British Journal of Pharmacology (2000) 131, 1227–1235

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Effects of chronic and acute aminoguanidine treatment on tail artery vasomotion in ageing rats

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1 This study was designed to evaluate the effects of aminoguanidine, a selective inhibitor of the inducible isoform of nitric oxide synthase (iNOS), on the reactivity and intracellular calcium ($[Ca^{2+}]_i$) mobilization induced by noradrenaline in the perfused tail artery from aged WAG/Rij rats. Global mean internal diameter was 350 ± 15 microns and wall thickness 161 ± 3 microns. The influence of the endothelium on these responses was also analysed. The intracellular dye fura-2 for $[Ca^{2+}]_i$ measurements was used.

2 Noradrenaline-induced vasoconstriction decreased progressively from 3 to 20 and 30 months. Removal of the endothelium attenuated vasoconstriction in 20 and 30 month-old rats ($P<0.05$) but not in young rats.

3 Chronic administration of aminoguanidine (50 mg kg^{-1} day^{-1}, p.o.) to WAG/Rij rats from 20 to 30 months enhanced ($P<0.01$) the $[Ca^{2+}]_i$-sensitivity of noradrenaline-induced vasoconstriction.

4 Aminoguanidine (300 μM) *in vitro* significantly shifted the concentration-vasoconstriction curve to noradrenaline to the left ($P<0.01$) in denuded vessels from both 20 and 30 month-old rats. The acute inhibitory effect of aminoguanidine was also observed after chronic aminoguanidine treatment. Aminoguanidine failed to modify vasoconstriction in the presence of the endothelium.

5 Acute aminoguanidine (300 μM) treatment did not modify vasoconstriction induced by noradrenaline in young rats.

6 Quantification of iNOS mRNA expression in tail arteries from 3 and 20 month-old WAG/Rij rats showed that expression was enhanced ($\times 2.1$, $P<0.01$) with age.

7 These results suggest that an inflammatory process develops in the media of the rat tail artery with age and that the subsequent increase in non-endothelial iNOS activity attenuates noradrenaline-induced vasoconstriction.

British Journal of Pharmacology (2000) **131,** 1227–1235

Keywords: Ageing; tail artery; endothelium; aminoguanidine; inducible NO synthase; noradrenaline; vasoconstriction; $[Ca^{2+}]_i$ mobilization

Abbreviations: AF, autofluorescence; AG, aminoguanidine; AG_{acute}, acute aminoguanidine treatment; AG_{chron}, chronic aminoguanidine treatment; b.p.m., beats per min; $[Ca^{2+}]_i$, intracellular calcium; cNOS, constitutive nitric oxide synthase; c.p.s., counts per second; F, fluorescence; i.d., internal diameter; IL, interleukin; i.p., intraperitoneal; iNOS, inducible nitric oxide synthase; o.d., outer diameter; p.o., oral; PSS, physiological saline solution; SNP, sodium nitroprusside; TNF, tumour necrosis factor

Introduction

Repeated inflammatory episodes occur during the ageing process leading to an increased systemic inflammatory response. Production and/or plasma concentrations of cytokines increase in both old animals and elderly humans (Foster *et al*., 1992; Wei *et al*., 1992). Elevated levels of endotoxin (lipopolysaccharide) or cytokines, such as interleukins (IL), interferon γ and tumour necrosis factor (TNF), stimulate expression of the inducible Ca^{2+}-independent isoform of NO synthase (iNOS) in a variety of cells, such as vascular smooth muscle (Busse & Mülsh, 1990; Fleming *et al*., 1991) and endothelial cells (Randomski *et al*., 1990; Michielsen *et al*., 1997). Several studies show that aminoguanidine, an L-arginine analogue, behaves as a potent and selective inhibitor of the iNOS in rat pulmonary (Griffiths *et al*., 1993) and mesenteric resistance arteries (Hasan *et al*., 1993; Heinemann & Stauber, 1995), and in rat aorta (Yen *et al*., 1995).

Functional consequences of expression of iNOs and NO-overproduction are vasodilatation and hyporesponsiveness to contractile agents such as α-adrenergic agonists (Wakabayashi *et al*., 1987; Griffiths *et al*., 1995; Thorin-Trescases *et al*., 1995). Isolated endotoxin-treated arteries show impaired contraction in response to phenylephrine (Griffiths *et al*., 1993), noradrenaline (Yen *et al*., 1995) or methoxamine (Heinemann & Stauber, 1995), that is restored by inhibitors of NO synthase, including aminoguanidine. Similar results have also been obtained in human arteries *in vitro* (Thorin-Trescases *et al*., 1995).

A study in the aorta of WAG/Rij rats showed increased cytokine production in the arterial wall with age (Belmin *et al*., 1995). It was proposed that enhanced formation in plasma and the arterial wall of advanced glycation end products, contributes to the increase in cytokine production

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via activation of macrophage-specific receptors and induction of TNF- and IL-1-secretion.

The aim of this study was to investigate whether aminoguanidine-sensitive iNOS activity played a role in vasomotion of the aged arterial wall. For this purpose, we propose: (a) to examine the impact of aminoguanidine treatment on the reactivity to noradrenaline in the isolated perfused tail artery from aged WAG/Rij rats; (b) to determine whether changes in noradrenaline-induced vasoconstriction by aminoguanidine are associated with differences in $[Ca^{2+}]_i$ mobilization, and (c) to evaluate the influence of the endothelium on these responses. We have previously shown that nitric oxide (of endothelial origin) modifies the $[Ca^{2+}]_i$-sensitivity of contraction without changing agonist-induced $[Ca^{2+}]_i$ mobilization (Tran *et al*., 1998).

