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Chronic nitroglycerine administration reduces endothelial nitric oxide production in rabbit mesenteric resistance artery

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1 We investigated whether 10 days' *in vivo* treatment with nitroglycerine (NTG) would inhibit nitric oxide production by the endothelial cells of resistance arteries *ex vivo* and, if so, what the underlying mechanism might be.

2 ACh increased the intracellular nitric oxide concentration ($[NO]_i$; estimated using the nitric oxide-sensitive fluorescent dye diaminofluorescein-2) within the endothelial cells of rabbit mesenteric resistance arteries. This effect was significantly smaller in arteries isolated from NTG-treated rabbits than in those from control rabbits. The reduction in endothelial $[NO]_i$ in NTG-treated rabbits was prevented when olmesartan (blocker of type 1 angiotensin II receptors (AT_1Rs)) was coadministered *in vivo* with NTG and also when the superoxide scavenger manganese (III) tetrakis-(4-benzoic acid) porphyrin (Mn-TBAP), the protein kinase C (PKC) inhibitor GF109203X or L-arginine (with or without the active form of folate (5-methyltetrahydrofolate)) was incubated with the arteries *in vitro*.

3 Endothelial cell superoxide production (estimated by ethidium fluorescence) was greatly increased in arteries from NTG-treated rabbits. This was normalized by *in vivo* coadministration of olmesartan with NTG and also by *in vitro* application of Mn-TBAP or GF109203X (but not of 5-methyltetrahydrofolate + L-arginine).

4 ACh increased the intracellular Ca^{2+} concentration (estimated using the Ca^{2+}-sensitive dye Fura 2) within endothelial cells, the increase being not significantly different between NTG-treated rabbits and control rabbits.

5 We conclude that in NTG-treated rabbits, endothelial nitric oxide production in mesenteric resistance arteries is reduced, possibly through a reduction in the bioavailability of L-arginine *via* an action mediated by superoxide. Activation of the AT_1R–PKC pathway may be involved in increasing superoxide production.
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Abbreviations: AT_1R, type-1 angiotensin II receptor; $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}; DAF-2, diaminofluorescein-2; L-012, 8-amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4-(2*H*,3*H*)dione sodium salt; L-NNA, N^{ω}-nitro-L-arginine; Mn-TBAP, manganese (III) tetrakis-(4-benzoic acid) porphyrin; 5-MTHF, 5-methyltetrahydrofolate; $[NO]_i$, intracellular concentration of nitric oxide; eNOS, endothelial nitric oxide synthase; NTG, nitroglycerine

Introduction

Nitroglycerine (NTG) is widely used in the management of such cardiovascular diseases as angina pectoris**,** acute myocardial infarction and congestive heart failure. Despite their beneficial haemodynamic and anti ischaemic effects, the usefulness of organic nitrates is limited by tolerance, which develops shortly after treatment starts (Parker *et al*., 1991). More importantly, long-term (e.g. 3–10 days) NTG therapy causes endothelial dysfunction in both the coronary and forearm arterial beds in humans (Caramori *et al*., 1998; Gori *et al*., 2001b). In rat and rabbit aortae, the relaxation responses not only to nitrovasodilators but also to endothelium-derived nitric oxide decline after 3 days' *in vivo* administration of NTG (hereafter referred to as 'crosstolerance'; Münzel *et al*., 1995; Laursen *et al*., 1996; Berkenboom *et al*., 1999). The mechanisms underlying this crosstolerance may be multifactorial and may involve neurohormonal adjustments (such as activation of the renin–angiotensin–aldosterone axis) as well as changes intrinsic to the vasculature itself (Gori & Parker, 2002a, b).

In conduit arteries, an increase in superoxide production *via* activation of angiotensin II and/or protein kinase C (PKC) may play a role in the development of the crosstolerance seen in NTG-treated animals (Cai & Harrison, 2000; Münzel *et al*., 2000; Gori & Parker, 2002a, b). In addition, on the basis of measurements of endothelium-dependent relaxation, nitrite- and/or nitrate production and cGMP-production, it has been suggested that nitric oxide production in vascular endothelial cells may be reduced in both animals and humans treated *in vivo* with NTG (Gori & Parker, 2002a, b). Superoxide and PKC have been implicated in depletions of the intracellular

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concentrations of tetrahydrobiopterin and L-arginine in endothelial cells (Graf *et al*., 2001; Verhaar *et al*., 2002; Krotova *et al*., 2003; Alp & Channon, 2004). This may be important since a deficiency of the substrate L-arginine or of the cofactor tetrahydrobiopterin not only reduces nitric oxide production by endothelial nitric oxide synthase (eNOS) but also increases superoxide production *via* 'eNOS uncoupling' (Vásquez-Vivar *et al*., 1998; Gori & Parker, 2002a, b; Kalinowski & Malinski, 2004). In patients with familial hypercholesterolaemia, 5-methyltetrahydrofolate (5-MTHF; an active form of folate) has been reported to restore *in vivo* endothelial function in forearm veins (in which 'eNOS uncoupling' has been suggested to develop) (Verhaar *et al*., 1998), although conflicting evidence has also been published (Woodman *et al*., 2004). Taken together, these findings suggest that superoxide may inhibit endothelial nitric oxide production through 'eNOS uncoupling' in large arteries and veins. However, to judge from the available evidence this may not be the full story since superoxide decreases both the bioavailability and effect of nitric oxide (Münzel *et al*., 2000; Gori & Parker, 2002a, b). Furthermore, it remains unclear whether 'eNOS uncoupling' develops in resistance arteries, as well as in conduit arteries and veins, following long-term *in vivo* administration of NTG since the characteristic features of crosstolerance have been suggested to differ among vessel types (Zelis & Mason, 1975; Bassenge & Stewart, 1986; Stewart *et al*., 1987; Münzel *et al*., 1996).

