British Journal of Pharmacology (1998) 125, 238–240
http://www.stockton-press.co.uk/bjp

SPECIAL REPORT

Increased response to big endothelin-1 in atherosclerotic human coronary artery: functional evidence for up-regulation of endothelin-converting enzyme activity in disease

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Overproduction of the potent vasoconstrictor peptide endothelin-1 (ET-1) is implicated in the pathogenesis of coronary artery disease. In endothelium-denuded human coronary arteries the response to big ET-1 was significantly enhanced in atherosclerotic arteries (coronary artery disease, CAD; $n=7$) with an EC_{50} value of 96 nM (57–161 nM, 95% C.I.) compared to 274 nM (205–365 nM) in non-diseased arteries (dilated cardiomyopathy, DCM; $n=10$) (Mann-Whitney U-test, $P<0.05$). Higher levels of immunoreactive endothelin (ET) could be detected by radioimmunoassay in bathing medium taken from CAD arteries than from DCM arteries (2.8 ± 0.5 nM, $n=5$ vs 1.1 ± 0.2 nM, $n=7$) (Student's two-tailed t-test, $P<0.05$). There were no differences in responses of arteries from either group to ET-1 (EC_{50} 10 nM, CAD vs 14 nM, DCM). The enhanced response of atherosclerotic human coronary arteries to big ET-1 appears to be due to up-regulation of endothelin-converting enzyme (ECE) activity rather than to an augmented response of the arteries to ET-1. This non-endothelial ECE may therefore be an important therapeutic target in coronary artery disease.

Keywords: Atherosclerosis; big endothelin-1; big ET-1; coronary artery disease; ECE; endothelin-1; endothelin-converting enzyme; ET-1; human coronary artery

Introduction The potent vasoconstrictor peptide ET-1 is synthesized by specific cleavage of its larger precursor, big ET-1, by one or more endothelin-converting enzymes (ECE) which have recently been cloned and sequenced (Xu *et al*., 1994; Emoto & Yanagisawa, 1995). In the human vasculature ET-1, big ET-1 and ECE-1 are localized to secretory and storage granules (Russell *et al*., 1998) within endothelial cells (Davenport *et al*., 1998). ET-1 is preferentially released abluminally, together with big ET-1, and it is possible that additional conversion of this released big ET-1 occurs on the surface of the underlying smooth muscle cells. This is predicted by the observation that big ET-1 infused into the human forearm significantly increases plasma levels of immunoreactive ET and decreases forearm blood flow in a phosphoramidon-sensitive manner (Plumpton *et al*., 1995). In this system the response to big ET-1 was too rapid for appreciable amounts of phosphoramidon to have penetrated cell membranes. Therefore the ECE responsible for the conversion of infused big ET-1 to ET-1 is probably an ectoenzyme. As endothelial ECE has a predominantly intracellular localization (Russell *et al*., 1998) expression of ECE on the surface of human smooth muscle cells is suggested. This is consistent with the observation that in isolated vascular preparations removal of the endothelium does not alter responses to big ET-1 (Fukuroda *et al*., 1990; Hisaki *et al*., 1993; Mombouli *et al*., 1993).

In support of this physiological role for smooth muscle ECE, it has been shown that in human umbilical vein this enzyme will convert big ET-1, big ET-2 and big ET-3 to their respective mature, biologically active forms (Maguire *et al*., 1997). ET-2 and ET-3 are not synthesized by the endothelium but their precursors are present in human plasma (Matsumoto *et al*., 1994). We have therefore postulated that big ET-2 and big ET-3 may be converted, together with big ET-1, at their target organs, for example by smooth muscle cells in the vasculature.

In coronary artery disease plasma levels of ET-1 are raised (Lerman *et al*., 1991) and there are reports of increased ET-1 production in human atherosclerotic lesions (Zeiher *et al*., 1995; Bacon *et al*., 1996). Recent data have also localized ECE isoforms to coronary artery atherectomy specimens (Minamino *et al*., 1997). It is possible that increased expression or activity of ECE on smooth muscle cells or other cell types present in atherosclerotic tissue may contribute to the increased plasma and tissue levels of the peptide seen in coronary artery disease.

We have therefore compared the effects of big ET-1 on coronary arteries from patients with either coronary artery disease who have extensive atherosclerosis or from patients with dilated cardiomyopathy whose coronary arteries are histologically normal (Dec & Fuster, 1994). To exclude contributions from endothelial cell ECE activity the arteries were all denuded of their endothelium.

Methods Human coronary arteries were obtained from the explanted hearts of patients undergoing cardiac transplantation for coronary artery disease (CAD; 7 male, 44–60 years) or dilated cardiomyopathy (DCM; 7 male, 3 female, 25–60 years). Drug therapy for both groups included diuretics, ACE inhibitors, nitrates, digoxin, antiarrhythmics, anticoagulants and aspirin. Coronary arteries were transported to the laboratory in cold oxygenated Krebs solution and experiments were performed on the same day.

