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Alternative pathways of angiotensin II production in the human saphenous vein

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1 The aim of our study was to demonstrate the existence, location and functional importance of an alternative angiotensin II-forming pathway other than angiotensin converting enzyme (ACE) in the human saphenous vein (SV).

2 Vascular reactivity studies using an *in vitro* organ bath technique showed that the SV ($n=20$) produced similar maximum contractions in response to angiotensin I (41.5 ± 5.4 mN) compared to those observed to angiotensin II (46.7 ± 10.9 mN). The response to angiotensin I could be significantly inhibited ($P<0.05$) by incubation with the AT_1 receptor antagonist losartan (1 μM).

3 Prior incubation of segments of SV with either captopril (1 μM) ($n=6$), quinaprilat (1 μM) ($n=7$), or the chymase inhibitor soya bean trypsin inhibitor (SBTI) (10 μM) ($n=7$) singularly failed to have any inhibitory effect on the response to angiotensin I. However when vessel segments ($n=7$) were co-incubated with quinaprilat (1 μM) and SBTI (10 μM), the SV exhibited a rightward shift in curve profile to angiotensin I and a markedly reduced maximum response 12.5 ± 2.4 mN, when compared to control (30.4 ± 7.6 mN), quinaprilat (24.5 ± 9.4 mN), and SBTI (31.6 ± 10.7 mN) on their own.

4 An immunohistochemical technique employing streptavidin biotin peroxidase localised ACE to both endothelial cells and smooth muscle cells while chymase was confined to mast cells in the adventitia of the vessel wall.

5 In conclusion, our results demonstrate the existence of an alternative angiotensin I converting pathway to that of ACE, involving chymase. Therefore, there is the capacity for a continuation of angiotensin II formation. in the presence of ACE inhibition.

Keywords: human saphenous vein; ACE; chymase; angiotensin II formation

Introduction

The physiological activity of angiotensin II has long been recognised to be dependent on conversion of its precursor angiotensin I by angiotensin converting enzyme (ACE). However, alternative angiotensin II forming pathways have been identified (Dzau, 1989). Angiotensin II formation has been shown to occur not only in the systemic circulation, but also in a number of local tissues including the human vasculature (Kifor & Dzau, 1987; Mizuno *et al*., 1988). This octapeptide, as a result of its many biological effects on vascular cellular components, is thought to play an important role in the development and progression of a number of cardiovascular diseases, such as hypertension and atherosclerosis (Griendling *et al*., 1994), as well as in vascular remodelling of vessels used as coronary artery bypass grafts (Rakugi *et al*., 1994). Pharmacological manipulation of the biochemical processes which result in angiotensin II formation has been shown to be of benefit in a number of these diseases (Osterrieder *et al*., 1991; Chobanian *et al*., 1990; The SOLVD Investigators, 1992). The mainstay of this therapy has been the use of ACE inhibitors. However it has been reported that following chronic ACE inhibitor therapy, patient serum angiotensin II concentrations return almost to pre-treatment levels (Mento & Wilkes, 1987). Also, blood pressure was further lowered in hypertensive patients prescribed angiotensin II receptor antagonists together with ACE inhibitors (Azizi *et al*., 1995). Such experimental evidence suggests the existence of other local angiotensin conversion pathways which may be responsible for angiotensin II formation in the cardiovascular system.

