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Aminoguanidine-provoked leukocyte adherence to rat mesenteric venules: role of constitutive nitric oxide synthase inhibition

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1 The effects of aminoguanidine on neutrophil adherence to venules and on the diameter of arterioles in the mesenteric vascular bed of the pentobarbitone-anaesthetized rat have been compared with those of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME).

2 Administration of L-NAME (1-10 mg kg^{-1}, i.v.) caused a dose-dependent increase in leukocyte adherence and a reduction in leukocyte rolling velocity in postcapillary venules of the rat mesentery over 1 h.

3 Likewise, aminoguanidine (10-100 mg kg^{-1}, i.v.) dose-dependently increased leukocyte adherence and decreased leukocyte rolling velocity over 1 h.

4 Both L-NAME and aminoguanidine caused a dose-dependent reduction in mesenteric arteriolar diameter and an increase in systemic arterial blood pressure.

5 The effects of aminoguanidine (50 mg kg^{-1}, i.v.) on leukocyte adherence, arteriolar diameter and on blood pressure were significantly reversed by pretreatment with L-arginine (300 mg kg^{-1}, i.v.).

6 These findings indicate that, like L-NAME, aminoguanidine can acutely promote leukocyte adherence to the mesenteric venular wall and reduce arteriolar diameter. Moreover, these acute effects were reversed by L-arginine, suggesting they are mediated through inhibition of constitutive NO synthase.

Keywords: Aminoguanidine; constitutive nitric oxide synthase; neutrophils; arteriolar diameter; nitric oxide

Introduction

Nitric oxide (NO), synthesized from L-arginine by a constitutive NO synthase (Moncada *et al.*, 1991), plays an important modulatory role in leukocyte behaviour in the vasculature Thus, NO prevents monocyte adhesion to vascular endothelium (Bath *et al.*, 1991) and inhibits neutrophil aggregation *in vitro* (McCall *et al.*, 1988). Moreover, inhibitors of constitutive NO synthesis (Rees *et al.*, 1990) such as N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME) promote leukocyte adherence to mesenteric postcapillary venules in the rat and cat (Kubes *et al.*, 1991; 1993; Arndt *et al.*, 1993).

In addition to its actions on constitutive NO synthase, L-NAME also inhibits the inducible isoform of NO synthase. The expression of the inducible isoform several hours after challenge with endotoxin (Salter *et al.*, 1991) is associated with the onset of microvascular injury in the intestine (Boughton-Smith *et al.*, 1993) and such injurious actions can be attenuated by L-NAME (Laszlo *et al.*, 1994a). Aminoguanidine has been described as a selective inhibitor of the inducible NO synthase by virtue of its apparent lack of effect on blood pressure *in vivo*, and hence on constitutive NO synthase, and its activity in a number of cell systems and vascular preparations *in vitro* (Corbett *et al.*, 1992; Misko *et al.*, 1993; Hasan *et al.*, 1993; Griffiths *et al.*, 1993; Joly *et al.*, 1994). However, recent studies suggest that aminoguanidine can also potently inhibit constitutive NO synthase in intestinal tissue *in vitro* and like L-NMMA and L-NAME, can promote acute intestinal tissue damage *in vivo* when administered at a time when expression of the inducible isoform of NO synthase is not detectable (Laszlo *et al.*, 1995a).

To clarify further the selectivity of action of aminoguanidine on NO synthase *in vivo*, the acute actions of aminoguanidine on neutrophil behaviour have been studied in single mesenteric postcapillary venules of the pentobarbitone anaesthetized rat and compared with those of L-NAME by use of intravital microscopy. Furthermore, the actions of aminoguanidine on mesenteric arteriolar diameter and systemic arterial blood pressure have also been compared to those of L-NAME.

A preliminary account of this work was presented to the British Pharmacological Society (Lopez-Belmonte *et al.*, 1995).

Methods

Measurement of systemic blood pressure

Male Wistar rats (230-270 g body weight) were deprived of food but not water for 18-20 h prior to the experiment. The animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and a tracheotomy performed to facilitate breathing during the experiment.

Mean systemic arterial blood pressure (BP) was measured from a cannula inserted into the right carotid artery and connected to a pressure transducer (Elcomatic) and a chart recorder (Grass Model 7D polygraph).

