British Journal of Pharmacology (2002) 136, 375–382
www.nature.com/bjp

Amlodipine, but not verapamil or nifedipine, dilates rabbit femoral artery largely through a nitric oxide- and kinin-dependent mechanism

[1,2]Biao Xu, [1]Li Xiao-hong, [1]Gao Lin, [2]Lindsay Queen & *[,2]Albert Ferro

[1]Department of Cardiology, Zhongda Hospital of Southeast University, Nanjing, People's Republic of China and [2]Centre for Cardiovascular Biology & Medicine, King's College London, London

1 We investigated the nitric oxide (NO) dependence of vasorelaxation in response to different calcium channel blockers (CCB), in rabbit femoral artery *in vivo*.

2 Anaesthetized rabbits underwent femoral artery ligation, and blood from the proximal artery was returned distal to the ligature through a constant infusion pump. The effects of local injection of CCB on perfusion pressure and plasma nitrite + nitrate (NO_x, which reflects local NO biosynthesis) concentration in this system were determined.

3 Intra-arterial verapamil, nifedipine or amlodipine 10 μmol kg^{-1} each reduced perfusion pressure. Pre-treatment with intra-arterial N^G-nitro-L-arginine methyl ester (L-NAME, a NO synthase inhibitor) 1 μmol kg^{-1} did not affect responses to verapamil or nifedipine, but attenuated the reduction in perfusion pressure to amlodipine, from $33.2 \pm 2.1\%$ to $22.5 \pm 1.6\%$ ($P = 0.002$).

4 Intra-arterial amlodipine – unlike verapamil or nifedipine – increased femoral venous NO_x, from 9.1 ± 0.4 μM to 14.1 ± 0.5 μM ($P = 0.005$).

5 The bradykinin B_2 receptor antagonist HOE 140, 30 mg kg^{-1}, attenuated the reduction in perfusion pressure and abolished the rise in venous NO_x concentration, following intra-arterial amlodipine.

6 Amlodipine potently inhibited serum angiotensin converting-enzyme (ACE) activity *in vitro*, as effectively as enalapril at similar concentrations.

7 These results suggest that the vasorelaxant effects of nifedipine and verapamil are NO-independent, whereas those of amlodipine are partly NO-dependent, in rabbit femoral artery *in vivo*. This effect of amlodipine occurs through B_2 receptor activation, and may be related to an increase in local bradykinin through inhibition of ACE.

British Journal of Pharmacology (2002) **136,** 375–382

Keywords: Calcium antagonists; amlodipine; nifedipine; verapamil; nitric oxide; bradykinin receptor; HOE 140; angiotensin converting-enzyme; vasorelaxation

Abbreviations: ACE, angiotensin converting-enzyme; ACh, acetylcholine; CCB, calcium channel blocker(s); HUVEC, human umbilical vein endothelial cells; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; NO_x, nitrite + nitrate

Introduction

Calcium channel blockers (CCB) are clinically useful vasodilators, used widely in the treatment of hypertension and ischaemic heart disease. Although their vasodilatory properties have traditionally been ascribed to their inhibitory action on voltage-gated calcium channels in vascular smooth muscle, some recent studies have suggested that amlodipine, a CCB of the dihydropyridine chemical class, has an additional vasodilatory effect through stimulation of nitric oxide (NO) release from blood vessels independent of its effect on calcium channels. Zhang and Hintze demonstrated that amlodipine, unlike nifedipine (another dihydropyridine CCB) or diltiazem (a chemically unrelated CCB of the benzothiazepine class), increases nitrite production from healthy canine coronary microvessels *in vitro*, reflecting an increase in NO biosynthesis, and that this increase is kinin-dependent (Zhang & Hintze, 1998). Similar results were obtained in coronary microvessels from dogs with pacing-induced heart failure (Zhang *et al*., 1999b). These same workers subsequently demonstrated in similar fashion that amlodipine can increase nitrite generation in coronary microvessels isolated from failing human hearts at the time of cardiac transplantation (Zhang *et al*., 1999a). This effect of amlodipine appears to be unconnected with its calcium channel-blocking activity, since this compound exists as two enantiomers, one of which possesses the ability to block L-type calcium channels whilst the other is responsible for its NO releasing property (Zhang *et al*., 2002).

Despite these *in vitro* findings, other groups have found no stimulatory effect of amlodipine on the cardiovascular NO system *in vivo* in a variety of models. Bennett *et al*. (1996) found that chronic amlodipine therapy reduced blood

*Author for correspondence at: Centre for Cardiovascular Biology & Medicine, King's College London, Guy's Hospital Campus, London Bridge, London SE1 1UL; E-mail: albert.ferro@kcl.ac.uk

pressure as effectively as chronic angiotensin converting-enzyme (ACE) inhibition in spontaneously hypertensive rats, but whereas ACE inhibition with quinapril or perindopril reversed the observed impairment of bradykinin-induced relaxation of mesenteric arteries in these rats amlodipine therapy did not. In a murine model of congestive heart failure, Wang *et al*. (1997) found that amlodipine had a protective effect against myocardial injury, and that this appeared to be in part the result of inhibition of NO production through suppressed expression of the inducible form of NO synthase (NOS) in myocardium.

Amlodipine may increase NO generation in the vasculature through an increase in kinin activity (Zhang & Hintze, 1998; Zhang *et al*., 1999a, b; 2000). Additionally, several CCB have been demonstrated to possess antioxidant and oxygen free radical-scavenging properties (Lupo *et al*., 1994; Hayashi *et al*., 1996; Napoli *et al*., 1996; Rojstaczer & Triggle, 1996; Lesnik *et al*., 1997; Sobal *et al*., 2001). Superoxide anion can remove NO by combining chemically with it, forming the potent oxidant and cytotoxic radical peroxynitrite. Thus, CCB may potentially increase NO availability through removal of superoxide anion.

In view of the lack of *in vivo* data regarding such effects, we wished to examine whether amlodipine increases vascular NO generation in an animal model, and the mechanism by which this may occur. For comparison, we also examined the NO dependency of the vascular effects of the related dihydropyridine CCB, nifedipine, and a chemically unrelated CCB, verapamil (a phenylalkylamine).

Methods

Experimental animals

New Zealand White rabbits were from the Nanjing Experimental Animals Centre of China, and were aged 6–8 months. All animal studies were carried out in accordance with the Declaration of Helsinki and according to the institutional regulations concerning animal experimentation of Southeast University, Nanjing, China.