Methods

The experiments were performed in 3, 20 and 30 month-old male normotensive WAG/Rij rats that were born and raised in the specific pathogen-free animal facility of the Centre d'Etudes de Saclay (Gif sur Yvette, France). The rats were housed at 20°C with a 12 h light/dark cycle, and allowed free access to food and water. At the age of 20 months, a subgroup was treated with aminoguanidine for 10 months. Aminoguanidine was given orally, added to the drinking water (1 g l^{-1}). The mean dose of aminoguanidine administered was 16 mg day^{-1} per rat, i.e. 50 mg day^{-1} kg^{-1} body weight.

Experiments were performed in accordance with the guidelines of the French Ministry of Agriculture (permit no. 005633).

Blood pressure in anaesthetized rats

Animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p., Sanofi Sante Nutrition Animale, Libourne, France). A polyethylene cannula (0.58 mm i.d. and 0.96 mm o.d., Jencons, Leigthon Buzzard, Bedfordshire, U.K.) was introduced into the left femoral artery and connected to a low-volume strain-gauge transducer (Baxter, Bentley Laboratories Europe, Uden, Netherlands) for measurement of blood pressure and heart rate. After an equilibration period of 30 min, blood pressure (systolic, diastolic and mean; mmHg) and heart rate (beats per min, b.p.m.) were measured during 15 min.

Effect of chronic aminoguanidine treatment on the $[Ca^{2+}]_i$-sensitivity of vasoconstriction

Simultaneous measurement of $[Ca^{2+}]_i$ mobilization and vasoconstriction were performed in the perfused tail artery using the technique described elsewhere (Capdeville Atkinson *et al*., 1993, 1995). The $[Ca^{2+}]_i$-sensitivity of noradrenaline-induced vasoconstriction was studied in the tail artery of 20 and 30 month-old aminoguanidine-treated and control rats in the presence or in the absence of the endothelium. The tail artery was dissected out under sodium pentobarbitone anaesthesia (60 mg kg^{-1}, i.p.; Sanofi). The proximal end of the vessel was cannulated with polyethylene tubing (0.4 mm i.d. and 0.8 mm o.d.; Jencons) and perfused *in situ* with a physiological salt solution (PSS, mM: NaCl 140; KCl 5; $CaCl_2$ 1.5; $MgCl_2$ 1; glucose 6; and HEPES 10; pH 7.40 ± 0.01) at a rate of 4 ml min^{-1} and an intraluminal pressure of 50 mmHg. The artery was thus removed from the tail under constant perfusion and placed in PSS at 22°C. A 1 cm segment was cannulated at both ends (intravascular portion of cannula, 0.2 mm) and mounted in a perfusion-cuvette system placed in a dual-wavelength spectrofluorometer (Fluorolog F1 T11; Spex, Edison, NJ, U.S.A.). A longitudinal tension of 0.5 g was used to restore the segment to its *in situ* length.

Arteries were perfused with PSS saturated with 100% O_2 (1.5 ml min^{-1}); the cuvette was filled with oxygenated PSS. Changes in perfusion pressure (ΔP, mmHg) were measured by a pressure transducer (Baxter) placed between the peristaltic pump and the arterial segment; the transducer was linked to a signal processing system (MacLab/MacBridge; World Precision Instruments Inc., Sarasota, FL, U.S.A.). All pressure values were corrected by subtraction of the pressure generated by the resistance of the tubing. After mounting, preparations were left to equilibrate for 30 min under constant perfusion with PSS. Towards the end of this equilibration period, an excitation spectrum of the non-loaded artery was determined at excitation wavelengths of 300–400 nm, and an emission wavelength of 510 nm. This provided values for basal autofluorescence [AF_{340} and AF_{380}, counts per second $\times 10^6$ (c.p.s. $\times 10^6$)]. Baseline perfusion pressure (mmHg) was also measured (19–22 mmHg; Table 1). The vessel was first stimulated three times with a high-potassium depolarizing solution (80 mM KCl, 2 min) in order to evaluate its reactivity, then vessels were contracted with noradrenaline (1 μM).

The arterial segment was perfused for 90 min with PSS containing the acetoxymethyl ester (AM) form of fura-2 (5 μM), previously dissolved in DMSO (1 mg ml^{-1}), Pluronic F127 (0.06%), and bovine serum albumin (0.01%). Loading was followed by a washout period of 20 min to remove any extracellular non-loaded ester and to allow completion of the intracellular hydrolysis of the dye. The segment was illuminated continuously at excitation wavelengths of 340 or 380 nm with a change from one to the other every second. A non-cumulative concentration-response curve to noradrenaline (0.1–30 μM) was constructed. For each stimulation, changes in intraluminal perfusion pressure and fluorescence were recorded. The fluorescence emitted by the artery (F_{340} and F_{380}, c.p.s. $\times 10^6$) was sampled and measured with a photomultiplier at an emission wavelength of 510 nm. An increase in $[Ca^{2+}]_i$ resulted in an increase in F_{340} and a decrease in F_{380} (increase in $R_{340/380}$). In all experiments, the changes in F_{340} and F_{380} recorded in the arterial preparations were in opposite directions. Basal autofluorescence values (F_{Mn}, see below) were substracted from the fluorescence values to give fluorescence signals (F'_{340} and F'_{380}). The background corrected ratio ($R'_{340/380}$) was representative of changes in intracellular $[Ca^{2+}]_i$. After washout, and a fall in tension to baseline, vessels were submitted to coperfusion of air for 10 min to disrupt the endothelium (Tatchum-Talom & Atkinson, 1997; Tran *et al*., 1998). Preparations were left to equilibrate for 20 min and the concentration-response curve to noradrenaline was repeated. The presence or absence of endothelial cells after and before air perfusion was assessed using the silver nitrate *en face* technique (Caplan *et al*., 1974). Silver nitrate *en face* staining confirmed the physical integrity of the endothelium in the arterial segments at the beginning of the experiments, showing the typical cobblestone pattern of this layer, air perfusion successfully removed endothelial cells from the luminal surface of the vessels (results not shown).

