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RADIOIMMUNOASSAY EVIDENCE THAT THE PRESSOR EFFECT OF BIG ENDOTHELIN-1 IS DUE TO LOCAL CONVERSION TO ENDOTHELIN-1

ROGER CORDER* and JOHN R. VANE

The William Harvey Research Institute, Medical College of St Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ, U.K.

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Abstract—Compared with endothelin-1 (ET-1), big endothelin-1 (big ET-1) is only weakly active on isolated vascular smooth muscle preparations. However, on systemic administration high doses of big ET-1 (1 nmol.kg^{-1}) are approximately equipotent to ET-1, indicating the existence of an endothelin converting enzyme in the circulation that rapidly converts big ET-1 to ET-1. In this study arterial blood levels of big ET-1 and ET-1 immunoreactivity were measured after bolus i.v. administration of big ET-1 (1 or 3 nmol.kg^{-1}) or ET-1 (1 nmol.kg^{-1}) in anaesthetised male Wistar rats. In addition, the effect of phosphoramidon (10 mg.kg^{-1}) on the pressor response to big ET-1 and its disappearance rate from the circulation were examined. After big ET-1 injection, circulating ET-1 concentrations did not exceed 2% of the big ET-1 level. Phosphoramidon reduced the pressor response to big ET-1 by 93%, but did not alter its rate of clearance from the circulation. Thus exogenous big ET-1 is converted locally in the vasculature and its disappearance from the circulation is not dependent on conversion to ET-1.

Key words: blood pressure; phosphoramidon; endothelin converting enzyme; endothelium; vascular smooth muscle

Shortly after the isolation of ET-1† [1], it was observed that although big ET-1 is relatively inactive on isolated blood vessels, systemic administration of big ET-1 induces a pressor response comparable in magnitude to that of ET-1 [2]. As with ET-1, this effect is substantially attenuated by the ET_A receptor antagonist BQ-123, indicating that it is mediated predominantly by ET_A receptors [3, 4]. However, in contrast to ET-1, the increase in blood pressure produced by big ET-1 can also be blocked by pretreatment with a high dose of the metalloprotease inhibitor phosphoramidon [5–10], which acts by inhibiting the enzymatic conversion of big ET-1 to ET-1 by the putative ECE [1]. Although it seems likely that the enzyme involved in the pressor response to exogenous big ET-1 is either on endothelial cells or on vascular smooth muscle cells [11], the nature of the endopeptidase and its precise location have yet to be unequivocally demonstrated. Moreover, its relevance to the *in vivo* biosynthesis of ET-1 has not been determined. To investigate further the site of conversion, we have used highly specific radioimmunoassays to measure the changes in plasma concentrations of big ET-1 and ET-1 after i.v. administration of big ET-1 in anaesthetised rats. In addition, the identity of the circulating immunoreactivity was also characterised by subjecting plasma samples to analysis by HPLC followed by immunoassay. Some of these findings were presented to the British Pharmacological Society [12].

MATERIALS AND METHODS

Male Wistar rats (250–330 g) from A. Tuck & Son (Battlebridge, U.K.), were anaesthetised with sodium thiopentone (120 mg.kg^{-1}, i.p.; Intraval, RMB Animal Health Care, Dagenham, U.K.). Intravascular catheters were inserted into the right femoral vein for peptide administration, and into the right femoral artery for measurement of MAP and/or withdrawal of blood samples. MAP was recorded using a Spectramed P23XL blood pressure transducer connected to a Grass 7D polygraph (Grass Instruments, Quincy, MA, U.S.A.). Each rat was used to study only one bolus injection of big ET-1 or ET-1. In animals used to study the blood pressure response, blood samples (0.3 mL) were collected at 1 and 3 min. These groups included rats administered big ET-1 (1 nmol.kg^{-1}) alone, or after phosphoramidon (10 mg.kg^{-1}, −1 min), and rats given ET-1 (1 nmol.kg^{-1}). To compare the pressor potencies of lower doses of these peptides, additional rats received either big ET-1 or ET-1 at 0.33 nmol.kg^{-1} without blood samples being collected.

