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V Niranjan, … , R D Gerard, M Yanagisawa

Research Article

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Systemic Hypertension Induced by Hepatic Overexpression of Human Preproendothelin-1 in Rats

Viswanathan Niranjan,‡ Sabine Télémaque,* Damiane deWit,* Robert D. Gerard,§ and Masashi Yanagisawa*

Howard Hughes Medical Institute and Department of Molecular Genetics, ‡Department of Pediatrics, and §Department of Biochemistry, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9050

Abstract

Endothelin-1 (ET-1) has been implicated in the regulation of vascular tone in various pathological conditions. To examine the effect of in vivo overexpression of the peptide in rats, we prepared recombinant adenovirus stocks encoding the human preproET-1 cDNA (Ad.ET-1) or *Escherichia coli lacZ* (Ad.βGal), each driven by cytomegalovirus early promoter. Ad.ET-1 or Ad.βGal was injected into the caudal vein of rats and the animals were studied under anesthesia 96 h later. Hepatic overexpression of the virus-derived human ET-1 mRNA was accompanied by a 13-fold elevation of liver ET-1 content in the Ad.ET-1 group. Circulating plasma ET-1 levels in the Ad.ET-1 group were sixfold higher than those in the Ad.βGal group. Mean arterial blood pressure was increased by 28 mmHg in the Ad.ET-1 group as compared with the Ad.βGal group. In the Ad.ET-1 group, intravenous infusion of the ET_A receptor antagonist FR 139317 reduced the blood pressure to levels seen in the Ad.βGal group, whereas the same antagonist did not significantly alter the blood pressure in the Ad.βGal group. Intravenous infusion of the ET_B receptor antagonist BQ-788 caused a small but significant increase in blood pressure in both groups. These findings demonstrate that endogenous overexpression of preproET-1, accompanied by an elevation of plasma ET-1 concentrations to the levels seen in pathophysiological states, can cause systemic hypertension through the activation of the ET_A receptor. (*J. Clin. Invest.* 1996. 98: 2364–2372.) Key words: adenovirus-mediated gene transfer • endothelin-converting enzyme • blood pressure • liver • endothelin antagonist

Introduction

Endothelins (ET)[1] are a family of 21 amino acid vasoactive peptides. Three known mammalian isopeptides, ET-1, ET-2, and ET-3 are expressed in a variety of vascular and nonvascular tissues (1, 2). These peptides are each produced from large prepro-polypeptide precursors that are cleaved to yield biologically inactive intermediates called big endothelins (big ET). Big endothelins are further cleaved by endothelin converting enzyme (ECE) to produce the active, 21-residue mature forms of endothelins (3). Two subtypes of endothelin receptors have been identified and termed endothelin-A (ET_A) and -B (ET_B) receptors (4, 5). The regulation of vascular tone by the endothelins is mediated by both receptors: ET_A and ET_B receptors can mediate ET-1–induced vasoconstriction, and ET_B receptors expressed on endothelial cells mediate vasodilatation via endothelin-induced nitric oxide release. The ET_B receptor accepts the three isopeptides equally, while the ET_A receptor demonstrates an affinity rank order of ET-1 ≥ ET-2 > ET-3.

The possible role of ET-1 in systemic hypertension has been the subject of extensive studies both in animal models and humans. In humans with essential hypertension, some studies demonstrate unaltered plasma levels of the peptide (6). However, other studies indicate that circulating ET-1 levels are increased in hypertensive subjects (7–9). Patients with ET-1–secreting tumors have been described to have elevated plasma ET-1 levels associated with systemic hypertension, which resolved after removal of the tumor (10). Immunoreactive ET-1 levels in circulating plasma have been reported to be unchanged in spontaneously hypertensive rats (SHR) and deoxycorticosterone (DOCA)-salt hypertensive rats (11). However, circulating ET-1 levels are elevated in experimental malignant hypertension induced by salt loading the SHR (12).

Studies with the ET_A antagonist BQ 123 and the ET_A/ET_B antagonist TAK-044 have shown that the endogenous generation of ET-1 contributes to basal vascular tone in humans (13, 14). SB 209670, a potent nonpeptide ET_A/ET_B receptor antagonist, significantly reduces blood pressure in the SHR (15). SB 209670 administered alone is equally efficacious to the angiotensin II receptor blocker losartan in decreasing blood pressure in the TGR(mRen-2)27 hypertensive transgenic rats, and shows an additive hypotensive effect when administered together with losartan (16). Likewise, an orally active sulfonamide ET_A antagonist decreases blood pressure by 25% in DOCA-salt rats (17). The ET_A antagonist BQ-610 partially reverses the systemic and renal vasoconstrictor effects of nitric oxide synthetase inhibition (18). Phosphoramidon, a potent inhibitor of neutral endopeptidase 24.11 (NEP) and a less potent

1. *Abbreviations used in this paper:* Ad.βGal, recombinant adenovirus containing the cytomegalovirus early promoter and the *E. coli lacZ* gene with a nuclear localization signal; Ad.ET-1, recombinant adenovirus containing the human preproET-1 cDNA driven by the cytomegalovirus early promoter; BUN, blood urea nitrogen; CHO, chinese hamster ovary; DOCA, deoxycorticosterone; ECE, endothelin converting enzyme; EIA, enzyme immunoassay; ET, endothelin; pfu, plaque-forming units; SHR, spontaneously hypertensive rat; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

inhibitor of ECE, lowers blood pressure in the SHR (19). CGS 26303, a nonpeptidic dual inhibitor of NEP and ECE, exhibits a markedly improved antihypertensive effect in the SHR compared with the selective NEP inhibitor CGS 24592. This implies that the inhibition of ET-1 production may result in a reduction of blood pressure (20).

In Dahl salt–sensitive rats, the gene encoding ET-2 cosegregates strongly with systolic blood pressure, whereas the genes for ET-1 or ET_A receptor do not (21). Mice heterozygous for a knocked-out ET-1 allele exhibit a reduced level of plasma ET-1 associated with a paradoxical elevation of blood pressure (22). Collectively, the involvement of endothelins in the regulation of blood pressure and pathophysiology of hypertension remains controversial.