A number of *in vitro* angiotensin II forming enzymes in the vessel wall other than ACE have been demonstrated, such as, tissue plasminogen activator (tPA), cathepsin G, tonin, neutralendopeptidase, and a chymostatin sensitive angiotensin II generating enzyme similar in structure to those of the chymase group of enzymes (Dzau, 1989). This latter enzyme has been identified as a highly specific serine protease (Urata *et al*., 1990b; Kinoshita *et al*., 1991), which distinguishes it from other angiotensin II forming enzymes, and is shown to be synthesised and stored in secreting granules of mast cells in the human myocardium, as well as in endothelial cells and mesenchymal cells (Urata *et al*., 1993, 1994). *In vitro* studies in the left ventricle of the human heart and in the coronary arteries (Urata *et al*., 1990a), have shown chymase to be responsible for approximately 80% of angiotensin II formation from angiotensin I, with similar results being observed with infused dog left anterior descending coronary arteries (Balcells *et al*., 1996). However, in contrast to these findings are data obtained from similar *in vivo* experiments, where tissue ACE appeared to be responsible for most of the angiotensin II formed across the human myocardial circulation (Zisman *et al*., 1995). In comparison, in monkey mesenteric artery and inferior epigastric strips, angiotensin I-mediated contraction was only partially inhibited with an ACE inhibitor, but completely inhibited by a combination of an ACE inhibitor and a chymostatin inhibitor (Okunishi *et al*., 1993). Therefore, it appears that significant differences in local angiotensin formation in cardiovascular tissues exists between humans and other species (Okunishi *et al*., 1993).

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The saphenous vein is of particular interest since it develops vein graft disease following its use as a coronary artery and peripheral bypass conduit, a process in which the local renin angiotensin system in the vessel wall has been implicated (Davies *et al*., 1995). Vascular remodelling, smooth muscle cell growth and intimal proliferation as well as altered vascular contraction are all features of this disease (Bulkley & Hutchins, 1978). It has been shown that angiotensin II has the capacity to mediate many of these characteristic features of vein graft disease via its local production in the vessel wall. The enzymes responsible for its production in the saphenous vein may therefore play a key role in mediation of the disease process. The aim of this study was to investigate the role of both angiotensin-converting enzyme and chymase in the contractile response observed to angiotensin I, the precursor of angiotensin II and to localise these enzymes within the wall of human saphenous veins.

Methods

Tissue collection and storage

Lengths of human saphenous vein (n = number of patients) ($n = 55$) were removed from male ($n = 48$; age range = 40–88 years), and female ($n = 7$; age range = 60–74 years) patients undergoing coronary artery bypass surgery for ischaemic heart disease. Immediately after removal, the vein was placed in sterile Hank's balanced salt solution, after which it was taken to the laboratory where it was either cleaned of excess connective tissue and used in organ bath studies, or mounted in OCT, and frozen in liquid nitrogen (N_2) for immunohistochemical studies. All patients were receiving a combination of one or more of the following drug therapies prior to surgery: nitrates, calcium channel blockers, anticoagulants, analgesics, antibiotics, lipid lowering drugs, β-blockers, or potassium channel blockers. Only 22% of patients were receiving an ACE inhibitor and no patient in the study was prescribed AT_1 receptor blockers. No single drug or combination of drugs appeared to influence the response of the saphenous vein in any of the protocols studied. The majority of patients undergoing surgery were male (87%), within a narrow age range, therefore it was considered inappropriate to attempt to correlate the sex and age of the patients with the experimental results observed.

In vitro *organ bath studies*

Segments of isolated saphenous vein were mounted in 5 ml organ baths containing Krebs Henseleit solution with the following composition (mM): NaCl 136.9; $NaHCO_3$ 11.9; KCl 2.7, NaH_2PO_4 0.4; $MgCl_2$ 2.5; $CaCl_2$ 2.5; glucose 11.1 and disodium EDTA 0.04. Organ chamber core temperature was maintained at 37°C and the krebs solution bathing the tissue was gassed continuously with 95% O_2 and 5% CO_2. For each vein segment, an initial stretch of 6 g was placed on the tissue. Following a period of relaxation, smooth muscle viability was tested by the addition of 90 mM KCl. When a maximum contraction had been reached, vessels were washed with fresh Krebs solution and again allowed to relax. Vessels were then further stretched by 1 g in a step wise manner until the final 90 mM KCl response was within 10% of the previous one. This was taken to be the optimum resting tension for an individual vein segment.

