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Nitric oxide-mediated modulation of the endothelin-1 signalling pathway in the human cardiovascular system

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1 We studied the ability of nitric oxide (NO) to physiologically antagonize endothelin-1 (ET-1) induced constrictions in human internal mammary artery (IMA). We also investigated the hypothesis that NO interacts directly with ET-receptor binding in human heart and aorta.

2 ET-1 potently contracted IMA (EC_{50} 6.86 nM, 95% CI: 3.5–13.4 nM; $n=12$). The constrictor response to 10 nM ET-1 was fully reversed by the NO-donor diethylamine NONOate (DEA/NO; EC_{50} 2.0 μM, 95% CI: 0.8–4.8 μM; $n=5$). The guanylate cyclase inhibitor ODQ (100 μM) reduced the response to DEA/NO but did not abolish it (E_{MAX} 50.9$\pm$8.5% in the presence of ODQ; 113.0$\pm$8.4%, control).

3 The increase in cyclic GMP by 30 μM DEA/NO was abolished in the presence of 100 μM ODQ ($n=6$).

4 In saturation binding experiments the NO-donor Diethyltriamine NONOate (DETA/NO; 1 mM) caused a 90% reduction in maximum binding of [^{125}I]-ET-1 in human heart, without affecting the affinity. This reduction in binding was abolished by haemoglobin. Pre-incubating either the radiolabel or the tissue with NO-donors did not reduce binding. A similar effect was observed in aortic smooth muscle.

5 We have shown that DEA/NO is able to reverse ET-1-induced contractions in the human vasculature. The binding studies suggest a direct interaction between NO and the ET receptor or receptor-ligand complex in human ventricular and aortic tissue. NO is released continuously *in vivo*, thus this apparent modification of ET-receptor binding may provide an additional mechanism by which NO counter-balances the effects of ET.

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Abbreviations: cyclic GMP, guanosine 3′5′-cyclic monophosphate; DEA, diethylamine; DEA/NO, diethylamine NONOate; DETA, diethyltriamine; DETA/NO, diethyltriamine NONOate; DTT, dithiothreitol; ET, endothelin; IMA, internal mammary artery; LV, left ventricle; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; SIN-1, 3-morpholino-sydnonimine; SNAP, *S*-nitroso-*N*-acetyl penicillamine; sGC, soluble guanylate cyclase

Introduction

The peptide endothelin-1 (ET-1) is a potent, long-acting vasoconstrictor of human blood vessels and has a positive inotropic effect on cardiac muscle (Davenport *et al*., 1989; Franco-Cereceda, 1989; Maguire & Davenport, 1995). ET-1 has been reported to bind irreversibly in animal tissue (Hilal-Dandan *et al*., 1997), however addition of an ET antagonist to human vessels pre-constricted with ET *in vitro* causes vasodilatation (Maguire *et al*., 1997). Most (>90%) of the ET-1 generated locally within the endothelium is released abluminally towards the media (Wagner *et al*., 1992). Both ET receptor subtypes are present in the vascular smooth muscle layer, although ET_A predominates and mediates vasoconstriction (Davenport *et al*., 1995). In the human vasculature ET is unusual amongst vasoactive peptides in that as well as being released from an inducible pathway it is also continuously released *via* a constitutive pathway (Russell *et al*., 1998). In agreement an infusion of a mixed ET antagonist *in vivo* causes vasodilatation in normotensive volunteers, unmasking a role for ET in making a significant contribution to the maintenance of normal vascular tone (Haynes *et al*., 1996).

Nitric oxide (NO) is also an endothelium-derived, locally acting vasoactive substance (Furchgott & Zawadzki, 1980) and there is increasing evidence for interaction between these opposing signalling pathways (Lüscher *et al*., 1990; Warner, 1999). The nitric oxide synthase (NOS) family of isozymes synthesise NO (Moncada *et al*., 1989), with endothelial NOS constitutively expressed in endothelial cells. Infusions of NOS inhibitors in humans *in vivo* produce vasoconstriction, by inhibiting the continuous production of endothelially-derived NO which contributes to the basal vasodilator tone (Vallance *et al*., 1989). Vasoconstriction is also observed on infusion of a selective ET_B antagonist and is thought to be because ET_B receptors expressed on the endothelium are coupled to the production of NO (Strachan *et al*., 1999).

