Characterization and cardiovascular actions of endothelin-1 and endothelin-3 from the American alligator

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Characterization and cardiovascular actions of endothelin-1 and endothelin-3 from the American alligator. Am J Physiol Regulatory Integrative Comp Physiol 282: R594–R602, 2002; 10.1152/ajpregu.00733.2000.—The structures and biological activities of the isoforms of endothelin (ET) in a reptile are unknown. ET-3, whose primary structure is identical to human ET-3 except for the substitution Phe4 → Tyr, and a peptide identical to human ET-1 were isolated from an extract of the lung of the alligator, Alligator mississippiensis. Bolus intravenous injections of alligator ET-3 (10, 30, and 100 pmol/kg) into anesthetized alligators produced dose-dependent decreases in systemic blood pressure (Psys) and systemic vascular resistance (Rsys) without change in heart rate (HR), systemic blood flow (Qsys), pulmonary pressure (Ppul), pulmonary vascular resistance (Rpul), or pulmonary blood flow (Qpul). At a dose of 300 pmol/kg, the initial vasodilatation was followed by an increase in Rsys and decreases in Qsys and Ppul. The response to intravenous human/alligator ET-1 (10, 30, 100, and 300 pmol/kg) was biphasic at all doses with initial decreases in Psys and Rsys being followed by sustained increases in these parameters. In the pulmonary circulation, ET-1 produced a dose-dependent decrease in Qpul and an increase in Rpul during the first phase of the response but no significant change during the second phase. There was no change in HR in response to ET-1. The vasodilator action of arginine, but not ET-1, was attenuated by N^-$-nitro-$-l-arginine methyl ester, indicating that the effect of the peptide is probably not mediated through increased synthesis of nitric oxide. The data demonstrate that the structure of the ET isoforms has been strongly conserved during the evolution of vertebrates but that cardiovascular actions differ significantly between the alligator and mammals, especially in the magnitude and duration of the hypotensive response.

IN MAMMALS, the endothelin (ET) family of peptides comprises three structurally related members: ET-1, ET-2, and ET-3 (6). The amino acid sequences of these peptides have been very strongly conserved among mammals with only mouse ET-2, which contains the substitution Ser^4 → Asn compared with human ET-2, representing a species variant (20). More recently, it has been shown that ETs are synthesized in the tissues of nonmammalian vertebrates, and existing data indicate that the primary structures of these peptides have also been strongly conserved. A peptide that is identical in structure to human ET-1 was isolated from an extract of the stomach of the European green frog Rana ridibunda, and a peptide that is identical to human ET-3 except for a single amino acid substitution (Phe^4 → Tyr) was purified from an extract of the liver in the same species (27). In contrast, only a single molecular component, which differs from human ET-1 by four amino acid substitutions at positions 4–7, was isolated from an extract of the kidney of the trout Oncorhynchus mykiss (26) (Fig. 1).

Preliminary physiological studies indicated that the biological activities of the ET peptides in nonmammalian species have also been well conserved across evolutionary lineages. In several species of mammals, ET-1 stimulates steroidogenesis in dispersed cells from the glomerulosa zone of the adrenal cortex (2), and it has been shown that synthetic frog/human ET-1 and frog ET-3 stimulate both corticosterone and aldosterone production by perifused interrenal slices from R. ridibunda (27). ET-1 was first identified on the basis of its powerful vasoconstrictor activity on vascular smooth muscle from a range of mammalian species (29), and synthetic trout ET potently constricts isolated rings of vascular tissue from trout efferent branchial artery, celiacomesenteric artery, and anterior cardinal vein (26). Studies in vivo have shown that bolus intrarterial injections of trout ET into unanesthetized trout produce complex hemodynamic effects involving increased ventral aortic and central venous pressure, increased gill resistance and systemic resistance, and decreased cardiac output, heart rate (HR), and stroke volume (5).

Before our study, the only ET-like peptides from a reptile to be characterized structurally were the sarafo-
toxins, a family of five isoforms isolated from the venom of the snake *Atractaspis engaddensis* (25). The sarafotoxins, like the ETs, comprise 21-amino acid residues, possess the same pattern of disulfide linkages, and exhibit strong vasoconstrictor activity (8), but the evolutionary relationships between the two families are unclear (9). The present study extends our understanding of the evolution of both structure and cardiovascular activity in the species of origin of ET-1 and ET-3 from the American alligator *A. mississippiensis*.

