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Comparison of vasodilators in human internal mammary artery: ghrelin is a potent physiological antagonist of endothelin-1

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1 The potential vasodilator function of the peptide ghrelin, recently identified as the endogenous ligand of the growth hormone secretagogue orphan receptor (GHS-R), was investigated in human endothelium-denuded internal mammary artery. The peptide endothelin-1 (ET-1) is a potent and long-lasting vasoconstrictor. Comparisons were made with established and putative endogenous vasodilators to determine if any could reverse ET-1-induced vasoconstriction in this vessel.

2 Ghrelin (0.1–300 nM) potently dilated 10 nM ET-1-induced constrictions (pD_2 8.39±0.29; E_{MAX} 63±5.6%; $n=9/14$, responders/total).

3 ANP (pD_2 7.75±0.14; E_{MAX} 106±2.0; $n=5/5$) and CGRP (pD_2 8.08±0.17; E_{MAX} 76±15% $n=5/6$) both produced complete reversal of the constrictor response to ET-1 (E_{MAX} not significantly different from 100%, $P>0.05$ one-sample t-test).

4 The following caused partial reversal of the ET-1 response: Adrenomedullin ($n=9/9$) and two peptides derived from proadrenomedullin, PAMP-12 ($n=6/7$) and PAMP-20 ($n=9/9$) (pD_2 values 7.63±0.28, 7.97±0.23 and 8.51±0.29; E_{MAX} 58±7.3, 54±10 and 51±7.8% respectively). Unexpectedly, amylin was only 2 fold less potent than CGRP, although there was less than 50% reversal of the ET-1 constriction (pD_2 7.86±0.30; E_{MAX} 41±5.4%; $n=7/9$). CNP ($n=6/6$) also partially reversed constrictions to ET-1 (E_{MAX} 53±6.3; pD_2 8.07±0.38).

5 BNP ($n=4/5$) and PGI_2 ($n=6/8$) were weak vasodilators, since concentration-response curves failed to reach a maximum within the range tested. PGE_2 caused a small dilatation in some vessels (E_{MAX} 17±2.1%; pD_2 8.63±0.36; $n=4/8$).

6 We have demonstrated ghrelin to be an effective, endothelium-independent vasodilator of the long-lasting constrictor ET-1 in human arteries producing responses similar to those of adrenomedullin ($P>0.05$, ANOVA).

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Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CGRP, calcitonin gene-related peptide; CNP, C-type natriuretic peptide; ET-1, endothelin-1; GHS-R, growth hormone secretagogue receptor; PAMP-12, proadrenomedullin N-terminal 12 peptide; PAMP-20, proadrenomedullin N-terminal 20 peptide; PGE_2, prostaglandin E_2; PGI_2, prostaglandin I_2

Introduction

The vascular endothelium continuously synthesises and releases the potent constrictor endothelin-1 (ET-1; Yanagisawa *et al*., 1988; Davenport *et al*., 1989; Franco Cereceda, 1989) *via* an unusual dual secretory pathway (Russell *et al*., 1998). The action of ET-1 is uniquely long lasting compared to other endogenous constrictors, with single concentrations of the peptide producing a vasoconstriction lasting several hours *in vivo* (Clarke *et al*., 1989). Furthermore, infusions of ET receptor antagonists cause vasodilatation in normotensive humans, demonstrating the contribution of the peptide to the maintenance of normal vascular tone (Haynes *et al*., 1996). Endothelin-1 is increased in many cardiovascular disease states and is thought to contribute to the heightened tone (Bacon *et al*., 1996; Lerman *et al*., 1991; Miyauchi *et al*., 1989). We have previously shown nitric oxide (NO) to be an effective physiological antagonist of the constrictor actions of ET-1 in human internal mammary artery (IMA; Wiley & Davenport, 2001b). However, single concentrations of NO-donors produce only transient vasodilatation (Wiley & Davenport, 2001c), indicating that NO must be continuously released *in vivo* to counterbalance the effects of ET-1. The NO signalling pathway is dysfunctional in atherosclerosis and this may not be limited to plaque-containing vessels (Reddy *et al*., 1994). It is therefore important to understand what other endogenous dilators could be important in reversing ET-1-mediated constrictions by direct action on the smooth muscle layer of human arteries. In addition to the known neuronal, endothelium-derived and hormonal vasodilators, there is also the possibility that other endogenous peptides recently paired to orphan G protein-coupled receptors (GPCR) may be involved in the regulation of vascular tone.

