Endothelin-1 (1-31) Is an Intermediate in the Production of Endothelin-1 After Big Endothelin-1 Administration In Vivo

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Abstract—The precursor of endothelin-1, big endothelin-1, can be hydrolyzed by chymase to generate endothelin-1 (1-31) in vitro. In the present study, we explored the processes involved in the production of endothelin-1 (1-31) as well as its pharmacodynamic characteristics in the rabbit in vivo. Endothelin-1 (1-31) (1 nmol/kg, injected into the left cardiac ventricle) induced a monophasic increase of mean arterial blood pressure similarly to big endothelin-1 (1-38), whereas endothelin-1 induces a biphasic response. Phosphoramidon, a dual neutral endopeptidase and endothelin-converting enzyme inhibitor, blocked both pressor responses to endothelin-1 (1-31) and big endothelin-1 but not those afforded by endothelin-1. Thiorphan, a neutral endopeptidase inhibitor, markedly inhibited the response to endothelin-1 (1-31) but only weakly reduced that of big endothelin-1. In contrast, CGS 35066, an endothelin-converting enzyme inhibitor, was significantly more efficient against the pressor response to big endothelin-1 than to endothelin-1 (1-31). Furthermore, injection of big endothelin-1 concomitantly with phosphoramidon induced an increase in endothelin-1 (1-31) plasma levels. Finally, intracardiac-administered endothelin-1 (1-31) induced an increase of endothelin-1 plasma levels, which are markedly reduced by phosphoramidon and thiorphan but not by CGS 35066. Our results thus demonstrate that endothelin-1 (1-31) is an alternate intermediate in the production of endothelin-1 after big endothelin-1 administration in the rabbit in vivo. (Hypertension. 2005;46:87-92.)

Key Words: endothelin ■ arterial pressure ■ rabbits ■ plasma

The potent vasopressor peptide endothelin-1 (ET-1) is generated from its precursor pro–ET-1 in a 2-step enzymatic pathway, the first involving subtilisin-like convertases and a carboxypeptidase to produce the 38-aa precursor big ET-1, which is subsequently cleaved to yield ET-1 via the activity of an endothelin-converting enzyme (ECE). On the other hand, pulmonary human mast cell-derived chymase cleaves the Tyr31–Gly32 bond of the precursor big ET-1 to yield ET-1 (1-31), a novel potent contractile peptide of vascular and nonvascular smooth muscle tissues. ET-1 (1-31) also stimulates vascular smooth muscle cell proliferation and induces calcium signaling in human coronary vessels similarly to ET-1.

Okishima et al reported the presence of ET-1 (1-31) in human lungs. In human airways, the calcium-increasing properties of ET-1 (1-31) are abolished by phosphoramidon or thiorphan, suggesting that the 31-aa peptide requires hydrolysis by the neutral endopeptidase 24.11 (NEP 24.11) to be active in vitro. Moreover, we reported recently that ET-1 (1-31) is a potent pressor and bronchoconstrictor agent in the guinea pig. These 2 latter properties of ET-1 (1-31) were found to be predominantly sensitive to ECE and NEP inhibitors, respectively.

From a pharmacological point of view, in vitro studies have shown that ET-1 (1-31) acts as a selective endothelin-A (ET\textsubscript{A}) or nonselective endothelin receptor ligand. Among those, smooth muscle contractile effects on the human coronary artery or NO induced release by endothelial cells are amenable to blockade of ET\textsubscript{A} or ET\textsubscript{B} receptors, respectively. Interestingly, whether it is ET-1 (1-31) or its conversion product ET-1 that actually binds to the endothelin receptors in vitro has not been confirmed with NEP or ECE inhibitors.

Considering that ET-1 (1-31) can be converted to ET-1 in vitro, we first established the pharmacological profile of the 31-aa peptide in the rabbit in vivo, in which the ET-1 system has been extensively studied in our laboratory. In addition, we investigated whether the pressor properties of ET-1 (1-31), similar to big ET-1, require a conversion into the mature peptide ET-1. We have also explored the putative contribution of NEP and ECE in that phenomenon in vivo.