**MATERIALS AND METHODS**

**Materials.** Alligator ET-3, identical in structure to frog ET-3, was synthesized by solid-phase methodology as previously described (27). Human ET-1, identical in structure to alligator ET-1, was supplied by Peninsula Laboratories (Belmont, CA).

Radioimmunoassay. ET-like immunoreactivity (ET-LI) was measured using an antiserum raised against human ET-1 that shows 60% cross-reactivity with human ET-2, 70% cross-reactivity with human ET-3, but only 0.1% reactivity with big human ET-1-(1-38) (24). 125I-labeled human ET-1 (Amersham Pharmacia, Piscataway, NJ; sp act 74 TBq/mmol) was used as tracer. The minimum detectable concentration using human ET-1 as standard was 1 fmol/tube.

**Tissue extraction.** Alligator tissues were obtained at the Rockefeller Wildlife Refuge (Grand Chenier, LA) from mature specimens (*n* = 8), hatched in 1972 or 1973, that had been euthanized and necropsied to investigate reproductive failure. Lung (1.5 kg) and kidney (0.6 kg) were frozen on dry ice and shipped to Creighton University, where they were stored at −80°C for 2 wk before extraction. The frozen tissues were separately homogenized with 10 vol of ice-cold 3:1 ethanol-0.7 M HCl (vol/vol) using a Waring blender. The homogenates were stirred for 3 h at 0°C and centrifuged (1,600 g, 30 min, 4°C), and ethanol was removed from the supernatants under reduced pressure. Peptide material was isolated from the extracts using Sep-Pak C18 cartridges (Waters Associates, Milford, MA) as previously described (26, 27). Bound material was eluted with acetoni-trile-water-trifluoroacetic acid (70:29:0.1 vol/vol/vol) and freeze-dried.

**Purification of ET-3 and ET-1 from lung.** The lung extract, after partial purification on Sep-Pak cartridges, was redissolved in 1 M acetic acid (8 ml) and chromatographed on a 100 × 2.5-cm column of Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 1 M acetic acid. The column was eluted at a flow rate of 48 ml/h, and fractions (8 ml) were collected. Absorbance was measured at 280 nm. The concentration of ET-LI in the fractions was determined by radioimmunoassay at a dilution of 1:30. Fractions containing ET-LI were pooled and pumped onto a 25 × 1-cm Vydac 218TP510 (C 18) reverse-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with 0.1% (vol/vol) trifluoroacetic acid-water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% over 10 min and to 49% over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm, and fractions (1 min) were collected. Alligator pulmonary ET-3 and ET-1 were purified to near homogeneity by successive chromatographies on 250 × 4.6-mm Vydac 214TP510 (C 18), Vydac 219TP54 (phenyl), and Vydac 218TP54 (C18) columns at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting
solvent was raised 21 to 42% over 40 min using a linear gradient.

**Purification of ET-3 from kidney.** The kidney extract, after partial purification on Sep-Pak cartridges, was chromatographed on a Sephadex G-25 column under the same conditions used for the renal extract. Pooled fractions containing ET-LI were chromatographed on Vydac C18, C4, and phenyl columns under the same conditions used for the purification of alligator pulmonary ET-3.

**Structural analysis.** The primary structures of the peptides were determined by automated Edman degradation.

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Fig. 2. Reverse-phase HPLC on a semipreparative Vydac 218TP510 (C18) column of an extract of alligator lung (A) and kidney (B), after partial purification on Sep-Pak cartridges and by gel-permeation chromatography. The fractions (1 min) containing ET-like immunoreactivity, denoted by the bar, were pooled for further purification. Dashed line shows the concentration of acetonitrile in the eluting solvent. ABS280, absorbance at 280 nm.