Arteries (4 mm rings) were denuded of endothelium (verified histologically) and set up in organ baths for isometric tension recordings as previously described (Maguire & Davenport, 1995). Cumulative concentration response curves (CCRC) were constructed to big ET-1 in the absence and presence of phosphoramidon (100 μM) or thiorphan (30 μM) which were added 30 min earlier. In some experiments CCRC

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were also determined for ET-1. Experiments were terminated by the addition of 50 mM KCl to produce a maximal contraction and agonist responses were expressed as a percentage of this maximum.

Individual CCRC were analysed using the curve fitting programme Fig P (Biosoft, Cambridge, U.K.). Values of EC_{50} were expressed as the geometric mean with 95% confidence intervals and statistical comparisons were made using the Mann-Whitney *U*-test. All other data were determined as arithmetic means ± s.e.mean and were compared using Student's two-tailed *t*-test. Significance was set at 95% ($P<0.05$). *n* values refer to the number of patients from whom arteries were obtained.

Samples of bathing medium were collected from tissue baths at the end of the experiment. To verify that conversion of big ET-1 to ET-1 is necessary for the observed response to big ET-1 these were analysed for the presence of mature ET using a specific radioimmunoassay as previously described (Plumpton *et al.*, 1996).

ET-1 and big ET-1 were from Peptide Institute (Osaka, Japan) and stock solutions (10^{-4} M) were made up in 0.1% acetic acid and stored at −20°C. Subsequent dilutions of the peptides were made in distilled water. Phosphoramidon (N-(α-Rhamno pyranosyloxyhydroxyphosphinyl)-L-Leucyl-L-Tryptophan, 2Na, $2H_2O$) was from Peptide Institute (Osaka, Japan) and made up in distilled water (10^{-2} M). Thiorphan was from Sigma-Aldrich Co. Ltd. (Poole, Dorset, U.K.) and was dissolved in dimethylsulphoxide (10^{-2} M). Krebs solution was of the following composition: (mM) NaCl 90, KCl 5, $MgSO_4.7H_2O$ 0.5, Na_2HPO_4 1, $NaHCO_3$ 45, $CaCl_2$ 2.25, glucose 10, Na pyruvate 5, fumaric acid 5, L-glutamic acid 5.

Results There were no differences in the tension developed to a maximal concentration of KCl in the CAD group or DCM group following CCRC to either big ET-1 (7.0 ± 1.2 g wt, $n=7$, and 7.2 ± 0.9 g wt, $n=10$, respectively, $P>0.05$) or to ET-1 (7.1 ± 2.3 g wt, $n=4$, and 6.3 ± 0.8 g wt, $n=8$, respectively, $P>0.05$). Maximum responses to the two peptides, expressed as a percentage of this KCl response, were similar; big ET-1 71 ± 6% ($n=7$) compared to ET-1 76 ± 13% ($n=4$) in CAD ($P>0.05$) and big ET-1 63 ± 9% ($n=10$) compared to ET-1 70 ± 5% ($n=8$) in DCM ($P>0.05$).

EC_{50} values for ET-1 were comparable in the two groups; 10 nM (4.0–27 nM, $n=4$) in CAD and 14 nM (6.0–35 nM, $n=8$) in DCM ($P>0.05$). EC_{50} values for big ET-1 were 96 nM (57–161 nM, $n=7$) in CAD and 274 nM (205–365 nM, $n=10$) in DCM (Figure 1). Therefore big ET-1 was a significantly ($P<0.05$) more potent constrictor of atherosclerotic coronary arteries than of non-diseased arteries.

Following the addition of phosphoramidon (100 μM), a combined ECE/neutral endopeptidase 24.11 inhibitor, the CCRC to big ET-1 in both groups were displaced to the right (Figure 2). Due to the limitation in the maximum concentration of big ET-1 that can be achieved in the bathing medium (700 nM), full CCRC could not be determined in the presence of phosphoramidon and therefore EC_{50} values for big ET-1 could not be calculated. However, the response to 300 nM big ET-1 was significantly attenuated by the inhibitor in both CAD ($P<0.02$) and DCM ($P<0.05$) arteries. The selective neutral endopeptidase inhibitor thiorphan (30 μM) was without effect on big ET-1 responses in either group (data not shown).