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NO was first thought to cause vasodilatation *via* the activation of soluble guanylate cyclase (sGC), expressed in the media, to produce the second messenger guanosine 3′5′-cyclic monophosphate (cyclic GMP; Rapoport & Murad, 1983). However evidence is emerging that NO can, in addition, directly modify a range of proteins including some ion channels and receptors (Weissman *et al*., 1990; Estrada *et al*., 1997; Galigniana *et al*., 1999). Goligorsky *et al*. (1994) demonstrated that the NO-donor 3-morpholino-sydnonimine (SIN-1) caused a reduction in ET-1 binding to ET receptors expressed in Chinese hamster ovary cells, but it is unclear whether NO would have a similar action against native human receptors. Unlike other widely used NO-donors such as SIN-1, NONOates have an advantage in mediating their actions solely *via* NO. This spontaneous production of NO closely correlates with vasodilator potency (Bohn & Schonafinger, 1989; Kowaluk & Fung, 1990; Morley & Keefer, 1993) and may be used to mimic the endogenous production of NO by the endothelium.

We hypothesized that NO can counter-balance the effects of ET-1 in human tissue both *via* a GC-dependent mechanism and by directly modifying receptor-ligand binding. We therefore examined the effects of NO on ET-1 mediated constrictions in human internal mammary artery (IMA) and ET-1 binding in human aorta and left ventricle (LV) *in vitro* using members of the NONOate class of NO-donor. Preliminary data from this study have been presented to the British Pharmacological Society (Wiley & Davenport, 2000a).

Methods

Tissue collection

Non-diseased IMA were obtained from 22 patients (19 male, 3 female) undergoing coronary artery bypass operations, mean age 62 years (range: 54–81 years). Patients were on a combination of therapies including anticoagulants, β-blockers, calcium channel blockers, nitrates and lipid-lowering drugs. Normal ventricular and aortic tissue was obtained from the explanted hearts of five transplant patients (1 male, 4 female), mean age 31 years (range 18–34 years). Tissue for binding studies and scintillation-proximity assays was frozen in liquid nitrogen and stored at -70°C; IMA for organ bath studies were kept at 4°C in Krebs' solution 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, 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. Output was recorded using a data acquisition system (Biopac Systems Inc., CA, U.S.A.). To obtain the optimal resting tension, 100 mM KCl was added at increasing levels of tension until no further increase in response was obtained. The preparations were then allowed to equilibrate to their own resting tension for at least 1 h before the start of the experiment. A submaximal concentration of ET-1 (10 nM) was added, and once the response had reached a plateau concentration-response curves to DEA/NO (10 nM–30 μM), alone and in the presence of 30 μM and 100 μM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; preincubated with the tissue for 20 min), were constructed. Control rings of artery were contracted with 10 nM ET-1 and the tension measured over the time course of the experiment. The inactive nucleophile diethylamine (DEA; 10 nM–30 μM) was used as a negative control. All experiments were terminated by 100 mM KCl and results were expressed as percentage relaxation of the constrictor response to ET-1.

Measurement of cyclic GMP levels

Three mm rings of IMA were incubated for 15 min in Krebs' solution (containing 1 mM isobutyl methylxanthine) with 10 nM ET-1 and 30 μM DEA/NO, alone and in the presence of 100 μM ODQ (pre-incubated with the tissue for 20 min) for 15 min at 37°C. Samples were immediately frozen in liquid nitrogen, homogenized using a sonicating probe in 6% tricloroacetic acid, and dried under vacuum. Cyclic GMP levels were then measured using a scintillation proximity assay (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Radioligand binding

Saturation binding experiments for [^{125}I]-ET-1 (4 pM–2 nM) were performed on 30 μm sections of human left ventricular free wall and aortic smooth muscle alone (control), in the presence of 1 mM DETA and 1 mM DETA/NO. Sections were washed with incubation buffer for 15 min before a 2 h incubation with the radioligand.

