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Phosphoramidon inhibition of the *in vivo* conversion of big endothelin-1 to endothelin-1 in the human forearm

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1 The vasoconstrictor peptide, endothelin-1 (ET-1) and a biologically inactive C-terminal fragment (CTF) are generated from an intermediate big ET-1 by a putative ET converting enzyme, sensitive to phosphoramidon. We have developed a procedure using selective solid-phase extraction and specific radioimmunoassays to measure the levels of immunoreactive (IR) big ET-1 and the products of conversion (ET-1 and CTF) in human plasma. These techniques have been used to determine the levels of the three peptides in venous plasma following local infusions of ET-1 and big ET-1, both alone and together with phosphoramidon.

2 Infusion of ET-1 into the brachial artery (5 pmol min^{-1}) significantly increased ($P<0.05$) IR ET levels from a basal level of 2.3 pM to 55.2 pM in plasma from the infused arm after 60 min of infusion. This corresponded with a marked decrease in forearm blood flow from a basal level of 2.6 ml dl^{-1} min^{-1} to 1.7 ml dl^{-1} min^{-1}. The levels of IR big ET-1 and CTF were unchanged. Co-infusion of phosphoramidon (30 nmol min^{-1}) with ET-1 had no significant effect on the plasma IR levels of ET, big ET-1, CTF, or blood flow.

3 Big ET-1 (50 pmol min^{-1}) significantly increased ($P<0.05$) venous concentrations of all three IR peptides after 60 min compared to basal (ET: from 2.2 to 7.7 pM, big ET-1: from 0 to 386.0 pM, CTF: from 0.2 to 37.0 pM). Forearm blood flow decreased significantly ($P<0.05$) from a basal level of 3.0 ml dl^{-1} min^{-1} to 1.6 ml dl^{-1} min^{-1}.

4 When phosphoramidon was co-infused with big ET-1, both the rise in IR ET and associated vasoconstriction were abolished. However, IR CTF was still detected, suggesting that either some conversion by phosphoramidon-insensitive enzyme(s) was occurring, and/or that CTF was being protected from further degradation by phosphoramidon.

5 These data show that in the human forearm the activity of a phosphoramidon-sensitive ET converting enzyme is at least in part responsible for the vasoconstrictor properties of exogenous big ET-1. Furthermore, because measurable levels of newly synthesized ET-1 are likely to be rapidly reduced in the blood/plasma through receptor binding, assay of IR big ET-1 and CTF may be a more sensitive measure of ET-1 generation in disease.

Keywords: Endothelin; big endothelin; endothelin converting enzyme; phosphoramidon; selective solid-phase extraction; radioimmunoassay; human brachial artery; vasoconstriction

Introduction

The vasoconstrictor peptide, endothelin-1 (ET-1, Yanagisawa *et al.*, 1988) is thought to be synthesized from a 212 amino acid precursor preproET-1, initially by removal of the signal sequence to form proET-1. Subsequently dibasic pair proteolysis, carboxypeptidase and possibly furin activity (Laporte *et al.*, 1993) forms the intermediate 38 amino acid peptide, big ET-1. The final stage of synthesis is an unusual cleavage catalysed by a putative ET converting enzyme (ECE) between residues 21 and 22 (tryptophan and valine) to form the biologically active, mature ET-1 and a C-terminal fragment (CTF) of big ET-1 (big $ET\text{-}1_{(22\text{-}38)}$) in an equimolar ratio. In support of this biosynthetic pathway, both mature ET and big ET-1 have been localized to the cytoplasm of endothelial cells from a range of human vascular beds (Howard *et al.*, 1992) and ET-1, big ET-1 and CTF have been identified in the conditioned medium from cultured endothelial cells (Sawamura *et al.*, 1989; Hexum *et al.*, 1990; Ikegawa *et al.*, 1990).

Big ET-1 shows little vasoconstrictor activity compared with ET-1 in human isolated blood vessels (Howard *et al.*, 1992). However, local brachial artery infusion of big ET-1 causes a slow onset, long-lasting vasoconstriction, with a potency about one tenth that of mature ET-1, suggesting an approximately 10% conversion of the luminally presented big ET-1 (Haynes & Webb, 1994). In addition, systemic intravenous infusion of big ET-1 in healthy human volunteers results in significant, long-lasting cardiovascular effects (Ahlborg *et al.*, 1991). Vasoconstrictor responses elicited by big ET-1 in animals and man can be blocked or inhibited by the metalloprotease inhibitor, phosphoramidon (Gardiner *et al.*, 1991; McMahon *et al.*, 1991; Bennett & Gardiner, 1994; Haynes & Webb, 1994). Moreover, in cultures of human endothelial cells, treatment with phosphoramidon simultaneously reduces the secretion of immunoreactive (IR) ET-1, whilst increasing that of the big ET-1, consistent with the inhibition of a phosphoramidon-sensitive ECE (Plumpton *et al.*, 1994). In further support of a phosphoramidon-sensitive enzyme, metalloprotease ECEs have recently been cloned from rat, bovine and human sources (Ikura *et al.*, 1994; Schmidt *et al.*, 1994; Shimada *et al.*, 1994; Xu *et al.*, 1994).