Intravital microscopy

Following induction of anaesthesia, rats were placed in a horizontal position on a transparent perspex microscope stage, and following a small midline incision, a segment of the ileo-coecal portion of the mesentery was exteriorised. This was then carefully spread over a perspex ring which was placed into an optically clear well in the stage. The exposed intestine was covered with saline-soaked gauze to minimize evaporation. The temperature of the perspex well was maintained at 37°C with a constant temperature circulator (Techne), while the exposed mesentery was superfused with warmed Krebs solution that was bubbled with a mixture of 95% O_2 and 5% CO_2. This surgical procedure took less than 10 min to perform. An intravital microscope (Nikon Optiphot-2) fitted with a ×25 objective lens (Leitz Wetzlar L25/0.35, Germany) and a ×10 eyepiece was used to visualize the mesenteric circulation, which was transilluminated with a 100W direct-current stabilized light

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source. A video camera (JVC TK-1280E 1/2" CCD colour camera) mounted on the microscope was used to display the images on a colour monitor, which were recorded for playback analysis using a videocassette recorder (Sony SLVE7).

Single unbranched mesenteric venules of 25–40 μm in diameter, and their associated arterioles (15–25 μm), were selected for study. Changes in arteriolar diameter were recorded by a photocell attached to the television screen by the method of Cardinal & Higgs (1980), with minor modifications. The photocell, which responds to changes in light intensity from the television screen, was connected to a pen recorder via a signal conditioning unit. A reference photocell placed close to the signal photocell was used to eliminate interference from background illumination levels.

The number of adherent neutrophils was determined during subsequent playback of the recorded video images. A leukocyte was considered adherent to the venular endothelium if it remained stationary in the same location for more than 30 s, and leukocyte adherence was expressed as the number of cells per 100 μm length of venule. Leukocyte rolling velocity, which decreases prior to adherence, was determined from the time required for a leukocyte to travel a preset distance (100 μm) along the length of the venule.

Effect of L-NAME and aminoguanidine

A 30 min post-surgical equilibration period was allowed before administration of either L-NAME, aminoguanidine or their vehicle (isotonic saline) to allow the preparation to stabilize following exteriorization and handling of the mesentery.

The effects of L-NAME (1–10 mg kg^{-1}, i.v.) or aminoguanidine hemisulphate (10–100 mg kg^{-1}, i.v.) on neutrophil adherence and rolling velocity in mesenteric venules and on mesenteric arteriolar diameter were then investigated for 1 h following bolus administration. Both drugs were dissolved freshly each day in isotonic saline and injected in a 1 ml kg^{-1} volume.

L-Arginine reversal of the effects of aminoguanidine

In a separate group of animals, L-arginine hydrochloride (300 mg kg^{-1}, i.v.) was injected 15 min prior to aminoguanidine (50 mg kg^{-1}, i.v.), and its effects on aminoguanidine-induced neutrophil rolling and adherence and on arteriolar constriction examined 30 min after aminoguanidine administration. Additionally, the effect of L-arginine pretreatment on the hypertensive action of aminoguanidine was also investigated.

Materials

Aminoguanidine hemisulphate, L-NAME, L-arginine hydrochloride and the Krebs solution (10-fold concentrate) were all obtained from the Sigma Chemical Co. (Poole, Dorset).

Statistical analysis

The data are expressed as mean ± s.e.mean from (n) rats per experimental group. For statistical comparisons, analysis of variance with the Bonferroni test for multiple comparisons was used; $P<0.05$ was taken as statistically significant.

Results

Effect of L-NAME and aminoguanidine on neutrophil adherence

Administration of L-NAME (1–10 mg kg^{-1}, i.v.) resulted in a time- and dose-dependent increase in the number of adherent neutrophils per 100 μm length of venule over 1 h, which commenced 5 min after its administration (Figure 1). Thus, L-NAME (5 mg kg^{-1}) increased neutrophil adherence by 526 ± 54% compared to control vehicle values, 30 min after its administration ($P<0.01$, $n=6$).