Materials, chemicals and drugs

Amlodipine was graciously provided by Pfizer Ltd (Sandwich, Kent, U.K.). All other drugs and chemicals were from Sigma-Aldrich Company Ltd (Poole, Dorset, U.K.). They were prepared on the day of study in normal saline, and kept at 4°C until use, at which time they were re-warmed to 37°C.

Rabbit femoral artery perfusion model

Cannulation procedure and initial studies Rabbit femoral artery responses were studied *in vivo* as described previously (Xu *et al*., 2000). Briefly, rabbits were anaesthetized with intraperitoneal sodium pentobarbital 25 mg kg^{-1}, following which both femoral arteries were dissected from the inguinal ligament to the distal 1/3 of the hind limb. One femoral artery was cannulated with a catheter connected to a pressure transducer (Gould P_{50}), for monitoring of systemic arterial pressure. Following administration of heparin 600 IU kg^{-1}, the femoral artery on the contralateral side was ligated 0.5 cm below the inguinal ligament, and a catheter was inserted into the medial femoral circumflex branch on this side, which was connected to another pressure transducer for monitoring of intra-arterial pressure. Two further catheters (of internal diameter 2 mm) were introduced into this femoral artery, one proximal to the ligature and one distal to it, and another catheter was inserted into the femoral vein; the two femoral arterial catheters were connected to the import and export tubes of a constant infusion rate pump respectively, which was used to regulate the flow of blood from the proximal to the distal parts of the femoral artery. Drugs were infused through the import tube by means of a Y-connector. The intra-arterial pressure on the ligated side was adjusted to the mean systemic blood pressure by regulating the flow rate, which was kept constant for a further 30 min prior to all experiments.

In initial experiments ($n=6$), we wished to confirm that vascular endothelial responses were intact by injecting a bolus of acetylcholine (ACh) 10^{-6} mol kg^{-1} into the femoral artery, 5 min following pre-treatment with either the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) 1 μmol kg^{-1} or vehicle into the same vessel, and measuring the resulting decrease in perfusion pressure as well as nitrite + nitrate (NO_x, an index of NO production) concentration in the femoral artery and vein using the Griess reaction (Green *et al*., 1982). Additionally, we wished to ascertain whether baseline perfusion pressure was affected by L-NAME, the bradykinin B_2 receptor antagonist HOE 140 or the antioxidant vitamin L-ascorbate. To this end, perfusion pressure was monitored following sequential injection (at 30 min intervals) of L-NAME 1 μmol kg^{-1}, HOE140 30 mg kg^{-1} and L-ascorbate 100 mg kg^{-1} ($n=6$ experiments).

Experimental protocol Following bolus intra-arterial injection of different CCB drugs (amlodipine, nifedipine or verapamil, each at a dose of 10^{-5} mol kg^{-1}), the effect on measured perfusion pressure over time was determined up to 40 min post-injection. Blood was also taken at different time points over the same period from both femoral artery and femoral vein on this side, to determine plasma NO_x concentration. Similar experiments were performed in other rabbits 5 min following pre-injection of the bradykinin B_2 receptor antagonist HOE 140 at a dose of 30 mg kg^{-1} or the NOS inhibitor L-NAME 1 μmol kg^{-1}. In other experiments, L-ascorbate 100 mg kg^{-1} was co-injected with CCB. Six rabbits were used for each experimental condition, and no rabbit underwent more than one injection of CCB.

Effect of amlodipine on NOS activity in cultured endothelial cells

Human umbilical endothelial cells (HUVEC) were isolated and cultured to confluence at passage 2 ($n=6$ experiments), as previously described (Ferro *et al*., 1999). Culture medium was Medium 199 with Earle's salts supplemented with penicillin 500 IU ml^{-1}, streptomycin 500 μg ml^{-1}, fungizone 1.25 μg ml^{-1}, glutamine 2 mM, fetal bovine serum 20%, heparin 0.01% (Grade 1A) and endothelial cell growth supplement 120 μg ml^{-1}. Following removal of the culture medium, washing $\times 3$ and equilibration for 60 min in balanced salt solution (BSS) buffer (composition in mM:

NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, HEPES 15, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8, glucose 5.5; pH 7.4), amlodipine 10^{-5} M, histamine 10^{-5} M (as a positive control) or vehicle were added, and incubation continued for 15 min. NOS activity was determined from the rate of conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline as previously described (Ferro *et al*., 1999). To check that measured L-[^{3}H]-citrulline counts truly reflected NOS activity, the inhibitory effect of L-NAME 100 μM on basal and histamine-induced counts was also assessed. Results were corrected for protein concentrations in the cellular extracts, as determined by the method of Lowry (Lowry *et al*., 1951).

Effect of amlodipine on serum ACE activity

The effect of amlodipine on ACE activity was determined using a proprietary ACE assay kit (Sigma Diagnostics, Germany). This assay is a spectrophotometric method using the synthetic tripeptide substrate N-[3-(2-furyl)acryloyl)-L-phenylalanylglycylglycine, whose hydrolysis to furylacryloyl-phenylalanine and glycylglycine is catalysed by ACE, resulting in a decrease in absorption at 340 nm. The ACE activity in a serum sample is determined by comparing the sample reaction rate to that obtained with the ACE Calibrator included in the kit, according to the manufacturer's instructions.

ACE activity was determined in a serum preparation termed ACE Control-E (supplied with the kit), in the absence or presence of amlodipine at different concentrations (10^{-10}–10^{-5} M). As a positive control, ACE activity was also determined in this same serum preparation, in the absence or presence of enalapril 10^{-10}–10^{-5} M.

Statistical analysis

Changes in intra-arterial pressure were expressed as a percentage of the baseline pressure; differences in per cent change in pressure or in measured NO_x concentration in response to vasodilators, in the absence or presence of L-NAME, HOE 140 or L-ascorbate, were analysed by two-way ANOVA. Effects of amlodipine and histamine on NOS activity in HUVEC were analysed by repeated measures one-way ANOVA. Where significant differences were detected by ANOVA, a *post hoc* Fisher's test was employed. Statistical significance was taken in all cases as $P<0.05$ (two-sided). Statistical analyses were performed using StatView version 5 (SAS). All data are expressed as mean ± s.e.mean.