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Fig. 3. Purification by reverse-phase HPLC of pulmonary alligator ET-3 on a semipreparative Vydac C18 column (A), analytic Vydac C4 column (B), analytic Vydac phenyl column (C), and analytic Vydac C18 column (D). Individual peaks were collected by hand, and arrows indicate where peak collection began and ended. Alligator pulmonary ET-1 and renal ET-3 were purified under similar conditions of chromatography. ABS214, absorbance at 214 nm.
using an Applied Biosystems Precise 491A sequenator (Foster City, CA). Mass spectrometry of the peptide was performed on a Voyager RP MALDI-TOF instrument (Perspective Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction, and the accelerating voltage in the ion source was 25 kV. Approximately 10 pmol of sample was used, and the accuracy of the mass determinations was at least 0.05%.

Surgery for cardiovascular studies. Alligators of both sexes (0.9–12.0 kg) were obtained from the Rockefeller Wildlife Refuge and were kept at 30°C on a 12:12-h dark-light cycle. The animals were not fed for 7 days before surgery. On the day of experimentation, the animals were anesthetized by intramuscular injection of 50 mg/kg pentobarbital sodium (Veterinary Laboratories, Lenexa, KS) ~2 h before surgery. Animals were placed in a supine position, tracheostomized, and ventilated with a gas mixture of composition 67% nitrogen-30% oxygen-3% carbon dioxide (3 breaths/min) at a tidal volume of 30–40 ml/kg (SAR-830 Ventilator, CWE, Ardmore, PA). Occlusive PE-50 polyethylene catheters were inserted into the right femoral artery and vein. The arterial catheter was connected to a Statham P23 pressure transducer, which was used for measurements of systemic arterial blood pressure. The venous catheter was used for injections of test substances. To access the central blood vessels, a 5- to 10-cm ventral incision was made through the skin, and the posterior one-third of the sternum was cut and separated with retractors. The vessels were carefully cleared from connective tissues. For measurements of blood flow, transit-time ultrasonic blood flow probes were placed on the left aorta (QLa, on one branch of the right aorta (QRAa), and on the left pulmonary artery (QLPa). The right pulmonary artery was nonocclusively cannulated using the Seldinger technique (28) for measurements of pulmonary arterial blood pressure. Body temperature was continuously monitored using a thermometer inserted 3–4 cm into the cloaca.

Hemodynamic measurements. All measurements were made on anesthetized animals at an ambient temperature of 30 ± 2°C. Signals from blood pressure transducers and blood flowmeters were continuously collected onto a computer with a commercial data-acquisition system (AcqKnowledge MP 100, Goleta, CA) sampling at 10 Hz. HR was calculated from the pulsatile systemic pressure signal. Experiments were begun 30 min after surgery. Baseline values of blood flow, blood pressures, and HR were recorded for 15 min. Increasing doses of alligator ET-3 (10, 30, 100, and 300 pmol/kg) were injected at intervals of between 15 and 60 min, depending on the time necessary to allow the cardiovascular parameters to stabilize between injections. Approximately 2 h after the last injection of alligator ET-3, ET-1 (10, 30, 100, and 300 pmol/kg) was injected following the same procedures used for ET-3. After the experimental protocol was complete, all animals were euthanized by intracardiac injections of KCl.

RESULTS

Purification of ET-3 and ET-1 from alligator lung. The extract of alligator lung, after partial purification on Sep-Pak cartridges, was subjected to gel-permeation chromatography on a Sephadex G-25 column. ET-LI was eluted as a broad peak with partition coefficient KAV between 0.55 and 0.75. These fractions were pooled and chromatographed on a semi-preparative Vydac C18 column, and the elution profile is shown in Fig. 2A. ET-LI was associated with two well-separated fractions denoted by the bars. Alligator pulmonary ET-3 was purified to near homogeneity, as assessed by peak symmetry, by successive chromatographies on a semi-preparative Vydac C4 column (Fig. 3A), an analytic Vydac C4 column (Fig. 3B), an analytic Vydac phenyl column (Fig. 3C), and an analytic Vydac C18 column (Fig. 3D). Alligator ET-1 was purified under the same conditions of chromatography. The final

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**Fig. 4.** Effect on systemic arterial blood pressure (Psys; in kPa) of bolus injections of increasing doses of alligator ET-3 (10, 30, 100, and 300 pmol/kg) in a single animal.
yields of pure peptides were ~30 pmol (ET-1) and 70 pmol (ET-3).