Using the same assay conditions, fixed concentration (0.11 nM [^{125}I]-ET-1) binding assays were carried out. Firstly, in the presence of 1 mM reduced haemoglobin, which acts as an NO scavenger; secondly, with a 1 h pre-incubation of the radioligand with 1 mM DEA/NO or DEA to determine if NO modifies the ET peptide (DEA/NO was used instead of DETA/NO in this experiment because it has a much shorter half-life and would have completely decomposed before incubation of the radiolabel with the tissue); thirdly, with a 1 h pre-incubation of the tissue with DETA/NO or DETA (1 mM) to investigate the reversibility of the effect of NO; and fourthly, with a 15 min pre-incubation of dithiothreitol (DTT; 1 mM). Preparations were then washed as before and counted on a gamma counter.

Determination of NO generation

Levels of NO generation were estimated using a modified Griess Assay. One mM DETA/NO and 1 μM ET-1 in HEPES buffer were incubated with sections of human LV for 2 h. Samples of buffer were then added to equal volumes of Griess reagents (2.5% w/v sulphanilamide and 2.5% N-(1-naphthlyl)ethyl-enediamine, both in 60% acetic acid) and measured for nitrite (NO_2^-) content using a spectrophotometer.

Determination of protein content

The protein content of samples from the binding experiments and cyclic GMP assays was calculated using a colorometric protein assay (Biorad, Hercules, CA, U.S.A.).

Mass spectrometry

In order to determine if the ET-1 peptide was modified by NO, mass spectrometry was performed using a Bruker Esquire-LC mass spectrometer with electrospray ionisation using Hewlett Packard ionsource (Bruker, Warwickshire, U.K.). Samples of ET-1 (10 μM) were analysed alone and in the presence of DETA/NO or DETA (10 mM; concentrations which gave the same ratio of ET-1 : DETA/NO as in the binding experiments). Analysis was made in positive ion mode (m/z) of both the single-charged ion, $(M+H)^+$ and doubled-charged ion $(M+2H)^+$.

Data analysis

EC_{50} values were determined for each curve using the iterative curve-fitting software Fig.P (Biosoft, Cambridgeshire, U.K.). EC_{50} values were expressed as geometric means with 95% confidence intervals (CI). Radioligand binding studies were analysed using the Kell suite of programs (Biosoft, Cambridgeshire, U.K.). K_D values were expressed as mean ± s.e.mean and compared with a Mann–Whitney *U*-test. All other data are expressed as arithmetic mean ± s.e.mean and are compared using Student's two-tailed *t*-test.

Materials

ET-1 (Peptide Institute Inc., Osaka, Japan) stock solutions (0.1 mM) were prepared in 0.1% acetic acid and stored at −20°C. DEA/NO and DETA/NO (Alexis Biochemicals, Nottinghamshire, U.K.) were stored under nitrogen at −70°C and dissolved in 0.01 M NaOH immediately prior to experiments. Stock solutions of ODQ (0.1 M; Tocris Bristol, U.K.) were prepared in dimethylsulphoxide. [^{125}I]-ET-1 and cyclic GMP scintillation proximity assay were from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). 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). The incubation buffer used in the binding study comprised (mM); HEPES, 50; $MgCl_2$, 5 and 0.3% w/v bovine serum albumin (pH 7.4), washing buffer was 50 mM tris (pH 7.4).

Results

Physiological antagonism

ET-1 (0.1–200 nM) potently contracted internal mammary artery (EC_{50} = 6.86 nM; 95% CI: 3.51–13.4 nM, n = 12, Figure 1). DEA/NO (0.01–30 μM fully reversed a 10 nM ET-1-induced constriction with an EC_{50} value of 1.96 μM (95% CI: 0.80–4.84 μM, n = 5, Figures 2b and 3), and maximal response of 113.0 ± 8.40% (a maximum response greater than 100% indicates that there was a small additional relaxation of the basal tone). DEA did not alter the ET-1 contraction (Figure 2a). Maximum relaxation to DEA/NO was only slightly reduced in the presence of 30 μM ODQ (relaxation to 30 μM DEA/NO: 87.6 ± 9.5%; data not shown). In the presence of 100 μM ODQ relaxation to DEA/NO was significantly attenuated at concentrations above 1 μM (P < 0.05, Student's *t*-test), but not abolished. At the highest concentration of DEA/NO tested the response was reduced to 50.9 ± 8.49% (Figure 3).