Results

Effect of L-NAME, HOE 140 and L-ascorbate on baseline perfusion pressure

Following the establishment of femoral artery perfusion in anaesthetised rabbits ($n=6$) and 30 min equilibration to systemic arterial pressure, we wished to ascertain whether the antagonists used in our studies affected baseline haemodynamics. Baseline perfusion pressure was 82 ± 6.2 mmHg; this was unaltered by sequential injection into the femoral artery of the NOS inhibitor L-NAME 1 μmol kg^{-1} (84 ± 6.9 mmHg), the bradykinin B_2 receptor antagonist HOE140 30 mg kg^{-1} (81 ± 7.6 mmHg) and the antioxidant vitamin L-ascorbate 100 mg kg^{-1} (86 ± 7.9 mmHg).

Endothelium-dependent vasorelaxation in perfused rabbit hindlimb vasculature

To confirm endothelial integrity in our perfused rabbit hindlimb model, we examined the effect of bolus intra-arterial injection of ACh 1 μmol kg^{-1}, 5 min following pre-treatment with L-NAME 1 μmol kg^{-1} or vehicle, on both haemodynamics and NO generation locally ($n=6$). ACh elicited a decrease in perfusion pressure, and an increase in femoral venous (but not femoral arterial) plasma NO_x concentration, both of which were blocked by pre-treatment with L-NAME (Figure 1), thus demonstrating that in our

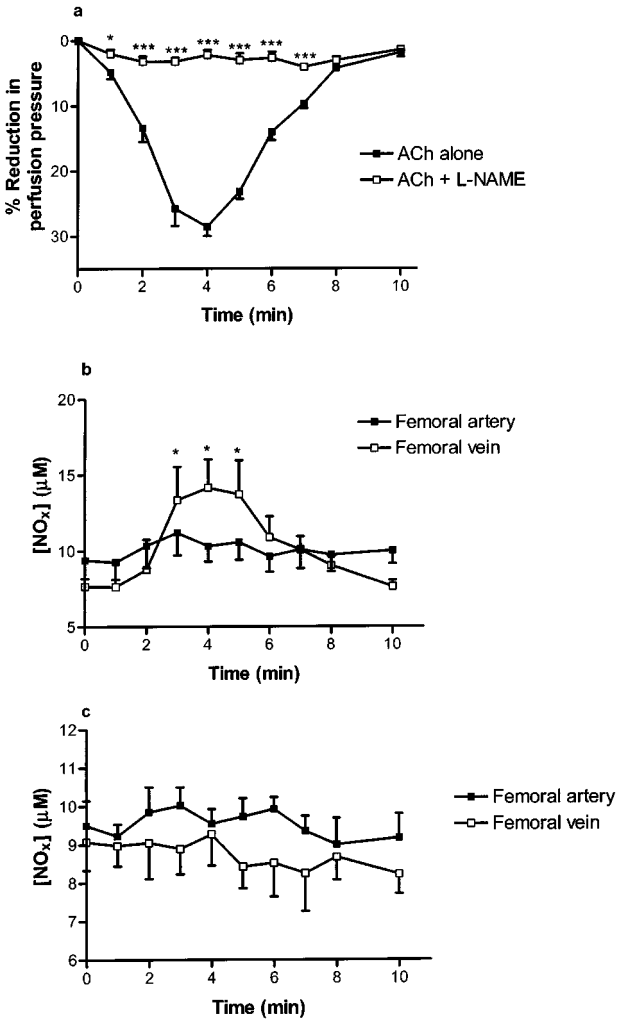

Figure 1 Responses to bolus injection of acetylcholine (ACh) 10^{-6} mol kg^{-1} into rabbit femoral artery, with or without pre-treatment with N^G-nitro-L-arginine methyl ester (L-NAME) 1 μmol kg^{-1}. (a) Per cent reduction in femoral artery perfusion pressure following ACh injection (at time 0). (b) Nitrite + nitrate (NO_x) concentration in femoral artery and femoral vein following ACh injection (at time 0), in the absence of L-NAME. (c) NO_x concentration in femoral artery and femoral vein following ACh injection (at time 0), in the presence of L-NAME. Data are mean ± s.e.mean. *$P<0.05$, **$P<0.005$, ***$P<0.001$ respectively, as compared with values in the absence of L-NAME.

preparation endothelium- and NO-dependent responses were intact. These results also show that L-NAME at this dose effectively suppresses vascular NO generation and NO-dependent relaxation.

Effect of calcium channel antagonists on perfusion pressure, and NO dependence

Following the establishment of femoral artery perfusion and equilibration as before, a bolus of amlodipine, nifedipine or verapamil (each at a dose of 10^{-5} mol l^{-1}) was administered into the femoral artery, and the resultant change in perfusion pressure was measured over time. Experiments were performed both in rabbits who had received an injection of L-NAME 1 μmol kg^{-1} and those who had received corresponding vehicle 5 min previously. Each CCB elicited a time-dependent reduction in measured perfusion pressure (Figure 2). The maximal reductions in perfusion pressure were: verapamil 30.7±3.7%, nifedipine 32.2±1.4%, amlodipine 33.2±2.1% (P=NS). L-NAME had no effect on the reduction in perfusion pressure induced by either verapamil or nifedipine; by contrast, following L-NAME, the maximal reduction in perfusion pressure in response to amlodipine was decreased to 22.5±1.6% ($P=0.002$ versus amlodipine alone).

Amlodipine is a longer-acting CCB, with a longer elimination half-life *in vivo*, than either nifedipine or verapamil. Of note, in our experiments, the time course of the changes in pressure differed between these drugs. The maximal reduction in perfusion pressure occurred at 4 min, 6 min and 20 min post-injection for nifedipine, verapamil and amlodipine respectively (Figure 2).