Purification of ET-3 from alligator kidney. The extract of alligator kidney, after partial purification on Sep-Pak cartridges, was subjected to gel-permeation chromatography on a Sephadex G-25 column. ET-LI was eluted as a broad peak in the same fractions as the pulmonary ET. These fractions were pooled and chromatographed on a semipreparative Vydac C₁₈ column, and the elution profile is shown in Fig. 2B. ET-LI was associated with the single fraction denoted by the bar. Alligator renal ET-3 was purified to near homogeneity, as assessed by peak symmetry, by successive chromatographies on semipreparative Vydac C₄, analytic Vydac C₄, analytic Vydac phenyl, and analytic Vydac C₁₈ columns under the same conditions used for the purification of ET-3 from lung (chromatograms not shown). The final yield of pure peptide was ~40 pmol.

Structural characterization. The primary structures of the alligator ET peptides were determined by Edman degradation using an automated microsequence analyzer. The amino acid sequence of alligator ET-1 was established as Xaa-Ser-Xaa-Ser-Ser-Leu-Met-Asp-Lys-Glu-Xaa-Val-Tyr-Phe-Xaa-His-Leu-Asp-Ile-Ile-Trp. No phenylthiohydantoin-coupled amino acid derivatives were detected during cycles 1, 3, 11, and 15, which is consistent with the presence of cystine residues at these positions. The structure of alligator ET-1, including the presence of two disulfide bridges, was confirmed by mass spectrometry. The observed molecular mass of the peptide was 2,493 ± 1 Da compared with a calculated average molecular mass of 2,492 Da for the proposed structure.

The primary structure of alligator pulmonary and renal ET-3 was the same and was established as Xaa-Thr-Xaa-Tyr-Thr-Tyr-Lys-Asp-Lys-Glu-Xaa-Val-Tyr-Tyr-Xaa-His-Leu-Asp-Ile-Ile-Trp. Again, no phenylthiohydantoin-coupled amino acid derivative was detected during cycles 1, 3, 11, and 15. The proposed amino acid sequence, including the presence of two cystine bridges, was confirmed by mass spectrometry (observed average molecular mass 2,659 ± 1 Da; calculated average molecular mass of 2,659 Da).

Hemodynamic effects of alligator ET-3. Intravenous injection of synthetic alligator ET-3 in the dose range 10–100 pmol/kg produced an immediate monophasic and concentration-dependent hypotensive response of short duration. The time course of the responses of Psys to increasing doses of the peptide in a single animal is shown in Fig. 4. The fall in Psys was accompanied by a decrease in Rsys, but there was no significant change in Qsys, Rps, pulmonary pressure (Pps; Fig. 5). At the highest dose tested (300 pmol/kg)
pmol/kg), the response was biphasic, with the initial fall in Psys being followed by significant rise in Rsys, leading to a small but nonsignificant increase in blood pressure. Qsys, Qpul, and Ppul decreased, but because of the variability of the response, there was no significant change in Rpul. There was no change in HR at any dose of ET-3 tested.

Hemodynamic effects of human/alligator ET-1. In contrast to the effects of ET-3, the response to synthetic ET-1 was biphasic at all doses tested. The time course of the response of Psys to increasing doses of ET-3 in a single animal is shown in Fig. 6. As shown in Fig. 7, intra-arterial injection of ET-1 produced an immediate fall in Psys that was significant even at a dose of 10 pmol/kg, but this was followed by a prolonged hypertensive response. The hypertensive phase lasted for approximately 10–15 min at the 10-pmol/kg dose and 45–60 min at the 300-pmol/kg dose, before the pressure returned to preinjection levels. During the initial hypotensive phase, Rsys decreased, and a significant increase in Qsys was observed at the 10- and 30-pmol/kg doses (Fig. 7). Qpul showed a dose-dependent decrease and mean Ppul did not change, indicating that Rpul increased. During the second hypertensive phase, Rsys increased and Qsys progressively decreased (Fig. 7). The only change in hemodynamic parameters was a small increase in Ppul at the highest dose (300 pmol/kg). As in the case of alligator ET-3, there was no change in HR after injection of ET-1 at any dose tested.