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As part of a strategy designed to identify these novel receptor systems in the human cardiovascular system we have synthesized radiolabelled peptides paired to orphan GPCR to screen for the presence of these receptors in the human vasculature. As a result, intense binding of the peptide ghrelin has been localized to the medial layer of human blood vessels (Katugampola *et al*., 2001). Ghrelin is an endogenous peptide that has recently been paired to the G protein-coupled growth hormone secretagogue receptor (GHS-R; Kojima *et al*., 1999). It was originally purified from the rat stomach and comprises 28 amino acids. The peptide has also been found in human stomach extract, where it differs by only two residues. The binding sites for iodinated ghrelin have been characterized in the human cardiovascular system (Katugampola *et al*., 2001) and bolus injections of ghrelin caused a significant decrease in the mean arterial pressure in healthy volunteers (Nagaya *et al*., 2001). However, to date, nothing is known about its direct action on the blood vessels of any species.

The vasodilator hormone adrenomedullin is derived from the 185 residue precursor preproadrenomedullin and two fragments, proadrenomedullin N-terminal 20 peptide (PAMP-20) and proadrenomedullin N-terminal 12 peptide (PAMP-12) have been shown to also have vasodilator function in animal tissues (Kitamura *et al*., 1993; Kuwasako *et al*., 1997). PAMP-20 and PAMP-12 are detectable in human plasma (Washimine *et al*., 1994; Kuwasako *et al*., 1999) and forearm infusions of PAMP-20 in healthy human volunteers causes vasodilatation (Nakamura *et al*., 1999). To date there is no information on the vasodilator potency of PAMP-12 in humans or on the ability of either fragment to directly relax human vascular smooth muscle.

Our objective was to determine if these novel endogenous peptides could reverse constrictions to ET-1 by directly acting on the smooth muscle layer of human IMA *in vitro*, and to compare the responses with established vasodilators. Preliminary data from this study have previously been presented to the British Pharmacological Society and British Atherosclerosis Society (Wiley & Davenport, 2001a, d).

Methods

Tissue collection

Histologically normal IMA were obtained (with local ethical approval) from 40 patients, mean age 66 years (range 37–79 years; eight female, 32 male) undergoing coronary artery bypass operations. Patients were on a combination of therapies including angiotensin-converting enzyme inhibitors, anticoagulants, β-blockers, calcium channel blockers, nitrates, and lipid-lowering drugs. Tissue samples were stored in Krebs' solution at 4°C overnight before use.

In vitro *pharmacology*

IMA were dissected free from surrounding tissue and cut into 3 mm rings. The rings were denuded of their endothelium using a blunt seeker (verified histologically; Maguire *et al*., 1997, 2001), mounted in 5 ml organ baths (Linton Instrumentation, Norfolk, U.K.) for the measurement of isometric tension (F30 force transducers; Hugo Sachs, March-Hugstetten, Germany) and bathed in oxygenated Krebs' solution at 37°C. To obtain the optimal resting tension, 100 mM KCl was added at increasing levels of basal tension until no further increase in response was obtained. Only tissue contracting to 100 mM KCl was used in the study.

Vessel segments were allowed to equilibrate to their own resting tension for at least 1 h before the start of the experiment. A sub-maximal concentration of ET-1 (10 nM) was added and once the response had reached a plateau, concentration-response curves to ghrelin (0.1–300 nM), calcitonin gene-related peptide (CGRP; 0.1–30 nM), amylin, adrenomedullin, PAMP-12, PAMP-20 (0.1–300 nM) atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP; 0.1–300 nM) were constructed. Control rings of artery were contracted with ET-1 (10 nM) and the tension measured over the time course of the experiment. All experiments were terminated with 100 mM KCl to ensure the viability of the tissue. Concentration response curves were expressed as a percentage of the constrictor response to 10 nM ET-1.

Data analysis

The negative log of the EC_{50} value (pD_2 value) was determined for each curve using iterative curve-fitting software fitting an asymmetric sigmoidal function (Biosoft, Cambridgeshire, U.K.). All data were expressed as arithmetic means ± s.e.mean. Concentration-response curves to PGI_2 and BNP did not always reach a maximum in the concentration range tested, consequently mean pD_2 and E_{MAX} values could not be calculated in these cases. Not all vessels responded to some of the vasodilators and only responders have been included in the mean data. The number of individuals, n, was expressed as responders/total. The slopes of the curves fitted by the software, pD_2 values and E_{MAX} values were analysed using ANOVA or a one-sample, two-tailed *t*-test as appropriate ($P<0.05$).