Conversion of big ET-1 to ET-1 appears to be essential to elicit the full haemodynamic effects observed *in vivo* with big ET-1; therefore human ECE may represent a useful therapeutic target in clinical conditions where ET-1 generation is increased. However, because ET-1 binds with high affinity to its receptors, we hypothesized that using circulating ET-1 concentrations as an index of the formation of ET-1 from exogenous big ET-1 may be misleading. A more complete picture would be obtained by measuring the precursor and both products of conversion. Our aim in the present study was to quantify plasma IR ET-1, big ET-1 and CTF following

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brachial artery infusion of ET-1 or big ET-1 in human volunteers and to compare the levels with the corresponding forearm blood flow responses. Secondly, we tested the effects of coinfusion of phosphoramidon with ET-1 or big ET-1 in the same system. A preliminary account of part of this work has been presented to the British Pharmacological Society (Plumpton *et al.*, 1995).

Methods

Clinical procedures

Six healthy males, aged 27–34 years, participated in this study with the approval of the local ethical committee, as previously described (Haynes & Webb, 1994). Briefly, all volunteers abstained from vasoactive drugs for one week, from alcohol for 24 h, and from food, caffeine-containing drinks and tobacco for 3 h before each infusion. Subjects rested recumbent in a quiet room maintained at a constant temperature of 24–26°C. A 27G cannula was inserted into the brachial artery of the non-dominant arm, under local anaesthesia, for drug infusion, and into the antecubital vein of both arms for the withdrawal of blood samples. ET-1, big ET-1 and phosphoramidon were dissolved in 0.9% saline and infused locally at a constant flow rate of 1 ml min^{-1}. Blood flow was measured in both forearms by venous occlusion plethysmography before and 60 and 90 min after dosing. Venous blood samples were taken before and 60 and 90 min after dosing where appropriate. Blood was collected into EDTA tubes and separated immediately. The resulting plasma was stored at −70°C until assayed.

On separate occasions, each subject received in random order, ET-1 (5 pmol min^{-1}) given alone for 60 min, ET-1 (5 pmol min^{-1}) given for 90 min with phosphoramidon (30 nmol min^{-1}) being co-infused for the first 60 min, big ET-1 (5, 15, or 50 pmol min^{-1}) given alone for 60 min, and big ET-1 (50 pmol min^{-1}) given for 90 min with phosphoramidon (30 nmol min^{-1}) being co-infused for the first 60 min. Drug doses were designed to have local activity in the infused forearm, but not systemically. The dose of phosphoramidon was chosen to achieve local concentrations in the forearm equivalent to the IC_{50} for ECE as determined by McMahon *et al.* (1991). None of the agents had any effect on systemic haemodynamics (forearm blood flow in the contralateral arm, arterial pressure or heart rate).

Sample preparation

After thawing, 4 ml EDTA-plasma samples were acidified by adding 1 ml hydrochloric acid 2 M, and clarified by centrifuging for 15 min at 2000 *g* at 4°C. The resulting supernatants were applied to activated 500 mg Spe-ed C_{18} (14% carbon coverage) disposable mini-columns using a vacuum manifold (Applied Separations, Laboratory Impex Ltd, Middx.). Unbound materials were washed from the mini-columns with 5 ml 0.1% trifluoroacetic acid and discarded. Immunoreactive CTF was eluted with 5 ml of 50% methanol, 0.1% TFA and immunoreactive (IR) ET and big ET-1 were separately eluted with a subsequent 2 ml of 80% methanol, 0.1% TFA. Eluates were evaporated to dryness in polypropylene tubes using a Savant sample concentrator (Life Sciences International (U.K.) Ltd, Basingstoke, Hants).