Our results argue in favor of ET-1 (1-31) being an alternate intermediate in the genesis of ET-1 from exogenously administered big ET-1.
Methodology

Hemodynamic Studies

All experimental procedures were approved by the ethical committee on animal research of the Université de Sherbrooke Medical School. Experiments were performed in ketamine/xylazine-anesthetized and ventilated (5 mL air/kg; 45 strokes/min; model 683; Harvard Apparatus) New Zealand White rabbits of either sex (1.25 to 1.75 kg) as reported previously.15,16

Briefly, a catheter (PE-90) was placed into the left ventricle via the right carotid artery for administration of the various agents and for blood sampling. Mean arterial blood pressure (MAP) and heart rate (HR) were constantly monitored via a heparinized cannula in the left femoral artery with a blood pressure analyzer (BPA-200A; Micro-Med).

Pressor responses to bolus injection of various agents (ET-1 [0.25 nmol/kg]; ET-1 (1-31) [0.01 to 2.5 nmol/kg]; big ET-1 (1-38) [0.01 to 2.5 nmol/kg]); were assessed for 30 minutes. In some experiments, inhibitors such as phosphoramidon17 (dual NEP/ECE inhibitor [0.01 to 10 mg/kg]), thiorphan18 (NEP inhibitor [0.01 to 1 mg/kg]), or CGS 3506619 (ECE inhibitor [0.1 and 1 mg/kg]) were administered 5, 15, or 30 minutes, respectively, before agonist injection.

In another series of experiments, the selective antagonists BQ-78820 (ETα receptor [0.25 mg/kg]), or BQ-12321 (ETβ receptor [1 mg/kg]) were administered 5 minutes before ET-1 (1-31) or big ET-1 (1 nmol/kg each).

Blood Sample Preparation

Blood samples (900 μL) taken before, 1, 2, and 5 minutes after injection of big ET-1 or ET-1 (1-31) were collected in trisodium citrate (3.5%) in a 9:1 ratio (vol/vol). Samples were centrifuged for 1 minute at 19 800 g for plasma separation and stored at −80°C until assayed.

After thawing, plasma samples (400 μL each) were acidified by addition of an equal volume of 0.2% trifluoroacetic acid (TFA) and clarified by centrifugation for 5 minutes at 19 800 g. The resulting supernatants were purified through a methanol-activated 500-mg Amprep C2 disposable cartridge (Amersham). The column was then washed with 6 mL of 0.1% TFA to discard the unbound material. Thereafter, elution with 2.5 mL of 60% acetonitrile/0.1% TFA for high-performance liquid chromatography (HPLC) was accomplished by ultraviolet absorbance (210 nm) and confirmed by RIA and EIA.

Desiccated residues were reconstituted in 1 mL of 10% acetonitrile/1% TFA for high-performance liquid chromatography (HPLC) analysis. A reversed-phase HPLC was performed using a Zorbax 300SB-C18 column (Agilent Technologies). Samples were eluted with a 35-minute linear gradient of acetonitrile (from 28% to 40%) in 0.1% TFA at a flow rate of 1 mL/min using a Waters model 510 pump. Thirty-second fractions (500 μL) were separated in two for subsequent determination of ET-1 and ET-1 (1-31) levels, evaporated to dryness, and analyzed by radioimmunoassay (RIA) and enzyme immunoassay (EIA), respectively. For calibration of elution parameters, synthetic ET-1 or ET-1 (1-31) was run separately or conjointly, whereas positions of the eluted standards were determined by ultraviolet absorbance (210 nm) and confirmed by RIA and EIA.

RIA and EIA Analysis

Immunoreactive endothelin (IR-ET) was measured by an RIA kit (RPA 555; Amersham) as reported previously.18 IR–ET (ET-1 (1-31)) was measured with an EIA kit (Immuno-Biological Laboratories).