Materials

Adrenomedullin, amylin, ANP, BNP, CGRP, CNP, ET-1, ghrelin, PAMP-12, PAMP-20 (Peptide Institute Inc., Osaka, Japan), PGI_2 and PGE_2 (Alexis Biochemicals, Notts., U.K.), stock solutions (0.1 mM) were prepared in 0.1% acetic acid (ET-1) and distilled water (adrenomedullin, amylin, ANP, BNP, CGRP, CNP, PGE_2, PAMP-12, PAMP-20, ghrelin) and stored at −20°C. PGI_2 stock solutions (1 mM) were prepared in distilled water and stored in the dark at 4°C. All other reagents were from Sigma-Aldrich Ltd. (Dorset, U.K.) or BDH Ltd. (Dorset, U.K.). Krebs' solution comprised (mM); NaCl 90, $NaHCO_3$ 45, KCl 5, $MgSO_4.7H_2O$ 0.5, $Na_2HPO_4.2H_2O$ 1, $CaCl_2$ 2.25, fumaric acid 5, glutamic acid 5, glucose 10, sodium pyruvate 5 (pH 7.4).

Results

Vasoreactivity

Internal mammary artery produced a maximal contractile force of 13.0 ± 0.6 mN mm^{-1} (response to 100 mM KCl; 123 segments from 40 patients). All segments tested produced a

sustained constriction to a sub-maximal concentration of ET-1 (10 nM), with a mean force of 10.6 ± 0.6 mN mm^{-1}.

The novel vasodilator ghrelin

Ghrelin (0.1 – 300 nM) potently dilated IMA previously constricted with 10 nM ET-1. The peptide was tested on rings of IMA from 11 individuals and nine of the rings responded. In responding tissues, the vasodilatation produced by ghrelin was slow in onset and sustained (Figure 1A). Cumulative concentration-response curves to the peptide (Figure 2) produced a mean maximum response of $63 \pm 5.6\%$ and a pD_2 of 8.40 ± 0.26.

Comparison with other vasodilators

The vasodilatation caused by ghrelin was compared with known vasodilators from the major families of endogenous vasoactive molecules (Figure 3), which were ranked in order of maximum response (Table 1). Three of the vasodilators were very weak dilators. Firstly, BNP (0.1 – 200 nM) produced a response in only four of the five segments tested and failed to reach a maximum in two of the responding tissues. Secondly, concentration-response curves to the prostanoid PGI_2 also failed to reach a maximum in some of the responding tissues, ($n = 6$ responders/8 total). Consequently, mean E_{MAX} and pD_2 values could not be calculated for these two dilators. Thirdly, PGE_2 (0.1 – 300 nM) caused a small, concentration dependent relaxation in four of the eight tissues tested (Figure 4A, Table 1). In the other four artery segments, PGE_2 caused vasodilatation at low concentrations that was overcome by vasoconstriction at higher concentrations (Figure 4B). pD_2 values could not be calculated for the second group owing to the complex nature of the response.

ANP ($n = 5/5$) and CGRP ($n = 5/6$) both produced complete reversal of the constrictor response to ET-1 (E_{MAX} not significantly different from 100%, $P > 0.05$ one-sample *t*-test). All the other vasodilators tested caused partial reversal of the ET-1-mediated constriction. The slopes of this medium efficacy group (ghrelin, adrenomedullin, CNP, PAMP-20, PAMP-12, and amylin) were not significantly different ($P > 0.05$, ANOVA), and neither were the pD_2 values or E_{MAX} values ($P > 0.05$, ANOVA).