Analysis of the bathing medium taken from arterial segments in which CCRC were constructed to big ET-1 clearly demonstrated the presence of mature ET. Significantly higher concentrations of mature ET were detected in medium bathing

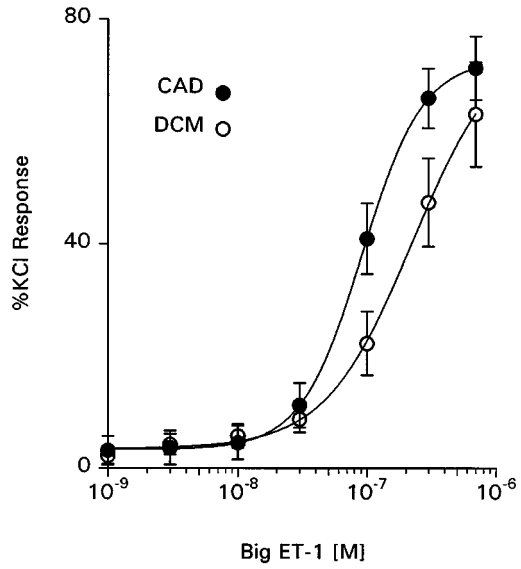

Figure 1 Cumulative concentration response curves to big ET-1 in atherosclerotic (CAD, $n=7$) and non-atherosclerotic (DCM, $n=10$) human coronary arteries. Data points are means ± s.e.mean.

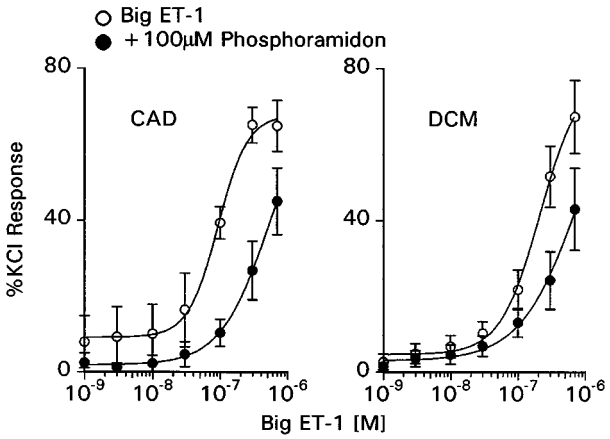

Figure 2 Effect of 100 μM phosphoramidon on responses to big ET-1 in atherosclerotic (CAD, $n=3$) and non-atherosclerotic (DCM, $n=7$) human coronary arteries. Data points are means ± s.e.mean.

CAD arteries than DCM arteries (2.8 ± 0.5 nM, $n=5$ and 1.1 ± 0.2 nM, $n=7$; $P<0.05$).

Discussion We have shown that the ability of endothelium-denuded atherosclerotic coronary arteries (CAD) to convert big ET-1 to mature ET is up-regulated compared to coronary arteries which are histologically normal (DCM). This was suggested by both the enhanced functional response of the CAD arteries to big ET-1 and the significantly higher levels of mature ET detected in the bathing medium surrounding them. It was interesting that there was no significant difference in the response of the two groups of coronary arteries to exogenously applied ET-1. This suggests that there have been no marked pathophysiological changes in the ET receptor systems that mediate vasoconstriction. This confirms that the augmented response to big ET-1 in atherosclerotic vessels is due to the enhanced ability of this tissue to convert big ET-1 to ET-1 rather than to an enhanced response to ET-1 itself.

This is the first report of a functional increase in ECE activity in human atherosclerosis. The implications from animal experiments are that this may be due to both enhanced expression of ECE protein levels and to an increase in enzyme activity. In a rabbit model of early atherosclerosis intense ECE immunoreactivity was localized to neointimal smooth muscle cells, macrophages and endothelial cells and local tissue levels (but not plasma levels) of ET-1 were raised (Grantham *et al*., 1998). In the rat balloon-injury model both ECE-1 mRNA levels and ECE activity were increased following vascular injury and the resulting neointimal proliferation was reduced by phosphoramidon (Minamino *et al*., 1997). Histological analysis is required to determine the precise cellular source of this augmented non-endothelial ECE activity in disease but the recent observation by Minamino *et al*., (1997) in two human coronary atherectomy samples suggests that ECE-1 immunoreactivity was present in both smooth muscle cells and macrophages.

The observation in our study that exogenously applied big ET-1 is converted in a phosphoramidon-sensitive manner by denuded human coronary arteries suggests that this enzyme is localized extracellularly. This enzyme activity, that is increased in disease, may be a more accessible therapeutic target than the intracellular ECE of the vascular endothelium.

This work was supported by grants from The British Heart Foundation, Isaac Newton Trust and Royal Society. We thank Rhoda Kuc for excellent technical assistance with the RIA experiments. We are grateful to Jean Chadderton and the theatre and Consultant staff at Papworth Hospital.

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(Received June 1, 1998
Revised June 26, 1998
Accepted July 6, 1998)