At the end of the protocol, maximal and minimal fluorescent signals were determined by the internal calibration

Table 1 Body weight, blood pressure, heart rate, basal perfusion pressure and autofluorescence, and response to KCl in perfused tail arteries of young and old WAG/Rij rats

Age (months)	*3*	*20*	*30*			
AG_{chron}	−	−	−	+	P_{age}	$P_{AGchron}$
Body weight (g)	285±10 (5)	383±15 (11)	371±13 (10)	352±8 (12)	<0.05	>0.05
Blood pressure (mmHg)					>0.05	>0.05
Systolic	131±3	131±5	125±5	119±6		
Diastolic	105±3	106±4	95±5	90±5		
Mean	119±3 (9)	118±4 (7)	109±5 (10)	103±5 (7)		
Heart rate (b.p.m.)	365±11 (9)	324±9 (7)	309±7 (10)	316±13 (7)	>0.05	>0.05
Basal						
Perfusion pressure (mmHg)	19±1 (10)	22±1 (11)	22±1 (13)	22±1 (12)	>0.05	>0.05
Autofluorescence (cps × 10^6)						
AF_{340}	ND	1.95±0.19	1.82±0.12	1.80±0.12	>0.05	>0.05
AF_{380}	ND	1.80±0.16 (6)	1.83±0.10 (6)	1.76±0.12 (6)	>0.05	>0.05
ΔP (mmHg) following KCl (80 mM, 2 min)	96±11 (10)	111±11 (11)	86±6 (13)	88±10 (12)	>0.05	>0.05

AG_{chron} = chronic aminoguanidine treatment (50 mg kg^{-1} day^{-1}, p.o.). Number of rats or segments are given in brackets. ND: not determined.

method described by Scanlon *et al.* (1987). R'_{max} was estimated from the background corrected ratio of F'_{340}/F'_{380} determined in the presence of PSS containing 4 mM of Ca^{2+} (5 min). R'_{min} was estimated from the background corrected ratio of F'_{340}/F'_{380} determined in the presence of calcium-free PSS [ethyleneglycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA); 10 mM, 10 min]. For the determination of R'_{max} and R'_{min}, the perfusate contained ionomycin (10 μM, pH = 7.40±0.01).

$[Ca^{2+}]_i$ was calculated using a modification (Capdeville-Atkinson *et al.*, 1995) of the equation of Grynkiewicz *et al.* (1985) and Scanlon *et al.* (1987):

$$[Ca^{2+}]_i = [(R' - R'_{min})/(R'_{max} - R')]\beta'$$

in arbitrary units (a.u.), where $R' = R'_{340/380}$; R'_{max} is the ratio of fluorescence in the presence of a saturating concentration of calcium ($CaCl_2$, 4 mM) and the calcium ionophore ionomycin (10 μM, 5 min); R'_{min} is the ratio of fluorescence in the presence of Ca^{2+}-free PSS containing EGTA (10 mM) and ionomycin (10 μM, 7 min); and β' is the ratio of F'_{380} at zero and saturating calcium concentrations.

The absence of $[Ca^{2+}]_i$-insensitive, fluorescence metabolites of fura-2 was checked by perfusing with Ca^{2+}-free PSS containing manganese chloride (1 mM) and ionomycin (10 μM) at the end of the experiment for 3 min. F_{Mn} was used to determine the extent of fura-2-$[Ca^{2+}]_i$ fluorescence (Scanlon *et al.*, 1987) and for background correction. Parameters were determined using intraluminal perfusion.

$[Ca^{2+}]_i$-sensitivity of vasoconstriction was estimated as: vasoconstriction/$[Ca^{2+}]_i$ mobilization ($\Delta P/\Delta[Ca^{2+}]_i$, mmHg a.u.$^{-1}$).

Acute aminoguanidine treatment

In a different series of experiments, the *in vitro* effects of aminoguanidine on noradrenaline-induced vasoconstriction was studied. The same protocol described above was applied in perfused tail artery segments from all experimental groups of animals plus a group of 3 month-old rats. The loading period with the fluorescent dye was replaced by perfusion of aminoguanidine (300 μM) added to the PSS, for 90 + 20 min, then throughout the experiment.

To test whether the effects of aminoguanidine were related to inhibition of NO synthesis rather than other non-specific effects, we further studied the effects of blockade of NO-production with L-N^G-nitroarginine methyl ester (L-NAME, 10 μM) on contractile responses to noradrenaline in endothelium-denuded arteries from young (3 months) and old (20 months) WAG/Rij rats. The same protocol as described for acute aminoguanidine treatment was applied.

Reactivity to NO-donor

The impact of the NO-donor sodium nitroprusside (100 μM) on the $[Ca^{2+}]_i$-sensitivity of 5-Hydroxytryptamine-induced vasoconstriction was performed in endothelium-denuded arteries using a protocol similar to that described above. Following loading with fura-2 and washout, 5-Hydroxytryptamine (3 μM) was perfused until a plateau vasoconstrictor response was obtained (4 min), then sodium nitroprusside was added for 4 min.