The vasoactive effects of angiotensin I and II were tested by the addition of increasing concentrations (0.01 nM–1 μM) of either peptide, in a cumulative manner. Only one peptide was used on any one vessel segment at any time. The angiotensin I effect in the human vasculature has been shown to be a result of its conversion to angiotensin II by an angiotensin converting enzyme. Hence we examined this possibility in the vein by pre-incubating vessel segments for 30 min with the ACE inhibitor captopril (1 μM). Other segments were incubated with either the ACE inhibitor quinaprilat (1 μM), or the mast cell serine protease inhibitor soya bean trypsin inhibitor (SBTI) (10 μM), on their own, or in combination at the same concentrations. In the absence or the presence of these inhibitors, a concentration response curve to angiotensin I (0.01 nM–1 μM) was carried out. The concentrations of captopril (Crabbe *et al*., 1996), and quinaprilat (unpublished data) used were both shown to be able to totally inhibit tissue ACE activity in segments of SV, while that of SBTI, had previously been shown to completely inhibit human heart chymase activity (Urata *et al*., 1990b).

Angiotensin II has been shown to mediate its contractile effects in the saphenous vein via the AT_1 receptor (Borland *et al*., 1996). Therefore we examined the role of the AT_1 receptor in mediating the contractile response to angiotensin I. Vessel segments were incubated with one of three concentrations of either the AT_1 receptor antagonist losartan (0.01, 0.1 or 1 μM), or the AT_2 receptor antagonist PD123319 (0.01, 0.1 or 1 μM) for 30 min. Then in the absence or the presence of either inhibitor the response to increasing concentrations of angiotensin I (0.001 nM–1 μM) was examined.

ACE and chymase localisation

Vessel segments previously snap frozen in liquid nitrogen, were sectioned transversely (6 μm thick), using a cryostat, placed on polylysine coated slides and allowed to air dry at room temperature before being wrapped in foil and stored at −20°C. Sections were removed from the freezer and allowed to equilibrate to room temperature before being fixed for 10 minutes in acetone and air dried. Sections were then incubated in either mouse anti-ACE antibody (1.3 μg/μl), or mouse-anti-chymase antibody (1.6 μg/μl), or an irrelevant mouse antibody of the same IgG subclass as a negative control, for 30 min. All antibodies were diluted in 0.005 M Tris buffered saline, pH 7.6 (TBS). Subsequently, sections were washed thoroughly in TBS and biotinylated rabbit anti-mouse $F(ab'_2)$ antibody (2.6 mg/ml) diluted in TBS containing 1% human AB serum was then applied for 30 min. After further washing in TBS the sections were incubated for 30 min in streptavidin biotin-peroxidase complexes diluted in 0.05 M Tris buffer; pH 7.6. Sections were washed again in TBS before the application of a solution of diaminobenzidine tetrahydrochloride as the visualisation agent. The slides were then rinsed in water, counterstained in Mayer's haematoxylin, dehydrated through graded alcohols, cleared in CNP30 and mounted in DPX. Mast cells were demonstrated in frozen sections by fixation in acetone, followed by incubation in 0.5% toluidine blue in 50% isopropanol for 30 min, blotted, then rinsed in isopropanol, followed by CNP30 and mounted in DPX.

Statistical analysis

In all organ bath studies, responses are expressed as either the mean of the absolute mN value ± the standard error of the mean (s.e.mean), or, as the mean response expressed as a percentage of the 90 mM KCl effect ± s.e.mean. Statistical analysis was carried out by means of a one way analysis of variance (ANOVA) followed by a Bonferroni t-test. Significance was taken as, $* = P < 0.05$.