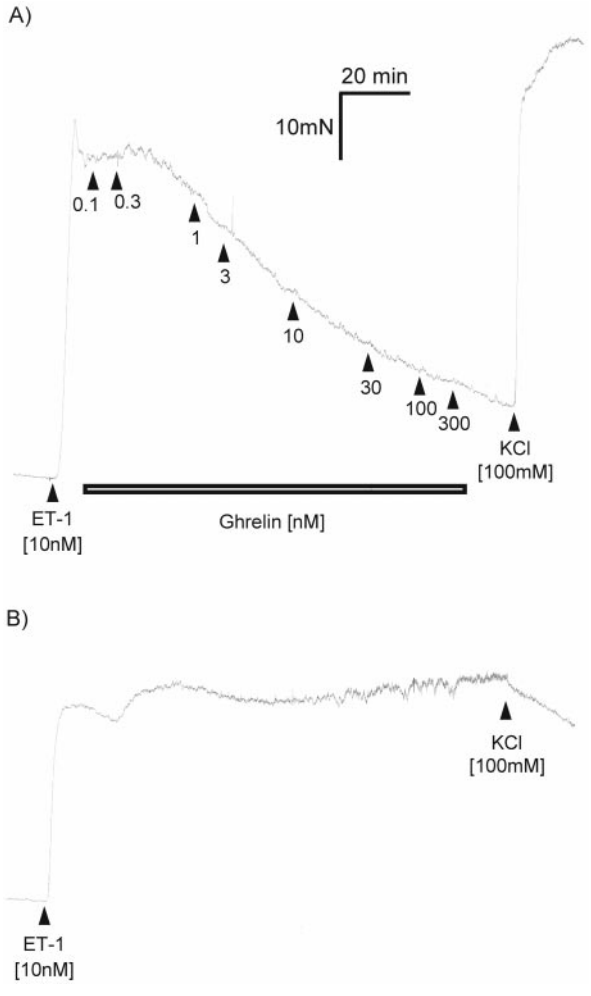

Figure 1 An original trace recording of a single experiment depicting (A) the novel vasodilator ghrelin reversing an ET-1-mediated constriction in a segment of human internal mammary artery and (B) a control constriction to ET-1 in an adjacent ring of tissue from the same patient. The phasic contractions are spontaneous activity of the tissue.

Discussion

Ghrelin, the endogenous peptide recently coupled to the GHS-R, was found to be a potent, endothelium-independent vasodilator of human IMA, effectively reversing ET-1 mediated constrictions *in vitro*. The vasodilatation observed in response to ghrelin was similar in potency and maximum response to that caused by adrenomedullin. Vasodilator responses to ghrelin were slow, taking between 10 and 20 min to plateau. This is 2.5 – 5 times slower than response times for the nitric oxide donor DEA/NO in human internal mammary artery constricted with the same concentration of endothelin-1, which reach a plateau in approximately 4 min (Wiley & Davenport, 2001b). Ghrelin is present in human plasma at approximately 100 pmol l^{-1} (Kojima *et al*., 1999), a concentration considerably higher than other vasoactive peptides. For example both CGRP and ANP are present at

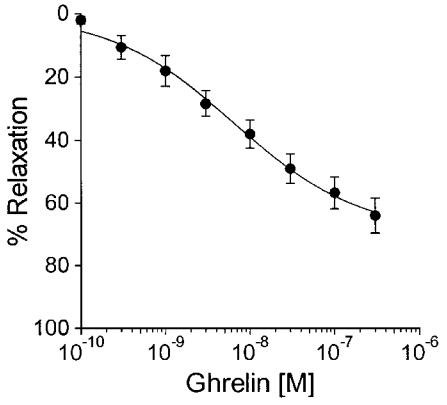

Figure 2 Cumulative concentration-response curve to ghrelin reversing constrictions induced by ET-1 (10 nM) ($n = 9/11$ responders/total). Results were expressed as percentage constrictor response to ET-1 (mean ± s.e.mean).

