British Journal of Pharmacology (2002) 135, 87–92

www.nature.com/bjp

Angiotensin AT_1 receptor signalling modulates reparative angiogenesis induced by limb ischaemia

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1 The concept that angiotensin II exerts pro-angiogenic activity is not universally accepted. We evaluated whether inhibition of the renin-angiotensin system (RAS) would influence reparative angiogenesis in a murine model of limb ischaemia.

2 Perfusion recovery following surgical removal of the left femoral artery was analysed by laser Doppler flowmetry in mice given the ACE inhibitor ramipril (1 mg kg^{-1} per day), the AT_1 antagonist losartan (15 mg kg^{-1} per day), or vehicle. Muscular capillarity was examined at necroscopy. Ramipril-induced effects were also studied under combined blockade of kinin B_1 and B_2 receptors. Furthermore, the effects of ischaemia on AT_1 gene expression and ACE activity were determined.

3 In untreated mice, muscular AT_{1a} gene expression was transiently decreased early after induction of limb ischaemia, whereas AT_{1b} mRNA was up-regulated. ACE activity was reduced in ischaemic muscles at 1 and 3 days. Gene expression of AT_1 isoforms as well as ACE activity returned to basal values by day 14. Spontaneous neovascularization allowed for complete perfusion recovery of the ischaemic limb after 21 days.

4 Reparative angiogenesis was negatively influenced by either ramipril ($P<0.02$) or losartan ($P<0.01$), leading to delayed and impaired post-ischaemic recovery (50–70% less compared with controls). Ramipril-induced effects remained unaltered under kinin receptor blockade.

5 The present study indicates that (a) expression of angiotensin II AT_1 receptors and ACE activity are modulated by ischaemia, (b) ACE-inhibition or AT_1 antagonism impairs reparative angiogenesis, and (c) intact AT_1 receptor signalling is essential for post-ischaemic recovery. These results provide new insights into the role of the RAS in vascular biology and suggest cautionary use of ACE inhibitors and AT_1 antagonists in patients at risk for developing peripheral ischaemia.
British Journal of Pharmacology (2002) **135,** 87–92

Keywords: Angiogenesis; renin angiotensin system; ACE-inhibitors; AT_1 antagonists; post-ischaemic recovery; skeletal muscle

Abbreviations: ACE, angiotensin coverting enzyme; AT_{1a}, Ang II AT_1 receptor, isoform a; AT_{1b}, Ang II AT_1 receptor, isoform b; B_1 , BKB_1 receptor; B_2 , BKB_2 receptor; BK, bradykinin; DALBK, des-Arg^9-[Leu^8]-BK, B_1 antagonist; Icatibant, D-Arg,[Hyp^3,Thi^5D-Tic^7,Oic^8]-BK, B_2 antagonist; KKS, kallikrein-kinin system; RAS, renin-angiotensin system

Introduction

The biological functions of the renin-angiotensin system (RAS) are mainly mediated by interaction of angiotensin II with specific receptors, classified as type 1 (AT_1) and 2 (AT_2). A growing body of evidence indicates that, besides acting as a vasopressor agent, angiotensin II plays a fundamental role in the regulation of cardiovascular growth and remodelling.

One approach to investigate the physiological relevance of endogenous angiotensin II is by interfering with generation of the peptide or with binding to receptors. In the clinical field, this strategy proved to exert significant cardiovascular protection (Materson & Preston, 1994; Pfeffer *et al*., 1992; Burris, 1995; Garg & Yusuf, 1995; Braunwald, 1998; Dickstein *et al*., 1995; McKelvie *et al*., 1999). This was ascribed to the fact that, when angiotensin II formation is suppressed by angiotensin converting enzyme (ACE) inhibition or blocked by selective AT_1 receptor antagonism, the hypertrophying and damaging actions of angiotensin II on heart and vessels are prevented. Furthermore, long-term ACE-inhibition was shown to promote significant myocardial neo-vascular (Olivetti *et al*., 1993), apparently beyond blood pressure control (Gohlke *et al*., 1997). However, the latter findings are in apparent contradiction with the pro-angiogenic effect exerted by angiotensin II *via* stimulation of AT_1 receptor signaling (Le Noble *et al*., 1993; Munzenmaier & Greene, 1996; Walsh *et al*., 1997; Fujiyama *et al*., 2001). Consistently, Wang & Prewitt (1990) reported that ACE inhibition by captopril blocks microvascular development in normotensive and hypertensive rats. Moreover, Amaral *et al*. (2001) found that muscular angiogenesis induced by electrical stimulation of limb skeletal muscles is mediated by both