A number of methods have been developed for the direct measurement of the intracellular concentration of nitric oxide ($[NO]_i$) within endothelial cells (Christodoulou *et al*., 1996; Nagano, 1999). However, the detection of nitric oxide *ex vivo* is hampered by its low production and rapid decomposition in vascular preparations. Although methods for the bioimaging of nitric oxide using electronic paramagnetic resonance and chemiluminescence assays have been developed, they are limited by serious technical drawbacks or low spatial resolution (Leone *et al*., 1996, Yoshimura *et al*., 1996). In recent years, the nitric oxide-sensitive fluorescence dye diaminofluorescein-2 (DAF-2) has been employed for the direct estimation of $[NO]_i$ (Kojima *et al*., 1998; Nakatsubo *et al*., 1998; Dedkova & Blatter, 2002; Murata *et al*., 2002; Pittner *et al*., 2003). We recently developed the ability to measure $[NO]_i$ by the use of DAF-2 in the endothelial cells of rabbit mesenteric resistance arteries *ex vivo*.

To clarify whether under *ex vivo* conditions, endothelial nitric oxide production in resistance arteries is reduced following long-term (10 days) *in vivo* treatment with NTG, we first observed the increase in endothelial $[NO]_i$ (estimated by DAF-2 fluorescence) induced by ACh in mesenteric resistance arteries isolated from NTG-untreated (control) and -treated rabbits. We next studied the effect of *in vivo* coadministration of the type-1 angiotensin II receptor (AT_1R) blocker olmesartan (Mizuno *et al*., 1995) with the NTG on the ACh-induced increase in endothelial $[NO]_i$. We also observed the *in vitro* effects of the following agents on the ACh-induced increase in endothelial $[NO]_i$: the superoxide scavenger manganese (III) tetrakis-(4-benzoic acid) porphyrin (Mn-TBAP) (Quijano *et al*., 2001), the PKC inhibitor GF109203X (Toullec *et al*., 1991; Nakano *et al*., 2004), the nitric oxide-synthase substrate L-arginine (either alone or together with 5-MTHF) and the tetrahydrobiopterin precursor sepiapterin (Shimizu *et al*., 1999).

Methods

Animals

All experiments performed in this study conformed to Guidelines on the Conduct of Animal Experiments issued by the Graduate School of Medical Sciences in Nagoya City University and were approved by the Committee on the Ethics of Animal Experiments in that institution. Male Japan White albino rabbits (supplied by Kitayama Labes, Ina, Japan), weighing 2.5–3.0 kg, were treated by applying transdermal NTG patches (Nitroderm TTS, Novartis Pharma, Tokyo, Japan) to a shaved dorsal thoracic area of the body. Such patches were present continuously for a period of 10 days (each patch being replaced daily with a new one) ('NTG-treated rabbits'). The theoretical delivery of NTG was $5\,mg\,24\,h^{-1}$. Using this protocol, we previously demonstrated the presence of 'nitrate-tolerance' by showing that the relaxing response to NTG was significantly reduced in the smooth muscle of mesenteric resistance arteries taken from such NTG-treated rabbits (Nakano *et al*., 2004). In some of the present NTG-treated rabbits, olmesartan medoxomil (referred to hereafter as olmesartan; $1\,mg\,kg^{-1}$) suspended in 0.15 % carboxymethylcellulose solution was administered orally once a day for the same 10-day period ('NTG + olmesartan-treated rabbits'). Male rabbits of a similar body weight served as controls ('control rabbits').

Blood pressure measurement

In some rabbits, mean arterial blood pressure was measured *via* an ear-artery catheter under light anaesthesia (pentobarbitone sodium $20\,mg\,kg^{-1}$ given intravenously (i.v.)). The pressure was continuously recorded for over 15 min and the mean pressure was averaged over the last 5-min period.

Tissue preparation

Rabbits were anaesthetized by injection of pentobarbitone sodium ($40\,mg\,kg^{-1}$ given i.v.), then killed by exsanguination. The third and fourth branches of the mesenteric artery distributing to the region of the ileum (diameter, approximately 120–150 μm) were immediately excised and placed in Krebs solution, then cleaned by removal of connective tissue. After each artery had been cut along its long axis using a small scissors, circularly cut strips were carefully prepared so as not to damage the endothelium. In some experiments, the endothelium was carefully removed by gentle rubbing of the intimal surface of the vessel using small pieces of razor blade, as previously described (Nakano *et al*., 2004).

Fluorescence detection

Fluorescence signals were detected using a CCD camera (C6790; Hamamatsu Photonics, Hamamatsu, Japan) fitted to an inverted fluorescence microscope (ECLIPSE TE300; Nikon, Tokyo, Japan), which allowed superfusion with the experimental solution. The microscope was equipped with objective lenses of a high numerical aperture (N.A.) (for $\times 20$ objective, N.A. = 0.75, Nikon; for $\times 40$ oil immersion objective, N.A. = 1.3, Nikon) and with filters appropriate for fluorescence microscopy.

An endothelium-intact strip was placed in a chamber of 1 ml volume with the luminal side down. Then, each end of the preparation was fixed using a small tungsten wire (diameter 0.02 mm), great care being taken to keep the endothelium intact. After the strip had been loaded with an appropriate fluorescent dye, the chamber was transferred to the fluorescence microscope. The focus was adjusted to reveal individual endothelial cells and the experiment was started after a 30-min perfusion with Krebs solution (at a flow rate of about 1 ml min^{-1}) under dark conditions. The images were captured and analysed using commercial software (AquaCosmos; Hamamatsu Photonics). These experiments were performed in such a way that control and test images were captured under the same conditions.