Materials

All salts used to make the modified Krebs solution were obtained from BDH (Poole, U.K). Angiotensin I was purchased from Sigma (Poole, U.K). Losartan was a gift from Merck (Rahway, U.S.A.), while PD123319 and quinaprilat were gifts from Parke-Davis (Ann Arbor, U.S.A). Streptavidin biotin peroxidase was purchased from Dako (High Wycombe, Bucks). ACE and chymase antibodies were purchased from Chemicon International Inc (Temecula, CA, U.S.A.). All drugs except quinaprilat were initially dissolved in distilled water. Further dilutions were made in Krebs Henseleit solution (composition of which is described above in methods section). Quinaprilat was initially dissolved in dimethyl sulphoxide, further dilutions again being made in Krebs Henseleit solution.

Results

Organ bath studies

Both angiotensin I and II produced concentration-dependent contractions in the saphenous vein. There was a rightward shift in the angiotensin I curve, which was less potent ($EC_{50} = 69.0 \pm 17$ nM), when compared to that of the angiotensin II response, ($EC_{50} = 39.0 \pm 32$ nM). Similar maximum contractile responses were observed with both angiotensin I (41.5 ± 5.4 mN) and angiotensin II (46.7 ± 10.9 mN) (Figure 1).

The ACE inhibitor captopril (1 μM) showed no inhibitory effect on the angiotensin I response at all concentrations of the peptide (Figure 2). In a similar manner, neither the tissue specific ACE inhibitor quinaprilat (1 μM) nor the chymase inhibitor SBTI (10 μM) on their own were able to inhibit contractions to angiotensin I throughout the concentration range in segments of saphenous vein. However, when both quinaprilat (1 μM) and SBTI (10 μM) were used in combination, contractions to angiotensin I were markedly inhibited and a pronounced rightward shift in the curve was observed when compared to control vessels (Figure 3).

The AT_1 receptor antagonist losartan was able to significantly inhibit contractions to angiotensin I in the saphenous vein in a concentration dependent manner. Significant differences ($P < 0.05$) were observed in maximum effect of angiotensin I in those vessels incubated with either 0.1 μM, or, 1 μM losartan (Figure 4A). In contrast, the AT_2-receptor antagonist PD123319 at all three concentrations showed no inhibitory effect on the angiotensin I response throughout the concentration-response curve (Figure 4B).

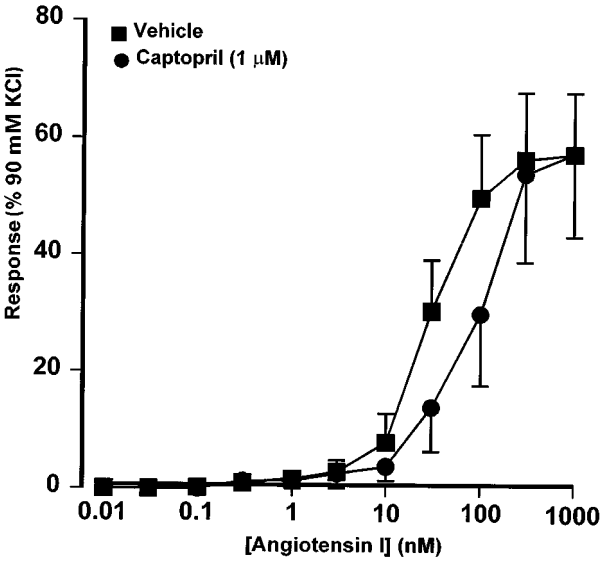

Figure 2 The contractile response of the human saphenous vein to increasing concentrations of angiotensin I (0.01 – 1000 mM) either in the absence of ($n = 6$ patients) or in the presence of the ACE inhibitor captopril (1 μM) ($n = 6$ patients). Mean contractile values are expressed as a % of the response to 90 mM KCl ± s.e.mean.