In rats used to study peak plasma concentrations of big ET-1 or ET-1, MAP was not recorded. Blood was collected from these rats at 15, 30 and 45 sec, and 1, 3 and 5 min (0.3 mL per sample). Plasma concentrations of big ET-1 were measured after administration of 1 or 3 nmol.kg^{-1} big ET-1. ET-1

* Corresponding author. Tel. 0171 982 6083; FAX 0171 251 1685.

† Abbreviations: ET-1; endothelin-1; big ET–1, big endothelin-1; MAP, mean arterial blood pressure; ECE, endothelin converting enzyme; RIA, radioimmunoassay; CTF, C-terminal fragment; TFA, trifluoroacetic acid.

levels were measured in these groups, and also for comparison in rats administered ET-1 (1 nmol.kg^{-1}). ET-1 and big ET-1 were dissolved in 10 mM sodium bicarbonate to give a stock concentration of 100 μM, and then diluted appropriately in 0.9% sodium chloride containing 0.2% rat albumin (Sigma, St Louis, MO, U.S.A.) to give an injection volume of 0.3 mL. Heparinised blood was collected into chilled plastic tubes, centrifuged, and plasma was removed and frozen at −30° until assay.

RIA of big ET-1 was performed, after dilution of the plasma in RIA buffer (50 mM sodium phosphate buffer, pH 7.4, containing 0.15% BSA, 0.005% Triton X-100 and 0.01% sodium azide), using a rabbit antiserum raised against the C-terminal sequence of human big ET-1$_{[22-38]}$. ^{125}I-human big ET-1 was used as tracer and dilutions of human big ET-1 were used as standard. Human big ET-1 and its CTF (big ET-1$_{[22-38]}$) show full cross-reactivity in this assay (100%), whereas the cross-reactivity with ET-1 is <0.001% [13]. For ET-1 measurement, plasma samples (0.1 mL) were acidified by dilution with 1 mL 50 mM HCl and extracted using disposable octadecasilylsilica columns (Techelut SPE C18, HPLC Technology, Macclesfield, U.K.). Immunoreactivity was then eluted with 80% acetonitrile containing 0.1% TFA, and after drying down, samples were reconstituted in assay buffer and subjected to RIA using a rabbit antiserum raised against the C-terminal ET-1$_{[16-21]}$ sequence (crossreactivity with big ET-1 <0.015%) [13]. Extraction efficiency was reproducibly 80–90%. ^{125}I-ET-1 was used as tracer and dilutions of ET-1 were used as standard. For *in vivo* administration, RIA, and standardisation of the HPLC system described below, ET-1 and human big ET-1$_{[1-38]}$ were purchased from Peptide Institute Inc. (Osaka, Japan). ^{125}I-ET-1 and ^{125}I-human big ET-1 were from Amersham International plc (Amersham, U.K.).

HPLC identification of plasma ET-1, big ET-1 and CTF immunoreactivity was performed using a column of 5 μm TSK gel ODS-120T (4.6 × 250 mm; TOSOH Corporation, Japan, from Anachem, Luton, U.K.). Separate pooled samples of plasma (0.5 mL) were prepared with samples collected during 15–60 sec and from 2–3 min from the groups of rats receiving big ET-1 at 1 and 3 nmol.kg^{-1} and ET-1 at 1 nmol.kg^{-1}. These samples were first acidified and extracted using TechElut SPE C18 columns, eluted with 1 mL 80% acetonitrile containing 0.1% TFA, concentrated to 0.2 mL under a stream of N_2, and injected on to the HPLC system. The HPLC column was eluted at a flow rate of 1 mL/min with a gradient of acetonitrile in 0.1% TFA using a Pharmacia LKB model 2249 gradient pump. The acetonitrile gradient used was 0–20% over 3 min, 20–32% over 12 min, 32–40% over 15 min, and 40–50% over 10 min. Fractions (30 sec) were collected and dried down for identification of the immunoreactivity present using the RIAs described above. Using this system, elution times for standard peptides were: CTF, 15.1 ± 0.1 min (N = 4); big ET-1, 27.5 ± 0.1 min (N = 10); and ET-1, 30.1 ± 0.1 min (N = 7).

Statistical analysis was performed by analysis of variance or Student's *t*-test as appropriate.