Radioimmunoassay

Plasma IR ET, big ET-1 and CTF were determined by radioimmunoassay as previously described (Plumpton *et al.*, 1993) using rabbit antisera raised against the C-termini of ET (ET-$1_{(15-21)}$) and big ET-1 (big ET-$1_{(31-38)}$). Briefly, plasma extracts were reconstituted in assay buffer (50 mM sodium phosphate, 0.25% bovine serum albumin (BSA), 0.01% Tween 20, 0.05% sodium azide, pH 7.4) and incubated in duplicate with diluted antisera overnight at 4°C. Following a further overnight incubation with ~10,000 c.p.m./tube tracer ([^{125}I]-ET-1 or [^{125}I]-big ET-1, Amersham International plc, Amersham, Bucks), bound counts were separated using Amerlex-M reagent (Amersham International plc, Amersham, Bucks) and radioactivity determined in a gamma counter (Canberra Packard, Pangbourne, Berks). Immunoreactivity was calculated by reference to standard curves (0.5–1000 fmol/tube) of authentic ET-1 (Peptide Institute, Scientific Marketing Associates, Barnet, Herts) or Novabiochem Ltd, Nottingham), or big ET-1 (Peninsula Laboratories Ltd, St. Helens, Lancs). For both assays, ED_{50} values were 20–25 fmol/tube, inter- and intra-assay coefficients of variation were <13% in the range 6–30 fmol/tube and the sensitivities of detection (defined as two s.d. above zero standard) were <1.25 fmol/tube (equivalent to sample sensitivities of <1.56 pM under the current extraction procedure). The recoveries of ET-1, big ET-1 and CTF were 57.5%, 39.8% and 76.6%, respectively ($n=4$). Plasma IR peptide concentrations are shown uncorrected for recovery.

The mature ET RIA cross-reacted 100% with ET-1, ET-2 and ET-3 as expected since the immunogen contained the seven C-terminal residues of ET-1 common to all three mature ET isoforms. Cross-reactivities with ET-$1_{(1-20)}$, big ET-$1_{(22-38)}$, big ET-1, big ET-2 and big ET-3 were <0.02%. The big ET-1 RIA showed <0.007% cross-reactivity with the mature ETs, big ET-2 and big ET-3 and cross-reacted 143% with big ET-$1_{(22-38)}$ thus allowing the quantification of CTF following fractionation. Neither of the assays showed any detectable cross-reactivity (<0.000014%) with phosphoramidon. Furthermore phosphoramidon did not interfere with either the ET or big ET-1 assays as indicated by superimposable standard curves at concentrations an order of magnitude higher than that of the infusate. No cross-reactivity was detected (<0.005%) at the highest concentrations tested with unrelated vasoactive peptides such as angiotensin II, atrial natriuretic factor and α-calcitonin gene-related peptide.

Drugs

The ET-1, big ET-1 and phosphoramidon for infusion were obtained from Clinalfa AG, Laufelfingen, Switzerland.

Statistical analysis

Results are given as the mean of six individuals ± s.e.mean. There was no evidence for non-normality of the data using the Shapiro-Francia test (Shapiro *et al.*, 1968) and so results were analysed by analysis of variance.

Results

Sample extraction

The extraction characteristics for synthetic ET-1, big ET-1 and CTF were determined by applying each peptide to a range of disposable reverse-phase minicolumns with differing packing chemistries and from various manufacturers. Immunoreactive peptides, determined by RIA, were eluted with a stepwise gradient of increasing concentrations of methanol. The C_{18} minicolumns obtained from Applied Separations gave the best resolution of the three peptides and were therefore chosen for further optimisation of conditions. The final extraction procedure described in the methods resulted in 96.0% of IR CTF eluting in the 50% methanol fraction and 99.7% and 96.9% of IR ET-1 and big ET-1, respectively eluting in the 80% methanol fraction ($n=6$).

ET-1 infusions

Infusion of ET-1 at 5 pmol min^{-1} resulted in a marked increase in the levels of IR ET above basal in the infused arm (Figure 1b) and was accompanied by a significant decrease in forearm blood flow again only in the infused

arm (Figure 1a). Coinfusion of phosphoramidon had no effect on the blood flow or IR ET levels both after 60 min coinfusion and 30 min after withdrawal of phosphoramidon (Figure 1e and f). The endogenous levels of IR big ET-1 and CTF were below the detection limits of the RIA in almost all cases.

