; Orita *et al.*, 1981; Yokozawa *et al.*, 1989). MG is structurally related to

the functional group of L-arginine, the natural substrate for NO-synthase. In a previous paper, we have shown that MG reduced endothelium-dependent, but not endothelium-independent relaxations of the rabbit aorta *in vitro* (Sorrentino *et al.*, 1993).

In this study we describe the effect of acute intravenous administration of MG on systemic blood pressure in urethane-anaesthetized rats.

Methods

Male Wistar rats were obtained from Charles River Italia. Upon arrival the rats were housed in an environment with controlled temperature (21–24°C) and lighting (12:12 h light-darkness cycle). Standard laboratory chow and drinking water were provided *ad libitum*. A period of seven days was allowed for acclimatization of the rats before any experimental manipulation was undertaken. At the time of the experiments, the body weight ranged from 200 to 300 g.

A tracheotomy was performed after urethane anaesthesia (1 g kg⁻¹, i.p.). A polyethylene cannula (PE-50) was placed in the internal jugular vein for administration of drugs. A PE-50 catheter was inserted into the left carotid artery and connected to a Bentley 800 Trantec (Basile, Comerio, Italy) for blood pressure monitoring. The lines were filled with heparinized saline (10 units ml⁻¹) to maintain patency. Blood pressure was recorded by a Thermal Arraycorder WR 7400 (Grampotec, Tokyo). After surgery, animals were divided into two groups: normal saline-treated rats and hexamethonium (5 mg kg⁻¹, i.v.)-treated rats. After 30 min of stabilization, MG was administered i.v. at cumulative doses (1, 3, 10 and 30 mg kg⁻¹) with 2 min intervals. At 5 min after the last MG injection, cumulative doses of L-arginine or D-arginine were administered (30 and 150 mg kg⁻¹, i.v.) with an interval of 2 min between injections. To evaluate the preventive and stereospecific effect of L-arginine on the increase in blood pressure induced by MG, the amino acid or its enantiomer (D) was administered 2 min before MG injection.

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Drugs

L-Arginine and D-arginine methylguanidine, hexamethonium, urethane were purchased from Sigma Chemical Co. Italy. All drugs were dissolved in normal saline (NaCl 0.9% w v⁻¹) and prepared daily.

Statistics

Results are expressed as mean $\pm$ s.e.mean of mean arterial blood pressure (MABP) of number (*n*) of experiments. Statistical comparisons were carried out by using analysis of variance (ANOVA, two-way) and Bonferroni multiple comparisons test. Results were considered significant at a *P* value <0.05 .

Results

The MABP of anaesthetized rats was 102 ± 1 mmHg (*n* = 50) with an heart rate (HR) of 385 ± 13 beats min⁻¹. In animals treated with the ganglionic blocking agent, hexamethonium, blood pressure was (within 2 min) reduced to 71 ± 2 mmHg (*n* = 36) and HR to 373 ± 20 beats min⁻¹ reaching a plateau of 87 ± 3 mmHg and an HR 401 ± 16 beats min⁻¹ within 25 min.

Effect of MG on MABP

Cumulative bolus injections of MG (1, 3, 10 and 30 mg kg⁻¹, i.v.) induced a dose-related increase in MABP ($P < 0.01$, *n* = 12; Figures 1 and 2). The increase in MABP induced by MG was rapid in onset, reaching a plateau within 2 min without any significant change in heart rate (406 ± 31 beats min⁻¹). The hypertensive effect of MG (30 mg kg⁻¹, i.v.), lasted for about 1 h (53 ± 10 min). Administration of cumulative doses of L-arginine (30 and 150 mg kg⁻¹, i.v.), which had no direct effect on blood pressure, significantly ($P < 0.05$ and $P < 0.01$ respectively; *n* = 6) reversed the increase in MABP induced by MG in a dose-dependent manner (Figure 3a). In contrast, D-arginine did not reverse the pressor effect elicited by MG ($P > 0.05$; *n* = 6; Figure 3a).

Pretreatment of rats with L-arginine (150 mg kg⁻¹, i.v.) or vehicle (control) 2 min before MG, did not significantly modify MABP and HR (L-arginine: 97 ± 3 mmHg and 399 ± 23 beats min⁻¹, *n* = 4; D-arginine: 98 ± 4 mmHg and 389 ± 38 beats min⁻¹, *n* = 4; vehicle 102 ± 2 mmHg and 385 ± 42 beats min⁻¹, *n* = 4). In these experiments, the time-course of the MG-induced increase of MABP was monitored for 30 min. Pretreatment of rats with L-arginine significantly ($P < 0.001$, versus normal saline; *n* = 4) prevented the pressor effect of MG (30 mg kg⁻¹, i.v.; Figure 4a). In contrast, pretreatment of rats with D-arginine did not affect the degree of the pressor response caused by the subsequent injection of MG ($P > 0.05$, *n* = 4). Thus, the rise in MABP caused by MG in rats pretreated with D-arginine was significantly greater than the one caused by MG in rats pretreated with L-arginine ($P < 0.001$, *n* = 4; Figure 4a).