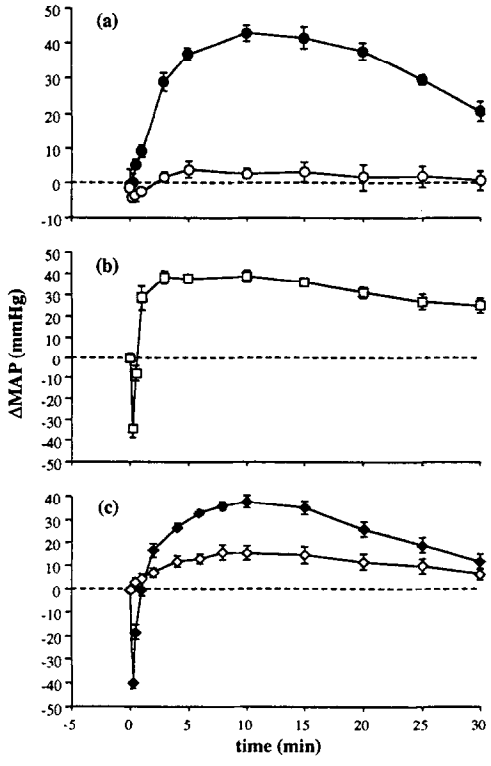

Fig. 1. (a) Changes in mean arterial blood pressure (MAP) induced by big ET-1 (1 nmol.kg^{-1}) administered alone, or after pretreatment with phosphoramidon (10 mg.kg^{-1}). Big ET-1 (●, N = 6, basal MAP 114 ± 4 mmHg); big ET-1 with phosphoramidon (○, N = 4, basal MAP 113 ± 4 mmHg). (b) Blood pressure response to ET-1 (1 nmol.kg^{-1}, □, N = 5, basal MAP 105 ± 2 mmHg). Blood samples were collected from each rat at 1 and 3 min. (c) Comparison of MAP responses to big ET-1 (◇, N = 5, basal MAP 115 ± 3 mmHg) with ET-1 (◆, N = 6, basal MAP 107 ± 3 mmHg) administered at doses of 0.33 nmol.kg^{-1}.

RESULTS

Basal blood pressure was similar in all groups where MAP was recorded (see legend to Fig. 1). Administration of phosphoramidon did not affect basal blood pressure but completely blocked the increase in MAP induced by big ET-1 (+10 min Δ = 43 ± 2 mmHg for big ET-1 alone, compared to 3 ± 1 mmHg with phosphoramidon; $P < 0.001$). In agreement with previous studies, the magnitude of the pressor response to big ET-1 at 1 nmol.kg^{-1} was not significantly different from the effect of ET-1 (1 nmol.kg^{-1}). However, when lower doses of the two peptides were compared (0.33 nmol.kg^{-1}), the pressor response to big ET-1 was only 40–45% of that to ET-1. This was true for the maximum pressor response (+15 ± 3 mmHg compared to 38 ± 3 mmHg, $P < 0.001$) and for the area of the response in arbitrary units (350 ± 78 vs 779 ± 57, $P = 0.0014$).

After bolus administration of big ET-1, peak plasma concentrations occurred within 30 sec. These were 12.9 ± 1.3 nM and 52.1 ± 3.0 nM for doses of big ET-1 at 1 and 3 nmol.kg^{-1} (Fig. 2). At 1 and 3 min these levels had declined to 6.8 ± 0.7 nM and

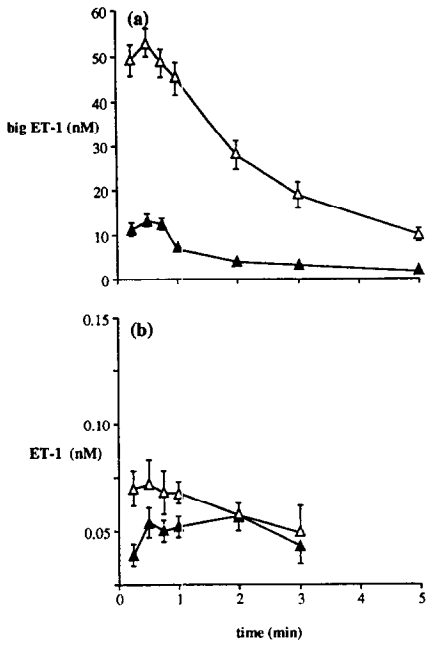

Fig. 2. Plasma concentrations of (a) big ET-$1_{[22-38]}$ and (b) ET-1 immunoreactivity in the same samples after bolus i.v. administration of big ET-1 (1▲, or 3△, nmol.kg^{-1}) to anaesthetised rats (N = 4 per group).