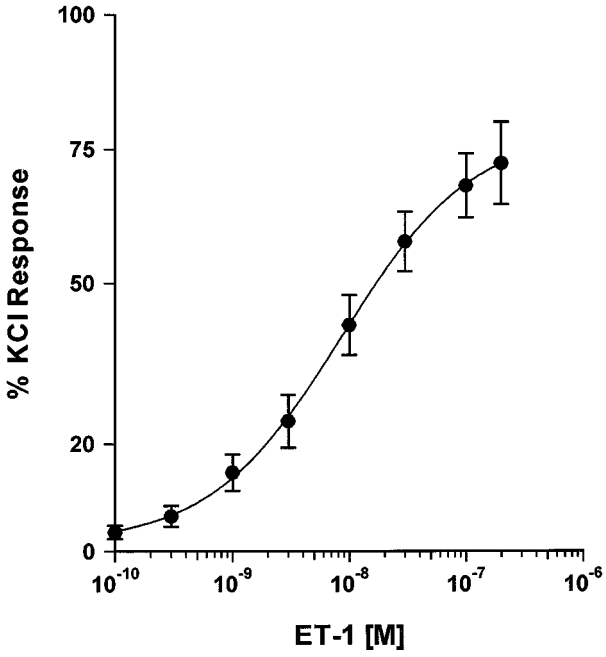

Figure 1 Cumulative concentration-response curve to ET-1 in internal mammary artery (n = 12). Responses expressed as mean ± s.e.mean per cent KCl response.

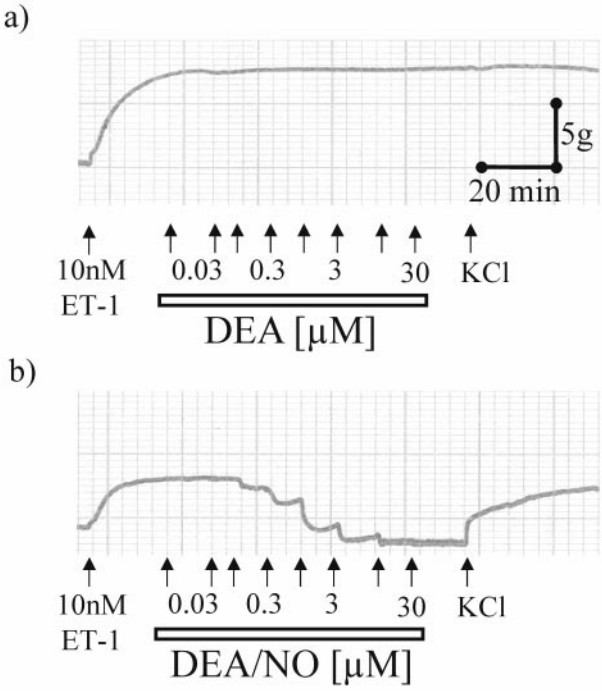

Figure 2 An original chart recording showing the constrictor effect of 10 nM ET-1 on IMA. The precursor nucleophile DEA has no effect on this constriction (a). DEA/NO concentration-dependently reversed the ET-1-mediated constriction (b).

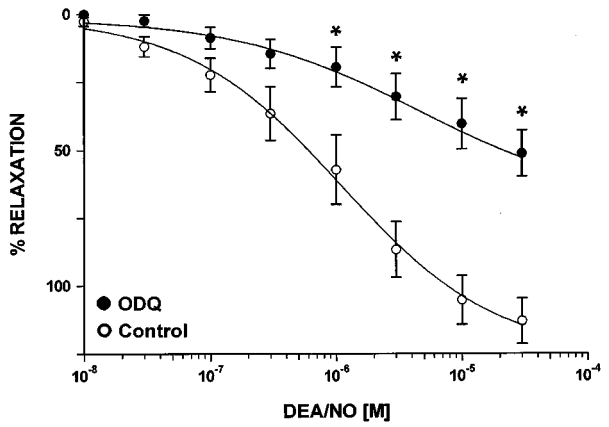

Figure 3 Cumulative concentration response curves to DEA/NO in internal mammary artery, alone and in the presence of 100 μM ODQ (*$P<0.05$, Student's t-test). Relaxations expressed as a percentage of the constrictor response and are means$\pm$s.e.mean ($n=5$).