A bolus intravenous injection of ET-1 in anesthetized rats results in an initial transient decrease in blood pressure followed by a potent sustained increase (23). A continuous intravenous infusion for up to 6 d of mature ET-1 in rats and dogs results in a sustained elevation of blood pressure, with the plasma ET-1 at pathophysiological levels (24–26). Thus, both acute and chronic exogenous administration of the mature peptide results in systemic hypertension. However, the effect of endogenous overexpression of preproET-1 in vivo has yet to be studied. Our previous efforts to generate transgenic mice that overexpress the human preproET-1 mRNA from its native promoter or a heterologous (metallothionein) promoter was hampered because of the apparent toxicity of ET-1 overproduction during development, which is lethal to the embryo (our unpublished observations). To elucidate the effect of endogenously elevated ET-1 production on systemic blood pressure, we used the adenovirus-mediated gene transfer and established a rat model for tissue overproduction of the peptide precursor. We show that adenovirus-mediated overexpression of the preproET-1 mRNA results in an elevation of blood pressure through the activation of the ET_A receptor, at plasma ET-1 levels in the pathophysiological range.

Methods

Preparation of recombinant adenovirus. Recombinant adenovirus containing the human preproET-1 cDNA driven by the cytomegalovirus early promoter (Ad.ET-1) was prepared by homologous recombination as described (27). Briefly, the EcoRI insert of pHET4-3 plasmid (28), containing the entire coding region for preproET-1, was ligated to pACCMVpLpA at its unique EcoRI site. The resulting construct was cotransfected with pJM17 plasmid into 293 cells by the calcium phosphate method. Viral plaques were purified to clonality by two rounds of plaque purification, verified by restriction digests of viral DNA and ET-1 production assay (see below), and used to infect cells for large scale preparation of the virus. Recombinant adenovirus containing the cytomegalovirus promoter and the *Escherichia coli lacZ* gene with a nuclear localization signal (Ad.βGal) was prepared similarly (27). For large-scale production of recombinant adenovirus (27), 293 cells in culture dishes (11×600 cm^2) were lysed 48 h after infection in 0.1% Nonidet P-40. Virus-containing extracts were centrifuged at 12,000 *g* for 10 min to remove debris. Virus particles in the supernatant were precipitated by adding 0.5 vol 20% (wt/vol) polyethylene glycol/2.5 M NaCl. The virus was collected by centrifugation at 12,000 *g* for 10 min and resuspended in 20 mM Tris-HCl (pH 8.0). The virus was further concentrated by CsCl density gradient ultracentrifugation as described (27), and desalted with a PD10 column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) equilibrated in 20 mM Tris-HCl (pH 8.0). The virus was stored in 10% (vol/vol) glycerol in 20 mM Tris-HCl (pH 8.0) and its titer determined by plaque assay

on 293 cells. We typically obtained final viral stocks of $\sim 10^8$ plaque-forming units (pfu) per microliter.

Determination of ET-1 and big ET-1 levels. We used previously characterized sandwich-type enzyme immunoassays (EIA) for mature and big ET-1 (29), with human ET-1 and human big ET-1 as standards. There is no detectable cross-reactivity between the ET-1 and big ET-1 assays. The mature ET-1 assay fully cross-reacts with rat mature ET-2, but not detectably with ET-3. The big ET-1 assay fully cross-reacts with human and rat big ET-1, but not detectably with rat big ET-2 or big ET-3. For assaying culture supernatants from adenovirus-infected Chinese hamster ovary (CHO) cells, cells were infected with viral lysate from 293 cells, and the medium was changed after 6 h. The medium was conditioned for an additional 72 h and directly analyzed for big ET-1 in triplicate by EIA. For plasma assay, rat blood samples were collected in chilled tubes containing Na$_2$-EDTA (2 mg/ml blood) and aprotinin (300 kallekrein-inhibiting units/ml blood), and plasma was separated immediately by centrifugation at 4°C. ET-1 and big ET-1 were extracted with Sep-pak C18 cartridges (Millipore Corp., Bedford, MA) as described (29), and assayed by EIA in duplicate. For tissue assay, peptides were extracted from rat liver (2 g) by homogenization in chloroform:methanol as described (30), and subjected to EIA after preextraction with Sep-pak C18 cartridges.

Animal preparations. All animal experiment procedures were reviewed and approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center. Male Wistar rats (8–15 wk old, 250–400 g body wt) were obtained from Simonson Laboratories (Gilroy, CA). Animals were anesthetized with 1–2 ml of methoxyflurane (Pitman-Moore, Mundelein, IL) in a closed gas chamber for 2–5 min. The rats were weighed, placed in an animal restrainer, and 2.5 ml of blood was withdrawn from the caudal artery to determine basal plasma ET-1 or big ET-1 levels. The rats were then injected with Ad.ET-1 (1, 5, or 10 × 10^9 pfu), Ad.βGal (5 × 10^9 pfu), or saline. The requisite volume (10–100 μl) of viral stock ($\sim 10^8$ pfu/μl) was diluted with saline to 600 μl and injected into the caudal vein. The catheter was flushed with an additional 400 μl saline. After recovery from anesthesia, the animals were housed normally.

Blood pressure measurement. Ad.ET-1 or Ad.βGal (5 × 10^9 pfu) was injected as described above into the caudal vein of the rats. Blood was not drawn for the determination of basal ET-1 levels in these groups of animals. 96 h after virus injection, the animals were reanesthetized with xylazine and ketamine (15 and 90 mg/kg, i.m.), which were supplemented as required. The rats were laid supine on a heating pad maintained at 37°C. The left external jugular vein and the right carotid artery were exposed and catheterized with PE-50 polyethylene tubings. The trachea was isolated and intubated (PE-240) to facilitate respiration. Arterial blood pressure and heart rate were monitored using a pressure transducer (P-23; Gould Inc., Valley View, OH) and recorded on a physiograph (2300R; Gould Inc.). The animals were allowed to equilibrate for at least 30 min after surgery, and the blood pressure and heart rate were recorded at steady state.

Effect of ET_A and ET_B antagonists on blood pressure. To study the effect of FR 139317 and BQ-788, separate randomized groups of rats were injected with Ad.ET-1 or Ad.βGal (5 × 10^9 pfu) as described above. A similar surgical procedure was used except that the left femoral vein was cannulated instead of the left external jugular vein. During the equilibration period, while arterial blood pressure was monitored, saline infusion was started intravenously at a rate of 20 μl/min using a syringe infusion pump (22; Harvard Apparatus Inc., South Natick, MA). After recording the basal blood pressure at steady state, the saline infusion was switched to FR 139317 (0.2 mg/kg per min at 20 μl/min) or BQ-788 (0.1 mg/kg per min at 20 μl/min). 30 min after starting antagonist infusion, blood pressure was recorded again at steady state. Blood was then collected from the arterial catheter for plasma ET-1 determination.