In order to ascertain whether part of the observed effect of CCB on perfusion pressure was mediated through scavenging of oxygen-derived free radicals, experiments were also performed consisting of co-injection of L-ascorbate 100 mg kg^{-1} with each CCB. The reduction in perfusion

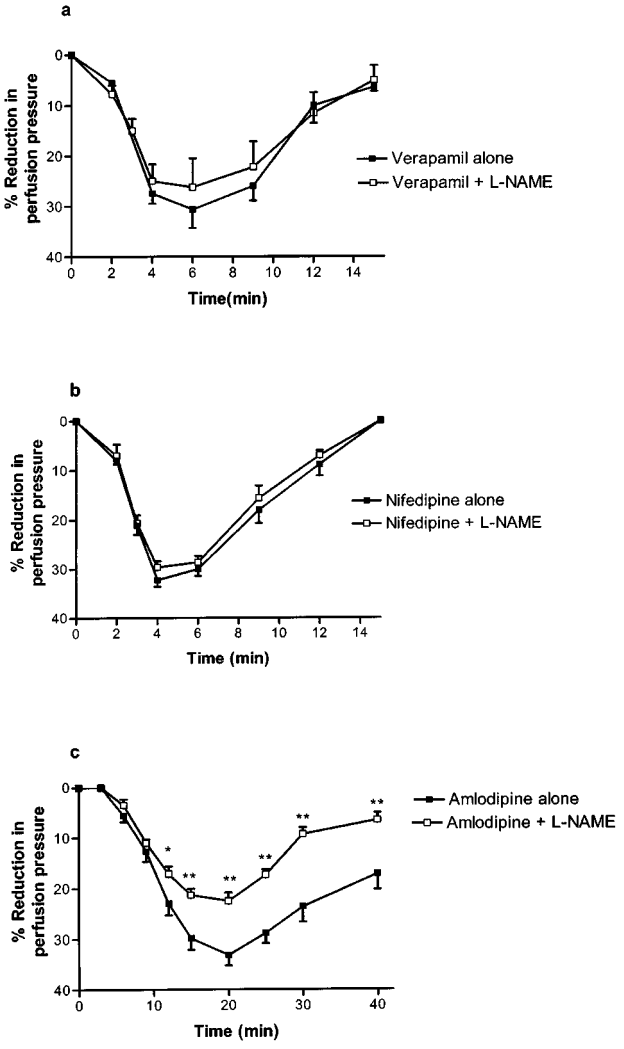

Figure 2 Per cent reduction in femoral artery perfusion pressure following bolus injection of verapamil (a), nifedipine (b) and amlodipine (c), each at a dose of 10^{-5} mol kg^{-1} (at time 0). Responses are shown with and without pre-treatment with intra-arterial N^G-nitro-L-arginine methyl ester (L-NAME) 1 μmol kg^{-1}. Data are mean±s.e.mean. *$P<0.05$ and **$P<0.005$ respectively, as compared with values in the absence of L-NAME.

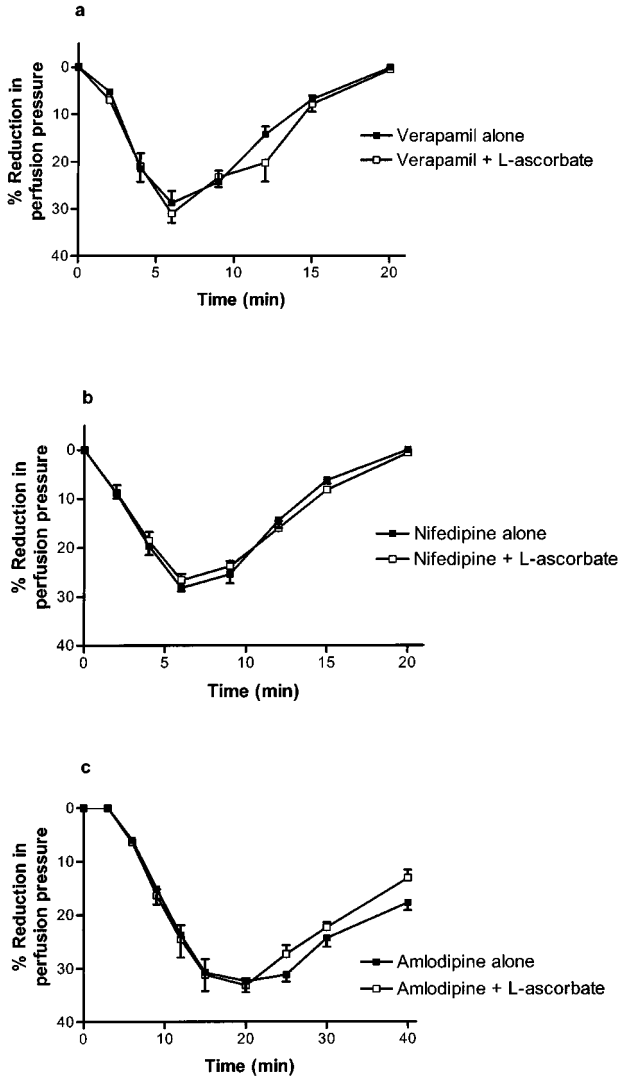

Figure 3 Per cent reduction in femoral artery perfusion pressure following bolus injection of verapamil (a), nifedipine (b) and amlodipine (c), each at a dose of 10^{-5} mol kg^{-1} (at time 0). Responses are shown with and without co-treatment with intra-arterial L-ascorbate 100 mg kg^{-1}. Data are mean±s.e.mean.

pressure with each CCB was unaffected by L-ascorbate (Figure 3).

Effect of amlodipine on NOS activity in HUVEC

To establish whether amlodipine could directly increase NOS activity in vascular endothelial cells, confluent HUVEC at passage 2 were incubated with amlodipine 10^{-5} M or vehicle; parallel experiments were performed with histamine 10^{-5} M, as a positive control known to increase endothelial cell NOS activity. NOS activity was measured by the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline, and corrected for protein in the cell extracts. As expected, histamine increased L-[^{3}H]-citrulline in HUVEC significantly above basal (Figure 4); both basal and histamine-stimulated NOS activity were inhibited by L-NAME. By contrast, amlodipine did not affect measured L-[^{3}H]-citrulline production as compared with basal levels.

Effect of calcium channel antagonists on vascular NO generation

Following intra-femoral injection of amlodipine, nifedipine or verapamil as above, with or without L-NAME, blood was taken from both the femoral artery and the femoral vein at different time points; NO_x concentration was measured in plasma by the Griess reaction, and changes in venous and arterial NO_x concentration (reflecting changes in local NO production) determined from these measurements. In all experiments, baseline arterial plasma NO_x concentration was greater than venous; this probably reflects a higher degree of NO production by arterial endothelium as compared with venous, with progressive degradation of NO through the local circulation, although another possible explanation is that NO is partitioned from plasma into erythrocytes to a greater degree in veins as compared with arteries due to differences in pCO_2 and bicarbonate concentrations, as previously suggested (Recchia *et al*., 2000). Nifedipine and verapamil elicited no change in femoral venous NO_x, whereas amlodipine increased venous NO_x from a baseline level of 9.1 ± 0.4 μM to a maximal level of 14.1 ± 0.5 μM at 12 min post-injection, $P = 0.005$ (Figure 5). The increase in venous NO_x following amlodipine persisted up to 25 min post-injection, but returned to baseline by 30 min. Arterial NO_x concentration did not change significantly in response to any CCB. In parallel experiments, following prior treatment with L-NAME, amlodipine failed to elicit this increase in venous NO_x (Figure 6); notably, L-NAME significantly reduced basal arterial NO_x concentration, from 10.4 ± 0.4 μM to 8.1 ± 0.5 μM, $P = 0.02$.