iNOS mRNA measurements by semi-quantitative polymerase chain reaction

Total RNA was extracted from tail arteries of 3 month- and 20 month-old WAG/Rij rats by using Trizol solution and a polytron homogenizer. Comparative reverse transcription-polymerase chain reaction was performed as described elsewhere (Challah *et al.*, 1997). One μg of total RNA was used to perform reverse transcription in a buffer containing 20 pmoles of oligo(dT)-(12–18) (Amersham Pharmacia Biotech, Uppsala, Sweden), 12 U of RNase inhibitor, dNTP 2.5 mM, DTT 5 mM and 160 U of MMLV RTase. iNOS mRNA was quantified by coamplifying GAPDH (17 cycles) and iNOS (35 cycles) with Taq polymerase 1 U, dNTP 0.1 mM, 0.1 μCi of α^{32}PdCTP using 1/10th of the reverse transcription mixture and 10 pmoles of each primers. The primers used were A2885 (5′-TGCTTTGTGCGGAGTGT-CAGT) and B3112 (5′-CGGACCATCTCCTGCATTTCT) for iNOS and GAPDH5′ (5′-ACCACAGTCCATGCCAT-CAC) and GAPDH3′ (5′-TCCACCACCCTGTTGCTGTA). Polymerase chain reaction products were separated on a 6%

polyacrylamide gel and quantified, after gel drying, using a phosphorimager (Bio-Rad, Hercules, CA, U.S.A.).

iNOS mRNA expression was calculated by normalizing iNOS mRNA to GAPDH mRNA.

Data analysis

Vasoconstriction was calculated as increments above the basal perfusion pressure (ΔP, mmHg) and $[Ca^{2+}]_i$ mobilization as increments above basal $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$, a.u.). Each individual set of concentration-effect (E/[A]) curve data was fitted to a logistic function (pragmatic logistic curve fitting) $E = \alpha\ [A]^m/[EC_{50}]^m + [A]^m$ in which E and [A] are the pharmacological effect and the concentration of agonist, respectively; α, EC_{50} and m are the asymptote, midpoint and slope parameters, respectively. Results are given as pD_2 (= negative logarithm to base 10 of the EC_{50}) and E_{max} (= α).

Statistical analysis

Results are expressed as means ± s.e.mean. Statistical evaluation was carried out using multivariate analysis of variance (ANOVA) to investigate the effects of age, aminoguanidine treatment and endothelium on noradenaline-induced vasoconstriction or $[Ca^{2+}]_i$ mobilization. A two-factorial ANOVA was used to analyse the effects of L-NAME on noradrenaline-induced vasoconstriction and of sodium nitroprusside on 5-Hydroxytryptamine-induced vasoconstriction. Statistical analysis for iNOS mRNA levels was performed using the non-parametrical method of Mann and Whitney. The level of significance was $P < 0.05$.

Drugs

Noradrenaline bitartrate, aminoguanidine hemisulphate, L-N^G-nitroarginine methyl ester, HEPES, fura-2 AM, bovine serum albumin, ionomycin, EGTA, sodium nitroprusside, 5-Hydroxytryptamine creatinine sulphate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pluronic F127 was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA, U.S.A.). NaCl, KCl, $CaCl_2$, $MgCl_2$ and glucose were purchased from Merck (Darmstadt, Germany). Trizol, RNase inhibitor, MMLV Rtase, Taq polymerase and dNTP solutions were from Life Technologies (Life Technologies Inc., Rockville, MD, U.S.A.). Ascorbic acid (100 μM) was added to the stock solution of noradrenaline (10 mM) to prevent oxidation. All other drugs were prepared daily by dissolving them in distilled deionized water, further dilutions were made in PSS.

Results

Body weight increased (33%) from 3 to 20 months but no further increase was observed at 30 months; aminoguanidine treatment had no effect on body weight. Neither ageing nor chronic aminoguanidine treatment had any effect on blood pressure or heart rate. Basal perfusion pressure did not differ between the groups and remained stable throughout the experiment. Chronic aminoguanidine treatment did not modify autofluorescence of arterial segments. The increase in perfusion pressure following stimulation with KCl was similar in all groups. These results are summarized in Table 1.

Concentration-response curves for noradrenaline-induced vasoconstriction are represented in Figures 1, 2 and 3; parameters estimated by logistic curve fitting are shown in Tables 2–4. Vasoconstrictor responses induced by noradrenaline significantly declined with age in intact and denuded vessels. Removal of endothelium produced a decrease of vasoconstriction elicited by noradrenaline in old animals (Figure 1). Chronic aminoguanidine treatment increased contractile responses (Figure 3 and Table 3).

Noradrenaline-induced $[Ca^{2-}]_i$ mobilization increased in a concentration-related manner. Ageing did not modify the amplitude of $[Ca^{2+}]_i$ mobilization either in the presence or the absence of the endothelium (Table 2). Chronic aminoguanidine treatment had no effect on the noradrenaline-induced $[Ca^{2-}]_i$-signal. Since in denuded vessels, contractile responses to noradrenaline were enhanced in aminoguanidine-treated