β-Galactosidase histochemistry. To stain adenovirus-infected CHO cells, the medium was aspirated, and the cells were rinsed with PBS

and incubated at 4°C for 5 min in fixing solution (2% formaldehyde/0.2% glutaraldehyde in PBS). The cells were then washed with PBS and stained in PBS containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 1.0 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal) for 4 h at 37°C (31). Cells were considered positive for the expression of *lacZ* product if they exhibited the characteristic nuclear and perinuclear blue staining. For tissue staining, ice-cold fixing solution (as above) was used to perfuse the animals via the external jugular vein. Tissue slices of 2–3-mm thick were postfixed, washed with PBS, and stained as above for 36 h at room temperature. Tissues were then embedded in paraffin, sectioned, and lightly counterstained with eosin (31).

Kidney and liver function. Blood was drawn 96 h after administration of virus for evaluation of hepatic and renal function. Serum aspartate and alanine transaminases, bilirubin, blood urea nitrogen (BUN), and creatinine were determined by colorimetric assays at the Department of Clinical Chemistry, Children's Medical Center of Dallas (Dallas, TX).

Northern blots. Tissue RNA was extracted with the RNAzol reagents (Tel-Test Inc., Friendswood, TX). Total RNA (20 μg per lane) was separated in a 1.1% agarose gel, transferred to a nylon membrane, and hybridized in QuikHyb solution (Stratagene Inc., La Jolla, CA). A 700-bp AvaI-EcoRI fragment of human ET-1 cDNA was random primed, ^{32}P labeled, and used as probe. This probe did not crosshybridize with the rat ET-1 mRNA. The membranes were washed in 15 mM NaCl/1.5 mM sodium citrate/0.1% SDS at 60°C and exposed to an x-ray film for 36 h with an intensifying screen. A mouse β-actin cDNA probe was used as loading control.

Statistical analysis. We used nonparametric tests throughout this study, considering the probable nonnormal distribution of data (32). Comparisons between multiple groups were made by the Kruskal-Wallis test. The Mann-Whitney U test with the Bonferonni correction was then used to compare two groups. All comparisons between the Ad.ET-1 and Ad.bGal groups in the latter part of the study were made by the Mann Whitney U test. We used the Wilcoxon signed rank test to compare the paired body weight measurements within the Ad.ET-1 group. Spearman rank correlation was used to compare big ET-1 and ET-1 levels from individual animals in the Ad.ET-1 group. $P < 0.05$ was considered significant. Unless otherwise stated, all values in the text are expressed in the following format: median; 25–75 percentiles (33). All data were processed by InStat (Graph-PAD Software for Science, San Diego, CA) and StatView for Macintosh (Abacus Concepts, Inc., Berkeley, CA) statistical packages.

Results

We constructed recombinant adenovirus encoding the cDNA for human preproET-1 (Ad.ET-1) and *E. coli lacZ* (Ad.βGal,

as negative control) by homologous recombination in 293 cells with a replication-deficient, *E1*-deleted adenovirus 5 genomic construct (Fig. 1). To assess functional activity of recombinant virus stocks, we infected cultured CHO cells with Ad.ET-1 or Ad.βGal. CHO cells do not produce ET-1 and have no detectable endogenous ECE activity. We assayed the culture supernatant for big ET-1 levels, and stained the cells for β-galactosidase activity. The big ET-1 concentration in medium from Ad.ET-1–infected CHO cells was 10±1 pM (mean±SEM), whereas it was below the detection threshold of EIA (< 0.004 pM) in medium from Ad.βGal-infected cells. CHO cells infected with Ad.βGal showed strong nuclear β galactosidase staining in > 80% of the cells, whereas cells infected with Ad.ET-1 did not (data not shown).

In a preliminary series of studies, we optimized experimental protocols for the adenovirus-mediated overexpression of human preproET-1 in vivo in rats. We injected Ad.ET-1 (1, 5, or 10×10^9 pfu) into the caudal vein, and measured plasma ET-1 levels both before and the designated number of hours after administration of the virus (Fig. 2 *A*). 48 h after injection of 5×10^9 pfu Ad.ET-1, plasma ET-1 levels were threefold higher than the preinjection basal values (1.2; 0.6–1.5 pM, $n = 6$ vs. 0.36; 0.29–0.57 pM, $n = 73$, $P = 0.0008$). 72 h after injection, plasma ET-1 levels were 10-fold higher than the basal values (4.2; 1.9–4.9 pM, $n = 7$, $P = 0.0001$), and remained elevated until 120 h after injection. After 120 h, plasma ET-1 levels gradually declined towards basal values. For all doses of Ad.ET-1 tested (1, 5, or 10×10^9 pfu), plasma ET-1 levels were highest between 72–120 h (data not shown). Fig. 2 *B* depicts plasma ET-1 levels 96 h after administration of saline, different doses of Ad.ET-1, or 5×10^9 pfu Ad.βGal. At 10^9 pfu of Ad.ET-1, there was only slight elevation in plasma ET-1 levels (1.2 pM, $n = 2$, $P = 0.02$, not significant with Bonferonni correction). At 5×10^9 pfu of Ad.ET-1, we saw a significant (10-fold) increase over preinjection basal levels (3.5; 2.0–4.3 pM, $n = 7$, $P = 0.0001$). This was also sixfold higher than the plasma ET-1 levels after administration of 5×10^9 pfu of Ad.βGal (0.6; 0.5–0.6 pM, $n = 7$, $P = 0.0017$). At 10×10^9 pfu; however, only 2 of 10 animals injected with Ad.ET-1 survived. The deaths typically occurred within the first few hours after injection. Preliminary measurements 96 h after Ad.ET-1 injection showed a marked increase of the arterial blood pressure (see below). 7 d after Ad.ET-1 injection, when the plasma ET-1 concentration returned to basal values (1.3; 0.8–1.6 pM, $n = 4$),
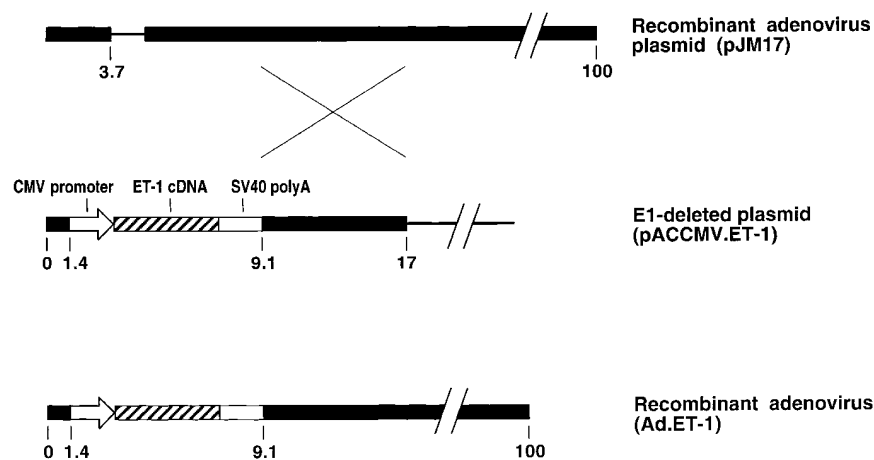