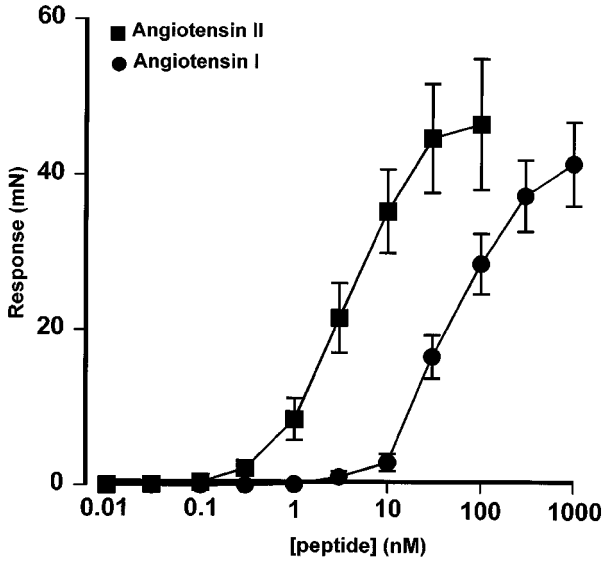

Figure 1 The contractile response of the human saphenous vein to increasing concentrations of either angiotensin I (0.01 – 1000 nM) ($n = 20$ patients), or angiotensin II (0.01 – 100 nM) ($n = 20$ patients), Values are expressed as the mean of the absolute contractile response (mN) ± the s.e.mean.

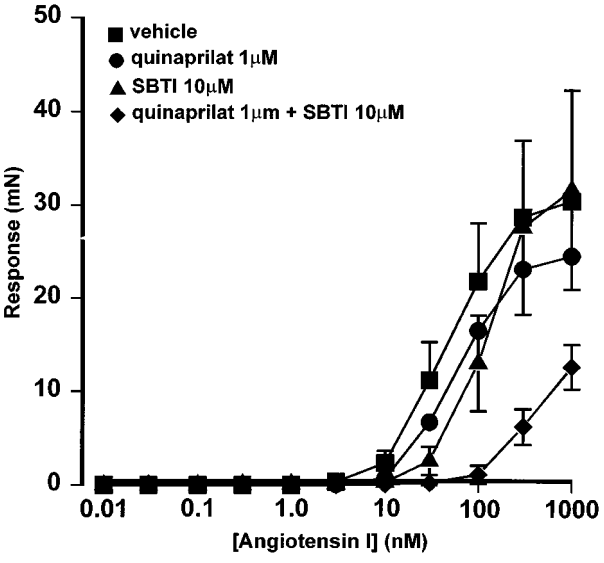

Figure 3 The contractile response of the human saphenous vein to increasing concentrations of angiotensin I (0.01 – 1000 nM) either in the absence of ($n = 7$ patients), or the presence of the ACE inhibitor quinaprilat (1 μM) ($n = 7$ patients), or the serine protease inhibitor SBTI (10 μM) ($n = 7$ patients), or both quinaprilat (1 μM) and SBTI (10 μM) combined ($n = 7$). Mean values are expressed as absolute mN values ± s.e.mean.

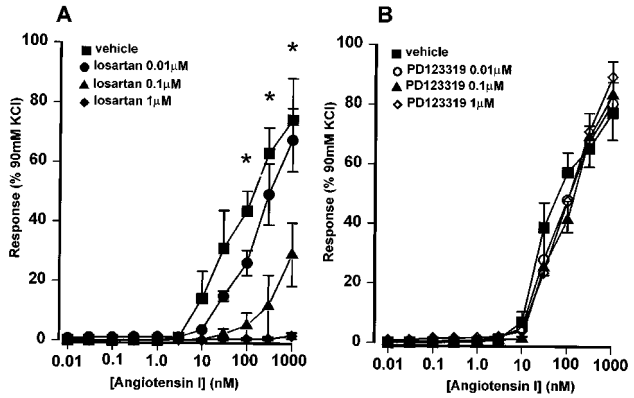

Figure 4 The contractile response obtained in the human saphenous vein to increasing concentrations of angiotensin I (0.01 – 1000 nM), in either (**A**) the absence of ($n=6$ patients), or the presence of one of three concentrations, 0.01 μM, 0.1 μM, or 1 μM, ($n=6$ patients), of the AT_1 receptor specific antagonist losartan, or (**B**) in the absence of ($n=5$ patients), or the presence of one of three concentrations, 0.01 μM, 0.1 μM, or 1 μM, ($n=5$ patients) of the AT_2 receptor specific antagonist PD123319. Mean values are expressed as a % of the response to 90 mM KCl $\pm$ s.e.mean. Significance was taken as $*=P<0.05$.