Measurement of $[NO]_i$

The $[NO]_i$ within endothelial cells was estimated from the increase in the fluorescence intensity of the nitric oxide-sensitive dye DAF-2 (Kojima *et al*., 1998; Nakatsubo *et al*., 1998). Endothelium-intact strips were exposed to membrane-permeable DAF-2 diacetate (10 μM) for 40 min at 37°C in Krebs solution, then washed with Krebs solution for 30 min. Next, ACh (3 μM) was applied for 15 min to the strips. DAF-2 was excited at 490 nm (half-width, 20 nm) at 60-s intervals and the DAF-2 fluorescence intensity within endothelial cells at any given time after the application of ACh (F) was normalized with respect to the fluorescence intensity just before the application of ACh (F_0) in the same experiment. Thus, changes in $[NO]_i$ are expressed as F/F_0. The mean fluorescence intensities obtained from five endothelial cells in each strip were averaged and this value (one value per strip) was used for the later analysis. All experiments were performed at 37°C.

The superoxide scavenger Mn-TBAP (40 μM), the PKC inhibitor GF109203X (3 μM), the eNOS substrate L-arginine (1 mM; either alone or together with the active form of folate, 5-MTHF (100 μM)), the tetrahydrobiopterin precursor sepiapterin (100 μM) or the nitric oxide-synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 0.1 mM) was applied to strips before and during the loading of DAF-2 (3 h application in total). ACh (3 μM) was then applied in the absence of the above agents.

Measurement of intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$)

The $[Ca^{2+}]_i$ was estimated using the ratiometric fluorescence Ca^{2+}-indicator Fura 2. Endothelium-intact strips were loaded with 5 μM Fura 2 acetoxymethyl ester (Fura 2-AM) in Krebs solution containing 0.001% Pluronic F-127 for 3 h at room temperature, then washed with Krebs solution for 30 min. Next, ACh (3 μM) was applied for 15 min to the strips. Fura 2 was excited by dual wavelengths (340 nm (F_{340}) and 380 nm (F_{380})) for 182 ms and collected through a 510 nm emission filter (half-width, 20 nm) at 15-s intervals. Changes in $[Ca^{2+}]_i$ were expressed as the change in the fluorescence ratio F_{340}/F_{380}. The mean fluorescence intensities obtained from five endothelial cells in each strip were averaged and this value (one value per strip) was used for the later analysis. All the experiments were performed at 37°C.

Superoxide production

The oxidative fluorescent dye dihydroethidium was used to detect superoxide production. Dihydroethidium, upon oxidation by intracellular superoxide anions, is converted to ethidium. This binds irreversibly to DNA, producing a bright red fluorescence. Segments (2.5 mm long) of endothelium-intact mesenteric resistance arteries were incubated with Krebs solution at 37°C for 3 h, then frozen in O.C.T. Compound (Tissue Tek; SAKURA Finetechnical, Tokyo, Japan). When the effects of Mn-TBAP (40 μM), GF109203X (3 μM) or L-arginine (1 mM) + 5-MTHF (100 μM) were to be examined, strips were incubated with the appropriate agent(s) for 3 h at 37°C before being frozen.

Transverse sections (10 μm thickness) were prepared on a cryostat (Microtome Cryostat HM 550; MICROM International GmbH, Walldorf, Germany) and placed on MAS-coated glass slides (Matsunami Glass, Kishiwada, Japan). They were then incubated in a light-protected chamber at 37°C for 30 min with 2 μM dihydroethidium. Images were obtained using a confocal laser-scanning microscope system (LSM5 PASCAL; Carl Zeiss, Jena, Germany). The excitation wavelength was 488 nm and emission fluorescence was detected through a 585 nm long-pass filter. Identical laser settings were used for the acquisition of images from different groups of rabbits. Fluorescence images were acquired as 12-bit files for subsequent analysis using commercial software supplied for the LSM5 PASCAL system (Carl Zeiss).

The changes in superoxide production in endothelium-intact strips of rabbit mesenteric resistance arteries (length 10–12 mm, width 0.5–0.6 mm) were also examined by using the superoxide-sensitive chemiluminescence dye 8-amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4-(2*H*,3*H*)dione sodium salt (L-012, 100 μM; Daiber *et al*., 2004) and a luminometer (Multi-biolumat LB 9505C; Berthold, Bad Wildbad, Germany).

Solutions

The ionic composition of the Krebs solution used was as follows (mM): 137.4 Na^+, 5.9 K^+, 1.2 Mg^{2+}, 2.6 Ca^{2+}, 15.5 HCO_3^-, 1.2 $H_2PO_4^-$, 134 Cl^- and 11.5 glucose. Nominal Ca^{2+}-free solution was prepared by replacing calcium chloride with magnesium chloride isosmotically (no EGTA added). The solutions were bubbled with 95% oxygen and 5% carbon dioxide.

Drugs

The drugs used in the current experiments were as follows: 5-MTHF and sepiapterin (Sigma Chemical Co., St Louis, MO, U.S.A.), Mn-TBAP (Alexis Biochemicals, San Diego, CA, U.S.A.), GF109203X (Tocris Cookson, Avonmouth, U.K.), dihydroethidium (Molecular Probes, Eugene, OR, U.S.A.), L-012 and L-arginine (Wako Pure Chemical, Tokyo, Japan), L-NNA (Peptide Institute Inc., Osaka, Japan), ACh-HCl (Daiichi Pharmaceutical, Tokyo, Japan), DAF-2 diacetate (Daiichi Pure Chemicals, Tokyo, Japan) and Fura 2-AM (Dojindo, Kumamoto, Japan). Olmesartan medoxomil (olmesartan) was kindly provided by Sankyo Pharmaceutical Co. (Tokyo, Japan).