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angiotensin II and vascular endothelial growth factor (VEGF) and completely abrogated by lisinopril or losartan (Amaral *et al*., 2001). It should be mentioned that all these studies were conducted under conditions of normoxya. From this it is difficult to predict the impact that interfering with RAS may have on spontaneous angiogenic response to ischaemia, particularly when considering that this condition reportedly up-regulates RAS expression and activity (Yang *et al*., 1997; Busatto *et al*., 1997). Noteworthy, although mot specifically prescribed for the treatment of ischaemia, ACE inhibitors or AT_1 antagonists are primarily used in conditions such as arterial hypertension and left ventricular hypertrophy, which are frequently complicated by atherosclerosis-induced vascular obstructions. To the best of our knowledge, only one study has addressed the angiogenic potential of ACE inhibitors in the context of peripheral ischaemia (Fabre *et al*., 1999). It was shown that quinaprilat is able to accelerate the rate of limb perfusion recovery through potentiation of angiogenic host defence response. Nevertheless, the role of angiotensin II in this favourable effect was not firmly established. In particular, not enough attention was paid to the fact that angiotensin II levels are not completely suppressed by ACE inhibition and to the possibility that kinins, recognized to be potent angiogenic agents (Emanueli *et al*., 2000b; 2001b), may have contributed to quinaprilat-induced beneficial effects. Finally, Fabre's protocol contemplates a 10 day latency between induction of limb ischaemia and ACE inhibition. It remains unknown if such a favourable action would be replicated by application of quinaprilat before or during the early phases of ischaemia, an experimental condition mimicking the situation of vascular occlusion occurring in patients under chronic ACE inhibition. To address these questions, we first evaluated if ischaemia caused by femoral artery excision modulates local AT_1 mRNA levels or ACE activity. Then, the angiogenic potential of ramipril, an ACE inhibitor shown to exert extensive target organ protection in clinical trials (Lonn *et al*., 2001), was challenged and compared with the action of the AT_1 receptor antagonist losartan. To assess possible interference of unrelated mechanisms, ramipril was also applied to ischaemia under combined pharmacological blockade of kinin B_1 and B_2 receptors.

Methods

Mice

Male Swiss mice (25–35 g body weight, Charles River, Milan) were housed at a constant room temperature ($24\pm1^{\circ}C$) and humidity ($60\pm3\%$) with a 12 h light/dark cycle and free access to tap water and chow.

All procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, U.S.A.) and were approved by the Local Institutional Committee.

Surgical induction of ischaemia

Mice were anaesthetized with 2,2,2-tribromoethanol (880 mmol kg^{-1} IP) and kept on a heating pad at 37°C. To induce unilateral limb ischaemia, the left femoral artery was exposed through a skin incision, dissected free from its proximal origin, closed with a microtip electro-coagulator, and excized.

Effect of limb ischaemia on AT_1 gene expression and ACE activity

Gene expression levels of AT_1 receptor isoforms, AT_{1a} and AT_{1b}, were evaluated by RT–PCR. To this aim, total RNA was isolated from frozen adductor skeletal muscles harvested at 0, 1, 3, 7, and 14 days following induction of ischaemia ($n=3-6$ each time point). Using RNAzol B method, cDNA was made from total RNA according to manufacturer's instructions (Stratagene). The primers used for amplification of a 287-bp product of mouse AT_{1a} receptor isoform were: AT_{1a} L (GAT AAT TAT GGC GAT TGT GC) and AT_{1b} R (TGC TCA TTT TCG TAG ACA GG). For amplification of a 303-bp product of AT_{1b} receptor isoform, the primers were: AT_{1b} L (ATT CAG TTT TCT GGA TGT GC) and AT_{1b} R (TCC ACT TCA AAA CAA TAC GC). For both sets of primers, the PCR amplification conditions were: denaturation at 95°C, annealing at 56°C, and elongation at 72°C for 30 cycle. AT_1 RNA levels were quantified with NIH Image J software program and normalized by GAPDH expression levels.