Figure 1. Schematic representation of recombinant adenovirus construct. Adenovirus was made by homologous recombination (*cross*) in 293 cells. Closed boxes represent viral DNA and the lines represent plasmid sequences. Crosshatched boxes represent human ET-1 cDNA or *E. Coli lacZ* cDNA. Numbers represent map units of viral DNA (1 map unit = 0.36 kb).
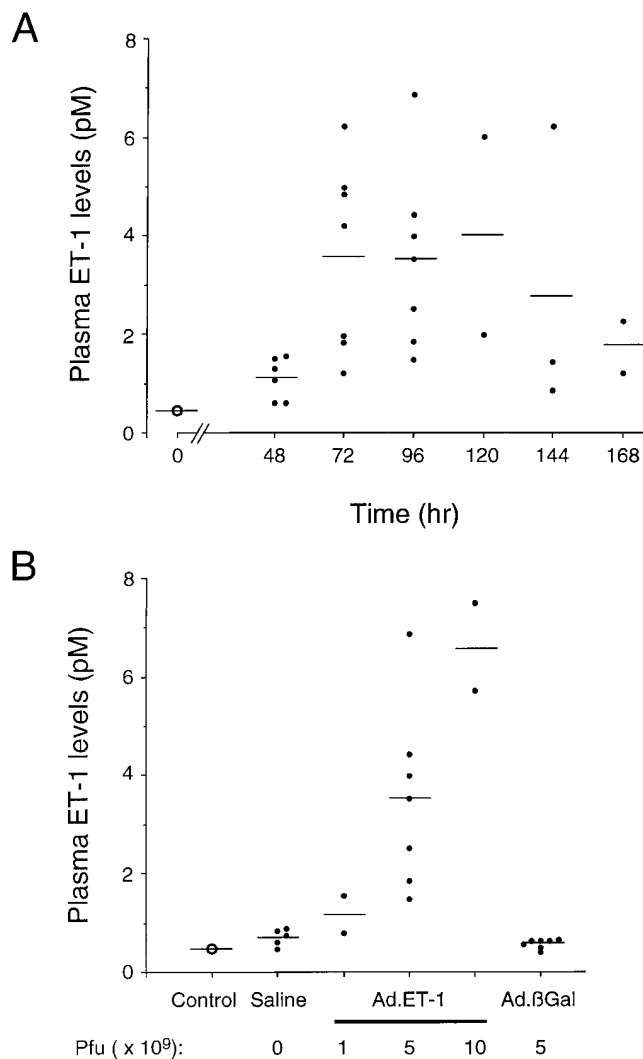
A

B

Figure 2. Plasma ET-1 levels after intravenous administration of adenovirus. Blood was drawn twice from each animal. Once before (basal) and once the designated number of hours after administration of virus or saline. Each dot represents a different animal. The control value (mean from a total of 73 animals) is represented as a single open circle for the sake of clarity. Horizontal lines depict mean values. (*A*) Time course of plasma ET-1 levels after 5×10^9 pfu of Ad.ET-1. (*B*) Plasma ET-1 levels 96 h after administration of different doses of virus or saline. At a dose of 10×10^9, only 2 of 10 animals survived.

Figure 3. Adenovirus-mediated expression of human preproET-1 mRNA in the liver. Total RNA (20 μg per lane) was extracted 96 h after intravenous administration of 5×10^9 pfu of adenovirus. Membranes were hybridized with a cDNA probe specific for human preproET-1 mRNA and later rehybridized with mouse β-actin cDNA.

the mean arterial pressure also returned to baseline (98; 95–105 mmHg, $n = 4$).

Based on these preliminary results, in the subsequent phase of the study we randomized a new cohort of animals into two groups: the Ad.ET-1 group (5×10^9 pfu, $n = 18$) and the Ad.βGal group (5×10^9 pfu, $n = 10$). We did not draw blood from these rats before virus injection to determine basal plasma peptide levels because we did not want to disturb body fluid homeostasis or induce anemia in these animals. 96 h after administration of the virus, the carotid artery was cannulated under anesthesia and the blood pressure was recorded at steady state, at 30–60 min after surgery. Arterial blood was then drawn for determination of plasma ET-1, big ET-1, and kidney and liver function studies. The animals were subse-
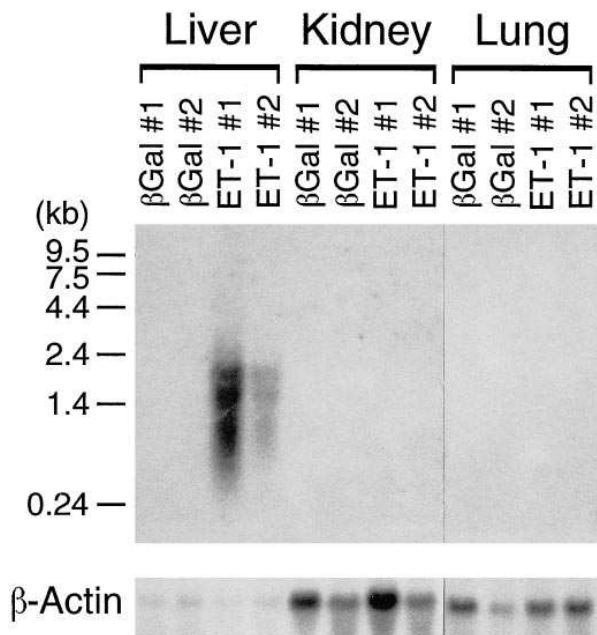
quently killed and the tissues collected for histological analyses, Northern blots, and ET-1 EIA.