Effect of MG on MABP in hexamethonium-treated rats

Hexamethonium (5 mg kg⁻¹, i.v.) pretreatment did not modify the changes in MABP caused by MG, although a reduction of basal MABP after hexamethonium treatment was observed. Indeed, in these animals cumulative bolus injections of MG (1, 3, 10 and 30 mg kg⁻¹, i.v.) induced a dose-related increase in MABP ($P < 0.01$, *n* = 12; Figure 2). As in rats, not treated with hexamethonium, L-arginine (30 and 150 mg kg⁻¹, i.v.) significantly reversed the pressor effect of MG ($P < 0.05$ and $P < 0.01$ respectively, *n* = 6; Figure 3b), while D-arginine was ineffective ($P > 0.05$, *n* = 6; Figure 3b). The duration of the increase in MABP elicited by MG (30 mg kg⁻¹, i.v.) in hexamethonium-treated rats was

significantly longer (183 ± 19 min; $P < 0.01$; *n* = 4) than in normal rats.

Also in hexamethonium-treated rats, L-arginine, D-arginine (150 mg kg⁻¹, i.v.) or vehicle administration did not significantly modify MABP and HR (L-arginine: 90 ± 5 mmHg and 391 ± 15 beats min⁻¹, *n* = 6; D-arginine: 89 ± 4 mmHg and 380 ± 24 beats min⁻¹, *n* = 6; vehicle 88 ± 7 mmHg and 387 ± 13 beats min⁻¹, *n* = 6). The subsequent (2 min) injection of MG (30 mg kg⁻¹, i.v.) caused a pressor effect which was similar to the one seen in rats not

Figure 1 Experimental records illustrating the dose-dependent increase in blood pressure to methylguanidine (MG) and its reversal by L-arginine but not D-arginine. Cumulative administration of MG (1, 3, 10, 30 mg kg⁻¹, i.v.; arrows pointing upwards) in anaesthetized rats followed by L-arginine (30, 150 mg kg⁻¹, i.v. (a), arrows pointing downwards) or D-arginine (30, 150 mg kg⁻¹, i.v. (b), arrows pointing downwards).

Figure 2 Dose-dependent increase of mean arterial blood pressure (MABP) induced by methylguanidine (MG, 1, 3, 10, 30 mg kg⁻¹, i.v.) in normal rats (○) and in rats treated with hexamethonium (5 mg kg⁻¹, i.v.; ●). Results are expressed as mean $\pm$ s.e.mean of 12 rats for both normal and hexamethonium-treated animals. There is no significant difference between normal saline and hexamethonium-treated rats ($P > 0.05$).

Figure 3 L-Arginine 30 (ii) and 150 (iii) mg kg^{-1} , i.v., but not D-arginine 30 (iv) and 150 (v) mg kg^{-1} , i.v., reduced the increase in mean arterial blood pressure (MABP) induced by methylguanidine (i) 30 mg kg^{-1} , i.v., in normal rats (a) and in rats treated with hexamethonium (5 mg kg^{-1}) (b). * $P < 0.05$, $n = 6$; ** $P < 0.001$, $n = 6$.

treated with hexamethonium. The pretreatment of rats with L-arginine significantly ($P < 0.001$, versus normal saline; $n = 6$) prevented this pressor effect of MG (Figure 4b). In contrast, pretreatment of rats with D-arginine did not affect the degree of the pressor response caused by the subsequent injection of MG ($P > 0.05$, $n = 6$; Figure 4b). Thus, the rise in MABP caused by MG in rats pretreated with D-arginine was significantly greater than the one caused by MG in rats pretreated with L-arginine ($P < 0.001$, $n = 6$; Figure 4b).

Discussion

In this study we analyzed the effect of MG, a product of protein catabolism structurally related to the guanidino group of L-arginine, on MABP and HR in the rat.

We demonstrated that MG caused dose-dependent increases in MABP in the anaesthetized rat. Similar increases in MABP induced by MG were shown in rats treated with hexamethonium.

L-Arginine, the substrate of the enzyme for the NO synthesis, reversed the increases in MABP caused by MG, whereas D-arginine was ineffective. These results suggest that the pressor effect produced by MG is due to inhibition of

Figure 4 In (a) is shown the effect of L-arginine (150 mg kg^{-1} ; $n = 4$; ●), D-arginine (150 mg kg^{-1} ; $n = 4$; Δ) and vehicle ($n = 4$; ○) administered i.v. 2 min before methylguanidine (MG) injection (30 mg kg^{-1} , i.v.) in rats. L-Arginine, but not D-arginine, significantly ($P < 0.05$) prevents the increase in mean arterial blood pressure (MABP) induced by MG. In (b) the effect of L-arginine (150 mg kg^{-1} ; $n = 6$; ●), of D-arginine (150 mg kg^{-1} ; $n = 6$; Δ) and of vehicle ($n = 6$; ○) administered i.v. 2 min before MG injection (30 mg kg^{-1} i.v.) in hexamethonium-treated rats is shown. L-Arginine, but not D-arginine, significantly ($P < 0.05$) prevents the increase in MABP induced by MG. Results are expressed as mean $\pm$ s.e.mean.

endothelial NO-synthase activity, confirming our previous *in vitro* study (Sorrentino *et al.*, 1993).