Immunohistochemistry

The immunohistochemical streptavidin peroxidase technique used to localise ACE, demonstrated strong positive staining, localised mainly to the endothelial cells lining the lumen of the saphenous vein (Figure 5B), as well as in those endothelial cells in the vasa vasorum in the adventitia (Figure 5C), when compared to the control vessel (Figure 5A). A pale and more diffuse staining was also detected in the medial smooth muscle layer (Figure 5B and C). Using a similar technique, the cross section of the vein also showed some strong positive staining for human mast cell chymase (Figure 5D) which was co-localised with mast cells by means of a toluidine blue stain, and was present mainly in the adventitia layer of the blood vessel, with a few cells staining positive in the media.

Discussion

This study has demonstrated the function of an alternative pathway other than ACE for the generation of angiotensin II in the human saphenous vein. We have also shown that the

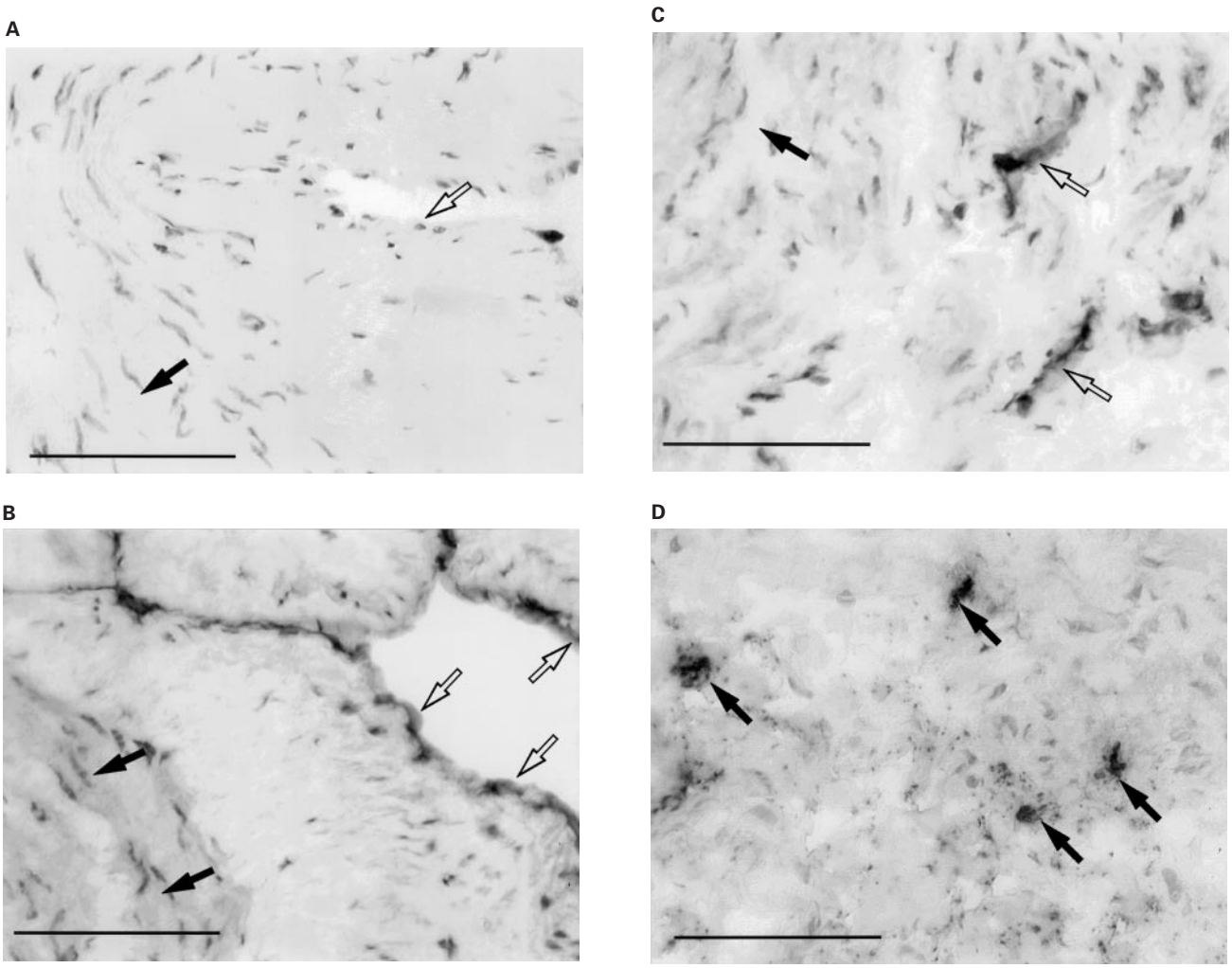

Figure 5 Cross sectional staining of the human saphenous vein, by means of an immunohistochemical technique employing streptavidin biotin peroxidase, showing (**A**) no staining in control sections (**B**) positive staining for ACE (open black arrows) localised to the endothelial cells lining the vessel lumen, and paler staining in the smooth muscle layer (filled black arrows), as well as (**C**) those endothelial cells lining the vasa vasorum (filled black arrows). Section (**D**) demonstrates positive staining for chymase present both in the granules released from the mast cells and those granules within the mast cells (filled black arrows). All sections are $\times 20$ magnification. The scale bar represents 100 microns.

profile of the contractile response to angiotensin I, is similar to that of angiotensin II, and also mediated by the AT_1 receptor (Borland *et al*., 1996). The effect of angiotensin I was dependent upon the function of both ACE and chymase. We were further able to localise ACE to the endothelial cells lining the blood vessel lumen and the vasa vasorum in the adventitial layer, as well as the smooth muscle cell layer, while chymase was co-localised with mast cells in the adventitia.