At the same time points of gene expression analysis, ACE activity was evaluated in plasma and in both ischaemic and contralateral gastrocnemious skeletal muscles by using the synthetic tri-peptide FAPGG kit (Sigma) as previously described (Fabre *et al*., 1999). For determination of plasma ACE, blood was sampled by direct puncture of the heart of anaesthetized mice. On the same occasion, limb muscles were harvested, weighed, and homogenized in PBS. Tissue homogenates were then centrifuged and the supernatant used for measurements. ACE activity was expressed as international unit (IU) per l of plasma (IU l^{-1}) or gram of tissue (IU g^{-1}).

Effect of ACE inhibition or AT_1 receptor antagonism on post-ischaemic haemodynamic recovery and reparative angiogenesis

The day before surgery, mice were randomly allocated to one of the following treatments: AT_1 antagonism by losartan (15 mg kg^{-1} per day, $n=10$), ACE inhibition by ramipril (1 mg kg^{-1} per day, $n=7$), or vehicle (tap water, $n=6$). An additional group ($n=7$) received ramipril in combination with bradykinin (BK)B_1 receptor antagonist des-Arg^9-Leu^8-BK (DALBK, 50 nmol kg^{-1} per day) and B_2 receptor antagonist D-Arg,[Hyp^3,Thi^{5}D-Tic^7,Oic^8]-BK (Icatibant, 1 μmol kg^{-1} per day). The antagonists were initially injected subcutaneously, limited to the day before induction of ischaemia, and then infused by the use of osmotic minipumps (Alza Co., Palo Alto, CA, U.S.A.), implanted into the abdomen on the occasion of femoral artery removal.

Haemodynamic measurements

Systolic blood pressure (SBP) and hindlimb blood flow (BF) were sequentially measured before and at 7, 14, and 21 days after femoral artery removal. SBP was measured in conscious mice by tail-cuff plethysmography (Madeddu *et al*., 1997).

The animals were then anaesthetized and placed on a heating plate at 37°C for 5 min for measurements of hindlimb blood flow by laser Doppler flowmetry (Laser Perfusion Imager System [LDPI], Lisca Inc., North Brunswick, NJ, U.S.A) (Emanueli *et al*., 2000b; 2001a). After completion of scanning procedure, a colour-coded image representing the microvascular blood flow distribution was captured on the monitor. The perfusion values were then stored for subsequent data analysis. Ischaemic to contralateral foot hindlimb BF ratio was taken as an index of post-ischaemic blood flow recovery (Emanueli *et al*., 2001a).

Analysis of capillary density

Capillary density was determined in ischaemic and contralateral adductor muscles at 21 days following surgery. Hindlimbs of anaesthetized mice were perfused with PBS (1 min), followed by 10% buffered formalin (10 min) at 100 mmHg *via* the abdominal aorta. Hindlimb muscles were placed in formalin for 48 h. After paraffin embedding, 3 μm-thick sections were cut from each sample with muscle fibres oriented in a transverse direction, stained with haematoxilin and eosin, and examined at 200 × magnification. The analysis of the capillary network was then performed using an ocular reticle (9604-μm^2 area) at 1000 × magnification. For each area of tissue section, 25 fields were randomly counted in a blind fashion. The number of capillary profiles (n_{cap}) was used to compute the capillary numerical density per mm^2 of muscle according to the following equation; $n_{cap}/mm^2 = n_{cap}$ in total fields/total field area (Emanueli *et al*., 2000b; 2001a). The number of myofibers per mm^2 of muscle was also evaluated and capillary to myofiber number ratio was then calculated (Emanueli *et al*., 2001b).

Drugs

Ramipril and Icatibant were generous gifts from Aventis-Pharma (Frankfurt, Germany), losartan was purchased from Merck (Milan, Italy), and DALBK from Sigma-Aldrich (Milan, Italy). Doses were chosen according to previous studies performed by our group (Emanueli *et al*., 2000a,b; 2001a) or others (Gohlke *et al*., 1997).