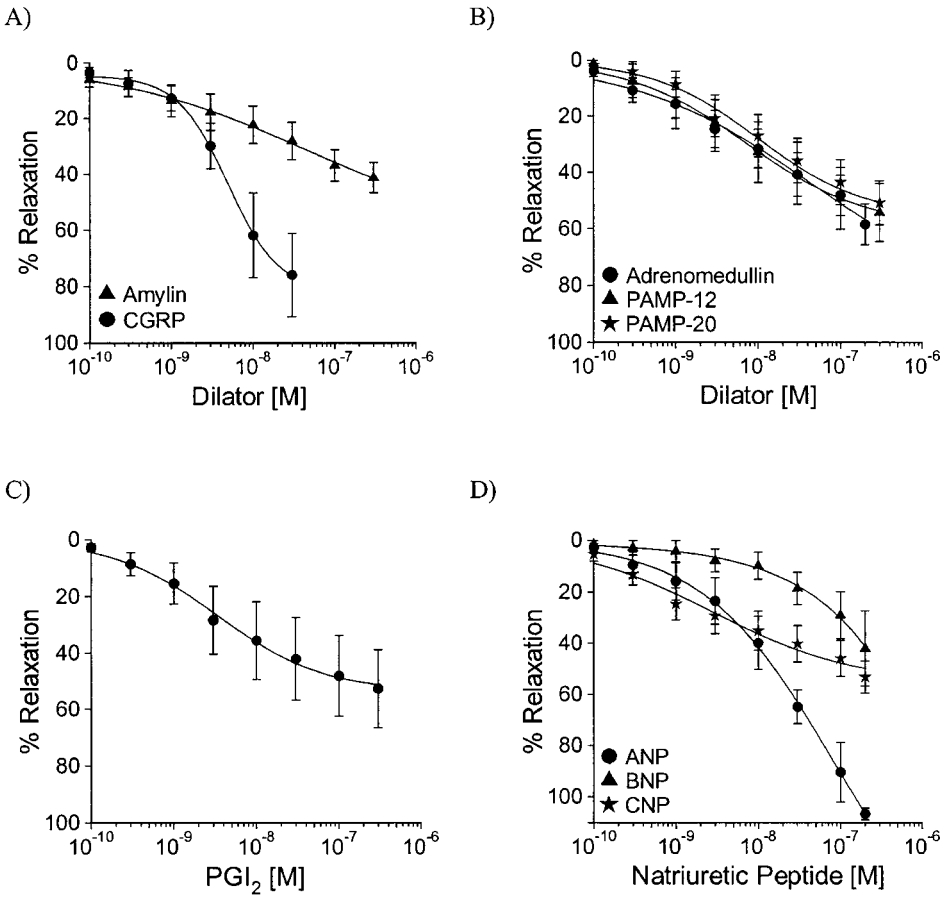

Figure 3 Cumulative concentration-response curves to (A) amylin (0.1–300 nM; $n=6/8$) and CGRP (0.1–30 nM; $n=5/6$), (B) adrenomedullin ($n=9/9$) and two peptide fragments of proadrenomedullin; PAMP-12 ($n=6/7$) and PAMP-20 ($n=9/9$), (C) PGI_2 ($n=6/8$; 0.1–300 nM), (D) atrial natriuretic peptide (ANP; $n=5/5$), brain natriuretic peptide (BNP; $n=4/5$) and C-type natriuretic peptide (CNP; $n=6$) in human IMA. Results were expressed as percentage ET-1 constrictor response (mean ± s.e.mean).

Table 1 Vasodilators in human internal mammary artery ranked in order of maximum response

	E_{MAX} (% relaxation)	pD_2	n (responders/total)
ANP	106 ± 2.0	7.75 ± 0.14	5/5
CGRP	76 ± 15	8.08 ± 0.17	5/6
Ghrelin	63 ± 5.6	8.40 ± 0.26	9/11
Adrenomedullin	58 ± 7.3	7.63 ± 0.28	9/9
PAMP-12	54 ± 10	8.07 ± 0.38	6/7
CNP	53 ± 6.3	8.51 ± 0.29	6/6
PAMP-20	51 ± 7.8	7.97 ± 0.23	9/9
Amylin	41 ± 5.4	7.86 ± 0.30	7/9
PGE_2	17 ± 2.1	8.63 ± 0.36	4/8
PGI_2	NC	NC	6/8
BNP	NC	NC	4/5

pD_2 values (the negative log of the concentration of an agonist producing a response equal to 50% of the maximum response of the tissue to that agonist) and E_{MAX} values (the maximal response to vasodilator as a percentage of the constrictor response to ET-1) were expressed as mean ± s.e.mean. Rings of artery from some individuals did not response to some of the dilators tested. These data were not included in the mean data presented (n is the number of individuals expressed as responders/total). NC, not calculated; some concentration-response curves to PGI_2 and BNP failed to reach a maximum, consequently the pD_2 values and E_{MAX} could not be calculated.

between 1 and 35 pmol l^{-1} (Lechleitner *et al*., 1992; Buckley *et al*., 1993). In man, intravenous bolus injection of ghrelin produces a sustained decrease in mean arterial pressure lasting over 100 min. GHR density is comparable to that of AT_2 receptors in human coronary artery (Katugampola & Davenport, 2002) and receptor density is reported to increase in atherosclerotic vessels (Katugampola *et al*., 2001). This, together with the data presented here would suggest that the ghrelin-GHSR signalling pathway is involved in the regulation of vascular tone in man and may also have a pathophysiological role in atherosclerosis.