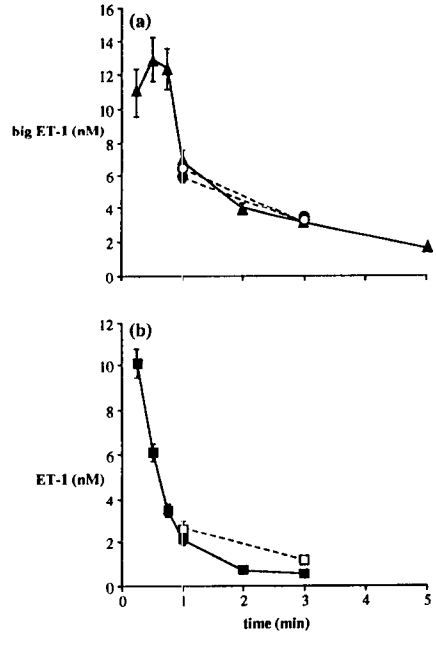

Fig. 3. Comparison of the time course of plasma concentrations following administration of (a) big ET-1 (▲) or (b) ET-1 (■) at doses of 1 nmol.kg^{-1}. The values for samples collected during studies where MAP was recorded are also indicated (Fig. 1); big ET-1 (●), big ET-1 with phosphoramidon (○) and ET-1 (□).

3.2 ± 0.2 nM after the 1 nmol.kg^{-1} dose, and 44.7 ± 3.4 nM and 18.3 ± 2.4 nM after the 3 nmol.kg^{-1} dose. Similar values were observed at 1 and 3 min in rats administered big ET-1 (1 nmol.kg^{-1}) where MAP was also recorded (Fig. 3). Although treatment with phosphoramidon completely blocked the pressor effect of big ET-1 it did not alter the clearance rate of big ET-1 from the circulation (Fig. 3). After ET-1 injection, peak plasma levels occurred within 15 sec of administration (10.1 ± 2.5 nM), and these decreased rapidly to 2.4 ± 0.2 and 1.2 ± 0.1 nM at 1 and 3 min, respectively. Comparable levels were also observed in animals where MAP was recorded. After big ET-1 administration, ET-1 was barely detectable in plasma (0.04–0.07 nM) and at no time point exceeded 2% of the big ET-1 concentration. ET-1 immunoreactivity was not detectable in plasma collected 5 min after big ET-1 administration.

HPLC characterisation of the plasma from rats administered big ET-1 showed that the big ET-$1_{[22-38]}$ immunoreactivity was predominantly intact big ET-1 with some CTF (Fig. 4). With both doses of big ET-1, the peak of CTF (eluting at 15 min in the HPLC gradient) was greater in the 2–3 min plasma pools than in the 1 min pools. HPLC results are shown only for big ET-1 at 3 nmol.kg^{-1} (Fig. 4); very similar results were obtained with the 1 nmol.kg^{-1} dose (data not shown). Namely, the profile of big ET-1 and ET-1 immunoreactivities was almost identical, simply that the levels were proportionally lower with the 1 nmol.kg^{-1} dose. After both doses of big ET-1, there was only a very small peak of ET-1 immunoreactivity identified in the pooled samples of arterial blood. In rats given ET-1 a single peak of immunoreactivity was identified corresponding to the authentic molecule. The HPLC peak area from 0.5 mL of the 1 min plasma pool was 1947 fmol, some 23 times greater than the corresponding peak area of ET-1 immunoreactivity for rats administered 1 nmol.kg^{-1} big ET-1. This demonstrates that the low levels of ET-1 obtained after big ET-1 injection are a true index of the circulating concentrations.