We did not observe an inhibitory effect to angiotensin I contractions in the vein in the presence of either the ACE inhibitors captopril or enalaprilat, although we have previously demonstrated that ACE activity in the saphenous vein is blocked by an ACE inhibitor (Crabbe *et al*., 1996). We were also not able to see any significant effect of the chymase inhibitor SBTI on its own, however when combined with an ACE inhibitor, there was significant inhibition of the angiotensin I-induced contraction. The combination of ACE and chymase inhibitors did not completely inhibit the maximum effect of angiotensin I, maximally reducing it by 70–80%. This may either suggest the existence of other non-ACE/non-chymase converting enzymes in the wall of the saphenous vein responsible for angiotensin II formation, or that lack of total inhibition may be a function of our *in vitro* experimental model. Our findings are supported by those of others who have shown the existence of ACE-independent angiotensin II-forming pathways, which are sensitive mainly to either broad spectrum serine protease inhibitors aprotinin and chymostatin, or to SBTI. Such experimental findings have been demonstrated in human uteroplacental arteries, where it was suggested that angiotensin I may have a direct effect on AT_1 receptors, or that there may exist a non-ACE pathway (Svane *et al*., 1991). Similarly in the ischaemic dog heart, an increase in angiotensin II concentration measured in the coronary sinus, was not reduced by pre-treatment with ACE inhibitors but instead by aprotinin (Gondo *et al*., 1989). Our findings suggest the existence of a shunting mechanism by which angiotensin II production may continue even in the presence of an ACE inhibitor, via the action of a vascular chymase enzyme. However when both pathways are blocked, the vessel wall has little capacity to form angiotensin II.