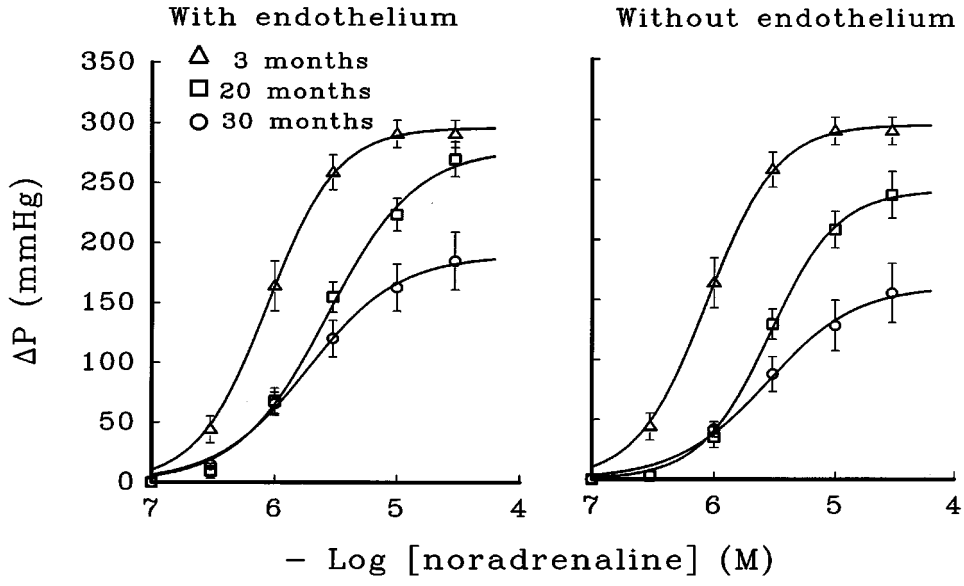

Figure 1 Concentration-response curves for noradrenaline-induced vasoconstriction in intact and endothelium-denuded tail artery segments from WAG/Rij rats. Results are expressed as increases in perfusion pressure (ΔP, mmHg). The lines drawn through the data were obtained by logistic curve fitting (see Methods). Results are the mean of 5–6 experiments; vertical lines show s.e.mean. ANOVA: $P_{age} < 0.001$; $P_{endothelium} < 0.05$; $P_{age \times endothelium} < 0.05$.

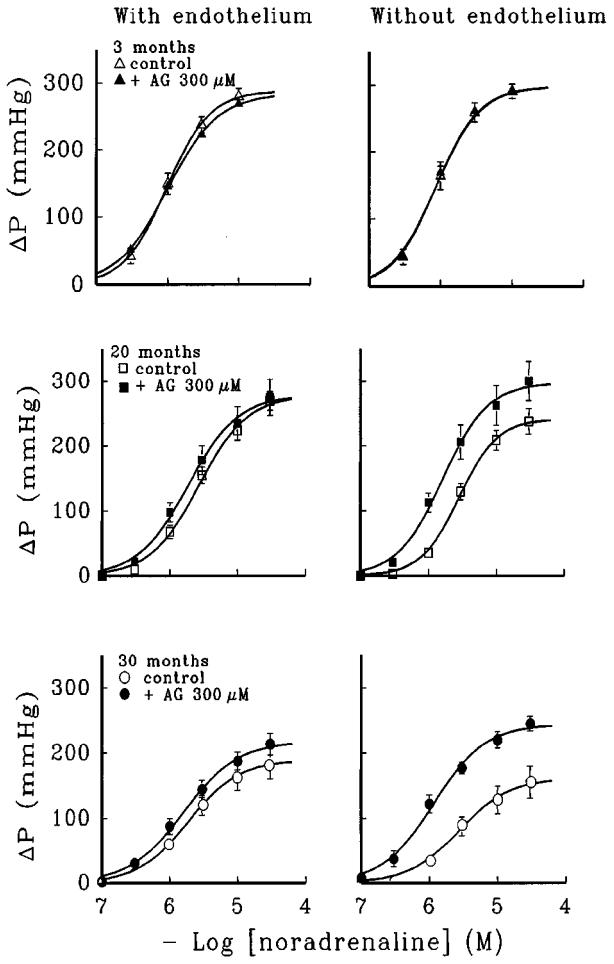

Figure 2 Effect of acute aminoguanidine treatment on noradrenaline-induced vasoconstriction in perfused tail artery segments with or without endothelium from WAG/Rij rats. Results are expressed as increases in perfusion pressure (ΔP, mmHg). The lines drawn through the data were obtained by pragmatic logistic curve fitting (see Methods). Results are the mean of 6–7 experiments; vertical lines show s.e.mean. ANOVA: $P_{age}<0.001$; $P_{AGacute}<0.001$; $P_{endothelium}>0.05$; $P_{age\times AGacute}<0.001$; $P_{age\times endothelium}>0.05$; $P_{AGacute\times endothelium}<0.001$.

animals with no change in $[Ca^{2+}]_i$, the $[Ca^{2-}]_i$-sensitivity of vasoconstriction, increased. The $[Ca^{2-}]_i$-sensitivity of vasoconstriction decreased with age (Table 2).

The effects of acute aminoguanidine treatment on the noradrenaline E/[A] curve were examined in tail arteries from all experimental groups, taking into account the presence or the absence of the endothelium. Basal perfusion pressure was not altered by aminoguanidine (300 μM) *in vitro*. Ninety minutes exposure to the iNOs inhibitor shifted the E/[A] to noradrenaline to the left in denuded vessels from 20 and 30 month-old rats. In contrast, in young rats, aminoguanidine failed to modify the concentration-response curves to the agonist (Figure 2, Tables 1 and 2).

Perfusion with aminoguanidine of tail artery segments from animals that previously underwent chronic aminoguanidine treatment led to a further enhancement of noradrenaline-induced vasoconstriction, that was more pronounced in the absence of the endothelium (Figure 3).

Treatment of tail artery segments with L-NAME (10 μM, 90 min) in the absence of the endothelium, significantly increased vasoconstrictor responses to noradrenaline in 20 month-old rats, whereas it was without effect in younger (3 months) animals (Figure 4).

Sodium nitroprusside (100 μM) produced a similar degree of vasorelaxation of tail arteries, in the absence of endothelium, at all ages (3: −43%, 20: −46%, 30 month-old: −42%) (Table 5).

iNOS mRNA expression was significantly increased ($\times 2.1$, $P<0.01$) in the tail artery of 20 month-old (3.00 ± 0.41, $n=8$) as compared with 3 month-old (1.43 ± 0.16, $n=10$) rats (Figure 5).

Discussion

The present study analysed vasoconstrictor responses induced by noradrenaline in the perfused rat tail artery with regard to ageing and treatment with aminoguanidine, a selective inhibitor of the iNOs, taking into account the influence of the vascular endothelium.