We histologically examined the tissue distribution of the virus-infected cells in animals that were randomly selected from each group ($n = 2$ per group). Tissue sections were stained with X-gal and examined for nuclear β-galactosidase activity. In the Ad.βGal animals, virtually all hepatocytes were strongly positive, similar to previous reports (27) (data not shown). Other cell types in the liver were negative for β-galactosidase staining. Fewer than 0.1% of cells in the spleen were also stained, and 1–2 cells per several lung sections were stained. No other tissues examined (brain, heart, kidney, intestine, pancreas, skeletal muscle, or testis) demonstrated detectable nuclear β-galactosidase staining. In particular, β-galactosidase staining was undetectable in blood vessels in all of the organs examined. Nuclear X-gal staining was absent from all tissues examined in rats injected with Ad.ET-1. We also compared hematoxylin-eosin–stained slices of the above tissues in both groups ($n = 2$ per group) and found no appreciable differences between the groups (data not shown). There was no overt necrosis of hepatocytes, although there was evidence of mild inflammation in the liver as suggested by the occasional infiltration by a solitary monocyte.

To verify endogenous overproduction of preproET-1 mRNA, we performed Northern blot analysis and determined tissue ET-1 levels in animals randomly selected from each group ($n = 2$ per group). Northern blots with a human ET-1–specific probe revealed abundant adenovirus-derived human ET-1 mRNA in the liver from animals in the Ad.ET-1 group (Fig. 3). A long exposure of the blots showed small amounts of the human ET-1 mRNA in the lungs of Ad.ET-1–injected animals. Human ET-1 mRNA was not detected in the kidney of the
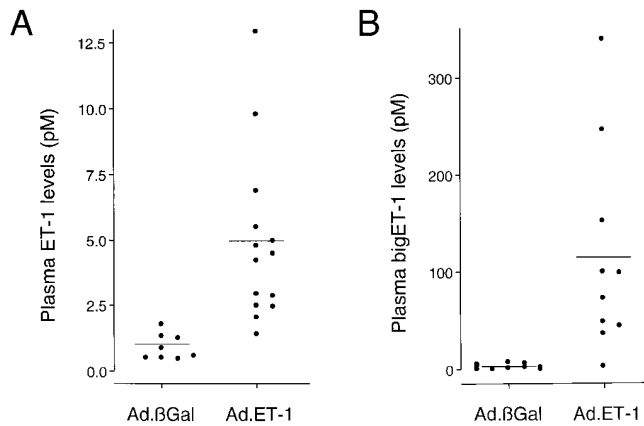
Figure 4. Comparison of plasma ET-1 (A) and big ET-1 (B) levels in Ad.ET-1 and Ad.βGal groups. Each dot represents a different animal whose blood pressure was measured (see Fig. 5). In these groups of animals, blood was drawn only once after monitoring blood pressure. Horizontal lines represent mean values. Note that concentrations of big ET-1 are ∼ 20-fold higher than those of ET-1 in the Ad.ET-1 group.

Ad.ET-1 animals or in any of the tissues examined (liver, lung, and kidney) from the Ad.βGal animals. To confirm hepatic biosynthesis of human preproET-1, we determined tissue ET-1 levels in the liver. Hepatic mature ET-1 levels were ∼ 13-fold higher in the Ad.ET-1 animals than in the Ad.βGal animals (mean: 0.67 vs. 0.05 fmol/g tissue, $n = 2$). The plasma mature ET-1 levels in the Ad.ET-1 group were sixfold greater than those in the Ad.βGal group (4.4; 2.5–5.5 pM, $n = 14$ vs. 0.8; 0.5–1.3 pM, $n = 8$, $P = 0.0002$) (Fig. 4 A). Plasma big ET-1 levels were 29-fold higher in the Ad.ET-1 group compared with the Ad.βGal group (86.6; 45.6–153.3 pM, $n = 10$ vs. 3.0; 1.4–6.3 pM, $n = 10$, $P = 0.0005$) (Fig. 4 B). We observed significant positive rank correlation between the plasma big ET-1 and ET-1 levels in individual animals from the Ad.ET-1 group ($P = 0.023$ by Spearman rank correlation test).

We compared the body weights of the animals before and after administration of the virus in both groups. There was no significant difference in weights between Ad.ET-1 and Ad.βGal groups before administration of virus (313; 279–381 g, $n = 15$, vs. 333; 317–390 g, $n = 10$, $P = 0.11$). However, body weights were significantly different between the groups 96 h after administration of the virus (292; 241–349 g in Ad.ET-1 group vs. 330; 314–395 g in Ad.βGal group, $P = 0.015$). In the Ad.ET-1 group, there was an ∼ 7% decrease in body weight after administration of the virus ($P = 0.0007$ by Wilcoxon signed rank test). The loss of body weight was associated with significantly higher BUN levels in the Ad.ET-1 group compared with the Ad.βGal group (33; 29–36 mg/dl, $n = 11$ vs. 25; 21–27 mg/dl, $n = 8$, $P = 0.0072$). However, serum creatinine values were similar in both groups (0.4; 0.3–0.7 mg/dl, $n = 11$ vs. 0.4; 0.3–0.7 mg/dl, $n = 8$, $P = 0.80$). Since the virus was localized in the liver, we also measured serum transaminases and bilirubin levels at the end of the study. There was no significant difference in the aspartate transaminase levels (265; 208–231 U, $n = 11$ vs. 292; 184–380 U, $n = 8$, $P = 0.62$) and alanine transaminase levels (129; 87–279 U, $n = 11$ vs. 93; 60–109 U, $n = 8$, $P = 0.076$) between the Ad.ET-1 and Ad.βGal groups. Total bilirubin levels were not significantly different between
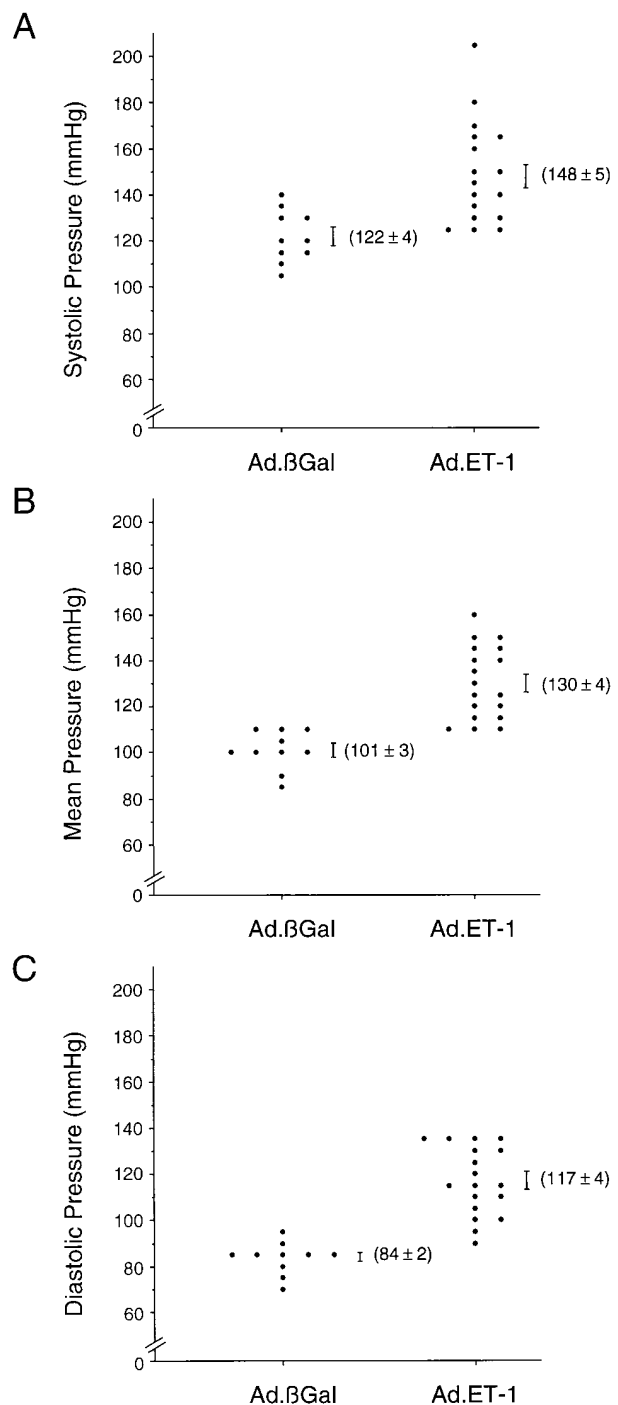