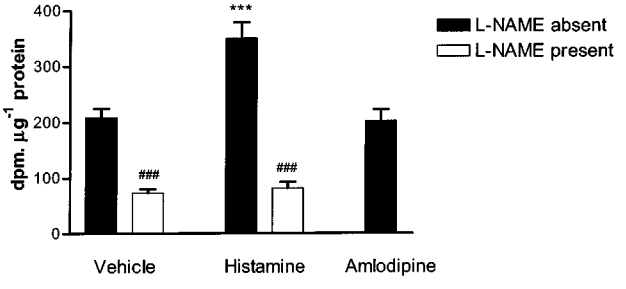

Figure 4 Nitric oxide synthase activity, expressed as ^{3}H counts in the L-citrulline fraction corrected for cellular protein, in human umbilical vein endothelial cells treated with vehicle, histamine 10^{-5} M (as a positive control) or amlodipine 10^{-5} M. Responses for vehicle and histamine are shown in the absence and presence of N^G-nitro-L-arginine methyl ester (L-NAME) 10^{-4} M. Data are mean $\pm$ s.e.mean. $^{***}P < 0.001$ as compared with vehicle. $^{###}P < 0.001$ as compared with values in the absence of L-NAME.

Kinin dependence of the effect of amlodipine on perfusion pressure and on NO generation

To determine whether the decrease in perfusion pressure and the increase in NO in response to amlodipine could be explained by an increase in kinin generation, amlodipine 10^{-5} M was injected into rabbit femoral arteries with or without prior injection of 30 mg kg^{-1} HOE 140. HOE 140 significantly attenuated the decrease in perfusion pressure to amlodipine (Figure 7a): the maximal decrease in pressure was $24.6 \pm 3.5\%$ in the absence of HOE 140, and $9.0 \pm 2.1\%$ in its presence ($P = 0.004$). Additionally, HOE 140 abolished the increase in venous NO_x in response to amlodipine (Figures 7b

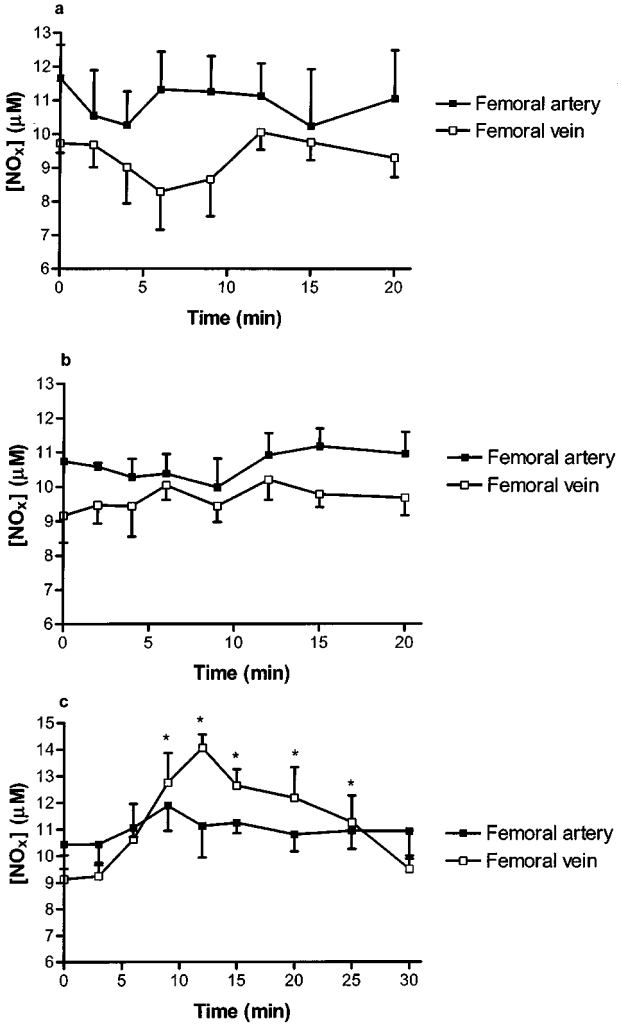

Figure 5 Nitrite + nitrate (NO_x) concentration in femoral artery and femoral vein following bolus injection of verapamil (a), nifedipine (b) and amlodipine (c), each at a dose of 10^{-5} mol kg^{-1} (at time 0). Data are mean $\pm$ s.e.mean. $^{*}P < 0.05$ as compared with baseline.

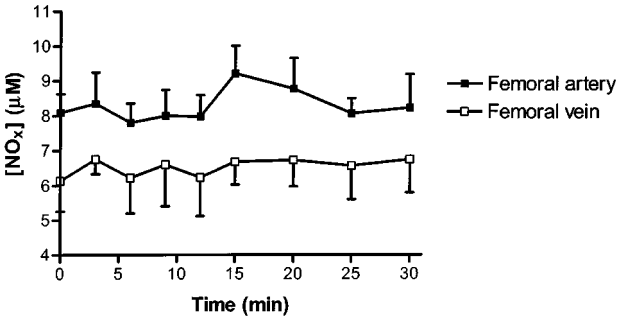

Figure 6 Nitrite + nitrate (NO_x) concentration in femoral artery and femoral vein following bolus injection of amlodipine 10^{-5} mol kg^{-1} (at time 0), in rabbits pre-treated with intra-arterial N^G-nitro-L-arginine methyl ester (L-NAME) 1 μmol kg^{-1}. Data are mean ± s.e.mean.

