Purification, structural characterization, and myotropic activity of endothelin from trout, Oncorhynchus mykiss

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Endothelin (ET) from a nontetrapod species has never been characterized. In the present study, the only nonmammalian ET-like peptide to be characterized structurally were the sarafotoxins, a family of five isoforms, isolated from the venom of the snake, Atractaspis engaddensis (30). The sarafotoxins, like the endothelins, comprise 21-amino-acid residues, possess the same pattern of disulfide linkages, and exhibit strong vasoconstrictor activity (3,12). However, structural similarity between genes encoding the biosynthetic precursors of the sarafotoxins (29) and the ETs (8) is minimal outside the bioactive region so that the evolutionary relationships between the two families are unclear.

Evidence that tissues of nonmammalian vertebrates express ET receptors has also been obtained. With the use of 125I-labeled sarafotoxin-b as tracer, specific binding sites for ET-related peptides were identified in membrane preparations from the heart and brain of the Torpedo and tilapia (35). Similar studies using 125I-labeled porcine ET-1 revealed the presence of specific binding sites in gill, heart, liver, kidney, and intestine of the trout (17). High-affinity ET-1 binding sites have also been identified in liver (20), heart (14), and oocytes (15) of the toad Xenopus laevis. Two Xenopus ET-receptor subtypes have been cloned and characterized: ET_A from heart (14) and ET_C from dermal melanophores (9), and a novel ET-receptor subtype (termed ET_B) in quail has also been cloned and characterized (16).

Functional studies have shown that the vasculatures of fish and amphibia are extremely sensitive to ET, thereby suggesting that the cardiovascular role of this peptide has been highly conserved during the course of vertebrate evolution. For example, human ET-1 potently (EC_{50} < 10 nM) constricts isolated vascular rings prepared from arteries and veins of the frog, Rana pipiens (22), toad, Bufo viridis (3), catfish, Amiurus melas (22), trout, Oncorhynchus mykiss (21), and spiny dogfish, Squalus acanthias (4). Bolus injection of low doses of porcine ET-1 (500 ng/kg body weight) into unanesthetized trout produced transient depressor and sustained pressor responses, as in mammals, with higher doses (1,500 ng/kg body weight) producing a triphasic (pressor-depressorpressor) response (21). In isolated heart preparations of the elasmobranch, Tor-
pedo ocellata and the teleost, Oreochromis niloticus (tilapia), concentrations of ET-1 and sarafotoxin-b as low as 50 ng/ml induced positive inotropic effects, reduction of the contraction rate, and arrhythmia (35).

In most mammalian tissues, ET is secreted by the constitutive pathway (19) with the result that steady-state concentrations of the peptide in tissues are very low. This poses a challenge to the peptide chemist to obtain sufficient pure material to permit structural characterization, but recent advances in the instrumenta-
tion of microsequence analysis allow amino acid sequence determination of very low picomole amounts of peptide. This study extends our understanding of the evolution of the ET family of peptides by describing the purification, structural characterization, and chemical synthesis in the biologically active form of ET from a teleost fish, the steelhead trout (Onchorhyncus mykiss).

MATERIALS AND METHODS

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) (31). 125I-labeled human ET-1 (Amersham Life Science, Arlington Heights, IL; specific activity 74 TBq/mM) was used as tracer. The minimum detectable concentration using human ET-1 as standard was ~1 fmol/tube.

Tissue extraction. In a preliminary experiment, whole brain, heart, intestine, gill, liver, kidney, and caecalomesen-
teric artery (CMA) from three specimens of adult rainbow trout were pooled and extracted with 10 volumes of ice-cold 3:1 ethanol/0.7 M HCl (by vol) using a Polytron homogenizer. Pooled plasma (10 ml) from three specimens was extracted by the same procedure. After centrifugation (1,600 g, 30 min, 4°C), an aliquot of each extract was freeze-dried and reconsti-
tuted in radioimmunoassay buffer (0.1 M sodium phosphate, pH 7.4, containing 0.4% bovine serum albumin) to give a final concentration equivalent to ~100 mg tissue/ml.