Figure 5. Effect of endogenous overproduction of preproET-1 on systolic (A), mean (B), and diastolic (C) arterial pressure in anesthetized rats. Vertical lines depict SEM. The blood pressure is shown in increments of 5 mmHg (the maximum resolution for the physiograph used).

the two groups (0.5; 0.1–0.9 mg/dl, $n = 11$ vs. 0.1; 0.1–0.2 mg/dl, $n = 8$, $P = 0.072$).

Systemic blood pressure was measured directly by catheterization of the carotid artery under xylazine/ketamine anesthesia in these two groups of adenovirus-injected animals. Systolic, diastolic, and mean arterial pressures for individual animals in each group are shown in Fig. 5. In the Ad.βGal

group, the mean arterial blood pressure ranged between 80 and 110 mmHg, while in the Ad.ET-1 group it was 110 and 160 mmHg. In the Ad.ET-1 group, the mean arterial pressure (128; 115–145 mmHg, $n = 18$) was significantly higher than in the Ad.βGal group (100; 100–110 mmHg, $n = 10$, $P = 0.0001$). The systolic pressure in the Ad.ET-1 group was significantly higher than in the Ad.βGal group (143; 130–165 mmHg, $n = 18$ vs. 120; 115–130 mmHg, $n = 10$, $P = 0.0016$). Likewise, the diastolic pressure in the Ad.ET-1 group was significantly higher than in the Ad.βGal group (115; 105–130 mmHg, $n = 18$, vs. 85; 80–85 mmHg, $n = 10$, $P = 0.0001$). There was no significant difference in pulse pressures between the two groups (30; 25–35 mmHg, $n = 18$ vs. 35; 30–45 mmHg, $n = 10$, $P = 0.19$). The heart rate in the Ad.ET-1 group was not significantly different from that in the Ad.βGal group (250; 225–275 bpm, $n = 18$ vs. 265; 240–290 bpm, $n = 10$, $P = 0.20$). We did not detect a significant correlation between the blood pressure and plasma ET-1 (or big ET-1) levels in the animals within the Ad.ET-1 group.

To examine which endothelin receptor subtype(s) are involved in the systemic hypertension seen in the ET-1–overexpressing animals, we intravenously infused the ET_A-selective antagonist FR 139317 (34) or the ET_B-selective antagonist BQ-788 (35) in separate randomized cohorts of the Ad.ET-1 rats ($n = 5$ for FR 139317, $n = 6$ for BQ-788) and Ad.βGal rats ($n = 4$ for FR 139317, $n = 6$ for BQ-788) (Fig. 6). Continuous infusion of FR 139317 (0.2 mg/kg per min) for 30 min resulted in a significant reduction of mean arterial pressure in the Ad.ET-1 group (from 130; 120–135 mmHg to 95; 71–103 mmHg, $n = 5$, $P = 0.008$) (Fig. 6 A). In contrast, infusion of FR 139317 did not alter mean blood pressure in the Ad.βGal group (from 83; 75–85 mmHg to 80; 75–83 mmHg, $n = 4$, $P = 0.7$). Infusion of FR 139317 did not affect the plasma concentration of ET-1 either in the Ad.ET-1 or Ad.βGal group (data not shown). Intravenous infusion of BQ-788 caused a small but significant increase in mean blood pressure both in the Ad.ET-1 group (from 130; 125–135 mmHg to 145; 140–150 mmHg, $n = 6$, $P = 0.03$) and in the Ad.βGal group (from 83; 80–90 mmHg to 95; 90–100 mmHg, $n = 6$, $P = 0.03$) (Fig. 6 B). In the Ad.βGal group, BQ-788 did not significantly affect plasma levels of ET-1 (0.8; 0.7–1.2, $n = 6$). However, in the Ad.ET-1 animals, the 30-min infusion of the ET_B-receptor antagonist caused a marked elevation of plasma ET-1 (19.6; 7.4–33.3 pM, $n = 6$, $P = 0.003$) as compared with the corresponding values of Ad.ET-1 animals without antagonist treatment described above (4.4; 2.5–5.5 pM, $n = 14$).