CGRP reversed constrictions to ET-1 with a similar potency to that described previously (Luu *et al*., 1997). Surprisingly, the pD_2 value for amylin was only 2 fold less potent than CGRP. Amylin has been found to be 100 times less potent than CGRP in increasing blood flow in rabbits *in vivo* (Brain *et al*., 1990) and 10 times less potent in mediating vasodilatation in the rat mesenteric arterial bed (Westfall & Curfman-Falvey, 1995).

ET-1-mediated constrictions were partially reversed by a direct dilator action of adrenomedullin on the vascular smooth muscle. Measurements of forearm blood flow in man have previously shown that adrenomedullin is a potent vasodilator of human arteries (Cockcroft *et al*., 1997;

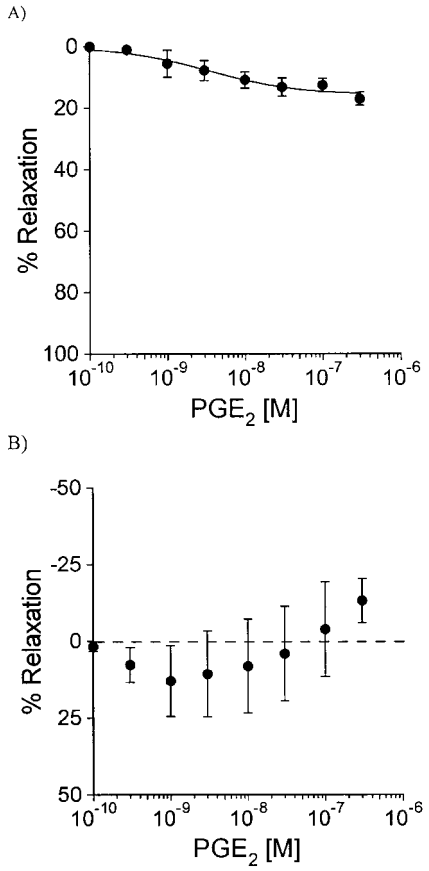

Figure 4 Cumulative concentration-response curves to prostaglandin E_2 (PGE_2) (0.1–300 nM) in human internal mammary artery. (A) Dilator subgroup ($n=4$ individuals) and (B) Constrictor subgroup ($n=4$ individuals). Vasodilatation was expressed as a percentage of the constrictor response to 10 nM ET-1 (mean ± s.e.mean). Constrictions to PGE_2 were also expressed as a percentage of the constrictor response to ET-1 (mean ± s.e.mean), to enable comparison with the dilator responses.

Nakamura *et al*., 1997). The main mechanism of adrenomedullin-mediated vasodilatation is *via* stimulation of medial adenylate cyclase (Ishizaka *et al*., 1994). Adrenomedullin also stimulates endothelial nitric oxide production (Shimekake *et al*., 1995), so part of the vasodilatation seen in the *in vivo* infusions would have been mediated by the indirect actions of adrenomedullin on the endothelium. Endothelial denudation reduced the vasodilatation to approximately 20% in human coronary arterioles (Terata *et al*., 2000) but the disparity in response could be explained either by the different vascular bed or by the size of artery studied.

The proadrenomedullin fragments PAMP-12 and PAMP-20 reversed ET-1 mediated constrictions with similar potencies and maximum responses to adrenomedullin. Two groups (Nakamura *et al*., 1999; Wilkinson *et al*., 2001) have found PAMP-20 to be 60–100 times less potent than adrenomedullin when infused into the human forearm, suggesting that PAMP-20 has a greater effect on conductance vessels such as the IMA than the peripheral resistance vasculature. PAMP-12 caused a dose-dependent hypotensive effect in anaesthetized rats that was similar to that seen with PAMP-20 (Kuwasako *et al*., 1997). The PAMP peptides are not thought to exert their physiological effects *via* the adrenomedullin, amylin or CGRP receptors, however the putative PAMP receptor has yet to be identified (Belloni *et al*., 1999).