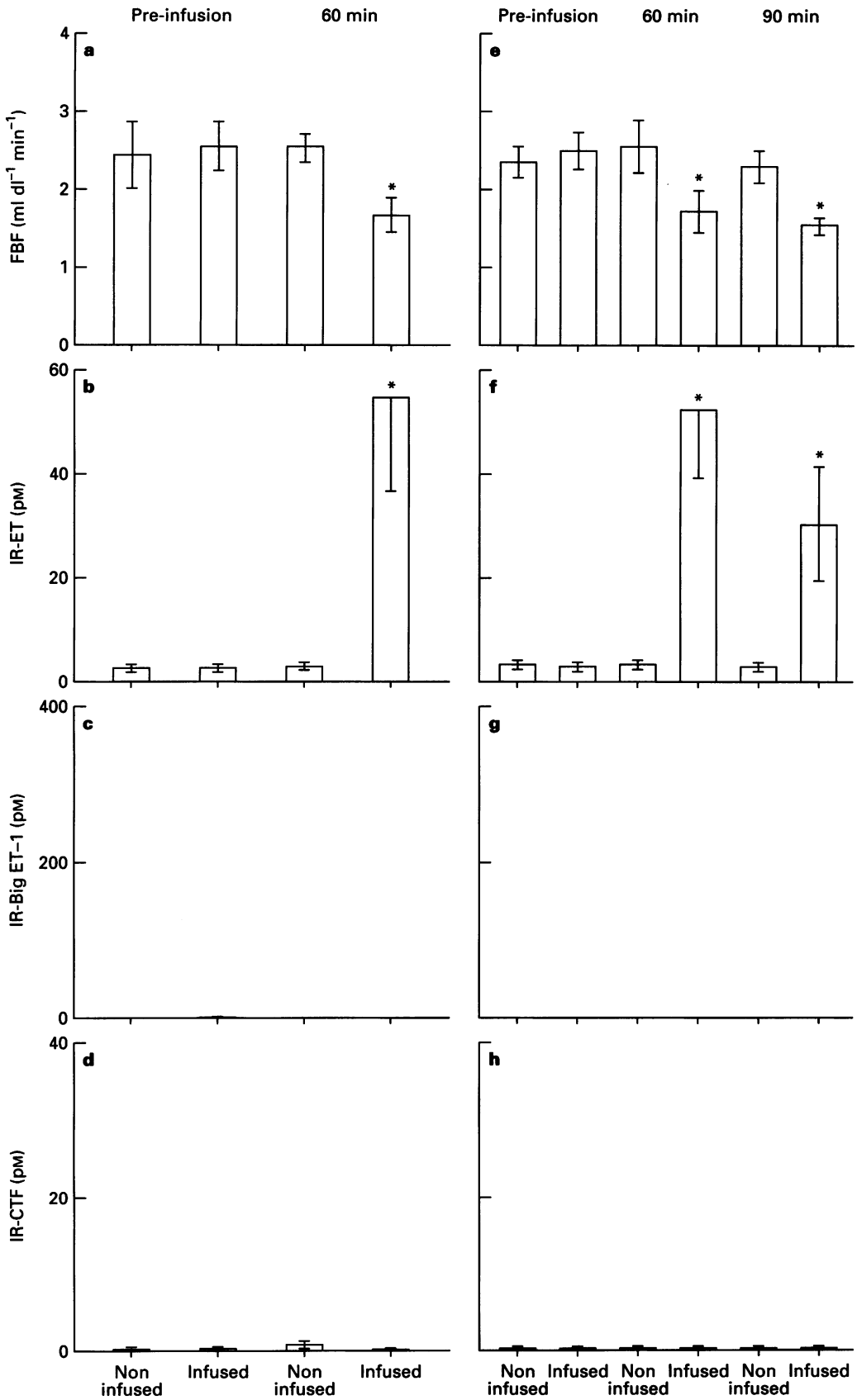

Figure 1 Effect of brachial artery infusion of ET-1 (5 pmol min^{-1}) for 60 min (a,b,c,d) and ET-1 (5 pmol min^{-1}) for 90 min with co-infusion of phosphoramidon (30 nmol min^{-1}) for the first 60 min (e,f,g,h). Forearm blood flow, plasma immunoreactive ET, big ET-1 and CTF are shown in (a,e), (b,f), (c,g) and (d,h), respectively. Mean values ($n=6$) are shown ± s.e.mean. *$P<0.05$ compared to basal.