We can exclude the possibility that the increase in MABP induced by MG is due to a direct or indirect action on the peripheral nervous system, as the treatment of rats with hexamethonium, a classical ganglionic blocker, neither modified the effect of MG nor the actions of L-arginine or D-arginine. The response to MG in hexamethonium-treated rats differed only in the duration of the hypertensive response. This last finding could imply that there is a neuronal adjustment of blood pressure following the MG-induced increase in MABP, ruling out rapid metabolism or excretion of MG.

Endogenous MG is normally excreted in the urine. In patients with chronic renal failure, who have a reduced, if any, urine production, plasma MG concentration increases (Giovannetti *et al.*, 1973; Matsumoto *et al.*, 1976; Orita *et al.*, 1981; Yokozawa *et al.*, 1989). This increase is either due to reduced excretion or due to increased production. Indeed, it

has been proposed that in patients with chronic renal failure, an increase in MG production may arise from the conversion of creatinine to MG (Orita *et al.*, 1978; Yokozawa *et al.*, 1990; Yokozawa *et al.*, 1991). Furthermore, Natelson & Sherwin (1979) suggested that urea could be converted to creatine, via canavanine in a supposed 'guanidinic cycle', and subsequently converted in creatinine to be included in the formation of MG.

The plasma concentration of MG in patients with chronic renal failure is still in dispute (Pfiffner & Myers 1926; Giovannetti *et al.*, 1968; Stein *et al.*, 1971; Baker & Marshall 1971; Menichini *et al.*, 1971). Some investigators questioned the different levels of MG that have been found citing methodological difficulties, including the production of MG during assay procedure (Orita *et al.*, 1981). Furthermore, it is noteworthy that MG can accumulate intracellularly, resulting in intracellular concentrations which are 5 to 7 times higher than the ones reported in the plasma (Matsumoto *et al.*, 1976; Orita *et al.*, 1981; Yokozawa *et al.*, 1989). Indeed, MG easily accumulates in cells and in tissues, such as erythrocytes, liver, kidney and muscle, either because of the basic characteristic of molecule and/or the systemic acidosis which occurs in this disease (Kikuchi *et al.*, 1981; Yokozawa *et al.*, 1990). At the moment, it is uncertain to what extent MG accumulates in endothelial cells, but here we have shown that MG, acting as an inhibitor of NO-synthase, could be considered as an uraemic toxin. The doses of MG used in our study may well lead to theoretical plasma concentrations ($16\text{--}468\text{ }\mu\text{g ml}^{-1}$) which are higher than the ones reported

for rats with chronic renal failure ($3\text{ }\mu\text{g ml}^{-1}$, Orita *et al.*, 1981; Yokozawa *et al.*, 1989), but they are not so high if we consider the accumulation as μg per g of tissue in rats with chronic renal failure estimated by the report of Orita and coworkers (1981) (our dosage: $1\text{--}30\text{ }\mu\text{g g}^{-1}$; rats with chronic renal failure: red blood cells, $24\text{ }\mu\text{g g}^{-1}$, liver, $28\text{ }\mu\text{g g}^{-1}$; muscle, $20\text{ }\mu\text{g g}^{-1}$; colon, $21\text{ }\mu\text{g g}^{-1}$). Furthermore, our range of doses was similar to the ones used by other investigators. In fact, chronic administration of MG (30 mg kg^{-1}) in dogs causes a uraemia-like syndrome (Giovannetti *et al.*, 1969), and chronic MG administration in rats ($5\text{--}50\text{ mg kg}^{-1}$) produces a dose-dependent decrease in survival rate (Yokozawa *et al.*, 1989).

In patients with chronic renal failure, other endogenous molecules have been found, such as canavanine, N^G -methyl-L-arginine, N^G , N^G -dimethylarginine (asymmetrical) and guanidine, all of which are inhibitors of NO-synthase (Schmidt *et al.*, 1988; Vallance *et al.*, 1992; Sorrentino *et al.*, 1993). We cannot exclude a synergism between MG and these endogenous inhibitors.

These endogenous inhibitors of NO-synthase could be part of the aetiological mechanism which predisposes patients with chronic renal failure to hypertension and impaired host defence. Furthermore our results may lead to an improved understanding of the role of NO-synthase in this pathology, and possibly suggest a novel therapeutic approach.

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