GF109203X (10 mM) and sepiapterin (100 mM) were each dissolved in DMSO to make stock solutions, while Mn-TBAP was dissolved in ethanol (as a 50 mM stock solution). All other drugs were dissolved in ultra-pure Milli-Q water (Japan Millipore Corp., Tokyo, Japan). The stock solutions were stored at −80°C and diluted in Krebs solution to the required final concentrations immediately before use.

Statistical analysis

All results are expressed as the mean ± s.e.m. The *n* values represent the number of rabbits used. A two-way repeated-measures ANOVA (followed by Scheffé's *F*-test for *post hoc* analysis) or a Student's unpaired *t*-test with an *F*-test were used for statistical analysis. The level of significance was set at $P<0.05$.

Results

Blood pressure

The mean arterial blood pressure in NTG-treated rabbits (86.3 ± 2.5 mmHg, $n=3$) was not significantly different from that in NTG-untreated control rabbits (85.3 ± 1.9 mmHg, $n=3$; $P>0.1$). The blood pressure tended to be lower, although not significantly, in NTG + olmesartan-treated rabbits (80.7 ± 3.2 mmHg, $n=3$; $P>0.1$ *versus* control rabbits).

Nitric oxide production by endothelial cells

Using an objective lens with a relatively high numerical aperture, the focus was adjusted to reveal the intimal surface of a mesenteric resistance artery. Under our experimental conditions, DAF-2 fluorescence was only visible within the endothelial cells. ACh (3 μM) progressively increased the DAF-2 fluorescence ratio until about 15 min after its application (Figure 1A and B). The nitric oxide synthase inhibitor L-NNA (0.1 mM) abolished the ACh-induced increase in the DAF-2 fluorescence ratio in control rabbits (the ratio = 1.01 ± 0.03 at 15 min after ACh application, $n=7$; Figure 2a). ACh did not modify the DAF-2 fluorescence ratio in endothelium-denuded strips or in endothelium-intact strips in nominal Ca^{2+}-free solution (data not shown). The above ACh-induced increase in the DAF-2 fluorescence ratio was significantly smaller in arteries from NTG-treated rabbits ($n=17$) than in those from control rabbits ($n=13$) ($P<0.001$, two-way repeated-measures ANOVA; Figure 1Ab and 1B). The *in vivo* administration of the AT_1R blocker olmesartan with the NTG largely prevented the above effect of NTG ($n=10$; $P<0.05$ *versus* control rabbits and $P<0.01$ *versus* NTG-treated rabbits, two-way repeated-measures ANOVA; Figure 1Ac and 1B). By contrast, in control rabbits, the ACh-induced increase in nitric oxide production was not significantly modified by the *in vivo* administration of olmesartan for 10 days (the ratio = 1.25 ± 0.05 at 15 min after ACh application, $n=3$; $P>0.1$ *versus* olmesartan-untreated control rabbits).

Effects of Mn-TBAP, GF109203X, L-arginine (with or without 5-MTHF) and sepiapterin on nitric oxide production by the endothelial cells

In arteries from control rabbits, various treatments (the superoxide scavenger Mn-TBAP (40 μM), the PKC inhibitor GF109203X (3 μM) and the nitric oxide-synthase substrate