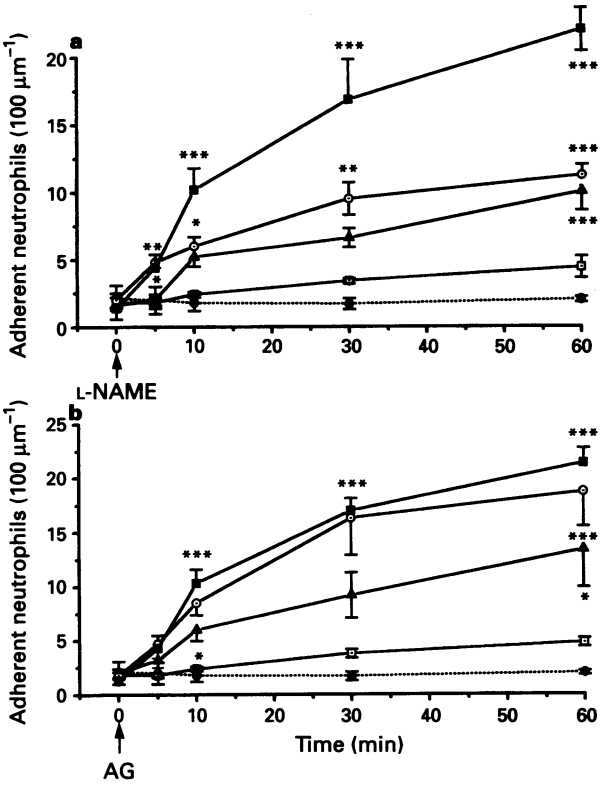

Figure 1 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) at 1(□), 2(▲), 5(○) and 10(■) mg kg^{-1}, i.v. (a) or aminoguanidine (AG), at 10(□), 25(▲), 50(○) and 100(■) mg kg^{-1}, i.v. (b) on neutrophil adherence to rat mesenteric postcapillary venules. Results, shown as the number of adherent neutrophils per 100 μm length of venule, are expressed as the mean ± s.e.mean of 5–6 experiments in each group. Significant difference from control vehicle infusion (●) is shown as $*P<0.05$; $**P<0.01$; $***P<0.001$.

Aminoguanidine (10–100 mg kg^{-1}, i.v.) also caused a time- and dose-dependent increase in adherent neutrophils over 1 h, which commenced 10 min after its administration (Figure 1). Thus, aminoguanidine (50 mg kg^{-1}) increased neutrophil adherence to postcapillary venules by 861 ± 200%, compared to control values, when determined 30 min after its administration ($P<0.001$, $n=6$).

Administration of the vehicle, isotonic saline did not result in any significant increase in the number of adherent neutrophils over the 1 h experimental period (Figure 1).

Effect of L-NAME and aminoguanidine on neutrophil rolling velocity

L-NAME (1–10 mg kg^{-1}, i.v.) caused a time- and dose-dependent decrease in neutrophil rolling velocity in postcapillary mesenteric venules of the rat, starting 5–10 min after administration, depending on the dose. With L-NAME (5 mg kg^{-1}), the reduction in neutrophil rolling velocity was 57 ± 13% when compared to vehicle administration values 30 min after its administration ($P<0.001$, $n=6$; Figure 2).

Similarly, aminoguanidine (10–100 mg kg^{-1}, i.v.) induced a time- and dose-dependent reduction in neutrophil rolling velocity (Figure 2), which at the higher dose started 5 min after administration. Aminoguanidine (50 mg kg^{-1}) reduced the rolling velocity of neutrophils by 56 ± 8% compared to vehicle values when determined 30 min after its administration ($P<0.001$, $n=6$).

Administration of the vehicle, did not significantly affect neutrophil rolling velocity over the 1 h experimental period (Figure 2).

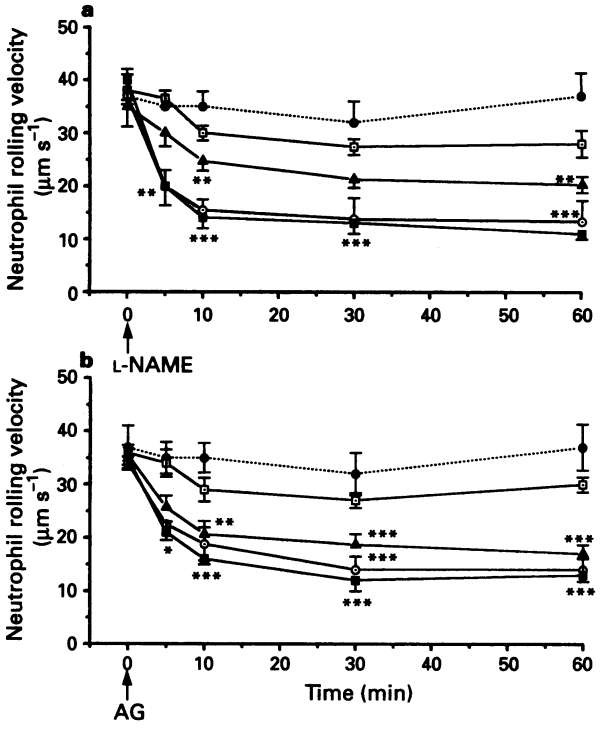

Figure 2 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) at 1(□), 2(▲), 5(○) and 10(■) mg kg^{-1}, i.v. (a) or aminoguanidine (AG), at 10(□), 25(▲), 50(○) and 100(■) mg kg^{-1}, i.v. (b) on neutrophil rolling velocity in rat mesenteric postcapillary venules. Results, shown as neutrophils rolling velocity (μm s^{-1}), are expressed as the mean ± s.e.mean of 5–6 experiments in each group. Significant difference from control vehicle infusion (●) is shown as $*P<0.05$; $**P<0.01$; $***P<0.001$.