Effects of L-NAME. As shown in Fig. 8, separate intravenous injections of ET-1 (300 pmol/kg) and L-arginine (150 mg/kg) produced a rapid and significant hypotensive response in animals equipped only with arterial and venous catheters. After administration of L-NAME, the mean arterial blood pressure was significantly elevated, but there was no difference in the hypotensive response to ET-1 before or after L-NAME injection. In contrast, the response to L-arginine was significantly attenuated after administration of L-NAME.

DISCUSSION

In most mammalian tissues, ET is secreted by the constitutive pathway (19), resulting in steady-state concentrations of the peptide that are very low. This poses a challenge to the peptide chemist wishing to obtain sufficient pure material to permit structural characterization, but recent advances in the instrumentation of microsequence analysis allow amino acid sequence determination of very low picomole amounts of a specific peptide. Studies in mammals have shown the lung (15) and kidney (1) not only synthesize ET but are sites of uptake of circulating ET that is internalized through receptor-mediated endocytosis. The alligator lung and kidney were chosen as the tissues from which to extract the peptides, but it is unclear to what extent the ET isoforms isolated in this study represent peptide that is synthesized in the tissues rather than material that has been taken up by the organ from the circulation. This article describes the purification of two components corresponding to ET-1 and ET-3 from lung, together with a single molecular form corresponding to ET-3 from kidney. The primary structures of these peptides are compared with those of previously characterized ET isoforms in Fig. 1.

Our study has demonstrated that the primary structures of the ET isoforms have been very strongly conserved throughout the evolution of tetrapods. ET-1 is structurally identical in an alligator, human, and frog. Alligator ET-3 is the same as in a frog and differs from human ET-3 by a single conservative amino acid substitution (Phe → Tyr). It has been proposed that the ET family of peptides arose from a series of gene duplication events (9). Duplication of the gene encoding an ancestral ET gave rise to a gene encoding ET-3 and a second gene encoding the ancestor of ET-1 and ET-2. A subsequent duplication of this latter gene gave rise to separate genes encoding ET-1 and ET-2. As all mammalian species yet studied synthesize the three ET isoforms, both gene duplications are presumed to have occurred before the appearance of mammals. Our failure to isolate ET-2 from the alligator tissues examined suggests, but of course does not prove, the hypothesis that the second gene duplication took place after the appearance of the reptiles, possibly within the mammalian lineage.

The biosynthesis of the ET isoforms in mammals is atypical in that posttranslational processing of preproendothelin at the site of dibasic amino acid residues by the well-characterized prohormone convertases produces a big ET, of between 38 and 41 amino acids depending on the species, that has low biological potency (19). Big ET is further processed by a highly selective enzyme, ET-converting enzyme, that exists in several isoforms and cleaves at the Trp21-Val22 bond in big ET-1 and big ET-2 and at the Trp21-Ile22 bond in big ET-3 (22). The isolation of alligator ET-1 and ET-3 in 21-amino acid residue forms indicates that preproendothelins are processed in alligators, as in a teleost fish.
and an amphibian (27), by a pathway similar to that in mammals.

The cardiovascular actions of the ET isoforms in mammals are mediated through interaction with two well-characterized receptors: the ETA receptor that is selective for ET-1 and ET-2 and the ETB receptor that exhibits similar affinities for all three isopeptides (23). Activation of the ETA receptor on vascular smooth muscle cells leads to vasoconstriction, whereas activation of the ETB receptor on endothelial cells elicits vasodilatation by a mechanism that involves the formation of nitric oxide (14). However, studies using ETB-selective agonists and antagonists have identified ETB receptors on vascular smooth muscle that also mediate vasoconstriction (13).