Statistical analysis

All results are expressed as mean ± standard error (s.e.mean). Multivariate repeated-measures ANOVA was performed to test from interaction between time and grouping factor. In multiple comparisons among independent groups in which ANOVA and *F*-test indicated significant differences, the statistical value was determined according to the Bonferroni's method. Differences within or between groups were determined using paired and unpaired Student's *t*-test, respectively. A value of $P<0.05$ was interpreted to denote statistical significance.

Results

Effect of limb ischaemia on muscular AT_1 gene expression

As shown in Figure 1, similar levels of GAPDH mRNA were detected in muscles of both experimental and control groups. AT_{1a} mRNA levels decreased by 3.1 fold in muscle harvested 3 days after femoral artery removal. In contrast AT_{1b} expression was about doubled early after ischaemia and returned to normal value by day 14.

Effect of ischaemia on ACE activity

ACE activity in the plasma or contralateral normoperfused muscle was not altered by unilateral limb ischaemia (data not shown). In contrast, muscular ACE activity was decreased from 634 ± 72 to 157 ± 45 IU gr^{-1} ($P<0.05$) at 3 days from induction of ischaemia. It returns to the basal value by day 14 from surgery (Figure 2).

Effect of ACE-inhibition or AT_1 antagonism on systemic and limb haemodynamics

As shown in Table 1, basal SBP did not differ among groups. SBP was not affected by limb ischaemia, ramipril, or losartan. As shown in Figure 3, hindlimb BF of vehicle-treated mice was dramatically reduced upon femoral artery removal and then recovered progressively over time, being completely re-established at day 21. The rate of haemodynamic recovery was severely impaired by either losartan or ramipril. At 21 days, hindlimb BF ratio of ramipril-treated mice was 50% less than that of controls. The effect was even more dramatic in the losartan group, in which no resolution of the haemodynamic deficit was manifested at the end of the experimental period (BF ratio: 70% less than in controls).

Pharmacological blockade of kinin receptors did not alter the local haemodynamic response to ACE inhibition.

Effect of ACE-inhibition or AT_1 blockade on capillary density of ischaemic muscles

Neither induction of left limb ischaemia nor pharmacological treatments altered the capillary density of contralateral, normoperfused adductors (data not shown). As shown in Figure 4A,B, induction of ischaemia promoted local neovascularization in vehicle-treated mice (1077 ± 145 *vs* 577 ± 65 cap mm^2, $P<0.02$ and 2.00 ± 0.16 vs 1.22 ± 0.11 cap/fibres, $P<0.05$). The angiogenic response was nullified

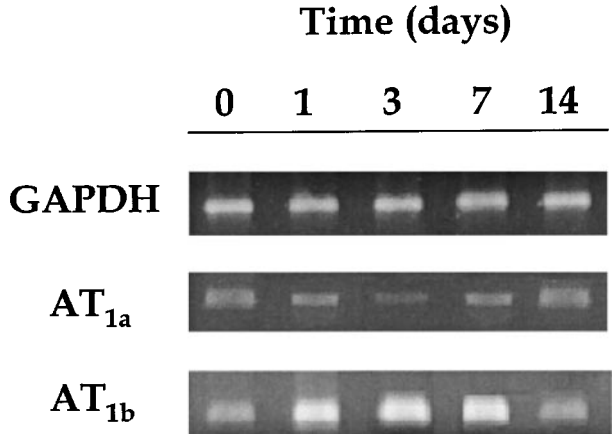

Figure 1 Expression of angiotensin II AT_{1a} and AT_{1b} receptors in muscles of mice at 0, 1, 3, 7, and 14 days following induction of limb ischaemia. Levels of GAPDH mRNAs are shown for comparisons.