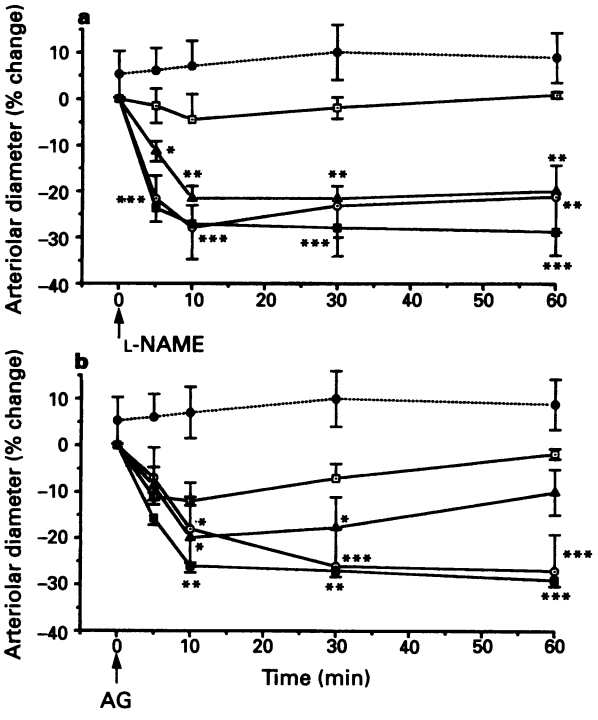

Figure 3 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) at 1(□), 2(▲), 5(○) and 10(■) mg kg^{-1}, i.v. (a) or aminoguanidine (AG), at 10(□), 25(▲), 50(○) and 100(■) mg kg^{-1}, i.v. (b) on rat mesenteric arteriolar diameter. Results, shown as % change in diameter from initial resting values, are expressed as the mean ± s.e.mean of 5–6 experiments in each group. Significant difference from control vehicle infusion (●) is shown as $*P<0.05$; $**P<0.01$; $***P<0.001$.

Effect of L-NAME and aminoguanidine on mesenteric arteriolar diameter

L-NAME (1–10 mg kg^{-1}, i.v.) caused a time- and dose-dependent reduction in mesenteric arteriolar diameter which was initiated 5–10 min after its administration, depending on the dose (Figure 3). Thus, L-NAME (5 mg kg^{-1}) reduced arteriolar diameter by 23 ± 7% after 30 min, as compared with initial resting values ($P<0.001$, $n=6$).

Aminoguanidine administration also induced a time- and dose-dependent reduction in arteriolar diameter, which was significantly different from control values within 10 min (Figure 3). Aminoguanidine (50 mg kg^{-1}) caused a reduction of arteriolar diameter of 27 ± 8%, when compared to initial resting values, 30 min after administration ($P<0.001$, $n=6$, Figure 3).

Administration of the vehicle did not significantly affect arteriolar diameter over the 1 h experimental period (Figure 3).

Effect of L-NAME and aminoguanidine on systemic arterial blood pressure

L-NAME (1–5 mg kg^{-1}, i.v.) induced a time- and dose-dependent increase in mean BP, which reached maximal values 10 min after its administration and which was well-maintained for the remainder of the 1 h observation period (Table 1).

Aminoguanidine also induced a dose-dependent increase in mean BP. However, this response was faster in onset and more transient than that of L-NAME, with maximum increases being observed within 1 min of its administration and with no significant effect remaining after 60 min (Table 1).