For the preparative study, frozen kidney (1,006 g) from steelhead trout (migratory Skamania strain) was homog-
ized with 10 volumes of ice-cold 3:1 ethanol/0.7 M HCl (by vol) using a Waring blender. The homogenate was stirred for 3 h at 0°C, centrifuged (1,600 g, 30 min, 4°C), and ethanol was removed from the supernatant under reduced pressure. Pep-
tide material was isolated from the extract using Sep-Pak C18 cartridges (Waters Associates, Milford, MA) as previously described (33). Bound material was eluted with a mixture of acetonitrile, water, and trifluoroacetic acid (70:0.29:9.01, by vol) and freeze-dried.

Purification. The tissue extract, after partial purification on Sep-Pak cartridges, was redissolved in 1% (by vol) acetic acid/water (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 concentra-
tion of ET-LI in the fractions was determined by radioim-
monoassay at a dilution of 1:100. Fractions containing ET-LI were pooled and pumped onto a 25 × 1-cm Vydac 218TP510 (C18) reverse-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with 0.1% (by 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. Absor-
bance was monitored at 214 and 280 nm, and fractions (1 min) were collected. The fraction containing ET-LI was rechromatographed on a 25 × 1-cm Vydac 214TP510 (C18) column equilibrated with a mixture of acetonitrile, water, and trifluoroacetic acid (21:0.78:9.01; by vol) at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 42% over 40 min using a linear gradient. Trout ET was purified to near homogeneity by successive chromatographies on 250 × 4.6-mm Vydac 214TP54 (C18), 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 to 21% over 40 min using a linear gradient.

Structural analysis. The primary structure of the peptide was determined by automated Edman degradation using a Perkin-Elmer Procise 491A sequenator. 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%.

Peptide synthesis. Trout ET was synthesized by solid-phase methodology on a 0.025 mM scale on an Applied Biosystems model 432A peptide synthesizer using a 4-(2,4-dimethoxy-
phenyl)-Fmoc-aminomethyl)phenoxycetamidemethyl resin (Perkin Elmer, Foster City, CA). Fmoc amino acid derivatives were activated with O-benzotriazol-1-yl-N,N,N’-tetra-
methyluronium hexafluorophosphate (1 equivalent), 1-hy-
droxy-benzotriazole hydrate (1 equivalent), and diisopropyl-
ethylamine (2 equivalents). Deprotection of the NH2 terminus by piperidine was monitored by online measurement of the conductance of the carbamate salt of the Fmoc group, and optimum coupling times were determined by the instrument in response to the deprotection times. The peptide was cleaved from the resin with a mixture of trifluoroacetic acid, water, thioanisole, and 1,2-ethanethiol (90:0.2:50:50:50) at 25°C for 3 h. The unpurified peptide was dissolved in 0.1 M ammonium acetate, pH 8.0 (500 ml) and subjected to air oxidation at room temperature for 24 h. The crude cyclized peptide material was isolated from the extract using Sep-Pak C18 cartridges (Waters Associates, Milford, MA) as previously described (33). Bound material was eluted with a mixture of acetonitrile, water, and trifluoroacetic acid (70:0.29:9.01, by vol) and freeze-dried.