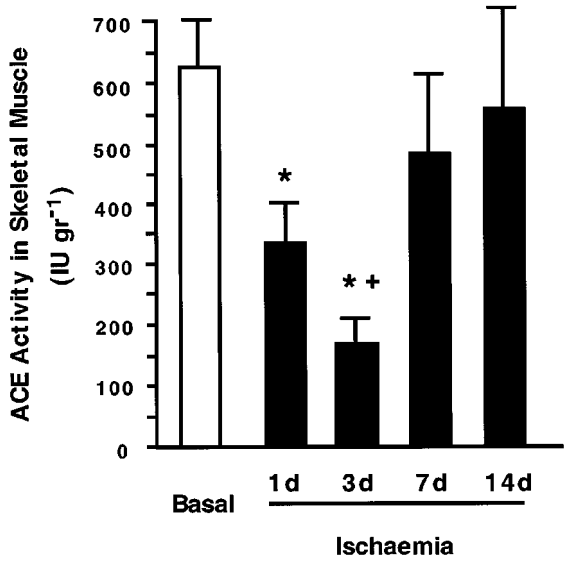

Figure 2 Bar graph shows the ACE activity in limb muscles of mice at 0, 1, 3, 7, and 14 days from induction of ischaemia ($n=4$-6 mice per each time points). Values are the mean ± s.e.mean. *$P<0.05$ vs basal; +$P<0.05$ vs day 1.

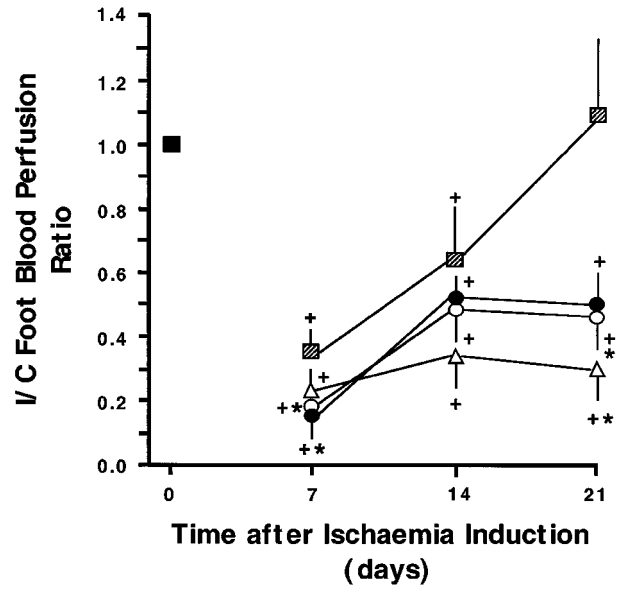

Figure 3 Line graph shows the time-course of perfusion recovery after induction of ischaemia evaluated by laser Doppler flowmetry. Pharmacological treatment started 1 day prior to surgery. Mice were given tap water to drink (vehicle, hatched squares) or water containing losartan (15 mg kg^{-1} per day, open triangles), or ramipril (1 mg kg^{-1} per day, open circles). Ramipril was also given in combination with IP bradykinin (BK) B_1 and B_2 receptor antagonists (des-Arg^9-Leu^8-BK at 50 nmol kg^{-1} and Arg,[Hyp^3,Thi^{5}D-Tic^7,Oic^8]-BK at 1 μmol kg^{-1} per day, respectively, full circles). Values are the mean ± s.e.mean and represent ischaemic to non-ischaemic perfusion ratio at the level of distal hindlimb. The perfusion ratio prior to induction of ischaemia (a full square) is shown as a reference. +$P<0.01$ vs basal (day 0); *$P<0.05$ vs vehicle.

Table 1 Effects of ACE Inhibition or AT_1 receptor blockade on systolic blood pressure in mice subjected to hindlimb ischaemia

Treatment	*Basal*	*7 days*	*14 days*	*21 days*
Vehicle	112±4	115±6	113±7	111±5
Ramipril	119±3	N.D.	111±4	104±6
Ramipril + B_1 and B_2 blockade	118±3	N.D.	103±2*	108±4
losartan	116±4	112±4	109±3	111±5

Tail-cuff systolic blood pressure (SBP,mmHG) under basal conditions and at 7, 14, and 21 days following induction of unilateral hindlimb ischaemia in mice under ACE inhibition (ramipril alone or in combination with kinin antagonists) or AT_1 blockade. Pharmacological treatment started 1 day before left femortal artery removal. Mice were given tap water to drink (vehicle) or water containing ramipril (1 mg kg^{-1} per day), ramipril in combination with IP bradykinin (BK) B_1 and B_2 receptor antagonists (des-Arg^9-Leu^8-BK at 50 nmol kg^{-1} and Arg,[Hyp^3, Thi^{5}D-Tic^7, Oic^8]-BK at 1 μmol kg^{-1} per day, repectively), or losartan (15 mg kg^{-1} per day). Each group consisted of at least $n=6$ mice. *$P<0.05$ vs basal SBP.

by either losartan or ramipril. The effect of ramipril remained unaltered under combined blockade of B_1 and B_2 receptors.