Big ET-1 infusions

When big ET-1 was infused at 5 or 15 pmol min^{-1}, only basal levels of ET were detected and there was no detectable change in forearm blood flow (Figure 2a,b,e and f). At 50 pmol min^{-1} significantly increased levels of ET were detected from the infused arm (Figure 3b) concomitant with a decrease in forearm blood flow comparable with that elicited by the 5 pmol min^{-1}

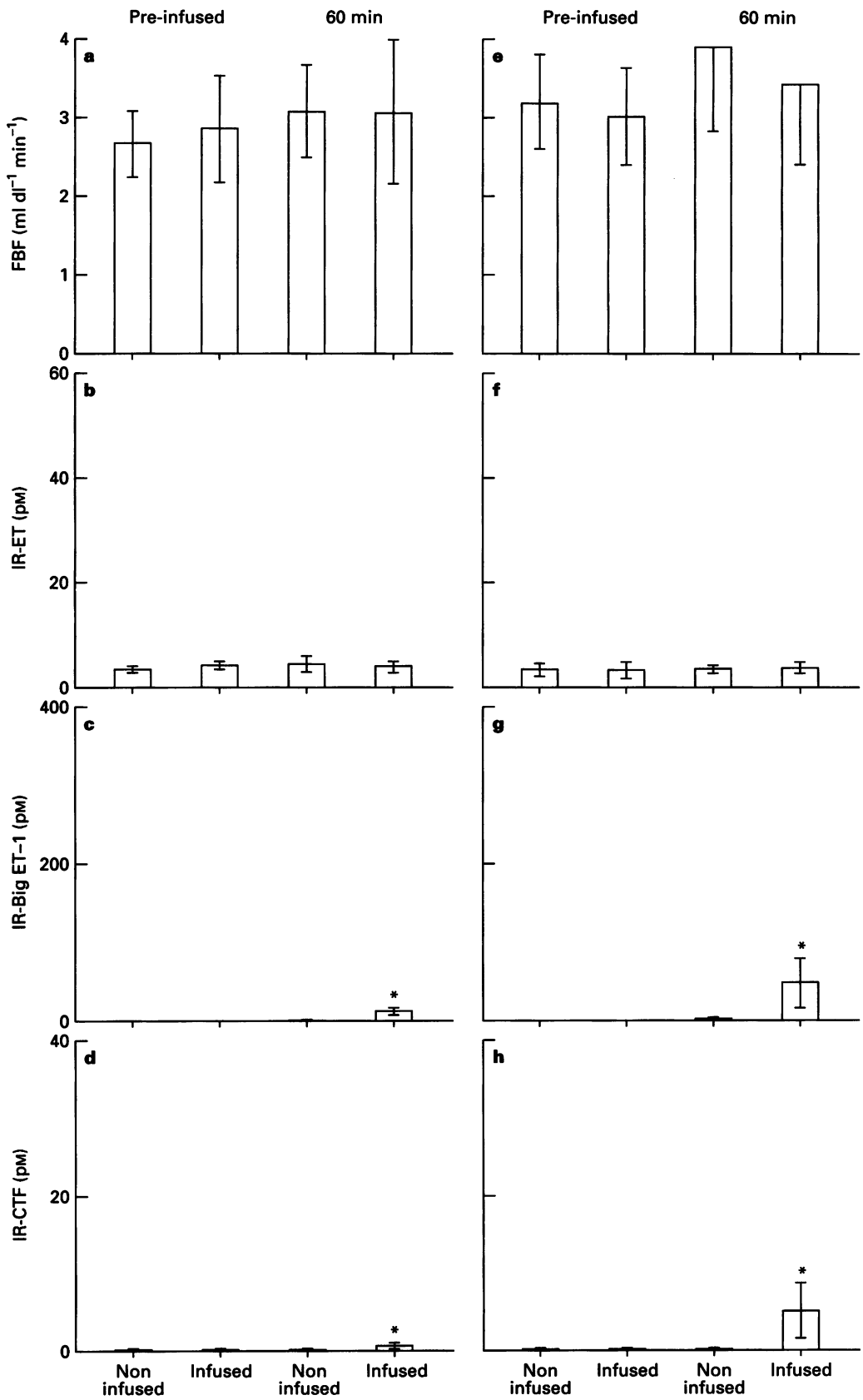

Figure 2 As Figure 1, except infusates were big ET-1 (5 pmol min^{-1}) for 60 min (a,b,c,d) and big ET-1 (15 pmol min^{-1}) for 60 min (e,f,g,h).

infusion of ET-1 (Figure 3a). The levels of IR big ET-1 and CTF increased in a dose-dependent fashion from both arms (Figures 2c,d,g,h and 3c and d), although the concentrations in the non-infused arm were negligible and accordingly were not associated with any detectable vasoconstriction (Figure 3a).