Isolated trout vessels. The CMA, the efferent branchial artery (EBA) from the third gill arch, and the anterior cardinal vein (ACV) were removed from anesthetized (0.4 g/l benzocaine) rainbow trout (kamloops strain; 0.3–0.6 kg) and placed in HEPES-buffered saline (HBS) at 4°C. With the use of a procedure previously described (21), loose connective tissue and blood were removed from the adventitia and 3- to 4-mm wide rings were cut from the center of each vessel. The vascular rings were suspended from 280-µm diameter steel hooks in individual 20-ml water-jacketed (12 ± 1°C) chambers containing HBS continuously gassed with room air. Tension was measured with Grass FT03C force-displacement transducers and recorded on a Gould series 8000s polygraph. The rings were equilibrated at a resting tension of 500 mg (200 mg for ACV) for 1 h, contracted with either 10−5 M epinephrine (CMA and EBA) or 10−3 M acetylcholine (ACV), washed three times with HBS, and reequilibrated at a
tension of 500 mg for 30 min. The effect of trout ET-1 (10^{-11} - 3 \times 10^{-7} \text{ M}) on the tension of the rings was measured by cumulative addition of the peptides. Comparisons between vessels were made with ANOVA (one way with Bonferroni adjustment) with the significance level set at P < 0.05. All values are means ± SE.

Isolated rat vessels. White male Wistar rats (300–500 g) were anesthetized with an intraperitoneal injection of nembutal (2 mg/kg body weight), and the abdominal aorta was exposed by a midline incision and removed. The vessels were cleared of blood, and 3-mm long aortic rings were placed in Krebs-Henseleit solution (37°C, pH 7.4) in the smooth muscle chambers described in isolated trout vessels and gassed with 95% O_{2}-5% CO_{2}. Approximately 300 mg of resting tension was applied for 1 h before; the vessels were then contracted with 40 mM KCl, rinsed, and baseline tension was reestablished for an additional 1 h prior to experimentation. Other procedures were identical to those used on the trout vessels.

Solutions. HBS consisted of (in mM): 145 NaCl, 3 KCl, 2 CaCl_{2}, 0.57 MgSO_{4}, 5 glucose, 7 HEPEES sodium salt, and 3 HEPEES (acid form); pH adjusted to 7.8. Krebs-Henseleit solution consisted of (in mM): 15 NaCl, 25 NaHCO_{3}, 1.38 NaH_{2}PO_{4}, 2.51 KCl, 2.46 MgSO_{4}, 1.91 CaCl_{2}, and 5.56 glucose; pH 7.4.

RESULTS

ET-LI in extracts of trout tissue. Extracts of trout whole brain, heart, intestine, gill, liver, and CMA, at a concentration of ~100 mg tissue/ml, and the extract of trout plasma did not contain detectable ET-LI. Only the extract of trout kidney contained material that inhibited the binding of \textsuperscript{125}I-labeled human ET-1 to the antiserum to human ET-1, but the immunoreactivity in serial dilutions of the extract did not diminish in parallel with the human ET-1 standard curve.

Peptide purification. The extract of trout 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 with a distribution coefficient (K_{AV}) between 0.55 and 0.75. These fractions were pooled and chromatographed on a semipreparative Vydac C_{18} column, and the elution profile is shown in Fig. 1. ET-LI was associated with the single fraction denoted by the bar. Trout ET was purified to near homogeneity, as assessed by peak symmetry, by successive chromatographies on a semipreparative Vydac C_{18} column (Fig. 2A), an analytical Vydac C_{18} column (Fig. 2B), an analytical Vydac phenyl column (Fig. 2C), and an analytical Vydac C_{18} column (Fig. 2D). The final yield of pure peptide was ~40 pmol, estimated from its absorbance at 214 nm.

Structural characterization. The primary structure of trout ET was determined by Edman degradation using an automated microsequence analyzer. The amino acid sequence of the peptide was established as Xaa-Ser-Glu-Asp-Lys-Glu-Xaa-Ala-Thr-Phe-Leu-Asp-Lys-Glu-Xaa-Val-Tyr-Phe-Xaa-His-Leu-Asp-Ile-Ile-Trp. No phenylthiohydantoin-coupled amino acid derivative was detected during cycles 1, 3, 11, and 15, which is consistent with the presence of cystine residues at these positions. The structure of trout ET, including the presence of four cystine residues, was confirmed by mass spectrometry. The observed molecular mass of the peptide was 2,507 ± 1 Da compared with a calculated mass of 2,506.0 Da for the proposed structure.