Discussion

The present study demonstrates that endogenous RAS is modulated by limb ishaemia and participates in the native growth of collateral vessels responsible for post-ischaemic recovery.

Muscular ACE activity was found significantly decreased early following ischaemia onset. In addition, we observed that the expression of AT_{1a} and AT_{1b} receptor genes is differently modulated in the ischaemic limb. This figure is apparently at variance with that observed in the ischaemic heart, in which both ACE activity and AT_1 receptor expression are increased (Yang *et al*., 1997; Busatto *et al*., 1997).

We adopted a pharmacological approach to ascertain the importance of endogenous angiotensin II in the reparative process occurring in response to ischaemia. The results obtained with losartan clearly indicates that AT_1 receptors plays a pivotal role in the spontaneous healing response. In fact, mice undergoing chronic blockade of this receptor subtype suffer from an insufficient blood flow recovery attributable to an impaired neo-vascularization. This in keeping with the observation that AT_1 receptor stimulation by angiotensin II is able to induce capillary proliferation (Le Noble *et al*., 1993; Munzenmaier & Greene, 1996; Walsh *et al*., 1997) by up-regulating the expression of VEGF and angiopoietin-2 (Amaral *et al*., 2001; Fujiyama *et al*., 2001). Administration of AT_1 antagonist in the setting of limb ischaemia, a condition characterized by local reduction in ACE activity, would result in the absolute abrogation of AT_1 signalling. However, we observed that ACE inhibition by ramipril was sufficient to negatively influence reparative angiogenesis. These results are in apparent contrast to those obtained by Fabre *et al*. (1999) who found that in normotensive rabbits the tissue ACE inhibitor quinaprilat improved haemodynamic recovery by increasing capillarity of the ischaemic limb. The discrepancy may be explained by substantial differences of the two experimental settings. In fact, in the case of Fabre's study, treatment schedule was