Drugs and Solutions

ET-1, ET-1 (1-31), big ET-1, and phosphoramidon were purchased from Peptide International, BQ-788 from American Peptide, and thiorphan from Sigma. BQ-123 was synthesized by Dr Witold Neugebauer (Department of Pharmacology, Université de Sherbrooke). Finally, CGS 35066 was supplied by Dr Arco Jeng (Novartis, Nuttley, NJ).

Drugs were dissolved in PBS, pH 7.4, except for BQ-123, BQ-788, thiorphan, and ET-1 (1-31), which were diluted in 10% dimethyl sulfoxide and subsequently in PBS and CGS 35066, in 0.25 mol/L of NaHCO3/PBS. Vehicles were systematically controlled and were without effects on all parameters studied in each series of experiments.

Statistical Analysis

Statistical analyses were performed by ANOVA, followed by the Tukey’s post hoc test for multiple comparisons in all applicable figures, whereas for data shown in Figure 3, a mixed regression with covariance structure was performed for comparison of 2 variables (time and treatment). Values of *P*<0.05 were considered significant.

Results

Pressor Responses to ET-1 (1-31) in the Anesthetized Rabbit

Basal MAP was averaged at 64.2±0.8 mm Hg and HR at 174.9±3.9 bpm in all animals tested in the present study. Bolus administration (intracardiac) of ET-1 (1-31) and big ET-1 (1 nmol/kg each) induced similar and protracted increases of MAP in the anesthetized rabbit (Figure 1a). On the other hand, ET-1 (0.25 nmol/kg) triggered a classical biphasic response, namely a transient hypotension, followed by a sustained increase in MAP (Figure 1a). In a separate series of experiments, no differences between male and female rabbits were found as far as the pressor response to ET-1 (1-31) (1 nmol/kg) is concerned (males 36.4±3.8; females 34.7±2.1 mm Hg; n=5 in each group).

On the other hand, similar profiles of dose-dependent pressor responses to ET-1 (1-31) and big ET-1 were observed as illustrated in Figure 1b.
Roles of ECE and NEP on Pressor Responses to ET-1 (1-31)

First, dose-dependent inhibitions were performed with phosphoramidon (NEP/ECE inhibitor; 0.01 to 10 mg/kg), thiorphan (NEP inhibitor; 0.01 to 1 mg/kg), and CGS 35066 (ECE inhibitor; 0.1 and 1 mg/kg) against the pressor responses to ET-1 (1-31) (1 nmol/kg). Threshold inhibitory effects of phosphoramidon and thiorphan against the response to ET-1 (1-31) (1 nmol/kg) were established at 0.1 mg/kg ($P < 0.05$ vs control; $n = 5$), whereas CGS 35066 was found inactive at the same dose. Maximal inhibitory doses for phosphoramidon, CGS 35066, and thiorphan were all obtained at 1 mg/kg against ET-1 (1-31) (data not shown).

On the other hand, pressor responses to big ET-1 (1 nmol/kg) were markedly reduced by phosphoramidon (10 mg/kg), and CGS 35066 (1 mg/kg) but not by thiorphan (1 mg/kg; Figure 2). Interestingly, at the same doses as described previously, responses to ET-1 (1-31) (1 nmol/kg) were abolished by phosphoramidon, slightly inhibited by CGS 35066, and thiorphan were all obtained at 1 mg/kg against ET-1 (1-31) (data not shown).

Contribution of ET Receptors to Pressor Responses to ET-1 (1-31)

In another series of experiments, the ETA antagonist BQ-123 (1 mg/kg) markedly reduced pressor responses induced by big ET-1 (1 nmol/kg), ET-1 (1-31) (1 nmol/kg), and ET-1 (0.25 nmol/kg). Each column represents the mean±SEM of at least 5 experiments. *$P < 0.05$ vs control; †$P < 0.05$.