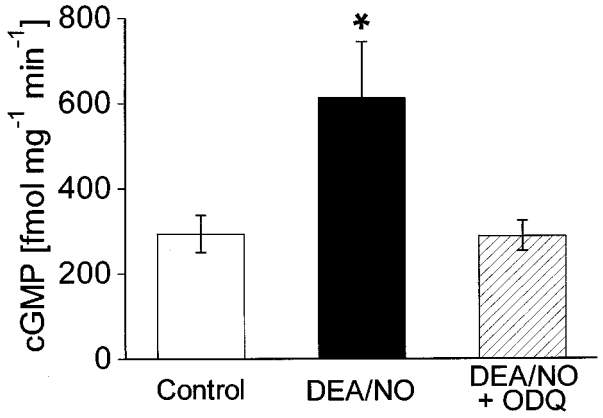

Figure 4 Cyclic GMP levels in rings of internal mammary artery stimulated with 30 μM DEA/NO, alone and in the presence of 100 μM ODQ ($n=6$; *$P<0.05$, Student's t-test).

Guanylate cyclase activity

In control IMA samples basal levels of cyclic GMP were 294.2 ± 44.0 fmol mg^{-1} min^{-1} (Figure 4; $n=6$). In the presence of 30 μM DEA/NO a significant increase in cyclase activity was observed to 614.3 ± 131.4 fmol mg^{-1} min^{-1} ($P<0.05$. Student's t-test). This increase was abolished by 100 μM ODQ.

Direct receptor modification

No significant difference in binding affinity or maximum binding intensity was observed between the control and DETA groups in human LV (Table 1; $n=3$). 1 mM DETA/NO caused a 90% reduction in B_{MAX} compared to control ($P<0.05$, Student's t-test, Table 1), with no change in affinity. A similar reduction in B_{MAX} was observed in sections of aortic smooth muscle ($P<0.05$, Student's t-test; Table 1), again with no significant alteration in affinity.

Fixed concentration binding assays were performed to investigate the nature of the NO-mediated reduction of [^{125}I]-ET-1 binding. The inhibition of binding by DETA/NO was abolished in the presence of the NO-scavenger reduced haemoglobin (1 mM; Figure 5a). The reduction in binding was not due to NO interacting with ligand because preincubation of the radiolabel with the short half-life NO-donor DEA/NO did not attenuate the binding (1 mM; Figure 5b). The modulation was found to be reversible as pre-incubating the tissue with NO before addition of the radiolabel did not cause a reduction in binding (Figure 5c), and pre-incubation of the tissue with disulphide bridge-reducing agent DTT (1 mM) caused a similar decrease in binding to DETA/NO ($P<0.05$, Student's t-test; Figure 5d).

Determination of nitric oxide generation by DETA/NO

A 2 h incubation of 1 mM DETA/NO in HEPES buffer at room temperature generated 21.24 ± 8.03 μM NO_2^-($n=3$). NO_2^- levels were below the level of detection in control and DETA treated groups.

Mass spectrometry

As expected, the mass spectrum of ET-1 contained two major peaks corresponding to the single charged ion (m/z: 2494 $[M+H]^+$) and double-charged ion (m/z: 1247 $[M+2H]^+$). No additional peaks were observed in those regions of the spectrum following addition of DETA/NO or DETA, indicating that NO was not co-ordinating with ET-1.

Discussion

ET-1 constricted human IMA with a similar potency to that described previously (Lüscher *et al.*, 1990; Wiley & Davenport, 2000b). DEA/NO completely and concentration-dependently reversed a constriction induced by a sub-maximal concentration of ET-1, an observation consistent with the hypothesis that NO mediates vasodilatation *via* an increase in the second messenger cyclic GMP. The compound DEA/NO was selected for these functional studies because it has a half-life of approximately 2.5 min at 37°C (Hrabie *et al.*, 1993) and would therefore continuously produce NO over the course of the experiment. Surprisingly the sGC inhibitor ODQ only reduced the effect of DEA/NO by 50%, even at a concentration of 100 μM. ODQ has been shown to completely inhibit sGC activity at concentrations ranging between 0.1 and 3 μM in a range of tissue types and preparations (Moro *et al.*, 1996; Onoue & Katusic, 1998; Weisbrod *et al.*, 1998). To further eliminate the possibility that ODQ was not inhibiting sGC effectively owing to poor tissue penetration, cyclic GMP levels were measured in the tissue. This showed that ODQ was inhibiting sGC activity completely, even at the highest applied concentration of NO-donor. The vasodilatation obtained in the presence of 100 μM ODQ is therefore due to non-cyclic GMP-mediated effects of NO.