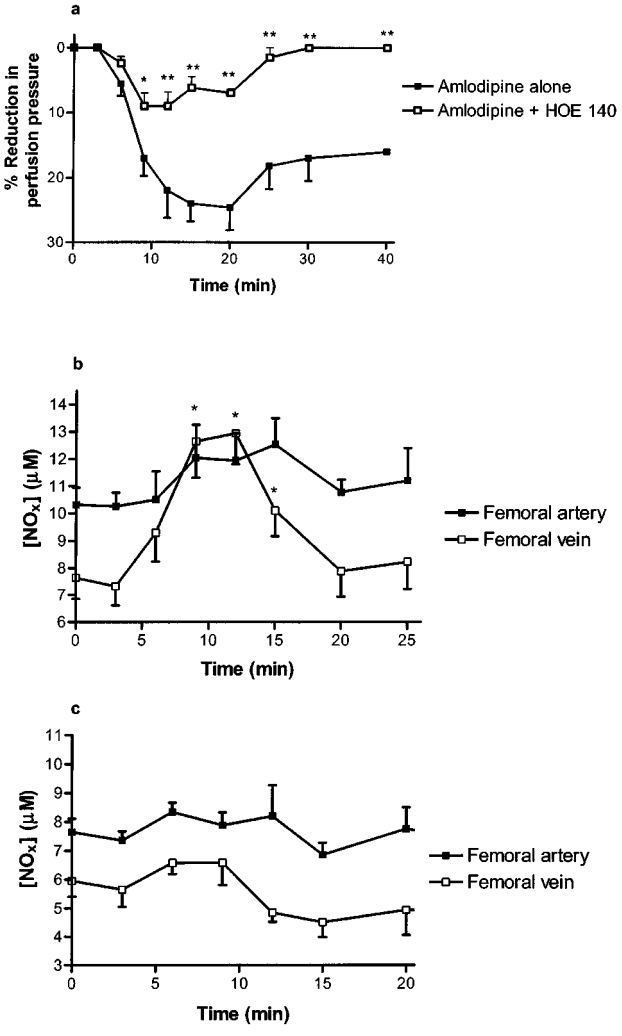

Figure 7 Effect of intra-arterial HOE 140 at a dose of 30 mg kg^{-1} on responses to amlodipine 10^{-5} mol kg^{-1}, injected at time 0, in rabbit hindlimb. (a) Per cent change in femoral artery perfusion pressure in the absence and presence of HOE 140. $^*P<0.05$ and $^{**}P<0.005$ as compared with values in the absence of HOE 140. (b) Nitrite + nitrate (NO_x) concentration in femoral artery and vein in the absence of HOE 140. $^*P<0.05$ as compared with baseline. (c) NO_x concentration in femoral artery and vein in the presence of HOE 140.

and 7c), as well as significantly reducing basal arterial NO_x, from 10.3 ± 0.6% to 7.6 ± 0.8% ($P=0.03$).

Effect of amlodipine on serum ACE activity

To ascertain whether the effect of amlodipine on B_2 receptor-mediated NO generation might be explained by an increase in local bradykinin concentration, through inhibition of its breakdown by ACE, the effect of amlodipine on serum ACE activity was determined using a proprietary ACE assay kit and a serum sample provided with this kit. In the absence of amlodipine, the ACE activity in the serum sample was 24.5 U/L. Although lower concentrations (10^{-10} M and 10^{-9} M) of amlodipine had a negligible effect, higher concentrations (10^{-8}–10^{-5} M) strongly inhibited ACE activity (Figure 8a), to an extent similar to the ACE inhibitor enalapril at similar concentrations (Figure 8b).

Discussion

We investigated whether the CCB amlodipine, nifedipine and verapamil mediated their vasorelaxant effects *in vivo* at least in part through modulation of vascular NO production. We found that nifedipine and verapamil elicited vasorelaxation of rabbit femoral artery independent of NO biosynthesis. By contrast, the vasorelaxant effect of amlodipine occurred partly through a NO-dependent mechanism. Whereas the per cent decrease in femoral artery perfusion pressure was roughly equivalent using equimolar doses of the three CCB (10^{-5} mol kg^{-1}), this decrease was unaffected by L-NAME in the case of nifedipine or verapamil, but was reduced by approximately

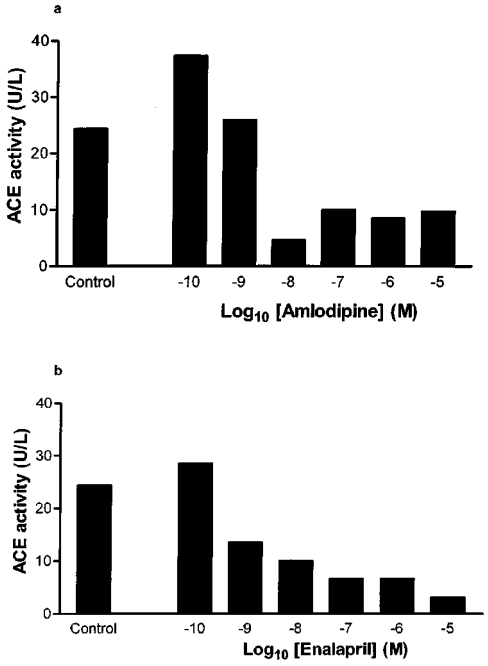

Figure 8 (a) Effect of amlodipine at different concentrations, on angiotensin converting-enzyme (ACE) activity in serum sample ACE Control-E. (b) Effect of enalapril at different concentrations, on ACE activity in this same serum sample.

one-third by L-NAME in the case of amlodipine. Measurement of NO_x concentration confirmed that amlodipine – but not verapamil or nifedipine – increased NO_x in femoral vein but not femoral artery, consistent with an increase in NO biosynthesis in the rabbit hindlimb, and that this increase was abolished by L-NAME. The effect of amlodipine on NO generation could not be explained by an antioxidant effect, nor by a direct stimulatory action on NOS in cultured endothelial cells. However, the bradykinin B_2 receptor antagonist HOE 140 also partly inhibited the vasorelaxant effect of amlodipine on rabbit femoral artery, and abolished the rise in femoral venous NO_x, *in vivo*. Our experiments indicate, therefore, that – unlike nifedipine or verapamil – amlodipine causes vasorelaxation partly through a kinin- and NO-dependent mechanism in this model. The lack of observed effect of amlodipine on NOS activity in HUVEC is likely to reflect a lack of kinin production when these cells are cultured in isolation from other components of the vasculature.