Conversion of Big ET-1 and ET-1 (1-31) to ET-1, Respectively

Reversed-phase HPLC analyses coupled to immunoassays were used to identify the immunoreactive components of the plasma samples. Elution profile of the samples after ET-1 (1-31) administration revealed a major peak at 35.5 minutes of elution time, which coincided with the retention time of the authentic ET-1 standard (Figure 4). Another major peak was identified at 37.0 minutes, which corresponds to the optimal retention time of the authentic ET-1 (1-31) standard.

Figures 5 and 6 depict plasma level variations of IR–ET-1 (1-31) after big ET-1 injection (1 nmol/kg) and IR–ET-1 after administration of ET-1 (1-31) (1 nmol/kg), respectively, in rabbits pretreated with vehicle or phosphoramidon. Noteworthy, we had demonstrated previously that intracardiac administration of big ET-1 generates a phosphoramidon-sensitive increase in plasma ET-1 concentration.

Interestingly, albeit low levels of ET-1 (1-31) were detected in the plasma of control rabbits, phosphoramidon (10 mg/kg) administered before big ET-1 unmasked a significant production of ET-1 (1-31) at 3 time points analyzed for these series of experiments (2.44, 2.32, and 2.01 fmol/mL plasma; Figure 5). Otherwise, cardiac administration of ET-1 (1-31) triggered an increase of ET-1 levels (42.84 fmol/mL plasma; mostly at the 1-minute time point) which was abolished by phosphoramidon (Figure 6). On the other hand, ET-1 (1-31) levels after intracardiac injection of the same
peptide were averaged at 1.53, 48.12, 8.78, and 4.04 fmol/mL plasma at time points 0, 1, 2, and 5 minutes, respectively.

Finally, increases in ET-1 plasma levels after ET-1 (1-31) administration were reduced by 65% in rabbits pretreated with thiorphan but not with CGS 35066 (Figure 7). In contrast, the increase in ET-1 plasma levels after intracardiac administration of big ET-1 were only reduced by 23% in rabbits pretreated with thiorphan (big ET-1/vehicle: 59.67 ± 2.33; + thiorphan: 45.72 ± 2.07 fmol/mL plasma; P < 0.05; n = 8 experiments).

**Discussion**

In the present study, we show for the first time that intracardiac-administered big ET-1 is transformed to ET-1 (1-31) and that the 31-aa intermediate administered by the same route generates ET-1 via NEP/ECE-dependent processes in vivo.

We had shown previously in this same animal model that big ET-1 was converted in the circulation to ET-1 via a phosphoramidon-sensitive process, suggesting that the precursor was inactive per se. Nakano et al reported the existence of ET-1 (1-31), and Hayasaki-Kajiwara et al suggested that this particular intermediate could generate ET-1 via an ECE/NEP process in human cultured bronchial smooth muscle cells. Furthermore, our group also showed that ET-1 (1-31) was a potent pressor and bronchoconstrictor peptide in vivo in the anesthetized guinea pig. The above-mentioned literature and the present study now allow us to suggest that ET-1 (1-31) may be an alternate intermediate in the genesis of ET-1 from big ET-1 in the circulation.

The pharmacological responses to ET-1 (1-31) are similar to those observed previously in the guinea-pig and closely mimic those afforded by big ET-1. Furthermore, in the rabbit, big ET-1 and ET-1 (1-31) are sensitive to ET_A and ET_B receptor antagonists in a similar fashion. Indeed BQ-123 and BQ-788 reduced and potentiated, respectively, the pressor responses to both agonists. Worthy of notice, ET-1 (1-31), similar to big ET-1, does not trigger the initial and transient hypotensive response seen after ET-1 injection in the rabbit model. We had suggested previously that big ET-1 was converted distally from the ET_B endothelial receptors and we can now extend that hypothesis to the intermediate ET-1 (1-31). We can further suggest that ET-1 (1-31), once converted to ET-1, activates in a nonselective fashion ET_A and ET_B receptors in the rabbit model in vivo.