In the presence of phosphoramidon, there was no significant increase in IR ET from the infused arm after 60 min (Figure 3f) and the associated vasoconstriction observed with big ET-1

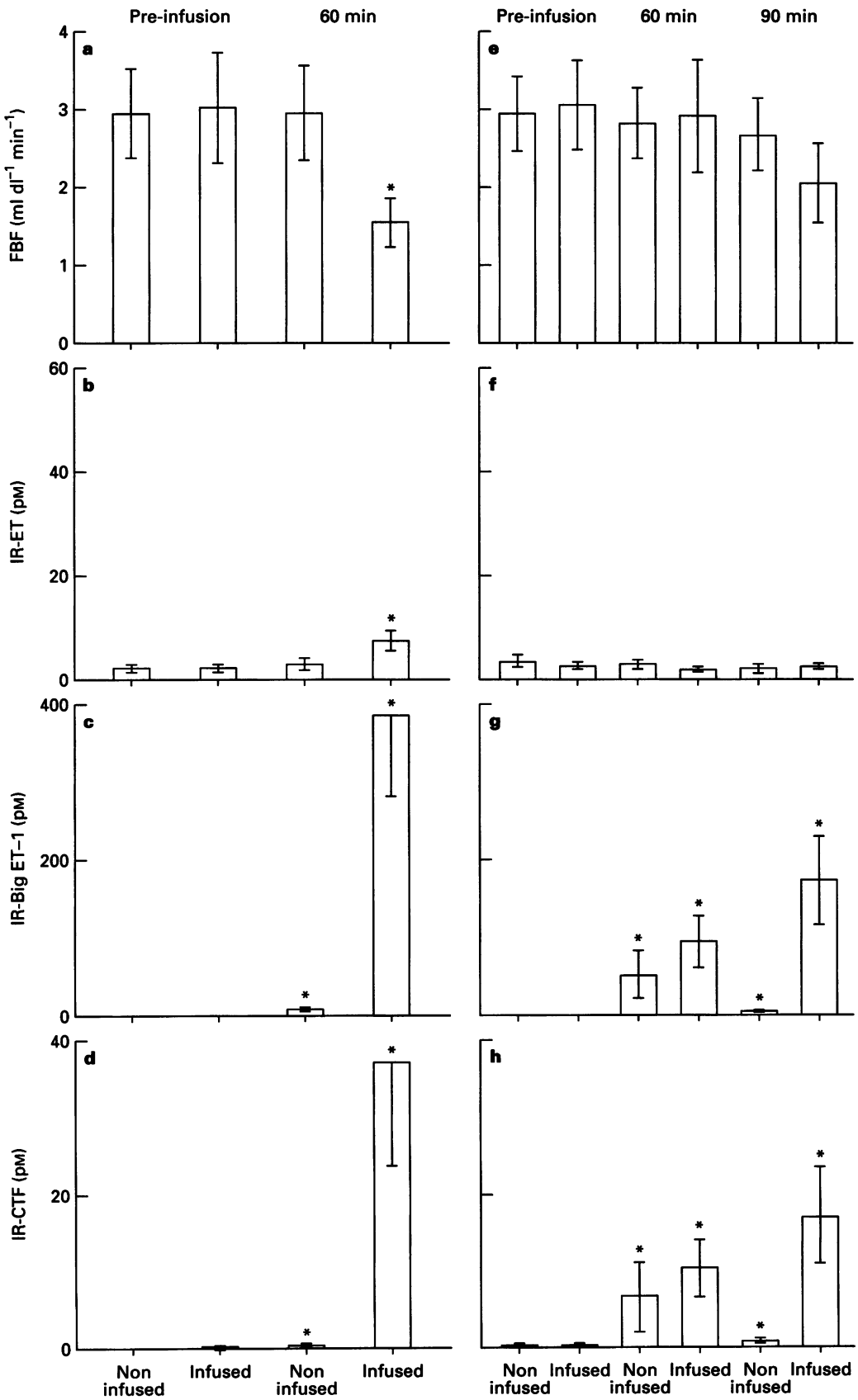

Figure 3 As Figure 1, except infusates were big ET-1 (50 pmol min^{-1}) for 60 min (a,b,c,d) and big ET-1 (50 pmol min^{-1}) for 90 min with co-infusion of phosphoramidon (30 nmol min^{-1}) for the first 60 min (e,f,g,h).

alone was also abolished (Figure 3e). The levels of IR big ET-1 from the infused arm were lower and the proportion detected from the non-infused arm higher than those in the absence of phosphoramidon (Figure 3g). However, 30 min after the withdrawal of phosphoramidon, the forearm blood flow and levels of IR big ET-1 show a return to the levels detected after 60 min without phosphoramidon (Figure 3e and g). The levels of IR CTF essentially mirrored those of IR big ET-1 despite the lack of vasoconstriction after 60 min (Figure 3h).