L-Arginine reversal of the effects of aminoguanidine

Pretreatment with L-arginine (300 mg kg^{-1}, i.v.) 15 min prior to aminoguanidine (50 mg kg^{-1}, s.c.) significantly inhibited the increase in neutrophil adherence (66 ± 2% inhibition, $P<0.05$; $n=4$) and reduction in rolling velocity (70 ± 5% inhibition,

Table 1 Effect of N^G-nitro-L-arginine methyl ester (L-NAME, 1–5 mg kg^{-1}, i.v.) or aminoguanidine (AG; 25–100 mg kg^{-1}, i.v.) on mean systemic arterial blood pressure (BP) over a 1 h period following administration

Treatment	ΔBP (mmHg)					
	0.5	1	5	10	30	60 min
L-NAME						
1 mg kg^{-1}	3 ± 2	9 ± 2*	19 ± 3**	19 ± 4**	23 ± 5***	19 ± 6**
2	7 ± 1	15 ± 2*	34 ± 3***	41 ± 5***	44 ± 9***	42 ± 9***
5	13 ± 4*	21 ± 3*	49 ± 5***	62 ± 3***	59 ± 5***	57 ± 6***
AG						
25 mg kg^{-1}	16 ± 3*	20 ± 3**	11 ± 2**	6 ± 2	8 ± 1*	−3 ± 1
50	19 ± 2*	28 ± 2***	12 ± 2***	9 ± 2**	10 ± 4**	1 ± 2
100	21 ± 3**	35 ± 2***	20 ± 5***	18 ± 3***	15 ± 4**	8 ± 4

Results are the mean of 5–6 experiments in each group, and are expressed as the ΔBP from basal resting values. Statistical significance from resting values is given as $*P<0.05$; $**P<0.01$; $***P<0.001$ (repeated measures ANOVA with Bonferroni test).

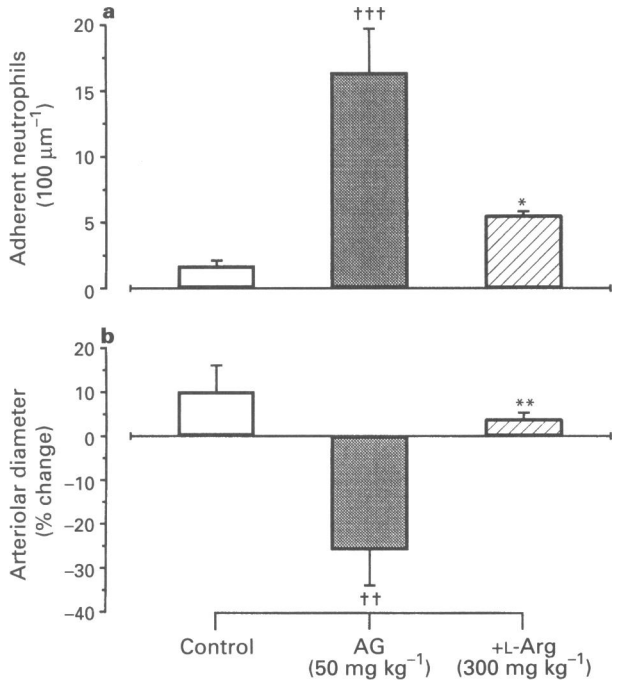

Figure 4 Reversal by pretreament (15 min) with L-arginine (300 mg kg^{-1}, i.v.) of the increase in neutrophil adherence (a) and decrease in arteriolar diameter (b) induced by aminoguanidine (AG, 50 mg kg^{-1}, i.v.). Results are shown as the number of adherent neutrophils per 100 μm length of venule and as the % change in arteriolar diameter from basal resting values, 30 min after administration of aminoguanidine. Results are the mean of 4 experiments in each group, where significant difference from control values (vehicle) is given as ††$P<0.01$; †††$P<0.001$. Significant difference from aminoguanidine values is given as *$P<0.05$; **$P<0.01$.

$P<0.05$; $n=4$) and abolished the reduction in arteriolar diameter ($P<0.001$; $n=4$) observed 30 min after aminoguanidine administration (Figure 4). Moreover, L -arginine pretreatment also significantly inhibited the maximum increase in mean BP induced by this same dose of aminoguanidine (42±9% inhibition, $P<0.01$, $n=4$).

Discussion

Inhibitors of constitutive NO synthesis promote leukocyte adhesion to postcapillary mesenteric venules and their subsequent emigration into the surrounding tissue (Kubes *et al.*, 1991; 1993; Arndt *et al.*, 1993). In the present study, it was confirmed that intravenous administration of L-NAME reduced neutrophil rolling velocity, an event that precedes leukocyte adhesion, as well as increased the number of adherent neutrophils in postcapillary mesenteric venules of the rat, over a 1 h experimental period.