Our data demonstrate that the hemodynamic responses of the alligator to ET-1 and ET-3 are qualitatively similar to the responses in mammals, but the magnitude and duration of the hypotensive phase are appreciably greater. For example, intravenous bolus injection of ET-1 (250 pmol/kg) into unanesthetized, normotensive rats produces a fall in mean arterial blood pressure of 3.5 kPa of 1-min duration followed by a sustained rise with a maximum increase of 3.7 kPa occurring 10 min after administration (12). The effects of alligator ET-1, at all doses tested, are biphasic with an initial systemic vasodilation and fall in Psys followed by a prolonged constriction of the systemic vasculature and hypertension. The changes in Qpul are probably reflections of changes in cardiac output rather than direct effects on the pulmonary vasculature and hypertension. The lack of effect of ET-1 on HR contrasts with the bradycardia seen in unanesthetized trout (5, 10) and is probably a consequence of the fact that
anesthetic used in this study abolishes the baroreceptor-mediated cardioinhibitory reflex in reptiles (3).

Alligator ET-3, at doses up to 100 pmol/kg, elicits dose-dependent decreases in R<sub>sys</sub>, leading to a fall in arterial blood pressure that persisted for up to 10 min. At the highest dose tested (300 pmol/kg), the sustained hypotensive phase (up to 20 min) was followed by a significant increase in R<sub>sys</sub>, but the effect on arterial blood pressure was not significant. By way of comparison, a bolus injection of ET-3 (1.1 nmol) into pentobarbital sodium-anesthetized cats produced a decrease in P<sub>sys</sub> of ~4.3 kPa whose duration was only 30–120 s (11). As found with ET-1, effects of ET-3 in the systemic circulation were more pronounced than in the pulmonary circulation. The only effects of ET-3 on the pulmonary circulation were a decrease in pressure and flow after the maximum dose, similar to the effects seen in the systemic circulation at the same time. The reduction in blood pressure was probably initiated by a decrease in R<sub>sys</sub>, leading to a decreased venous return to the heart and a subsequent reduction in stroke volume and cardiac output.

The circulating concentrations of ET-LI in the alligator are below the detection limit of the radioimmunoassay used in this study (unpublished data) so that injection of ET, even in a dose as low as 10 pmol/kg, probably produces plasma levels of the peptide that are supraphysiological. It may be argued, therefore, that the cardiovascular effects reported here may be more pharmacological than physiological in character. On the other hand, the study has shown that ET is synthesized in alligator lung and kidney and may also be made in the endothelial cells of vascular tissue, as in mammals (29). Consequently, the peptide may exercise its regulatory effects on cardiovascular function by a mechanism that involves a paracrine rather than an endocrine action.

**Perspectives**

Collectively, our data, together with earlier studies in the trout (5, 10) and amphibia (18), demonstrate that the hypertensive action of ET-1 probably arose early in vertebrate evolution, and its retention implies that it has an important regulatory function. For example, bolus injections of trout ET (667 pmol/kg) into unanesthetized trout result in a twofold increase in ventral aortic pressure (5), and human/frog ET-1 potently (EC<sub>50</sub> < 10 nM) constricts isolated vascular rings prepared from arteries and veins of the frog *Rana pipiens* (18). In contrast, the hypotensive actions of ET-1 and ET-3 were not observed in trout even at high doses and are appreciably less in those mammals yet studied compared with the alligator. The diverse actions of the ET isoforms on the cardiovascular system may involve activation of several effector systems, which include the phospholipase C, phospholipase A<sub>2</sub>, phospholipase D systems and the adenylate cyclase and guanylate cyclase transduction pathways (4). The involvement of nitric oxide in mediating, at least in part, the vasodilator action of ET in mammals is well established (14, 23), but it has been asserted that nonprostanoid endothelium-derived relaxing factors, such as nitric oxide, are not produced in trout vessels (16). Immunohistochemical studies demonstrated the presence of nitric oxide synthase in blood vessels in the submucosa and on the serosal surface of the intestine of the crocodile *Crocodylus porosus* (17). Several neuropeptides whose vasodilator actions are linked to nitric oxide synthesis, such as substance P, neurokinin A, and calcitonin gene-related peptide, produced relaxation of the celiac vascular bed in this species (7). The hypotensive action of L-arginine in the American alligator suggests that P<sub>sys</sub> in the animal is under tonic regulation by nitric oxide, but the lack of effect of L-NAME on the hypotensive action of ET-1 indicates that increased nitric oxide synthesis is probably not responsible for the fall in blood pressure produced by the peptide. Future studies will assess the involvement of other possible mediators of the hypotensive response to the ET isoforms, such as prostaglandins and leukotrienes.

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