Discussion

We have used the adenovirus-mediated gene transfer to overexpress human preproET-1 cDNA in vivo in rats. A single intravenous dose of recombinant adenovirus resulted in an increased preproET-1 mRNA expression in the liver associated with elevation in hepatic tissue ET-1 levels. Plasma ET-1 levels were increased sixfold over the Ad.βGal-injected control animals, and remained elevated at these levels 72–120 h after administration of the virus. Rats with tissue overexpression of preproET-1 mRNA provide a more pathophysiologically relevant model as compared with models involving infusion of exogenous peptides, since the present model more closely mimics the in vivo disturbances seen in the actual pathological conditions. Limitations of the current model, however, include the relatively transient nature of ET-1 overproduction and re-
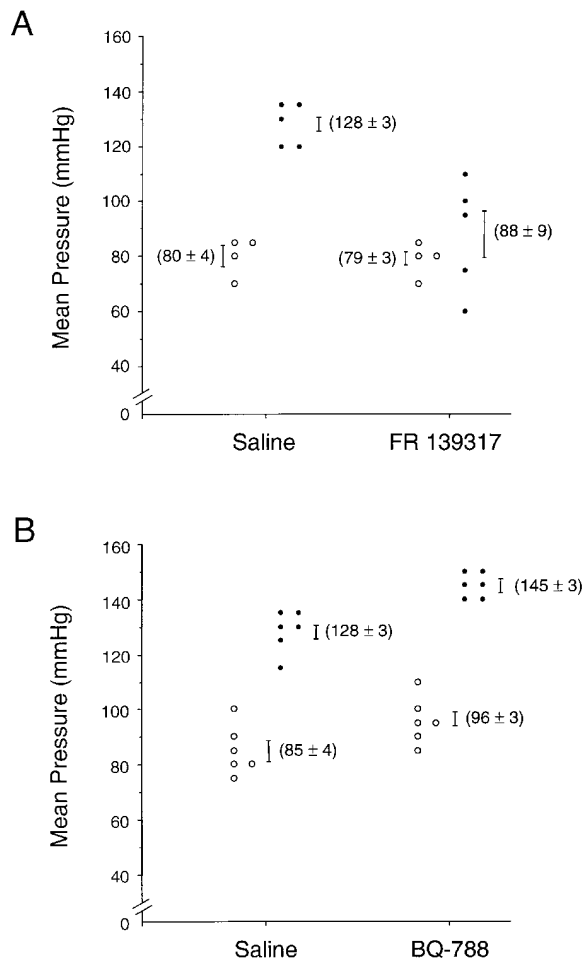

Figure 6. Effect of ET_A antagonist FR 139317 (0.2 mg/kg per min) (*A*) and ET_B antagonist BQ-788 (0.1 mg/kg per min) (*B*) on mean arterial pressure in adenovirus-injected rats. Arterial pressure was recorded at steady state twice from each animal, before (*Saline*) and 30 min after infusion of antagonist. Closed and open symbols represent individual Ad.ET-1 and Ad.βGal rats, respectively. Vertical lines depict mean±SEM.

sulting hypertension. These limitations may be partially overcome by the use of improved adenoviral vectors. One such vector contains no viral structural genes so that infection by the virus is less likely to induce cellular immune response (36).

Interestingly, circulating big ET-1 levels were ∼ 20-fold higher than the mature ET-1 levels in the Ad.ET-1 group (as compared with mature ET-1 to big ET-1 ratio of 1:4 in Ad.βGal group), suggesting that under the conditions of preproET-1 overexpression, ECE can be a rate limiting step in the production of mature ET-1 in vivo. It is therefore possible that overexpression of ECE in itself may result in systemic hypertension under certain circumstances. This also suggests that these rats may prove to be useful for in vivo screening of ECE inhibitors. Alternatively, the disproportionately high big ET-1 levels may be partly due to a slower rate of clearance and/or degradation of circulating big ET-1 as compared with the mature peptide (37, 38).

In the present study, overexpression of preproET-1 cDNA in vivo resulted in a significant decrease in body weight accompanied by a mild elevation in BUN levels. The decrease in

weight with elevated BUN and normal creatinine levels is consistent with a mild reduction of the intravascular volume. Low dose exogenous ET-1 can directly promote natriuresis (25) as well as increase free water clearance in the kidney (39, 40). It is also possible that hypertension associated with an ET-1–induced elevation of peripheral vascular resistance may result in reduced intravascular volume due to suppression of the renin-angiotensin-aldosterone system (see below). Since we did not determine blood electrolyte levels in this study, we do not know whether the small reduction of intravascular volume is due to a negative salt balance, a negative free water balance (with somehow decreased oral water intake), or both. Other physiological parameters not addressed in the present study are those related to the direct hepatic effects of the overexpression of ET-1. Parameters such as blood sugar and portal pressure will be needed to determine whether local overproduction of ET-1 has an effect on the physiological function of the liver.

The present study demonstrates the elevation of systemic blood pressure resulting from hepatic overexpression of preproET-1. The arterial blood pressure in the Ad.βGal control group in this study was similar to that in Wistar rats under xylazine/ketamine anesthesia in previous studies (41). In several rat models of hypertension (SHR and DOCA-salt rats), there is no elevation in circulating ET-1 levels (11). However, endothelin receptor antagonists and antiendothelin antibodies have been reported to reduce blood pressure in a number of distinct rat models of hypertension, including SHR, DOCA-salt, renin-overexpressing transgenic TGR(mRen-2)27, and L-NAME treatment (15–18, 42–46). The findings in the present study are complementary to these previous observations, indicating that in vivo overexpression of preproET-1 is sufficient to cause systemic hypertension under the conditions used in the present study.

The absolute levels of circulating mature ET-1 at which hypertension was observed in the present study are within or only slightly above the ranges seen in pathophysiological conditions in animal models and human patients. Basal ET-1 levels previously reported in Wistar-Kyoto (0.7 pM) and Wistar (0.4 pM) rats are similar to those in the present study (0.36 pM) (11, 30). In this latter study, plasma ET-1 levels increased fourfold over basal values within 10 min of reperfusion in a rat model for myocardial infarction. A number of pathological states are also associated with elevation of plasma ET-1 levels in humans. Plasma ET-1–like immunoreactivity in patients with World Health Organization Stage II or III essential hypertension are about twofold higher than age-matched controls (13.6 and 7.4 pM, respectively) (7). Plasma ET-1 levels are up to three- to fourfold higher in patients with advanced atherosclerosis as compared with control subjects (47). A threefold elevation in plasma ET-1 levels, as compared with age-matched normotensive pregnant women, is seen in patients with preeclampsia (0.25 to 0.75 pM) (48) and pregnancy-induced hypertension (0.24 to 0.76 pM) (49). A pathophysiological increase in plasma ET-1 levels is also seen in a myriad of acute life-threatening disease states. Within a few hours of myocardial infarction, plasma ET-1 levels increase sixfold (0.3 to 1.98 pM) (50). A fivefold increase of plasma ET-1 (2.1 to 9.8 pM) from control values is seen in the adult respiratory distress syndrome (51). A threefold elevation of plasma ET-1 (0.3 to 1.0 pM) has been described shortly after lung transplantation in humans (52). An increase in plasma ET-1 levels has

also been reported with trauma (1.5-fold, median 2.1 pM) (53), disseminated intravascular coagulation (twofold, 1.24 from 0.6 pM) (54), acute renal failure (sevenfold, 0.6 to 4.1 pM) (55), and cardiac failure (fivefold, 0.25 to 1.48 pM) (56).