In dog renal arterial strips the response to angiotensin I has been shown to be attenuated by ACE inhibitors and chymostatin combined when the endothelium was intact, but when removed, the sensitivity to ACE inhibitors was lost while that to chymostatin was preserved (Okunishi *et al*., 1987). We have not studied the effects of ACE and chymase inhibitors in the absence of the endothelium. Immunohistochemical staining for ACE was present on an almost intact endothelium, therefore insufficient endothelial ACE cannot be the explanation for the observed lack of effect of ACE inhibitors. Saphenous vein grafts removed after varying lengths of time following coronary artery bypass grafting retain similar ACE activity when compared to their native vessels, despite almost total absence of luminal endothelial cells (Crabbe *et al*., 1996). Moreover, we have also demonstrated the presence of ACE in the smooth muscle cells of the vein.

It has been suggested that as ACE is bound to the endothelial cell membrane with its catalytic site exposed to the extracellular surface (Balcells *et al*., 1996), this may make circulating angiotensin I more accessible to ACE. In contrast, chymase may contribute little to circulating angiotensin II formation, but may be of greater importance with regards to its functional effects on the smooth muscle cells in the blood vessel wall. Chymase containing mast cells may be disrupted during surgical preparation of the venous graft, releasing more of the enzyme and hence may be of great importance not only in an experimental model, but also in the short and long term following coronary artery bypass grafting.

There exists experimental evidence, in dog aorta (Heistad *et al*., 1981), rabbit carotid artery (Booth *et al*., 1989), and in the Yucatan mini-pig (Barker *et al*., 1993), to suggest that initiation of atherosclerosis may occur following occlusion of the vasa vasorum, rendering the vessel wall hypoxic, and resulting in initiation of medial necrosis, intimal proliferation, and formation of hyperplastic lesions (Martin *et al*., 1990). Vasa vasorum of the SV may become occluded due to structural damage following harvesting, as well as the action of a number of vasoactive factors, including angiotensin II the levels of which have been shown to be raised during cardiopulmonary bypass (Downing & Edmunds, 1992). Local production of the peptide by the vessel wall during the peri-operative period may be contributed to by both ACE and chymase enzymes, and may thus be insensitive to the action of ACE inhibitors alone. O'Donohoe *et al*., (1991), was able to localise increased levels of ACE to the intimal hyperplastic layer of the vein graft of New Zealand rabbits. However he also showed there to be an ACE resistant component of angiotensin I-induced contractions in both isolated native veins and vein grafts, suggesting the presence of non-ACE mechanisms in this model (O'Donohoe *et al*., 1991).

One of the possible limitations of our study, are potential differences which exist with regards to the involvement of either ACE or chymase in angiotensin II formation between in *vitro* and *in vivo* experimental models. For example in the human heart, *in vitro* experiments have shown chymase to be of great importance in angiotensin II formation (Urata *et al*., 1990a). In contrast, in patients with left ventricular hypertrophy, enaliprilat infused into the coronary artery, improved the diastolic function of the heart, in the absence of any contrary effects, suggesting an important role for ACE in intracardiac angiotensin II formation (Friedrich *et al*., 1994). Another limitation of our study is that the angiotensin I and enzyme inhibitors added are exposed equally to the luminal and abluminal surfaces of the vessel wall. This may overestimate the contribution of enzymes within the adventitia, since most angiotensin I *in vivo* would be exposed preferentially to the vascular endothelium.

In conclusion, we have demonstrated the existence of an alternative pathway responsible for the conversion of angiotensin I to angiotensin II in the human saphenous vein. Therefore our findings may suggest the requirement for the development of other pharmacological therapeutic agents in addition to ACE inhibitors which may help control the local production of angiotensin II, and hence the initiation of vein graft disease.

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