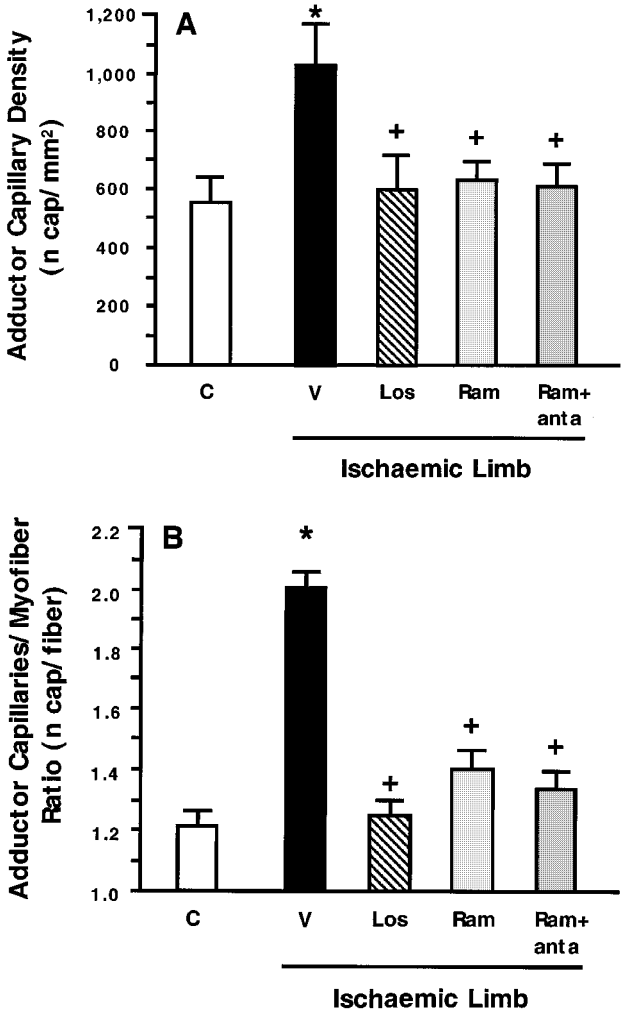

Figure 4 Bar graph shows the effect of chronic treatment with losartan (Los), ramipril (Ram), ramipril in combination with DALBK and Icatibant (Ram + anta), or vehicle (V) on the spontaneous angiogenic response to limb ischaemia. Capillary density per mm^2 of adductor (A) and the capillary to myofiber ratio (B) were evaluated in ischaemic adductors at day 21 after femoral artery removal. The capillary density of normoperfused adductors of untreated mice (C) was also counted. Values are mean ± s.e.mean. *$P<0.001$ vs normoperfused controls. +$P<0.01$ vs ischaemic adductors of vehicle-treated mice.

designed to allow spontaneous recovery to occur prior to ACE inhibition. In contrast, it was our peculiar purpose to reproduce an experimental setting mimicking the situation of patients in which treatment with RAS inhibitors is started previously of the occurrence of an ischaemic insult. Consequently, the importance of endogenous modulators acting during early phases of post-ischaemic healing could have been better appreciated in our study. Species-related differences should also be taken into consideration.

Previous studies from our group showed that kinins exert potent angiogenic activity and that potentiation of this mechanism by delivery of human tissue kallikrein stimulates neovascularization in normoperfused or ischaemic skeletal muscle (Emanueli *et al*., 2000; 2001a). Moreover, in the myocardium of stroke-prone spontaneously hypertensive rats (SP-SHR), life-long treatment with ramipril reportedly increases capillary density *via* a BK B_2-mediated mechanism (Gohlke *et al*., 1997). However, as inferred from results obtained in mice with combined blockade of B_1 and B_2 receptors, kinins do not appear to balance the negative impact of reducing the rate of angiotensin II formation. It remains to explain why the same ACE inhibitor (ramipril) exerts opposite microvascular effects in the myocardium and skeletal muscle. Long-term ACE inhibition leads to reduction, but not absolute suppression of angiotensin II levels, which could be beneficial for the heart of SP-SHR. In contrast, short-term ACE inhibition with superimposed ischaemia could critically influence the natural ligand available for the AT_1 receptor, thus resulting in detrimental effects for skeletal muscle. It is also possible that heart and skeletal muscle differ regarding the microvascular responses to angiotensin II.

Among possible mechanisms implicated in the adverse effects of ramipril on reparative angiogenesis, one may consider the reduction in the formation of angiotensin IV, an angiotensin II metabolite exerting a pro-angiogenic action (Wright & Harding, 1997). On the other hand, it is also possible that ramipril-driven increase in angiotensin I results in augmented synthesis of the anti-angiogenic derivate Ang (1–7) (Chappell *et al*., 2000). Both these effects may play a role in the final anti-angiogenic action of ACE inhibition.

Under AT_1 receptor blockade, all angiotensin II is available for AT_2 stimulation. Therefore, the possibility cannot be discarded that the effects exerted by losartan is in part attributable to AT_2 activation. Indeed, angiotensin II-induced angiogenesis in the rat cremaster muscle is enhanced by AT_2 antagonists (Munzenmaier & Greene, 1996), possibly due to interference on AT_2-mediated apoptotic effect on endothelial cells (Hu *et al*., 1997; Walsh *et al*., 1997). Further studies using AT_2 antagonists may help obtain deeper insights into the role of this receptor subtype in post-ischaemic recovery.

In conclusion, our findings indicate a pivotal role of angiotensin II in the process of reparative angiogenesis. In a clinical perspective, the occurrence of acute peripheral vascular occlusion in patients under medication with ACE inhibitors or AT_1 antagonists might require the most careful evaluation of the pros and contras of maintaining such a therapeautic regimen.

We are grateful to Aventis-Pharma (Frankfurt, Germany) for providing us with Icatibant and ramipril.

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(Received April 25, 2001
Revised October 26, 2001
Accepted October 26, 2001)