Aminoguanidine also reduced neutrophil rolling velocity and increased the number of adherent neutrophils following intravenous administration. This response was therefore similar to that obtained with L-NAME, although the latter was some 5 times more potent than aminoguanidine. The effect of aminoguanidine on neutrophil rolling and adherence was substantially inhibited by pretreatment with L-arginine, suggesting this action is mediated, to a greater extent, through inhibition of NO synthase. Since these effects of aminoguanidine were initiated within 5–10 min of administration, and the surgical procedures and stabilization period lasted only 45 min, there would be insufficient time for expression of the inducible isoform of NO synthase under these conditions (Salter *et al.*, 1991; Boughton-Smith *et al.*, 1993). Hence aminoguanidine is likely to exert these actions on neutrophil activity by inhibiting constitutive NO synthase.

These results further support recent findings in the conscious rat where aminoguanidine, like L-NAME, enhanced the plasma leakage induced in the rat ileum and colon by endotoxin over a 1 h period, at a time when expression of the inducible NO synthase would not be detected (Laszlo *et al.*, 1995a). Moreover, aminoguanidine inhibited both the constitutive and inducible enzymes in homogenates of these tissues, exhibiting only a two fold selectivity against the inducible isoform (Laszlo *et al.*, 1995a). The promotion of neutrophil adhesion by aminoguanidine is likely to contribute to the observed acute intestinal microvascular injury following its administration (Laszlo *et al.*, 1995a), as found with the vascular dysfunction seen with L-NAME (Kubes *et al.*, 1992; Laszlo *et al.*, 1995b).

The pro-adhesive action of NO synthase inhibitors, which can be reversed by a specific antibody to the CD-18 leukocyte adhesion glycoprotein, may involve a mast cell-dependent process (Kubes *et al.*, 1991; 1993) with the local release of mediators such as platelet activating factor and leukotriene B_4 (Arndt *et al.*, 1993). The acute microvascular leakage provoked by L-NAME in the intestine of endotoxin-treated rats also involves the release of these mediators (Laszlo *et al.*, 1994b; Laszlo & Whittle, 1995). The involvement of similar processes in the mechanisms underlying the acute actions of aminoguanidine on leukocyte adherence and acute microvascular injury thus warrants evaluation.

A decrease in hydrodynamic dispersal forces, such as shear rate, which normally tend to sweep neutrophils away from the vascular wall also contributes to the increase in neutrophil adhesion induced by L-NAME (Kubes *et al.*, 1991). In the present study, both L-NAME and aminoguanidine decreased mesenteric arteriolar diameter within 10 min of administration, which would consequently reduce venular blood flow, an effect abolished by pretreatment with L-arginine.

The acute increase in systemic BP observed following administration of inhibitors of NO synthase such as L-NMMA or L-NAME is considered to reflect inhibition of constitutive NO synthase in the vascular endothelium (Rees *et al.*, 1989; 1990; Whittle *et al.*, 1989). Thus, the apparent low hypertensive activity of aminoguanidine in previous studies, when compared with that of L-NMMA, has been taken as evidence of a minimal action on the constitutive enzyme (Corbett *et al.*, 1992; Hasan *et al.*, 1993). However, in the present study, intravenous administration of aminoguanidine resulted in a dose-dependent increase in BP, an action inhibited by pretreatment with L-arginine. Aminoguanidine was however less potent than L-NAME and in addition, its profile of action was different. Thus, aminoguanidine induced a rapid and transient increase in BP, reaching its maximal value within 1 min of administration, while the response to L-NAME was slower to develop but was well sustained throughout the 1 h experimental period. In a previous study, subcutaneous administration of aminoguanidine induced a slow and prolonged increase in BP over 1 h, with the maximal increase being of similar magnitude to that observed in the current study (Laszlo *et al.*, 1995a). Interestingly, the actions of aminoguanidine on BP were more rapid in onset and less maintained than on arteriolar diameter, or on neutrophil adhesion in the venules, suggesting a dissociation between its effects on the local and systemic circulation.

Aminoguanidine has a broad profile of pharmacological and biochemical actions, which include inhibition of diamine oxidase, an enzyme responsible for the metabolism of histamine (Ohrui *et al.*, 1992). This action could therefore result in an increase in endogenous histamine levels, which can itself induce neutrophil rolling (Lawrence & Springer, 1991). Such effects could thus contribute to the observed actions of aminoguanidine on neutrophil activity in mesenteric venules, along with inhibition of the constitutive NO synthase *in vivo*. Thus, the use of aminoguanidine as a selective inhibitor of inducible NO synthase *in vivo* and interpretation of the experimental findings with this compound should be approached with caution.

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