On the other hand, ET-1 (1-31) requires the activity of NEP and ECE to fully induce its pressor effects in vivo in the rabbit model. However, unlike the pressor effects of big ET-1, which are ECE dependent, we suggest that NEP is the predominant enzymatic pathway involved in the pharmacological properties of ET-1 (1-31). We found similar results in the anesthetized guinea pig with the notable exception that in this latter animal species, ECE and NEP were mainly involved in the pressor responses and respiratory resistance.
increases triggered by ET-1 (1-31), respectively.\textsuperscript{11} The hemodynamic properties of ET-1 (1-31), being markedly more sensitive to NEP inhibition in the rabbit than in the guinea pig,\textsuperscript{11} would suggests some interspecies variability.

Interestingly, the production of ET-1 from intracardiac administered ET-1 (1-31) is markedly reduced by phosphoramidon (ECE/NEP inhibitor) and by thiorphan (NEP inhibitor) but not by CGS 35066 (ECE inhibitor). These results further suggest that ET-1 (1-31) is transformed to ET-1 via an enzymatic pathway involving NEP and ECE to a lesser extent. In contrast, thiorphan moderately reduces the production of ET-1 in rabbits preadministered with big ET-1 (1–38), thus supporting a lesser role for NEP than ECE in the conversion of the 38-aa precursor.

We thus confirm, in an in vivo setting, the initial study of Okada et al\textsuperscript{22} demonstrating that the carboxyl-terminal sequence at residues 32 to 37 of big ET-1 is pivotal for a selective conversion by the ECE.

Furthermore, we have shown a dynamic increase of plasma ET-1 (1-31) levels after administration of big ET-1 only under conditions of phosphoramidon treatment (ie, inhibition of NEP and ECE). This result unmask an alternative conversion pathway involving perhaps chymase in vivo as shown previously in vitro by Nakano et al.\textsuperscript{2} However, to confirm this postulate, it will be required to monitor and quantify simultaneously ET-1 (1-31) and ET-1 productions in the circulation after administration of big ET-1 in the rabbit model. We are currently investigating this particular issue.

On the other hand, one must take into account that the present study addresses the production of ET-1 (1-31) or of ET-1 from exogenously administered big ET-1 or ET-1 (1-31), respectively. It is possible that endogenously, the processing of these peptides may be different from that observed in the present study.

Interestingly, ET-1 (1-31) has been reported recently to be elevated in atherosclerotic lesions of hypercholesterolemic hamsters.\textsuperscript{23} Furthermore, chymase (suggested to hydrolyze big ET-1 to ET-1 (1-31)) has been shown to be involved in cardiac fibrosis.\textsuperscript{24} Finally, inflammatory responses related to infiltrating neutrophils stained positive for NEP have been reported in patients with ruptured atherosclerotic lesions.\textsuperscript{25}

In the current experimental settings, our results suggest that ET-1 (1-31) is only involved in a minor fashion as an NEP-dependent intermediate product in the conversion of big ET-1 to ET-1. Indeed, the direct ECE-dependent production of the mature peptide from the 38-aa precursor big ET-1 remains the strongly predominant pathway. Whether the same postulate holds true in conditions of mast cell degranulation (and thus enhanced chymase activity), such as those found in hypercholesteremic animals,\textsuperscript{23} remains to be determined.

**Perspectives**

We have shown that ET-1 (1-31) is involved in the genesis of ET-1 from its precursor big ET-1 in vivo. In contrast to big ET-1, NEP and, to a lesser extent, ECE are involved in the production of ET-1 from the 31-aa intermediate. Thus, big ET-1 and ET-1 (1-31) are suggested to be converted by distinct enzymatic processes in vivo. The involvement of NEP and chymase in the genesis of ET-1 opens a new area of research toward drug design to efficiently interfere with the production of the potent pressor peptide. Albeit ET-1 (1-31) appears to be remotely involved in the production of ET-1 in pharmacological settings such as those put forward in the present study, the implication of the 31-aa intermediate may be greatly enhanced in inflammatory conditions related to cardiovascular diseases. Hence, the monitoring of ET-1 (1-31) as either a marker or putatively as one of the multiple causes of arterial diseases should perhaps be revisited.

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