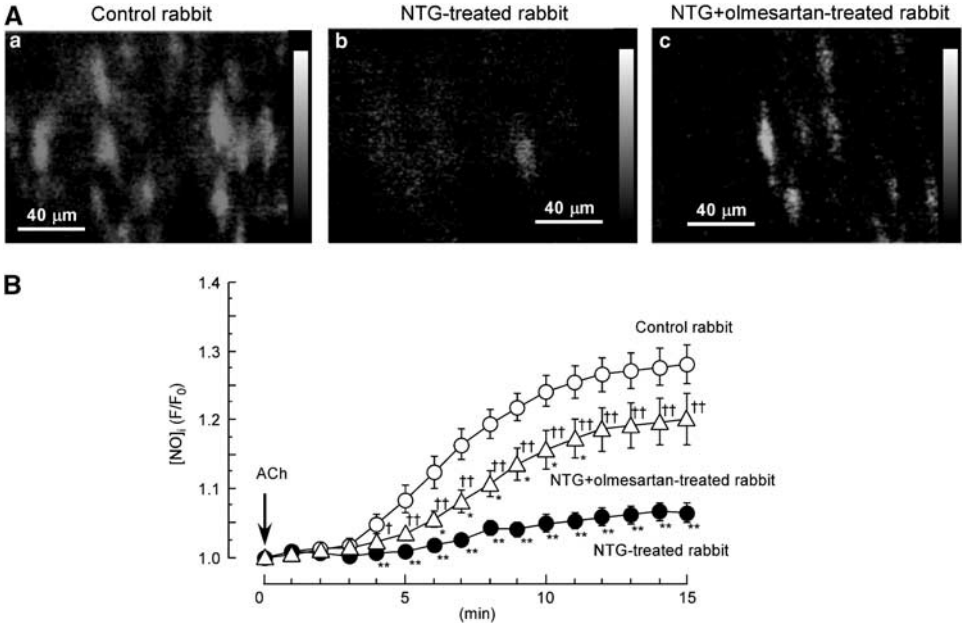

Figure 1 ACh-induced increase in $[NO]_i$ in endothelial cells of rabbit mesenteric resistance arteries. (A) Fluorescence ratio images of the nitric oxide-sensitive dye DAF-2 (taken at 15 min after application of 3 μM ACh) in endothelial cells. When used, olmesartan (blocker of type 1 angiotensin II receptors (AT_1Rs)) was coadministered *in vivo* with NTG. Fluorescence ratio was taken as the fluorescence intensity at 15 min after ACh application (F) divided by the fluorescence intensity just before ACh application (F_0) in each pixel. (B) Effect of ACh on DAF-2 fluorescence ratio as a function of time. $[NO]_i$ is expressed as the ratio of F (fluorescence intensity at a given time after ACh application) to F_0 (just before ACh application). Data are shown as mean ± s.e.m. $^{**}P<0.01$ *versus* 'Control rabbit'; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *versus* 'NTG-treated rabbit' (two-way repeated-measures ANOVA followed by Scheffé's *F*-test for *post hoc* analysis).

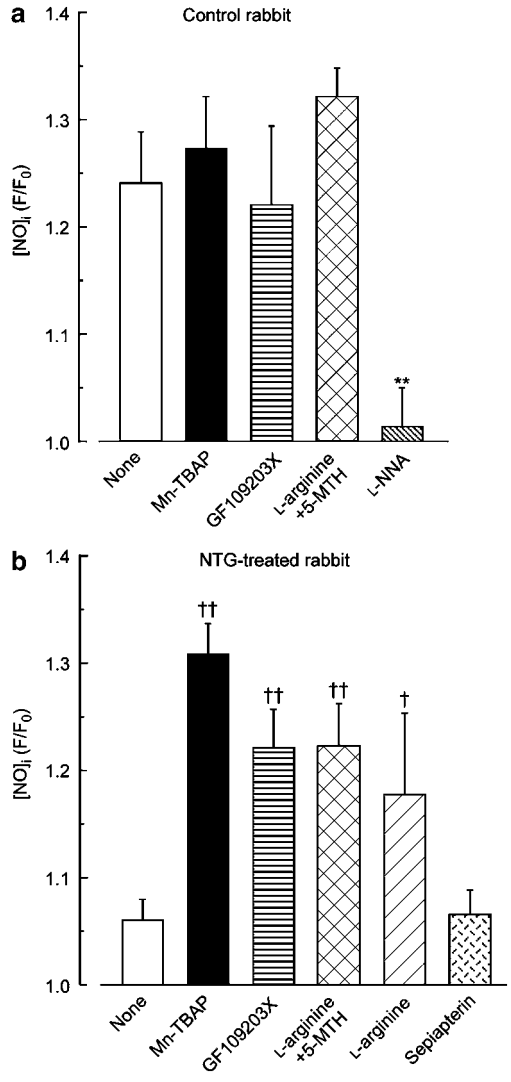

Figure 2 Effects of *in vitro* applications of L-NNA, Mn-TBAP, GF109203X, L-arginine (with or without 5-MTHF) and sepiapterin on ACh-induced increase in $[NO]_i$ in endothelial cells of rabbit mesenteric resistance arteries. (a) Control rabbits. (b) NTG-treated rabbits. Increase in $[NO]_i$ is expressed as ratio of F (fluorescence intensity at 15 min after ACh application) to F_0 (just before ACh application). Data are shown as mean ± s.e.m. $^{**}P<0.01$ *versus* 'None' in control rabbits; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *versus* 'None' in NTG-treated rabbits (Student's unpaired *t*-test).

L-arginine (1 mM) plus the active form of folate 5-MTHF (100 μM)) all failed significantly to modify nitric oxide production by endothelial cells at 15 min after an application of ACh (Figure 2a). By contrast, the reduced ACh-induced nitric oxide production seen in arteries from NTG-treated rabbits was significantly enhanced by all three of these treatments, towards the level seen in arteries from control rabbits (in each case, $n=7$; $P<0.01$ *versus* 'None' in NTG-treated rabbits; Figure 2b). Similarly, the reduced ACh-induced nitric oxide production seen in NTG-treated rabbits was restored by an application of L-arginine (1 mM) alone ($n=4$; $P<0.05$ *versus* 'None' in NTG-treated rabbits) but not by sepiapterin (100 μM; $n=6$; $P>0.1$; Figure 2b). In magnitude, the enhancement induced by L-arginine alone was similar to those induced by Mn-TBAP, GF109203X and L-arginine + 5-MTHF ($P>0.1$ in each case; Figure 2b).

Superoxide production within the vascular wall

In arteries from control rabbits, a faint superoxide production (estimated from ethidium fluorescence) was seen within a few endothelial cells (Figure 3Aa). In arteries from NTG-treated rabbits, superoxide production was very much increased within endothelial cells and was also seen in some smooth muscle cells (Figure 3Ab). It was normalized when olmesartan was coadministered *in vivo* with the NTG (Figure 3Ac). The increased superoxide production seen within the vascular wall in arteries from NTG-treated rabbits was greatly reduced when strips were coincubated for 3 h with either Mn-TBAP (Figure 3Bb) or GF109203X (Figure 3Bc) *in vitro*. By contrast, L-arginine + 5-MTHF had no such effect (Figure 3Bd).