As previously shown in the perfused tail artery from WAG/Rij rats (Capdeville-Atkinson *et al*., 1995), ageing produced a progressive decline in noradrenaline- (and 5-Hydroxytryptamine-) induced contraction but did not modify responses to KCl. These results suggest that the basic contractile machinery was not impaired by age but that a specific alteration of receptor stimulation exists. Different factors could explain the age-related hyporesponsiveness to α-adrenoceptor stimulation: (1) morphological alterations, (2) changes in α-adrenoceptor density or affinity, (3) alteration in post-receptor events. Differences in the wall structure, α_1-adrenoceptor density or affinity do not seem to play a major role in the differences in vasoconstrictor responses between young and old animals (Vila *et al*., 1997). Thus, the explanation for the age-related hyporeactivity to α-adrenoceptor agonists probably lies in changes in the signal transductional mechanisms. The age-associated diminution of noradrenaline-induced vasoconstriction observed in this and a previous study (Capdeville-Atkinson *et al*., 1995) was not accompanied by a diminution in $[Ca^{2+}]_i$ mobilization. In addition, age does not influence α_1-adrenoceptor mediated phosphoinositide hydrolysis in tail artery (Tabernero & Vila, 1995). These findings suggest that steps downstream of $[Ca^{2+}]_i$ mobilization in the intracellular amplification pathways are involved (Capdeville-Atkinson *et al*., 1995).

Removal of endothelium significantly reduced noradrenaline-induced vasoconstriction in old but not in young animals. There is a consensus in published reports that the presence of the endothelium attenuates noradrenaline-induced vasoconstriction in arteries from young animals and that this effect wanes or is even reversed in senescence (Atkinson *et al*., 1994; Kung & Lüscher, 1995; Mantelli *et al*., 1995; Tabernero & Vila, 1995). The effect of the removal of endothelium could be explained by the removal of a vasoconstrictor influence or the amplification of a vasodilator influence. Considering the possibility that production and release of an endothelial vasoconstrictor (s) increases with age, one candidate is endothelin. Whilst the endothelial immunoreactivity of endothelin does not change with age (at least in the rat aorta, Aliev *et al*., 1995), it is possible that endothelin release and catabolism are modified. Another possibility is that the production and release of endothelial constrictor cyclooxygenase products increase with age (Koga *et al*., 1988). It is unlikely that amplification of an endothelial dilator factor is involved as endothelial NO production and release fall with age (Tschudi *et al*., 1996). It was suggested that increased inactivation of NO is involved (Kung & Lüscher, 1995). It is possible that amplification of a vasodilator influence involves release from the endothelium of free radicals that are

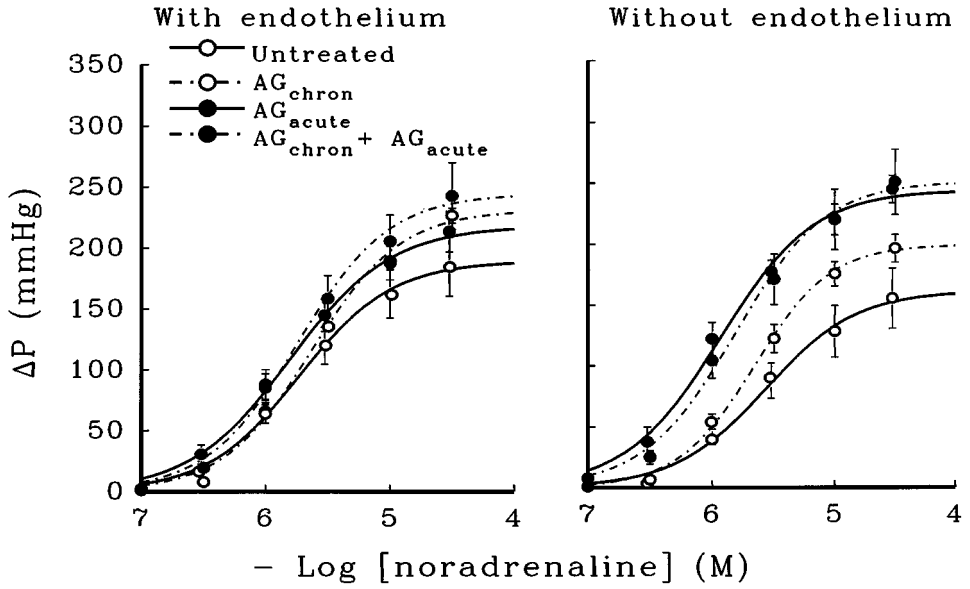

Figure 3 Noradrenaline-elicited contractions in intact and denuded tail arteries from 30 month-old WAG/Rij rats. Results are expressed as increases in perfusion pressure (ΔP, mmHg). The lines drawn through the data were obtained by pragmatic logistic curve fitting (see Methods). Results are the mean of 6–7 experiments; vertical lines show s.e.mean. ANOVA: $P_{AGchron}<0.05$; $P_{AGacute}<0.001$; $P_{endothelium}>0.05$; $P_{AGchron\ x\ AGacute}<0.05$; $P_{AGchron\ x\ endothelium}>0.05$; $P_{AGacute\ x\ endothelium}<0.001$.

Table 2 Noradrenaline-induced vasoconstriction, $[Ca^{2+}]_i$ mobilization and $[Ca^{2+}]_i$-sensitivity of vasoconstriction in perfused tail artery from control young and old WAG/Rij rats, in presence or absence of endothelium

Age (months)	*3*		*20*		*30*				
Endothelium	+	−	+	−	+	−	P_{age}	$P_{endothelium}$	$P_{age\times endothelium}$
Vasoconstriction (ΔP)							<0.001	<0.05	<0.05
pD_2	6.01±0.07	6.06±0.06	5.57±0.08	5.55±0.08	5.73±0.15	5.57±0.20			
α (E_{max})	288±18	296±17	278±18	241±19	189±21	161±25			
$\Delta[Ca^{2+}]_i$ (a.u.)							>0.05	>0.05	>0.05
pD_2	ND	ND	6.10±0.16	5.90±0.11	6.06±0.08	6.06±0.08			
α (E_{max})	ND	ND	0.57±0.05	0.58±0.04	0.60±0.08	0.66±0.04			
$\Delta P/\Delta[Ca^{2+}]_i$ (mmHg a.u.$^{-1}$)							<0.001	<0.001	=0.05
pD_2	ND	ND	5.90±0.06	5.80±0.07	6.13±0.08	5.93±0.12			
α (E_{max})	ND	ND	481±22	426±27	339±20	250±23			
n	5		6		6				

n: number of segments. Parameters were obtained by pragmatic logistic curve fitting (see Methods).