Chemical synthesis. A previous study (13) has shown that air oxidation of fully reduced human ET-1 at a pH > 7 and at high dilution results in formation of an oxidized product with disulfide bridges between Cys\textsuperscript{1} and Cys\textsuperscript{5} and between Cys\textsuperscript{3} and Cys\textsuperscript{11} (the pattern in naturally occurring ET) in ~75% yield. Cyclization of synthetic linear trout ET under the same experimental condition and subjecting the reaction mixture to reversed-phase HPLC led to the elution profile shown in Fig. 3. The three major peaks in the chromatogram (designated 1, 2, and 3) were analyzed by Edman degradation and shown to have the same amino acid sequence as the endogenous peptide. However, at a concentration of ~10^{-7} \text{ M}, only peak 2 produced a strong and sustained contraction of isolated vascular rings of the trout epibranchial artery. It is concluded, therefore, that peak 2 represents authentic trout ET, whereas peaks 1 and 3 represent incorrectly folded analogs. After a further chromatography on a Vydac C_{18} column, the final yield of pure trout ET was 3.1 mg. The identity of the synthetic peptide was confirmed by mass spectrometry (observed molecular mass 2,506 ± 1 Da; calculated molecular mass 2,506.0 Da).

Myotropic activity. The responses of trout vessels to trout ET and to rat or human ET-1 are shown in Figs. 4–6. Synthetic trout ET produced concentration-dependent constrictions of isolated vascular rings from EBA (pD\textsubscript{2} = 7.90 ± 0.06, n = 5), ACV (pD\textsubscript{2} = 8.57 ± 0.25, n = 4), and CMA (pD\textsubscript{2} = 8.03 ± 0.04, n = 4) (pD\textsubscript{2} = -log EC\textsubscript{50}). Trout ET also produced concentration-dependent constriction of the isolated rat abdominal aorta (AO; pD\textsubscript{2} = 8.86 ± 0.08, n = 7; Fig. 6). Rings from ACV were responsive to significantly (P < 0.001) lower concentrations of trout ET than either CMA or EBA. The EBA, ACV, and AO were more sensitive to mammal-
lian ET-1 (pD$_2$ for human ET-1: EBA = 9.12 ± 0.14; ACV = 9.90 ± 0.15; AO = 8.86 ± 0.08; all P < 0.001). However, there was no significant difference in the maximum tension produced by either peptide in these vessels.

**DISCUSSION**

The primary structure of trout ET is compared with the structures of three isoforms of human ET in Fig. 7. The amino acid sequence of each isoform has been

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**Fig. 2.** Purification by reverse-phase HPLC of trout ET on a semipreparative Vydac 214TP510 (C$_4$) column (A), analytical Vydac 214TP54 (C$_4$) column (B), analytical Vydac 219TP54 (phenyl) column (C), and analytical Vydac 218TP54 (C$_{18}$) column (D). Individual peaks were collected by hand, and arrows indicate where peak collection began and ended. ABS$_{214}$ absorbance measured at 214 nm.

**Fig. 3.** Purification by reverse-phase HPLC on a Vydac C$_{18}$ column of synthetic trout ET prepared by air oxidation of uncyclized peptide. Peak, delineated by arrows (peak 2), produced constriction of rings of vascular tissue from trout epibranchial artery and was used in further studies. Peaks 1 and 3 were inactive.