The percentage of NO-induced vasodilatation mediated by cyclic GMP appears to vary between species and vascular bed. Plane *et al.* (1998) found that 10 μM ODQ reduced relaxation responses to SIN-1 and *S*-nitroso-*N*-acetylpenicillamine (SNAP) by 92% in the rabbit carotid artery, whereas Onoue & Katusic (1998) saw a complete relaxation to DEA/

Table 1 Binding characteristics for [^{125}I]-ET-1 in human left ventricle and aorta alone and in the presence of DETA or DETA/NO

	Left Ventricle			*Aorta*	
	Control	*DETA*	*DETA/NO*	*Control*	*DETA/NO*
B_{MAX} (fmol mg^{-1})	139±24.2	145±35.4	14.3±5.46*	7.61±1.14	3.34±0.75*
K_D (nM)	0.47±0.13	0.37±0.07	0.33±0.16	0.23±0.05	0.13±0.07

Maximum binding (B_{MAX}) expressed as mean±s.e.mean, affinity (K_D) as pooled data±s.e.mean. *$P<0.05$ Student's t-test, $n=3$, Hill slopes close to unity.

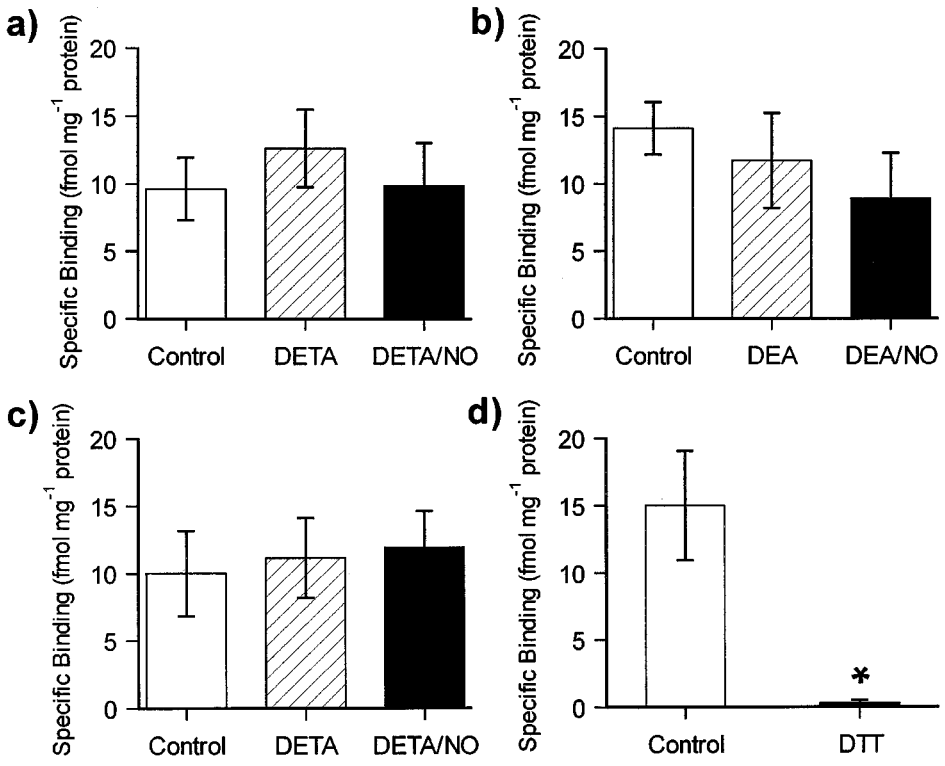

Figure 5 Fixed concentration binding assays in human left ventricle (0.11 nM [^{125}I]-ET-1). (a) Incubation in the presence of 1 mM haemoglobin ($n=4$), (b) pre-incubation of radiolabel with 3 mM DEA/NO ($n=4$), (c) pre-incubation of tissue with 3 mM DETA or DETA/NO ($n=4$), (d) pre-incubation of tissue with 1 mM dithiothreitol ($n=3$, *$P<0.05$, Student's t-test).