The release of ET-1 from endothelial cells is polarized towards the basolateral side, compatible with the idea that it functions as a paracrine factor rather than a systemically circulating hormone (1). Elevation of plasma ET-1 levels in disease states has therefore been considered as a mere spillover effect associated with local overproduction. In the present study, hepatic overexpression of preproET-1 mRNA leads to an active secretion of the peptide into the circulation from hepatocytes, resulting in elevated levels of plasma big and mature ET-1. We did not detect expression of the virus-derived mRNA in the peripheral vasculature. The direct causal relationship of the elevated plasma ET-1 levels and systemic hypertension seen in this study therefore provides evidence that ET-1 can act as a significant circulating factor under certain pathological conditions. Thus, one needs carefully to examine the possibility that the "spillover" ET-1 seen in the aforementioned disease states may play a role in the on-going pathophysiology. A causal role for ET-1 in the pathophysiology of different diseases needs to be further delineated in humans. The availability of nonpeptide antagonists (15, 17, 57, 58) will facilitate the establishment of pathophysiological roles of endogenous ET-1 in human patients.

The mechanism of the increase in blood pressure induced by endogenous overproduction of ET-1 in the present model needs to be investigated in detail. In dogs, low rate continuous infusion of mature ET-1 over 8 d resulted in an elevation of blood pressure associated with an increase in peripheral resistance and decrease in cardiac output (25). This suggests that systemic hypertension after the long-term infusion of ET-1 is due to peripheral vasoconstriction induced by the peptide. In this respect, the present model can be considered highly analogous to a slow, continuous systemic administration of big ET-1. Although we will need to determine cardiac output for a definitive conclusion, we feel that the hypertension seen in the present study is also due primarily to an elevated systemic vascular resistance.

A bolus injection of a pharmacological dose of ET-1 results in a rapid and transient decrease, followed by a sustained increase in blood pressure (23). BQ-123, an ET_A-receptor antagonist, potently inhibits the pressor response, but has no effect on the transient depressor response in rats (35). In the same report, BQ-788, a selective ET_B-receptor antagonist, abolished the transient depressor response, resulting in a rapid onset of apparently enhanced pressor response. We have taken advantage of the availability of selective ET_A and ET_B antagonists to delineate the role these two receptor subtypes play in the hypertensive state seen in our rat model. We have found that blockade of the ET_A receptor by FR 139317 abolishes the hypertensive effect of Ad.ET-1 injection, indicating that the hepatic overexpression of ET-1 exerts the systemic pressor effect through the activation of the ET_A receptor in the present model. In the present study, we did not detect any contribution of the "contractile" ET_B receptor (59, 60) to the elevation of arterial pressure.

Interestingly, BQ-788 exacerbated hypertension in these animals. The ET_B antagonist also caused a marked increase of the already elevated plasma ET-1 in the Ad.ET-1 animals. Previous studies demonstrated that the administration of BQ-

788 causes a significant elongation of plasma half-life of ET-1, indicating that the ET_B receptor is responsible for the rapid clearance of circulating ET-1 (61). These observations support the idea that the increment of blood pressure by BQ-788 in the ET-1–overexpressing animals may partly be due to the further elevation of circulating ET-1 levels due to the blockade of the "clearance" ET_B receptor. However, we have also observed a similar relative increase (by $\sim 10\%$) of blood pressure after BQ-788 in the control Ad.βGal rats. In these rats, BQ-788 did not appreciably increase circulating levels of ET-1. These latter observations strongly indicate the possibility that blockade of the ET_B receptor may also result in an elevation of blood pressure through the removal of vasodilatory mechanisms mediated by ET_B receptors, including the release of dilatory prostanoids and nitric oxide from the endothelium. These findings also suggest that the endogenous baseline release of ET-1 in these animals may contribute to an ET_B-mediated basal vasodilatory tone under these conditions.

Various counterregulatory mechanisms work in synchrony with vasoconstrictors in the regulation of systemic blood pressure. For example, the in vivo pressor effect of pharmacological doses of ET-1 has been demonstrated to be limited by the ET-1–induced release of prostacyclin and nitric oxide (62). The significant elevation of blood pressure seen in this study indicates that these counterregulatory mechanisms do not completely offset the effects of overexpressed preproET-1 in these rats. This provides an ideal model for studying the role of counterregulatory and synergistic factors under the state of endogenous overproduction of ET-1.

The present work conclusively demonstrates that peripheral overexpression of preproET-1 can result in systemic hypertension, accompanied by an elevation of plasma ET-1 levels within pathophysiological ranges. Our current results as well as previous studies with endothelin antagonists may appear contradictory to the observation that heterozygous ET-1 knockout mice have higher blood pressure than wild-type litter mates (22). However, a number of fundamental differences between these models should be noted: (*a*) the heterozygous knockout mice express a reduced level of ET-1 throughout their life, from embryonic stages into adulthood. In contrast, in the present and many other pathophysiological models, a relatively acute, de novo overproduction of ET-1 takes place in the adult animal. The state of various counterregulatory mechanisms may be fundamentally different in these situations. (*b*) In the heterozygous knockout mice, the reduction of ET-1 production occurs systemically, including, for example, the central nervous system. In contrast, in most pathophysiological states including the present model, the ET-1 overproduction is spatially limited. In the present model, for example, it is unlikely that the brain constitutes a major target for the peptide that is overproduced in the liver; ET-1 is known to be unable to cross the blood-brain barrier (63). In contrast, recent analyses suggest that the small elevation of blood pressure in heterozygous ET-1 knockout mice may be secondary to chronic arterial hypoxia due to a disturbance in the respiratory center, where endogenous ET-1 may play an important role (64). Further delineation of the role of the peptide in many acute and chronic debilitating illnesses is warranted.

Acknowledgments

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