In endothelium-intact strips, the chemiluminescence intensities of L-012 (100 μM, a superoxide-sensitive chemiluminescence dye) were not significantly different among the control rabbits, NTG-treated rabbits and NTG + olmesartan-treated rabbits. In the absence of SOD, the values (counts min^{-1} mg dry $weight^{-1}$) were 22,777 ± 1186, 20,707 ± 1402 and 23,730 ± 3843 for control, NTG- and NTG + olmesartan-treated rabbits, respectively ($n=3$ for each group, $P>0.1$). An addition of SOD (200 U ml^{-1}) caused total abolition of these signals (282 ± 321, −68 ± 983 and −73 ± 576 counts min^{-1} mg dry $weight^{-1}$ in control, NTG- and NTG + olmesartan-treated rabbits, respectively).

$[Ca^{2+}]_i$ increase within endothelial cells

The resting values obtained for the Fura 2 ratio in endothelial cells in rabbit mesenteric arteries were not significantly different between control rabbits (0.54 ± 0.02, $n=9$) and NTG-treated rabbits (0.58 ± 0.02, $n=6$, $P>0.1$). ACh (3 μM) induced a rapid transient increase in this ratio, followed by a tonic increase (with a slight decline as a function of time until 15 min) in arteries from both control rabbits and NTG-treated rabbits (Figure 4A and 4B). This ACh-induced increase was not significantly different between the two groups ($n=4$ for each group; $P>0.1$, two-way repeated-measures ANOVA).

Discussion

In the present study, we found that under *ex vivo* conditions, the ACh-induced increase in $[NO]_i$ in endothelial cells was significantly smaller in mesenteric resistance arteries from NTG-treated rabbits than in those from NTG-untreated control rabbits. We also found (a) that the intensity of the fluorescence due to the oxidative fluorescence dye dihydroethidium within vascular cells (including endothelial cells) was greatly increased in the NTG-treated group and (b) that this was normalized by the *in vitro* application of the cell-permeable superoxide scavenger Mn-TBAP. Although the assessment of superoxide production in the vascular wall using dihydroethidium is semiquantitative, these results suggest that superoxide production by vascular cells in mesenteric resistance arteries is increased in NTG-treated rabbits. Moreover, Mn-TBAP enhanced the ACh-induced increase in endothelial $[NO]_i$ in this resistance artery only in the NTG-treated group. These results indicate that long-term (10 days) *in vivo* administration of NTG reduces ACh-induced endothelial nitric oxide production by an action that is probably mediated

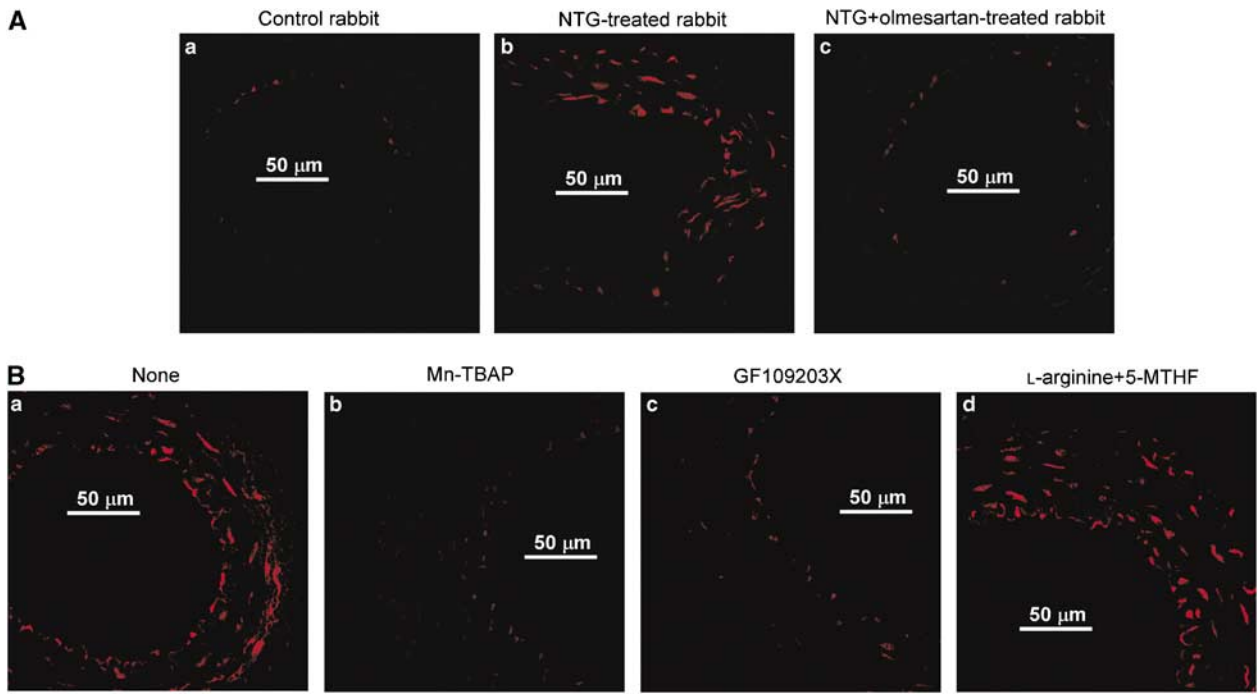

Figure 3 Superoxide production within vascular walls of rabbit mesenteric resistance arteries. (A) Superoxide production was estimated from the fluorescence intensity of the superoxide-sensitive dye dihydroethidium. (B) Effects of *in vitro* applications of Mn-TBAP, GF109203X and L-arginine + 5-MTHF on superoxide production in NTG-treated rabbits. For both (A) and (B), similar observations were made in other sections obtained from four preparations from four other animals.

via an increased cellular production of superoxide in rabbit mesenteric resistance arteries, just as it has been postulated to do in conduit arteries (Münzel *et al*., 1995; 1999; 2000; Kurz *et al*., 1999).