NO in the presence of 30 μM ODQ in isolated canine cerebral arteries. In both cases the concentration of ODQ used was sufficient to abolish cyclic GMP production in the preparation.

Another study on human IMA (Hamilton *et al.*, 1999), found that responses to carbachol were abolished in the presence of 10 μM ODQ. This apparently conflicting observation may be explained by the fact that at high concentrations (>30 μM) ODQ can inhibit other haem-containing enzymes including NOS (Feelisch *et al.*, 1999). As carbachol causes vasodilatation indirectly through activation of the endothelium to generate dilating factors including NO, the greater inhibition seen to carbachol-induced vasodilatation may to some extent be because ODQ is inhibiting both sGC and NOS. In the present study, endothelium-denuded rings of artery and directly acting NO-donors were used which precludes any effect of NOS inhibition.

A further explanation for the heterogenity of response to NO is a direct interaction with the signalling pathway for certain constrictors, but not others. In this study we used radioligand-binding to investigate if this was the case for the ET receptor. Owing to the small size of IMA it was not possible to collect enough receptor protein to carry out the study in this vessel. Therefore experiments were carried out in left ventricle which has a comparable affinity for [^{125}I]-ET-1 (Davenport *et al.*, 1993; Molenaar *et al.*, 1993). Aortic smooth muscle, which displays both a similar affinity for [^{125}I]-ET-1 and EC_{50} value for ET-1 to IMA, was used to show that similar effects occur with receptors from vascular tissue (Bacon & Davenport, 1996; Maguire & Davenport, 1995).

A continuous production of NO was achieved throughout the experiment using the NO-donor DETA/NO which has a half-life estimated at 56 h in physiological solution at room temperature (Hrabie *et al.*, 1993). This reduced the maximum

binding of [^{125}I]-ET-1 in both ventricular myocardium and aortic smooth muscle, B_{MAX} to native receptors was reduced by approximately 90% in the left ventricle and 60% in aorta. The affinities of [^{125}I]-ET-1 observed in these experiments were comparable to those in the literature (Molenaar *et al*., 1993; Bacon & Davenport, 1996). The affinity was not significantly altered in the presence of DETA/NO in either tissue tested, an observation which agrees with the study on transfected ET receptors in Chinese hamster ovary cells (Goligorsky *et al*., 1994).

The other product of the decomposition of DETA/NO, DETA, had no effect on the binding characteristics of [^{125}I]-ET-1. This indicates that the reduction in ligand binding was caused either by the DETA/NO adjunct or by the NO formed by its spontaneous decomposition. In agreement with this, the reduction in binding was abolished in the presence of reduced haemoglobin, a scavenger of NO, which suggests it was indeed the NO which modified receptor-ligand binding.

ET-1 was not modified by NO because binding was unaffected by incubating the radiolabel with NO. Further evidence in support of this is provided by the mass spectrometry data, where no change in the ET-1 spectra was seen on addition of either DETA or DETA/NO. The lack of effect when pre-incubating DETA/NO with the tissue before its exposure to the radiolabel implies that NO affects the receptor-ligand complex or, more likely, that the effect of NO on the receptor is reversible.

Although most research into the actions of NO in biological systems has focused on GC-mediated effects, there is increasing evidence for direct interactions with proteins. Enzymes such as the angiotensin-converting enzyme can be inhibited by NO (Ackermann *et al*., 1998) and a number of receptor-coupled ion channels are also thought to be regulated by NO, including the NMDA receptor coupled ion-channel (Hoyt *et al*., 1992; Lei *et al*., 1992; Manzoni & Bockaert, 1993), ryanodine-receptor (Xu *et al*., 1998; Suko *et al*., 1999; Hart & Dulhunty, 2000) and peripheral benzodiazepine receptor coupled anion channel (Weissman *et al*., 1990). Glucocorticoid receptors are inhibited by NO (Galigniana *et al*., 1999), however to our knowledge the ET receptor is the only G-protein coupled receptor found to be modulated by NO to date, and this is the first report of the direct interaction of NO with any receptor in human tissue. Fouchier *et al*. (1994) reported a reduction in iodinated vasoactive intestinal peptide binding in a human melanoma cell line, but as the NO was incubated with live cells for 1 h before the addition of the radiolabel, internalization of receptors cannot be excluded as a possible mechanism of action.