Discussion

This study has shown that in healthy human volunteers, brachial artery infusion of ET-1 resulted in a marked increase in local venous plasma IR ET. A significant increase in IR ET was also detected when the precursor big ET-1 was infused. Local brachial artery infusions of ET-1 have been shown to cause significant vasoconstriction (Clarke *et al.*, 1989; Pernow *et al.*, 1991; Haynes & Webb, 1994) and systemic intravenous infusion of ET-1 elicits a sustained pressor response (Weitzberg *et al.*, 1991). In addition, ET-1 is a potent constrictor of human isolated blood vessels *in vitro*, with an EC_{50} of 3–18 nM in a range of human isolated blood vessels (Davenport *et al.*, 1989; Maguire & Davenport, 1995). Infusion of big ET-1 also results in vasoconstriction, presumably by conversion to biologically active ET-1 (Ahlborg *et al.*, 1994; Haynes & Webb, 1994).

High-performance liquid chromatography coupled with immunoassays have identified ET-1, big ET-1 and CTF in extracts of cultured animal endothelial cells (Sawamura *et al.*, 1989; Hexum *et al.*, 1990; Ikegawa *et al.*, 1990) and demonstrated that CTF is the major form of IR big ET-1 in porcine lung (Kitamura *et al.*, 1990). In addition, in human subjects, we have detected significant IR CTF in conditioned medium from cultured endocardial endothelial cells and umbilical vein endothelial and smooth muscle cells (unpublished observations). Therefore in the present study we measured the plasma IR levels of all three peptides by RIAs. Measurements were made following local infusions of either ET-1 or big ET-1 both with and without phosphoramidon which blocks the functional responses to big ET-1 (Haynes & Webb, 1994). Two-site enzyme-linked immunosorbent assays previously used for quantifying ET-1 and big ET-1 could not be used to measure CTF because this peptide contains only one of the two epitopes necessary to generate a signal in these assay systems (Plumpton *et al.*, 1994). However, the big ET-1 RIA used in the present study showed a marked cross-reactivity with CTF and could be used to determine plasma IR CTF following a fractionation step. This is the first report of CTF measurement with this technique, which may be useful for assessing the effects of orally active ET antagonists on the levels of ET synthesis. The levels of IR ET detected in the basal samples were in good agreement with previously reported normal human plasma levels using a similar RIA technique (Davenport *et al.*, 1990a). Previously reported basal levels for plasma IR big ET-1 (Suzuki *et al.*, 1990) are below the detection limit of the RIA under these conditions, possibly explaining why endogenous IR big ET-1 was not detected.

Infusion of ET-1 into the brachial artery of the human forearm resulted in the expected significant rise in the levels of IR ET from the infused arm, with concomitant vasoconstriction. Since ET-1 has been shown to be a substrate for neutral endopeptidase -24.11 (EC. 3.4.24.11, NEP) (Sokolovsky *et al.*, 1990; Turner, 1993) causing rapid inactivation, co-infusion of phosphoramidon might have been expected to increase the levels of IR ET. However, no significant differences were observed indicating that other mechanisms controlling the levels of ET may be involved.

Big ET-1 infusion caused a dose-dependent increase in the levels of IR big ET-1, and decrease in blood flow in the infused arm. Furthermore there was a dose-dependent increase in the levels of IR CTF from the infused arm and at the highest dose, a small but significant increase in the IR ET. These data are consistent with local conversion of exogenous big ET-1 in the infused arm, in accord with animal studies (Gardiner *et al.*, 1991; Bennett & Gardiner, 1994; Corder & Vane, 1994). Although the vasoconstrictor effects were confined to the infused arm, there were detectable increases of IR big ET-1 and CTF from the contralateral arm indicating systemic increases in the levels of these peptides. Since the vasoconstrictor responses to both ET-1 and big ET-1 reached significance by 5 min (unpublished observations), the effects are unlikely to be due to *de novo* synthesis, although the processing of stored precursors cannot be excluded. We have been unable to detect binding sites for [^{125}I]-big ET-1 in human cardiovascular tissues at concentrations up to 1 nM (Davenport *et al.*, 1990b) suggesting that there are no specific high affinity receptors for big ET-1. In addition, 1 μM big ET-1 does not compete for the binding of [^{125}I]-ET-1 binding sites. In human isolated saphenous vein, CTF, at concentrations up to 1 μM, has no detectable vasoconstrictor action nor does it antagonize the actions of ET-1 (unpublished observations). Taken together, these results strongly suggest that the vasoconstrictor effects are due to the formation of biologically active ET-1 from exogenous big ET-1 rather than a direct effect of CTF or big ET-1 itself.