In our studies, we used only a single dose of each CCB intra-arterially, since the maximal reduction in pressure following injection of any particular dose of amlodipine occurred slowly (aproximately 20 min); therefore, it would be difficult to perform a full dose-response curve to this drug in any individual animal. We therefore chose to use the same molar dose of each CCB (verapamil, nifedipine and amlodipine), in order to enable direct comparison of their relative effects on the NO and kinin systems. We chose to use a dose of 10^{-5} mol kg^{-1}, since this is towards the higher end of the daily dose range of these drugs in clinical practice (when given orally). We also chose to use one particular dose of L-NAME intra-arterially, namely 1 μmol kg^{-1}, since we found that this dose abolished ACh responses in our model, thereby indicating effective abolition of NO-dependent responses by this dose.

We found that concomitant intra-arterial administration of L-ascorbate at a dose of 100 mg kg^{-1} did not affect the vasodilatory effect of any of the CCB tested, suggesting that the differential effect of amlodipine on NO-dependent responses as compared with verapamil or nifedipine is unlikely to be explained by a greater oxygen-derived free radical scavenging capacity, since we would then expect an augmentation by L-ascorbate of reduction in pressure with verapamil and nifedipine as compared with amlodipine, and this was not observed. This dose of L-ascorbate was chosen on the basis of previous studies in humans, where injection of similar doses (adjusted for body weight and time of injection) of L-ascorbate into the brachial artery improved forearm blood flow and indices of oxidative stress (Beckman *et al*., 2001; Taddei *et al*., 2001).

Bradykinin elicits vasorelaxation through stimulation of endothelial cell NO synthesis (Palmer *et al*., 1987). It is a nonapeptide formed together with kallidin by the cleavage of kininogens through the action of both plasma and tissue-derived serine proteases, termed kallikreins (reviewed by Nakanishi, 1987). Both bradykinin and kallidin are potent agonists at the B_2 receptor, which is constitutively present in most normal tissues, including the vascular endothelium. The B_1 receptor subtype selectively binds to the carboxyl-terminal des-arginine metabolites of bradykinin and kallidin, and is expressed at very low levels in normal vascular and other tissues, although it is upregulated by inflammation (Regoli & Barabé, 1980; Dray & Perkins, 1993). HOE 140 is a selective antagonist at the B_2 receptor subtype (Feletou *et al*., 1994). In view of previous findings that the NO-generating effect of amlodipine in canine coronary microvessels may be partly kinin-dependent *in vitro*, we sought to determine the effect of B_2 receptor blockade with HOE 140 in rabbit femoral artery *in vivo*. Our data demonstrate that, in the presence of B_2 blockade, the ability of amlodipine to cause vascular NO generation was abolished in this system. To investigate whether this effect of amlodipine might be attributable to an increase in local bradykinin concentration, secondary to inhibition of its breakdown by ACE, we examined the ability of amlodipine to cause ACE inhibition in a serum sample. We found that amlodipine can potently inhibit ACE activity, to a similar extent to enalapril, a well-established ACE inhibitor used widely in clinical practice. We infer, therefore, that the kinin- and NO-dependent component of the vasorelaxation induced by amlodipine in our model may indeed relate to its ability to inhibit ACE.

Our study provides the first *in vivo* demonstration that CCB differ in their capacity to cause vasorelaxation through activation of the vascular NO system. The clinical implications of our findings merit further investigation. NO biosynthesis by the vascular endothelium is generally thought of as beneficial, since NO – quite apart from its vasodilatory properties – exerts anti-atherogenic effects on the vascular wall (reviewed by Napoli & Ignarro, 2001). Thus, one might postulate that a CCB such as amlodipine, which stimulates NO biosynthesis, might exert an additional vasculoprotective effect not shared with other CCB which produce a similar degree of vasorelaxation but do not generate NO. On the other hand, patients with established atherosclerotic disease, as well as those at risk for atherosclerosis, exhibit impairment of vascular endothelial function, and an impaired capacity to generate NO (Ludmer *et al*., 1986; Vita *et al*., 1990; Zeiher *et al*., 1991; Egashira *et al*., 1993; Quyyumi *et al*., 1995). In such patients, one might expect from our data that amlodipine would exert a lesser vasorelaxant effect than an equimolar dose of nifedipine or verapamil. Further studies are needed to examine the relative therapeutic merits of different CCB in both animal models of vascular disease and in humans with coronary and other vascular disease.

In conclusion, we have found that the vasorelaxant effects of the CCB nifedipine and verapamil are independent of NO, whereas those of amlodipine are partly dependent on NO generation through the mediation of bradykinin B_2 receptors, in rabbit femoral artery *in vivo*. This in turn may relate to an increase in local bradykinin concentration, secondary to inhibition of ACE activity by amlodipine, an effect not previously described. If replicated in humans, our results may have important implications for the use of different CCB in the treatment of cardiovascular disease.

B. Xu is the recipient of a Wellcome Trust International Research Development Award. We are grateful to Pfizer Limited, UK, for kindly providing us with amlodipine for use in these experiments.

References

BECKMAN, J.A., GOLDFINE, A.B., GORDON, M.B. & CREAGER, M.A. (2001). Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. *Circulation,* **103,** 1618 – 1623.

BENNETT, M.A., HILLIER, C. & THURSTON, H. (1996). Endothelium-dependent relaxation in resistance arteries from spontaneously hypertensive rats: effect of long-term treatment with perindopril, quinapril, hydralazine or amlodipine. *J. Hypertens.,* **14,** 389 – 397.

DRAY, A. & PERKINS, M. (1993). Bradykinin and inflammatory pain. *Trends Neurosci.,* **16,** 99 – 104.

EGASHIRA, K., INOU, T., HIROOKA, Y., YAMADA A., MARUOKA, Y., KAI, H., SUGIMACHI, M., SUZUKI, S. & TAKESHITA, A. (1993). Impaired coronary blood flow response to acetylcholine in patients with coronary risk factors and proximal atherosclerotic lesions. *J. Clin. Invest.,* **91,** 29 – 37.

FELETOU, M., GERMAIN, M., THURIEAU, C., FAUCHERE, J.L. & CANET, E. (1994). Agonistic and antagonistic properties of the bradykinin B2 receptor antagonist, HOE 140, in isolated blood vessels from different species. *Br. J. Pharmacol.,* **112,** 683 – 689.

FERRO, A., QUEEN, L.R., PRIEST, R.M., XU, B., RITTER, J.M., POSTON, L. & WARD, J.P.T. (1999). Activation of endothelial nitric oxide synthase by β_2-adrenoceptors in human umbilical vein. *Br. J. Pharmacol.,* **126,** 1872 – 1880.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S. & TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. *Anal. Biochem.,* **126,** 131 – 138.