The primary mechanism suggested to explain the non-cyclic GMP mediated effects of NO on proteins is the *S*-nitrosylation of thiol groups by NO oxidant derivatives. Site-directed mutagenesis of residue Cys399 in recombinant NR2A subunits removes the ability of endogenous NO generation to regulate NMDA receptor-coupled ion channel activity (Choi *et al*., 2000). Further evidence to support the role of *S*-nitrosylation is provided by Castro *et al*. (1999) who have created a functional *S*-nitrosylation site by a single point mutation (Gly120Cys) in methionine adenoyltransferase.

ET_A and ET_B receptors contain 21 and 20 cysteine residues respectively, and point mutations at Cys174 or Cys255, residues located on the first and second extracellular loops of the ET_B receptor result in a complete abolition of specific ET-1 binding (Haendler *et al*., 1993). Cysteine residues either exist as free thiol groups or form disulphide bridges, and in our study DTT prevented all [^{125}I]-ET-1 binding, confirming the importance of disulphide bonds in conserving receptor-ligand binding. Nitrosylation of either a free thiol or disruption of a disulphide bond could interrupt receptor-ligand interactions. DTT caused an irreversible disruption of [^{125}I]-ET-1 binding whereas NO caused reversible reduction of [^{125}I]-ET-1 binding. This apparent contradiction can be explained by the fact that nitrosthiols formed in the presence of NO are less stable than the disulphide complex formed with DTT (Getz *et al*., 1999; Lynch, 1998). The lack of effect of NO on the ET-1 peptide, which also contains two disulphide bonds, might suggest that it is more likely that NO alters the binding capacity by modifying free thiol groups present on cysteine residues on the ET receptor.

The concentrations of NO or NO-donor used in the studies cited above are comparatively high, in the range of 0.05–1 mM, but the modulation of receptor ligand binding is unlikely to be non-specific, as several studies have shown no effect with equal or higher concentrations of NO with other receptor types including kainate and neuropeptide Y receptors (Dotsch *et al*., 1997; Lees *et al*., 1997). Differential effects of NO have also been described at the same receptor depending on the concentration on donor used (Hart & Dulhunty, 2000); 10 μM SNAP was found to activate rabbit skeletal ryanodine receptors whereas 1 mM reduced the frequency of channel opening in the same preparation.

The amount of NO generated from 1 mM DETA/NO during the course of the experiment was estimated using a Griess assay. NO decomposes to nitrate and nitrite in biological systems, however the Griess assay only measures nitrite, so the concentration recorded will be a slight underestimation of the total amount of NO generated. (The theoretical amount of NO generated by DETA/NO during 2 h at 22°C in physiological solution is 35.3 μM). Both of these figures, however, fall within the range of nitrite/nitrate levels reported in physiological and pathophysiological conditions in humans (Avontuur *et al*., 1999; de Werra *et al*., 1997; Pastor & Suter, 1998).

The inducible form of NOS, iNOS is up-regulated in many disease states, particularly septic shock, where the increase in NO production is thought to be the major cause of the hypotension and reduced cardiac contractility associated with the condition (Kumar *et al*., 1999; Titheradge, 1999). Several studies have also shown an increase in plasma levels of ET peptides in septicaemia (Voerman *et al*., 1992; Mitaka *et al*., 1998; Avontuur *et al*., 1999), which may either be due to increased synthesis by activated endothelial cells, or a reduced clearance of ET from the plasma. Conversely, in cardiovascular disease there is a decrease in NO production (Chester *et al*., 1990; Katz *et al*., 1999) and an increase ET receptor expression and plasma ET-1 (McMurray *et al*., 1992; Bacon *et al*., 1996), leading to an overall hypertension. We hypothesize that the direct modulatory effect of NO on ET receptor-ligand binding may provide an additional mechanism whereby the tonic production of NO counterbalances the constrictor effects of ET in normal physiological conditions. In inflammatory diseases the balance is tipped towards the dilator actions of NO and the nitrosylation of ET receptors could explain the

failure of the increased levels of ET-1 to reverse the hypotension. The reduced clearance of the peptide may also be because of the inactivation of ET_B receptors by NO in the kidney and lung. The hypertension associated with cardiovascular disease could, in some part, be because the reduced NO produced by endothelial NOS increases the number of available ET receptors on the vascular smooth muscle.

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