Table 3 Noradrenaline-induced vasoconstriction, $[Ca^{2+}]_i$ mobilization and $[Ca^{2+}]_i$-sensitivity in perfused tail artery from control and aminoguanidine-treated 30 month-old WAG/Rij rats, in presence or absence of endothelium

	Control		AG_{chron}				
Endothelium	+	−	+	−	$P_{AGchron}$	$P_{endothelium}$	$P_{AGchron\times endothelium}$
Vasoconstriction (ΔP)					<0.001	<0.01	>0.05
pD_2	5.73±0.15	5.57±0.20	5.63±0.04	5.66±0.06			
α (E_{max})	189±21	161±25	231±9	199±11			
$\Delta[Ca^{2+}]_i$ (a.u.)					>0.05	>0.05	>0.05
pD_2	6.06±0.08	6.06±0.08	6.20±0.09	6.14±0.12			
α (E_{max})	0.60±0.08	0.66±0.04	0.56±0.03	0.61±0.04			
$\Delta P/\Delta[Ca^{2+}]_i$ (mmHg a.u.$^{-1}$)					<0.01	<0.001	>0.05
pD_2	6.13±0.08	5.93±0.12	5.87±0.09	5.88±0.11			
α (E_{max})	339±20	250±23	438±31	345±28			
n	6		6				

n: number of segments. Parameters were obtained by pragmatic logistic curve fitting (see Methods). AG_{chron} = chronic aminoguanidine treatment (50 mg kg^{-1} day^{-1}, p.o.).

suppressing the effect of NO and this will be dealt with below. Factors other than endothelial factors must be involved, however, as in old animals, aminoguanidine has its greatest effect in the absence of endothelium. This could be explained by the fact that aminoguanidine acts as a free radical scavenger (Giardino *et al*., 1998; Tanaka *et al*., 1999) replacing the function of endothelial free radical scavengers. There are several arguments against this hypothesis. Firstly, L-NAME, an inhibitor of NOS, which is not a free radical scavenger, has an effect similar to that of aminoguanidine. Secondly, concerning the role of endothelial free radical scavengers, evidence for an increase with age in endothelial

Table 4 Noradrenaline-induced vasoconstriction in perfused tail artery from young and old WAG/Rij rats after acute aminoguanidine (300 μM) treatment, in presence or absence of endothelium

Age (months)	*3*		*20*		*30*				
Endothelium	+	−	+	−	+	−	P_{age}	$P_{endothelium}$	$P_{age \times endothelium}$
ΔP (mmHg)							<0.001	<0.001	>0.05
pD_2	6.00±0.04	6.07±0.03	5.73±0.12	5.79±0.10	5.80±0.10	5.94±0.07			
α (E_{max})	284±10	297±8	278±24	297±23	218±15	244±12			
n	5		5		7				

n: number of segments. Parameters were obtained by pragmatic logistic curve fitting (see Methods). For control values see Table 2.

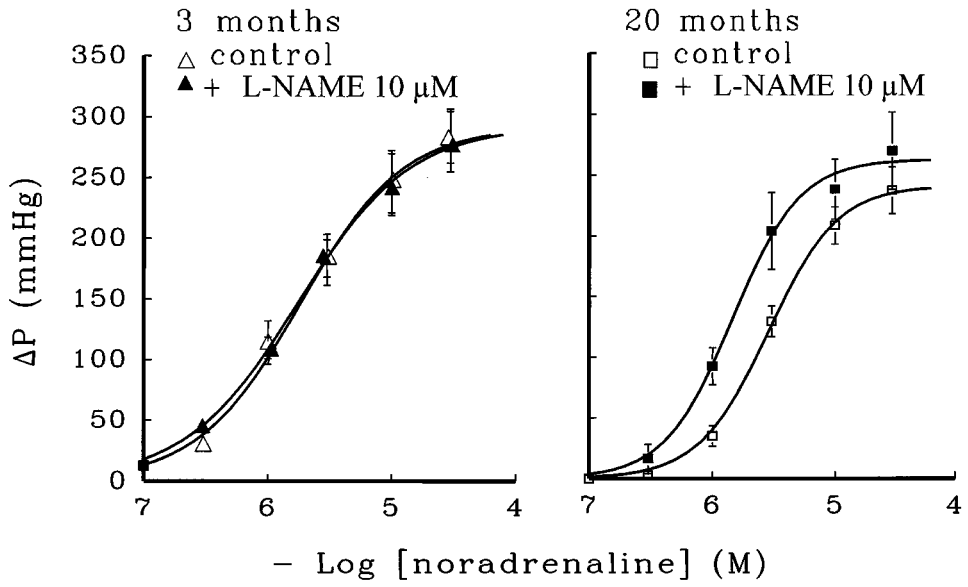

Figure 4 Concentration-response curves for noradrenaline-induced vasoconstriction in endothelium-denuded tail artery segments from young and old WAG/Rij rats in the absence or presence of L-NAME (10 μM). Results are expressed as increases in perfusion pressure (ΔP, mmHg). The lines drawn through the data were obtained by logistic curve fitting (see Methods). Results are the mean of 5–6 experiments; vertical lines show s.e.mean. Two-way ANOVA (20 months): $P_{L\text{-}NAME}<0.001$.