In the present experiments, the chemiluminescence intensities of L-012 (a superoxide-sensitive chemiluminescence dye) shown by the endothelium-intact strips of rabbit mesenteric resistance arteries were not significantly different among the control rabbits, NTG-treated rabbits and NTG + olmesartan-treated rabbits. Since addition of SOD caused total abolition of these signals, we believe that our system appropriately measured the production of superoxide, or at least that located in the extracellular space. Therefore, it may be that the chemiluminescence-based assay (using L-012) is not sensitive enough to detect changes in the production of superoxide in both the intracellular and extracellular spaces in such small segments of mesenteric resistance arteries from NTG-treated rabbits.

Reduced production of nitric oxide by endothelial cells in NTG-treated rabbits

eNOS contains two functionally distinct domains: an N-terminal oxygenase domain (containing binding sites for haem, tetrahydrobiopterin and L-arginine) and a C-terminal reductase domain (containing binding sites for FAD, FMN and NAD(P)H). These two domains are linked by a calmodulin-binding site. In this site, upon Ca^{2+} binding, calmodulin increases the rate of electron transfer from NAD(P)H to the reductase-domain flavins and from the reductase domain to the haem centre for oxidation of the cosubstrates O_2 and L-arginine, thereby producing nitric oxide and L-citrulline (Palmer *et al*., 1988; Moncada *et al*., 1991; Hemmens & Mayer, 1998). The presence of agonists or shear stress enhances the production of nitric oxide through an increase in $[Ca^{2+}]_i$ within endothelial cells and subsequently activates the cascade described above to produce nitric oxide. We found that in the endothelial cells of rabbit mesenteric resistance arteries, the increase in $[Ca^{2+}]_i$ induced by ACh was not altered in the NTG-treated group (compared with that in the NTG-untreated control group). This indicates that an abnormality in the downstream signals following the increase in $[Ca^{2+}]_i$ within the endothelial cell is responsible for the downregulation of nitric oxide production seen in rabbits treated *in vivo* with NTG.

Superoxide reduces $[NO]_i$ through its binding with nitric oxide to form peroxynitrite, which in turn uncouples eNOS through an oxidation of the eNOS cofactor tetrahydrobiopterin (Vásquez-Vivar *et al*., 1998; Gori & Parker, 2002a). Furthermore, long-term (3 days) *in vivo* administration of NTG may reduce the cellular concentration of the eNOS substrate L-arginine through an inhibition of its uptake into endothelial cells, as found in cultured bovine aortic endothelial cells (Abou-Mohamed *et al*., 2000). In conditions in which there is a deficiency of tetrahydrobiopterin or L-arginine, eNOS cannot catalyse the five-electron oxidation of L-arginine to nitric oxide, resulting in a decreased production of nitric oxide and, instead, an increased production of superoxide in high $[Ca^{2+}]_i$ conditions (Wever *et al*., 1997; Raman *et al*., 1998; Vásquez-Vivar *et al*., 1998). It has been suggested that the active form of folate, 5-MTHF, increases nitric oxide production through an enhanced binding of tetrahydrobiopterin to eNOS and/or an increased tetrahydrobiopterin availability (Stroes *et al*., 2000; Heller *et al*., 2001; Loscalzo, 2001; Verhaar *et al*., 2002). Moreover, it has been found that

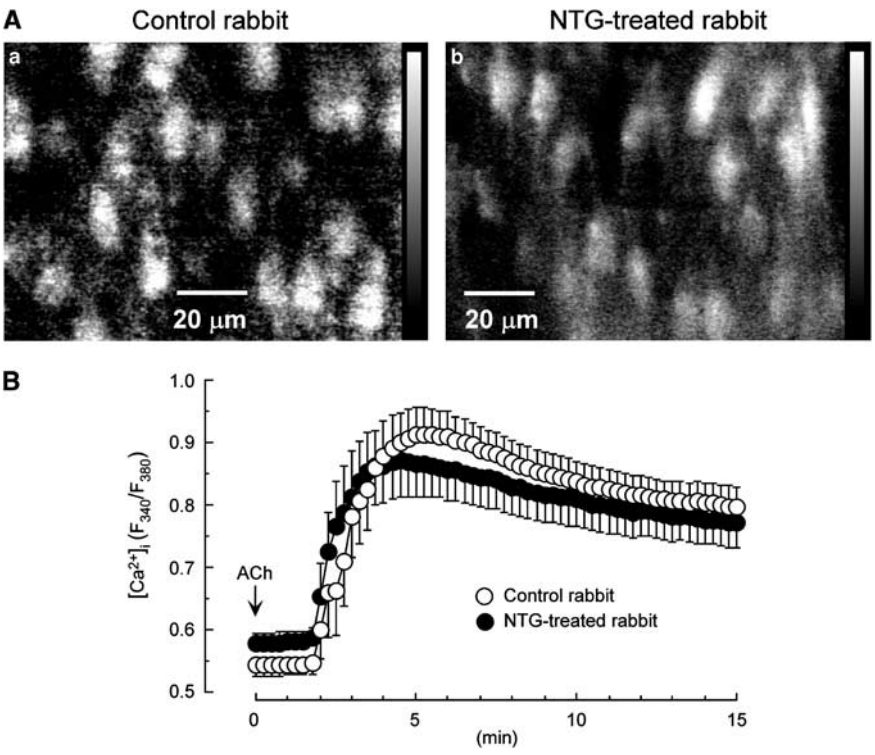

Figure 4 ACh-induced increase in $[Ca^{2+}]_i$ in endothelial cells of rabbit mesenteric resistance arteries. (A) Fluorescence ratio images of the Ca^{2+}-sensitive dye Fura 2 at 15 min after application of 3 μM ACh. (B) ACh-induced increase in $[Ca^{2+}]_i$ as a function of time. $[Ca^{2+}]_i$ is expressed as the Fura 2 ratio (F_{340}/F_{380}). Data are shown as mean $\pm$ s.e.m.