DISCUSSION

An early report of plasma levels of ET-1 measured 1 min after systemic administration of big ET-1 in rabbits showed that ET-1 levels reached 30% of the levels obtained with a comparable dose of ET-1 [14], suggesting that there was a rapid conversion of big ET-1 to ET-1 which then circulated for at least 5 min. The principal new finding, obtained from the studies described here in rats using highly specific RIAs, is that conversion of big ET-1 to ET-1 occurs locally in the vasculature. Therefore, it seems likely that the ET-1 immunoreactivity measured in the earlier study in rabbits was simply due to big ET-1 cross-reacting in the ET-1 assay used [14]. Consistent with the current findings, the response to big ET-1 is reported to be similar whether administered by intraarterial or intravenous injection, suggesting that big ET-1 is converted to ET-1 at its site of action [15]. Moreover, locally applied big ET-1 produces vasoconstriction in the hamster cheek pouch and rat

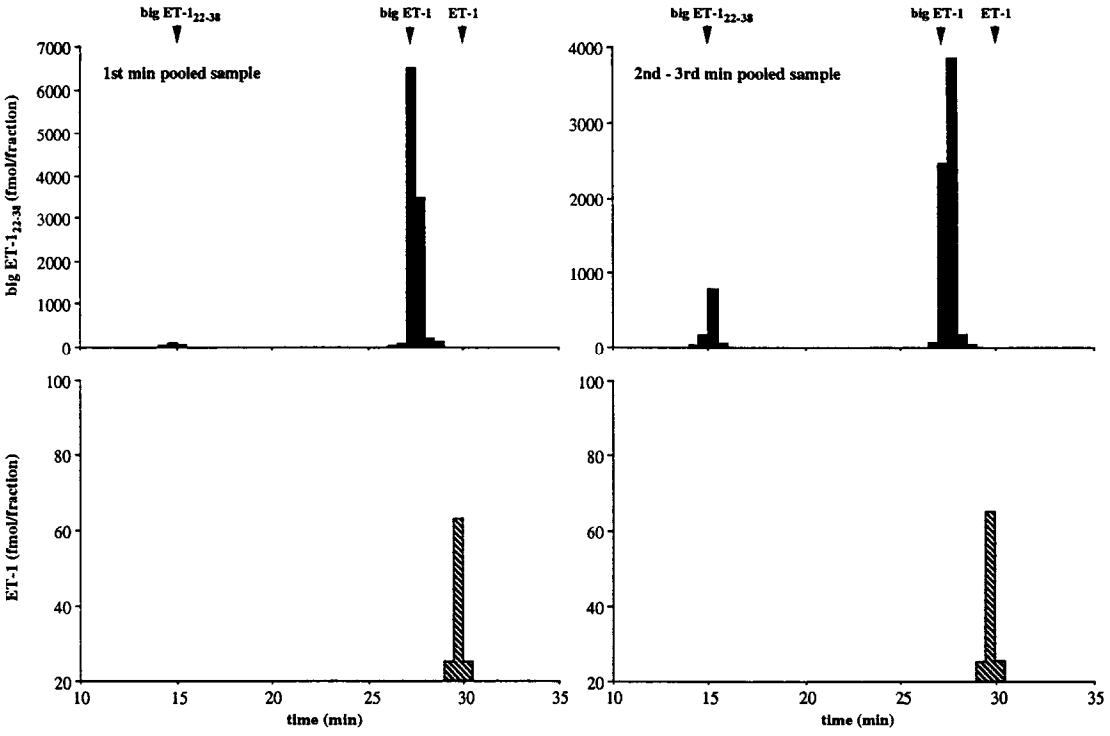

Fig. 4. HPLC characterisation of big ET-$1_{[22-38]}$ (top panels) and ET-1 (lower panels) immunoreactivity in plasma collected during the 1st min (left hand panels) and 2nd–3rd min (right hand panels) after big ET-1 administration (3 nmol.kg^{-1}). The elution positions of standard peptides are indicated.

skin [16]. Combined with the results from plasma measurements here, these studies show that big ET-1 is converted locally in the vasculature to ET-1, and that the ET-1 generated does not circulate to any extent. Furthermore, the fact that phosphoramidon did not alter the rate at which big ET-1 disappeared from the circulation suggests that conversion of big ET-1 to ET-1 is not the rate-limiting step for big ET-1 to be taken up in the vasculature. Hence, big ET-1 probably diffuses through the fenestrations of the endothelium, and is then converted to ET-1 in the blood vessel wall. In agreement with this hypothesis, it is interesting to note that the initial vasodilator response observed after bolus injection of the endothelins or sarafotoxins is generally absent when big ET-1 is administered [3], although a small effect is sometimes observed [17]. This indicates that the ET-1 generated does not attain sufficient levels in the vicinity of the endothelial ET_B receptor to evoke a vasodilator action [3].