The small rise in IR ET is in good agreement with animal studies where the levels of ET-1 have been measured (Hemsen *et al.*, 1991; Corder & Vane, 1994). In particular the apparent discrepancy between the levels of IR ET following local infusion of ET-1 and big ET-1 which resulted in similar fuctional responses are concordant with the results of systemic infusion of these peptides in human subjects (Weitzberg *et al.*, 1991; Ahlborg *et al.*, 1994). We propose that the detected increases in plasma IR ET were modest following infusion of big ET-1 owing to ET binding to its receptors with a high affinity immediately following synthesis (Davenport *et al.*, 1995) in support of a 'stoichiometric' model for ET-1 binding and action (Frelin & Guedin, 1994). Furthermore, once bound, the peptide dissociates slowly from its receptors in the vasculature (Molenaar *et al.*, 1993). These data question the ability of plasma IR ET estimations to provide relevant information. It is likely that measurement of big ET-1 and CTF in addition to ET will be of greater value in monitoring the generation of ET.

Co-infusion of phosphoramidon with big ET-1 results in the abolition of vasoconstriction and increase in IR ET in the infused arm. This is consistent with the inhibition of a phosphoramidon-sensitive ECE and in agreement with *in vitro* data from cultured animal (Ohnaka *et al.*, 1991; Sawamura *et al.*, 1991) and human endothelial cells (Plumpton *et al.*, 1994), and porcine (Fukuroda *et al.*, 1990) and human isolated blood vessels *in vitro* (Mombouli *et al.*, 1993). The presence of CTF following coinfusion of phosphoramidon suggests that other ECEs may still be active, for example an aspartyl protease that has been identified in human endothelial cell cultures (Plumpton *et al.*, 1994). In addition, phosphoramidon may protect CTF, generated from endogenous and exogenous big ET-1, from proteolysis by NEP as CTF has been shown also to be a substrate for this enzyme (Murphy *et al.*, 1994). Phosphoramidon caused an increase in the levels of big ET-1 from the non-infused arm suggesting a systemic protection against degradation and/or conversion. In contrast, the levels in the infused arm decreased when compared to the infusion of big ET-1 alone. We propose that this effect was due to the increased dilution effect of the relatively higher blood flow. This is supported by the return of blood flow and IR big ET-1 levels towards those found after big ET-1 infusion following the withdrawal of phosphoramidon.

The precise location of big ET-1 conversion in the present study is not clear. Watanabe *et al.* (1991) have demonstrated that whole human blood is not a major site of conversion. In addition, Fukuroda *et al.* (1990) and Mombouli *et al.* (1993) have shown that in porcine and human isolated blood vessels, phosphoramidon-sensitive conversion of big ET-1 is independent of the endothelium. The concentrations of phosphoramidon used in the present study were low compared with

reported IC_{50} values for ECE inhibition (Opgenorth *et al.*, 1992) and the inhibitor does not enter cells efficiently (Turner, 1993). It is therefore most likely that the ECE is located on the extracellular surface of the vascular smooth muscle cells of the resistance vessels of the forearm, proximal to target vasoconstrictor ET_A receptors (Davenport & Maguire, 1994; Davenport *et al.*, 1995; Maguire & Davenport, 1995). Specific antisera or other selective probes for ECE will undoubtedly aid the location of the ECE involved. We predict that this ECE is physiologically relevant as the local infusion of phosphoramidon alone resulted in vasodilatation presumably due to inhibition of endogenous ET synthesis (Haynes & Webb, 1994). However, it is possible that other ECEs contribute to endogenous ET synthesis.

In conclusion, we have shown that big ET-1-induced forearm vasoconstriction is associated with a significant increase in IR ET and CTF after 60 min. The increases in IR ET and vasoconstriction are blocked by coinfusion of phosphoramidon, suggesting that a phosphoramidon-sensitive ECE is at least in part responsible for the vasoconstrictor properties of big ET-1. This enzyme is, therefore, a valid target for therapeutic agents in human subjects. The development of more potent, specific and orally active ECE inhibitors may lead to specific vasodilator drugs use which may be of clinical benefit perhaps in addition to specific antagonists at ET receptors. Such drugs may have potential in lowering the levels of circulating ET-1 in cardiovascular diseases where the levels of ET-1 are raised. Measurable levels of newly synthesized ET-1 are likely to be reduced through rapid receptor binding, therefore assay of IR big ET-1 and CTF may be a more sensitive measure of the overexpression of ET-1 in disease.

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