Constrictions to ET-1 were partially reversed by PGI_2, although not all preparations responded to the prostanoid. Only a few studies have investigated the effects of exogenous PGI_2 on human arteries. Yang *et al*. (1989) also observed vasodilatation in IMA pre-constricted with ET-1, but the highest concentration applied (100 nM PGI_2) elicited vasoconstriction. This was not seen in the present study, or in human umbilical artery (Chaudhuri *et al*., 1993), however a small constrictor response has been reported at concentrations greater than 1 μM in human uterine artery and saphenous vein (Baxter *et al*., 1995; Schuller-Petrovic *et al*., 1997). As the constrictor response has only been reported at extremely high concentrations of PGI_2, there may not be any physiological relevance.

Responses to PGE_2 in IMA varied between patients, as the prostanoid only caused vasodilatation in 50% of vessels tested and at high concentrations this slight dilatation was overcome by vasoconstriction. Both constrictor and dilator responses to PGE_2 have been described in pre-constricted human arteries. Qian *et al*. (1994) observed constriction to PGE_2 in 13 of 15 pulmonary arteries tested and dilatation in the remaining two. Walch *et al*. (1999), also working on pulmonary arteries, and Kimura *et al*. (1995), working on uterine arteries, found PGE_2 to cause vasodilation at low concentrations and vasoconstriction at higher concentrations. Interestingly, another study describes the dilator activity of PGE_2 to be as effective as PGI_2, both completely reversing phenylephrine-induced constrictions in human uterine arteries (Baxter *et al*., 1995). These reports demonstrate that responses to PGE_2 vary considerably between and within vascular beds. This is unlikely to be caused by the absence or presence of a functional endothelium in the vessels studied as reports demonstrate that both constrictor and dilator responses to PGE_2 are endothelium independent (Kimura *et al*., 1995; Boersma *et al*., 1999). A more probable explanation is a differential distribution of EP receptor subtypes, although some actions of PGE_2 may also be mediated *via* other prostanoid receptors. The present study indicates that even where the dilator-coupled receptors dominate, PGE_2 is still not effective in reversing constrictions induced by ET-1 in IMA.

ANP fully reversed constrictions to ET-1. CNP was the most potent natriuretic peptide, but the maximum response was half that of ANP. This is surprising as CNP is the only natriuretic peptide not secreted as a circulating hormone by the heart, but is produced in the endothelium and has no known natriuretic properties (Hunt *et al*., 1994). BNP was the least potent, with concentration-response curves incomplete in the concentration range applied. These data differ from a previous report (Protter *et al*., 1996) where BNP was found to be the most potent vasodilator of the natriuretic peptides in human IMA, with the greatest maximum response. This may be explained by the removal of the endothelium in the present study as responses to BNP *in vivo* have been shown to be largely endothelium-dependent (Zellner *et al*., 1999). The plasma concentration of BNP is increased in cardiovascular disease (Saito *et al*., 1989; Troughton *et al*., 2000), but the ability of BNP to reverse

the heightened tone seen following coronary artery bypass may be compromised by a dysfunctional endothelium.

Some of the vasodilators only produced a response in some of the segments tested and this may reflect differences in receptor expression between individuals. The effect of previous therapies could provide an alternative explanation, however as every patient was on between three and nine treatments, it was not possible to test this hypothesis in the present study.

By systematic comparison, we have shown that of eight endogenous molecules previously reported to have vasodilator action, only ANP and CGRP can fully reverse the long-acting constrictor tone of ET-1 in human arteries. This provides further evidence of the potential benefit of dual neutral endopeptidase/endothelin-converting enzyme inhibitors in the treatment of cardiovascular disease. Such inhibitors prevent the synthesis of the potent constrictor ET-1, whilst concomitantly preventing the degradation of the effective vasodilator ANP. Our results also suggest that other fragments of proadrenomedullin, in particular PAMP-12 and PAMP-20 may prove to be as important in regulating vascular tone in humans as adrenomedullin.

Importantly, ghrelin, the newly discovered endogenous ligand of the GSH-R is a potent, directly acting vasodilator of human arteries and produces a similar maximum response to adrenomedullin with comparable potency. This, together with the high circulating levels of ghrelin in human plasma, hypotensive action *in vivo* and localization of GSH-R receptors to the human cardiovascular system, implies that ghrelin is likely to play a role in the regulation of vascular tone in man.

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