Thus after systemic injection of big ET-1, the vasoconstrictor effect is largely due to conversion to ET-1 by an endothelin converting enzyme located in the sub-endothelial matrix or indeed on the plasma membrane of vascular smooth muscle cells. Further support for this idea is provided by evidence that removal of the endothelium does not abrogate the vasoconstrictor activity of big ET-1 in the isolated and perfused mesentery of the rat [18].

Although HPLC characterisation of the immunoreactivity in plasma showed very little ET-1 immunoreactivity, the fact that the CTF was identified in significant quantities indicates that cleavage of big ET-1 had occurred at the Trp^{21}-Val^{22} bond, as would be expected for a true endothelin converting enzyme. Based on the HPLC data from the pooled samples of plasma (Fig. 4), there was a higher molar ratio of CTF to ET-1 when compared in the same sample, particularly in the 2–3 min pooled plasma. There are probably two reasons for this. Firstly, the ET-1 generated from big ET-1 binds rapidly to endothelin receptors, effectively preventing it from circulating, whereas there is no evidence for a receptor that binds CTF. Secondly, CTF may be less susceptible to enzymatic degradation than ET-1. Hence, as ET-1 is co-released with CTF and probably other fragments of the proendothelin precursor, it would seem likely that a highly selective plasma assay for CTF or other metabolically stable fragments may provide a more effective method of monitoring ET-1 release *in vivo* than measuring ET-1 itself.

Although all the studies of the *in vivo* actions of big ET-1 point to a rapid and effective conversion of high doses of big ET-1, the physiological importance of these effects is unclear. We have evaluated the effect of a lower dose of big ET-1 and found it to be substantially smaller than the same dose of ET-1. Similarly, others have reported that while doses of big ET-1 of 0.1 nmol.kg^{-1} or less are virtually inactive [17, 19, 20], ET-1 retains substantial cardiovascular effects at these doses. Moreover, pretreatment with a non-selective ET_A/ET_B endo-

thelin receptor antagonist (Ro 46–2005), at a dose ($10\,mg.kg^{-1}$) that caused a relatively greater antagonism of dilator ET_B receptors than vasoconstrictor receptors, resulted in a doubling of the magnitude of the response to ET-1 [21]. Therefore a true comparison of the pressor potencies of ET-1 to big ET-1 cannot be achieved without selective blockade of vasodilator ET_B receptors; for example, using BQ-788 which increases by almost three-fold the peak MAP response to ET-1 [22].

The conversion of low doses of exogenous big ET-1 does not seem to represent an efficient means of generating biological activity *in vivo*. This calls into question the physiological significance of this conversion of high doses of exogenous big ET-1. Indeed, most studies of cultured cells demonstrate the release of predominantly ET-1 with little big ET-1 [23], indicating that endogenously the conversion to ET-1 is mainly intracellular. High concentrations of phosphoramidon do inhibit the intracellular conversion of big ET-1 to ET-1 [23]. However, it is unclear how rapidly phosphoramidon enters cells to inhibit this enzyme, or whether the same enzyme is responsible for the intracellular conversion of endogenous big ET-1 as that generating the vasoconstrictor activity from exogenous big ET-1. Interestingly, a 30 min infusion of phosphoramidon at a dose that completely blocks the pressor effect of exogenous big ET-1 does not attenuate the release of endogenous ET-1 evoked by a number of stimuli [24]. The most convincing explanation for these findings is that conversion of exogenous big ET-1 occurs in the extracellular vascular environment by an enzyme readily inhibited by phosphoramidon. In contrast, the physiologically relevant endothelin converting enzyme, which may be the same or a different enzyme, cleaves endogenous big ET-1 intracellularly and is therefore not readily inhibited by 30 min infusion of phosphoramidon.

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