Table 5 5-Hydroxytryptamine (3 μM)-induced vasoconstriction, $[Ca^{2+}]_i$ mobilization and $[Ca^{2+}]_i$-sensitivity of vasoconstriction, in absence or presence of sodium nitroprusside (100 μM), in endothelium-denuded perfused tail arteries from young and old WAG/Rij rats

Age (months)	*3*		*20*		*30*				
Sodium nitroprusside	−	+	−	+	−	+	P_{age}	P_{SNP}	$P_{SNP \times age}$
Δ (mmHg)	295±8	168±10	160±26	87±11	172±19	99±8	<0.05	<0.05	>0.05
$\Delta P[Ca^{2+}]_i$ (a.u.)	0.55±0.02	0.57±0.05	0.51±0.02	0.49±0.03	0.54±0.01	0.58±0.04	>0.05	>0.05	>0.05
$\Delta P/\Delta[Ca^{2+}]_i$ (mmHg a.u.$^{-1}$)	536±25	295±35	314±28	178±32	319±20	171±26	<0.05	<0.05	>0.05
n	5		6		6				

n: number of segments.

Figure 5 mRNA levels of inducible nitric oxide synthase (iNOS) in tail arteries from 3 (left) and 20 (right) month-old WAG/Rij rats.

production of superoxide anions comes from the work of Rodriguez-Martinez *et al.* (1998). These authors showed that malondialdehyde reduced the endothelium-mediated vasodilator response of the rat tail artery to acetylcholine, and that this effect of malondialdehyde was abolished by superoxide dismutase (a superoxide anion scavenger).

They went on to show that the reduction in acetylcholine-induced relaxation required a 20 fold higher concentration of malondialdehyde in arterial segments from 30 month-old rats (compared to 6 month-old). Taken together these results suggest that, in the rat tail artery at least, endothelial superoxide anion production increases with ageing. However whether this modifies the NO system is less clear. Tschudi *et al.* (1996) showed that superoxide dismutase did not modify peak NO concentrations following the calcium ionophore, A23187, in aorta from old rats. On the basis of the above observations our working hypothesis is that smooth muscle cell iNOS production of NO following stimulation with noradrenaline increases with age so lowering the vasoconstrictor response to noradrenaline. Evidence to support this hypothesis is provided by the observation that iNOS expression increases with ageing in the arterial wall of the tail artery. The level of iNOS mRNA was 2.1 times higher in 20 month-old rats when compared with 3 month-old rats. Similar results have been described in the pulmonary artery

from WAG/Rij rats (Challah *et al*., 1997). In contrast, in the same strain of rats, iNOS level decreased with age in the aorta. Other authors have reported an enhanced expression of iNOS in the vessel wall of ageing aortas from Wistar rats (Cernadas *et al*., 1998). NO produced by iNOS may be partially scavenged by free radicals released from the endothelium but the balance between these two systems is in favour of increased levels of NO in old animals. Our conclusion is not modified by possible changes in smooth muscle cell reactivity to NO as experiments with sodium nitroprusside showed that the dilator effect of this NO-donor is similar at all ages.

Certain methodological provisos have to be raised which may affect the conclusions of our study. Firstly, it can be argued that a higher baseline perfusion pressure may be more appropriate. Higher perfusion pressures require much higher flow rates (Atkinson *et al*., 1986) and these reduce the duration of the viability of the preparation (results not shown). Use of the fura-2 technique requires a HEPES/O_2 perfusate (Grynkiewicz *et al*., 1985). This gives a high O_2 tension which may modify the scavenging of NO. It should be noted, however, that segments from animals of all ages were perfused with the same solution. Our hypothesis is based on the effects of aminoguanidine on noradrenaline-induced contraction and its lack of effect on KCl-induced contraction. However, full concentration-responses curves to KCl were not performed and the response to the single concentration of KCl used was lower than E_{max} for noradrenaline. Therefore non-specific changes in the contractility of vascular smooth muscle with ageing cannot be completely excluded on the basis of the results presented.

It is intriguing that chronic inhibition of iNOS with aminoguanidine does not raise blood pressure as has been shown to occur with chronic administration of other inhibitors of NOS. Several comments can be made on this question. Firstly, it should be noted that E_{max} to noradrenaline in the presence of aminoguanidine in old animals (200 mmHg) was lower than in the absence of aminoguanidine (300 mmHg) in young animals. Thus the possible *in vivo* change in diameter produced by aminoguanidine in old animals may not be great enough to increase blood pressure. Secondly, the greatest effect of aminoguanidine in old animals is seen following removal of endothelium which is not a physiological situation. Given that chronic aminoguanidine treatment does not raise blood pressure, what is the physiological or pathological relevance of our findings? It may be possible that changes in local iNOS activity with age are involved in local vasomotor regulatory mechanisms such as the shift in the lower limit of cerebral blood flow autoregulation with ageing (Lartaud *et al*., 1994). It is also possible that changes in local iNOS activity are involved in age-related changes in impedance rather than those in resistance. We have recently shown that chronic aminoguanidine treatment in old animals lowers the age-related increase in the rigidity of the aortic wall (results not shown).

Antonia Tabernero was supported by a post-doctoral fellowship from the Ministry of Culture and Education, Spain. The work was financed by the French Ministry of Education, Research and Technology (grant EA 3116). The authors wish to thank Caroline Cantini, Pascal Kieffer, Isabelle Lartaud-Idjouadiene (blood pressure measurement), Esther Spitzbarth-Régrigny (experiments on L-NAME), Laurence Oster, Nathalie Thorin-Trescases (experiments on sodium nitroprusside), Jean-Yves Jouzeau, Nathalie Presle (an attempt to measure NO release).

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(Received August 1, 2000
Accepted September 6, 2000)