HAYASHI, K., KUGA, Y., NOMURA, Y., OKURA, Y., TANAKA, K., YASUNOBU, Y., NOMURA, K., SHINGU, T., KUWASHIMA, J. & KAJIYAMA, G. (1996). Inhibition of lipid hydroperoxidation of low density lipoprotein by the Ca^{2+}-channel and α_1-adrenoreceptor antagonist monatepil maleate. *Arzneimittelforschung,* **46,** 378 – 381.

LESNIK, P., DACHET, C., PETIT, L., MOREAU, M., GRIGLIO, S., BRUDI, P. & CHAPMAN, M.J. (1997). Impact of a combination of a calcium antagonist and a beta-blocker on cell- and copper-mediated oxidation of LDL and on the accumulation and efflux of cholesterol in human macrophages and murine J774 cells. *Arterioscler. Thromb. Vasc. Biol.,* **17,** 979 – 988.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193,** 265 – 275.

LUDMER, P., SELWYN, A., SHOOK, T.L., WAYNE, R.R., MUDGE, G.H., ALEXANDER, R.W. & GANZ, P. (1986). Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N. Engl. J. Med.,* **315,** 1046 – 1051.

LUPO, E., LOCHER, R., WEISSER, B. & VETTER, W. (1994). *In vitro* antioxidant activity of calcium antagonists against LDL oxidation compared with α-tocopherol. *Biochem. Biophys. Res. Commun.,* **203,** 1803 – 1808.

NAKANISHI, S. (1987). Substance P precursor and kininogen: their structures, gene organizations, and regulation. *Physiol. Rev.,* **67,** 1117 – 1142.

NAPOLI, C., CHIARIELLO, M., PALUMBO, G. & AMBROSIO, G. (1996). Calcium-channel blockers inhibit low-density lipoprotein oxidation by oxygen radicals. *Cardiovasc. Drugs Ther.,* **10,** 417 – 424.

NAPOLI, C. & IGNARRO, L.J. (2001). Nitric oxide and atherosclerosis. *Nitric Oxide,* **5,** 88 – 97.

PALMER, R.M., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature,* **327,** 524 – 526.

QUYYUMI, A.A., DAKAK, N., ANDREWS, N.P., HUSAIN, S., ARORA, S., GILLIGAN, D.M., PANZA, J.A. & CANNON, 3RD. R.O. (1995). Nitric oxide activity in the human coronary circulation: impact of risk factors for coronary atherosclerosis. *J. Clin. Invest.,* **95,** 1747 – 1755.

RECCHIA, F.A., VOGEL, T.R. & HINTZE, T.H. (2000). NO metabolites accumulate in erythrocytes in proportion to carbon dioxide and bicarbonate concentration. *Am. J. Physiol. Heart Circ. Physiol.,* **279,** H852 – 856.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.,* **32,** 1 – 46.

ROJSTACZER, N. & TRIGGLE, D.J. (1996). Structure-function relationships of calcium antagonists. Effect on oxidative modification of low density lipoprotein. *Biochem. Pharmacol.,* **51,** 141 – 150.

SOBAL, G., MENZEL, E.J. & SINZINGER, H. (2001). Calcium antagonists as inhibitors of *in vitro* low density lipoprotein oxidation and glycation. *Biochem. Pharmacol.,* **61,** 373 – 379.

TADDEI, S., VIRDIS, A., GHIADONI, L., MAGAGNA, A., FAVILLA, S., POMPELLA, A. & SALVETTI, A. (2001). Restoration of nitric oxide availability after calcium antagonist treatment in essential hypertension. *Hypertension,* **37,** 943 – 948.

VITA, J.A., TREASURE, C.B., NABEL, E.G., MCLENACHAN, J.M., FISH, R.D., YEUNG, A.C., VEKSHTEIN, V.I., SELWYN, A.P. & GANZ, P. (1990). Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. *Circulation,* **81,** 491 – 497.

WANG, W.Z., MATSUMORI, A., YAMADA, T., SHIOI, T., OKADA, I., MATSUI, S., SATO, Y., SUZUKI, H., SHIOTA, K. & SASAYAMA, S. (1997). Beneficial effects of amlodipine in a murine model of congestive heart failure induced by viral myocarditis. A possible mechanism through inhibition of nitric oxide production. *Circulation,* **95,** 245 – 251.

XU, B., LI, J., GAO, L. & FERRO, A. (2000). Nitric oxide-dependent vasodilatation of rabbit femoral artery by β_2-adrenergic stimulation or cyclic AMP elevation *in vivo*. *Br. J. Pharmacol.,* **129,** 969 – 974.

ZEIHER, A.M., DREXLER, H., WOLLSCHLAGER, H. & JUST, H. (1991). Modulation of coronary vasomotor tone in humans: progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation,* **83,** 391 – 401.

ZHANG, H. & HINTZE, T.H. (1998). Amlodipine releases nitric oxide from canine coronary microvessels: an unexpected mechanism of action of a calcium channel-blocking agent. *Circulation,* **97,** 576 – 580.

ZHANG, X., KICHUK, M.R., MITAL, S., OZ, M., MICHLER, R., NASJLETTI, A., KALEY, G. & HINTZE, T.H. (1999a). Amlodipine promotes kinin-mediated nitric oxide production in coronary microvessels of failing human hearts. *Am. J. Cardiol.,* **84,** 27L – 33L.

ZHANG, X.P., LOKE, K.E., MITAL, S., CHAHWALA, S. & HINTZE, T.H. (2002). Paradoxical release of nitric oxide by an L-type calcium channel antagonist, the r+ enantiomer of amlodipine. *J. Cardiovasc. Pharmacol.,* **39,** 208 – 214.

ZHANG, X., RECCHIA, F.A., BERNSTEIN, R., XU, X., NASJLETTI, A. & HINTZE, T.H. (1999b). Kinin-mediated coronary nitric oxide production contributes to the therapeutic action of angiotensin-converting enzyme and neutral endopeptidase inhibitors and amlodipine in the treatment of heart failure. *J. Pharmacol. Exp. Ther.,* **288,** 742 – 751.

ZHANG, X., XU, X., NASJLETTI, A. & HINTZE, T.H. (2000). Amlodipine enhances NO production induced by an ACE inhibitor through a kinin-mediated mechanism in canine coronary microvessels. *J. Cardiovasc. Pharmacol.,* **35,** 195 – 202.

(Received February 18, 2002
Revised April 5, 2002
Accepted April 8, 2002)