(i) 5-MTHF supplementation prevents the endothelial dysfunction induced by the chronic administration of NTG in healthy subjects (Gori *et al*., 2001a) and (ii) L-arginine administration improves tolerance to NTG in patients with angina (Parker *et al*., 2002). These pieces of evidence suggest that in the setting of nitrate tolerance, a cellular deficiency not only of tetrahydrobiopterin but also of L-arginine may be involved in the reduced nitric oxide production that exists in human vascular endothelial cells. In the present experiments, we found that in mesenteric resistance arteries from NTG-treated rabbits, the ACh-induced $[NO]_i$ increase in endothelial cells was enhanced by an *in vitro* application of L-arginine alone to the same extent as by coapplication of L-arginine and 5-MTHF. Furthermore, the tetrahydrobiopterin precursor sepiapterin alone had no effect on the ACh-induced $[NO]_i$ increase in NTG-treated rabbits. These results suggest that a reduction in the availability of intracellular L-arginine (rather than of tetrahydrobiopterin) may be causally involved in the reduced ACh-induced $[NO]_i$ increase seen in the endothelial cells of mesenteric resistance arteries from NTG-treated rabbits.

Role of AT_1R in superoxide production

In animals and humans, *in vivo* administration of NTG activates the renin–angiotensin–aldosterone axis *via* activation of a neurohormonal counter-regulatory mechanism (Münzel *et al*., 1996; Gori & Parker, 2002a, b). Angiotensin II binds to AT_1R and enhances the activity and/or expression of membrane-bound forms of NAD(P)H oxidases, thus increasing superoxide production by vascular cells (Touyz & Schiffrin, 2000; Mollnau *et al*., 2002; Kalinowski & Malinski, 2004). In the present experiments, *in vivo* coadministration of the AT_1R blocker olmesartan with NTG normalized the increased superoxide production by the endothelial cells of mesenteric resistance arteries that was seen in the NTG-treated group. However, it has been reported that another AT_1R blocker, losartan, does not prevent the tolerance to NTG shown by blood pressure and forearm-venous-volume responses in humans (Milone *et al*., 1999). Furthermore, it has been noted that losartan needs to be given at a high dose (10–25 mg kg^{-1}) to be very effective at preventing the nitrate tolerance seen in the aorta of NTG-treated rabbits (Kurz *et al*., 1999), and high doses are also needed in the case of angiotensin-converting-enzyme (ACE) inhibitors (Muiesan *et al*., 1993; Münzel *et al*., 1996). Taken together, these results suggest that to prevent nitrate tolerance by modulating the renin–angiotensin system, a powerful inhibition needs to be produced (e.g. by high-dose ACE inhibition or high-dose AT_1R blockade).

It has been suggested that PKC may, to some extent, mediate the above action of angiotensin II (Münzel *et al*., 1995; Harrison, 1997; Heitzer *et al*., 1999). In the present study, we found that *in vitro* application of the PKC inhibitor GF109203X normalized superoxide production by endothelial cells in arteries from NTG-treated rabbits, suggesting that long-term *in vivo* administration of NTG enhances superoxide production *via* an activation of AT_1R–PKC pathways within the endothelial cells of rabbit mesenteric resistance arteries. It was recently found that both NTG (Abou-Mohamed *et al*., 2004) and the nitric oxide donor *S*-nitroso-*N*-acetyl-DL-penicillamine (Balafanova *et al*., 2002) activate PKC in endothelial cells *via* a mechanism apparently unrelated

to the renin–angiotensin system. Furthermore, the transport of L-arginine into endothelial cells is downregulated by activation of PKC (Graf *et al*., 2001; Krotova *et al*., 2003). Taken together, these results suggest that activation of PKC may play a pivotal role in the downregulation of endothelial nitric oxide production seen in the setting of nitrate tolerance.

It is known that NTG tolerance affects the relaxation responses to other nitrovasodilators, as well as to endothelium-derived nitric oxide ('crosstolerance'; Gori & Parker, 2002a, b). Several possible mechanisms need to be considered for this crosstolerance, such as (i) a decrease in the bioavailability of nitric oxide (White *et al*., 1997), (ii) a desensitization of soluble guanylyl cyclase (Molina *et al*., 1987; Mollnau *et al*., 2002) and/or (iii) a reduction in cGMP-mediated relaxation (Soff *et al*., 1997; Nakano *et al*., 2004). In the present experiments, we found that ACh-induced nitric oxide production is reduced in the endothelial cells of mesenteric resistance arteries from rabbits treated with NTG. In the setting of NTG-tolerance, however, there seem to be differential effects among various types of blood vessels and species in the responsiveness to NTG (Zelis & Mason, 1975; Stewart *et al*., 1986; 1987; Münzel *et al*., 1996). Thus, we must stress the need for caution before any attempt is made to extrapolate the present results to other vascular beds and/or different species, in which additional mechanisms not addressed by the present experiments may help to underpin nitrate tolerance.

In conclusion, 10 days *in vivo* administration of NTG leads to a reduction in nitric oxide production by the endothelial cells of the rabbit mesenteric resistance artery. It is suggested that a decrease in the bioavailability of L-arginine resulting from an increased concentration of superoxide (through an activation of AT_1R–PKC pathways) within the endothelial cells may be the underlying mechanism.

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