**Fig. 4.** Effects of increasing concentrations of synthetic trout ET and rat/human ET-1 on tension of isolated vascular rings from trout efferent branchial artery from third gill arch. Data are expressed as %maximum constriction produced by peptides (means ± SE; n = 5). Inset: maximum tension (mg tension/mg vessel wet wt.) produced by trout or rat/human ET.
strongly conserved among those mammalian species yet studied (human, pig, dog, rabbit, ox, and rat; reviewed in Refs. 23 and 26), with the only species-related sequence difference being the substitution Ser4 = Asn in mouse ET-2 (24). Trout ET contains three amino acid substitutions (Ala4 = Ser, Thr5 = Ser, and Phe6 = Trp) compared with human ET-2 and an additional substitution (Leu7 = Met) compared with human ET-1. On this basis alone, however, it would be unreasonable to claim that the trout peptide was the homolog of mammalian ET-2 rather than ET-1 as the mammalian kidney synthesizes both ET-1 and ET-2 (25). By way of comparison with a neuroendocrine peptide of comparable size, trout gastrin-releasing peptide contains nine amino acid substitutions and a four-residue deletion compared with human gastrin-releasing peptide (7). 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 prohormone convertases produces a “big” ET of between 38 and 41 amino acids, depending on the species, that has low biological potency (11). Big ET is further processed by the highly selective ET-converting enzymes that exist in several isoforms and cleaves at the Trp21-Val22 bond in big ET-1 and ET-2 and at the Trp21-Ile22 bond in ET-3 (25). The isolation of trout ET in a single 21-amino-acid form suggests that preproendothelin is probably processed in teleost fish by a similar pathway as in mammals. Our data do not exclude the possibility that big ET is also present in trout tissues as the antiserum used in this study for detection does not recognize big ET.

The origin of the ET isolated from the trout kidney is uncertain. Whereas the material may represent, at least in part, peptide that is synthesized in the kidney, it may also represent circulating ET that has been internalized by the organ through receptor-mediated endocytosis. ET-like immunoreactivity has been measured in the plasma of a range of teleost species (32), and receptor-mediated uptake of circulating ET-1 by the rat kidney has been described (1). In mammals, both paracrine and endocrine roles for ET in the regulation of renal blood flow, glomerular filtration rate, and sodium and water excretion have been proposed (26). However, continuous infusions of human ET-1 at rates up to 30 ng·kg⁻¹·min⁻¹ into unanesthetized trout had no effect on urine flow, osmolarity, and electrolyte concentrations (21). The failure to detect ET-LI in extracts of the other trout tissues examined was unexpected, especially in light of the observation that extracts of brain, pituitary, liver, kidney, stomach, and interrenal gland of the frog Rana ridibunda contained detectable concentrations of ET-LI measured using the same antiserum (Y. Wang and J. M. Conlon, unpublished observation). At this time, a plausible explanation for the discrepancy between the two species is not available, and clearly further studies are warranted to investigate the localization of ET in trout tissue by immunohistochemistry and/or in situ hybridization.

The actions of ET in mammals are mediated through interaction with two well-characterized receptors. The ETA receptor is selective for ET-1 and ET-2, whereas the ETB receptor exhibits similar affinities for all three
The study has demonstrated that the amino acid sequence of ET has been strongly conserved during the evolution of vertebrates. However, the isolation of only one single molecular form from trout tissues suggests that the putative gene duplications that have led to three ET isoforms in mammals may have taken place after the evolution of tetrapods. Consistent with this hypothesis, a related study has led to the purification from tissues of the frog Rana ridibunda of ET-1 with an amino acid sequence identical to human ET-1 and ET-3 that differs from human ET-3 by only one amino acid residue (Y. Wang, I. Remy-Jouet, C. Delarue, H. Vaudry, and J. M. Conlon, unpublished observation). The fact that the vasoconstrictor action of trout ET on trout vascular tissue is the same as the action of the mammalian ET isoforms on mammalian tissue is indicative of the fact that the role of ET in hemodynamic regulation has also been conserved across phylogenetic lines. The increased potency of human ET-1 relative to trout ET in constricting trout vessels was unexpected but may reflect the fact that human ET-1 is degraded more slowly than the native ET by peptidases in trout tissue. This result is not without precedent as, for example, salmon calcitonin is used therapeutically as a more potent and effective form of the hormone in the human.

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