Published in final edited form as:

Vascul Pharmacol. 2007 ; 47(5-6): 302–312. doi:10.1016/j.vph.2007.08.006.

Big ET-1 processing into vasoactive peptides in arteries and veins

Stephanie W. Watts, PhD, Keshari Thakali, PhD, Chuck Smark, BS, Catherine Rondelli, BS, and Gregory D. Fink, PhD

Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI 48824-1317

Abstract

The endothelin (ET) peptides are more potent in contracting veins than arteries. The precursor big ET-1 is metabolized by endothelin converting enzyme [ECE; to ET-1 (1–21)], matrix metalloproteases [MMPs; to ET-1 (1–32)] and chymase [to ET-1(1–31)]. We hypothesized that arteries and veins were differently dependent in conversion of big ET-1 to vasoconstrictors. Immunohistochemical, western, zymographic and isometric contractile assays in rat aorta and vena cava were used. Big ET-1 contracted aorta [$60 \pm 17\%$ phenylephrine contraction] but was more efficacious in vena cava [$478 \pm 61\%$ norepinephrine contraction]. ECE and its product ET-1(1–21) were detected in aorta and vena cava, and the ECE inhibitors phosphoramidon and CGS-26393 reduced big ET-1-induced contraction. ET-1 (1–32) contracted aorta and vena cava but inhibition of MMPs with minocycline or GM6001 did not reduce big ET-1 –induced contraction; zymography confirmed active tissue MMPs. Aorta and vena cava contracted to the product of chymase, ET-1 (1–31). Chymase was detected in aorta and only weakly in vena cava. Inhibition of chymase (chymostatin, 100 μ M) reduced arterial (19% control) but not venous constriction to big ET-1. These results suggest at least one potential significant difference— the role of chymase-- *in vitro* enzymatic processing of big ET-1 in arteries and veins.

Keywords

artery; vein; ET processing

1. Introduction

Veins and arteries differ qualitatively and quantitatively in response to ET-1. ET-1 typically has a lower threshold of contraction, higher potency and greater relative efficacy in veins than arteries. Veins constrict faster to ET-1 than do arteries and veins do not desensitize to acute or chronic elevations in ET-1 as do arteries [11,32,36]. These studies raise the idea that ET may be an important controller of endogenous venous tone. We have been interested in understanding the role of venomotor tone in control of blood pressure, specifically as it pertains to the ability of ET-1 to modify venous tone. The central hypothesis of this study is that veins and arteries differ in their enzymatic ability to metabolize big ET-1 and thus may be exposed to different profiles of vasoactive ET peptides.

Correspondence should be sent to: Stephanie W. Watts, B445 Life Sciences Building, Department of Pharmacology & Toxicology, Michigan State University, East Lansing, MI 48824-1317, USA, Telephone: 517 353 3724, Fax: 517 353 8915, E-mail: E-mail: wattss@msu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Endothelin [ET-1 (1–21)] is one of the most potent vasoconstrictors to be isolated from blood vessels. Biosynthesis of this peptide begins with the actions of a furin-like protease on pro-ET-1 to form the peptide big ET-1 (1–38) [6,14,18,29,30,38], with much of the proteolytic processing thought to take place in the vesicles of endothelial cells [8]. The most extensively studied processing pathway of big ET-1 is through the zinc-dependent endoprotease endothelin converting enzyme (ECE) to form ET-1 (1–21), commonly known as ET-1 [8,9,18,30,31]. In recent years, other pathways for big ET-1 processing have been discovered.

Matrix metalloproteases (MMPs) are serine proteases that can contribute to alteration in the extracellular matrix of blood vessels. Two intensively studied MMPs in the vasculature are MMP-2 and MMP-9, also referred to as gelatinase A and B. Their protease function allows dissolution of matrix and remodeling. Big ET-1 is acted upon by MMP-2 to form ET-1 (1–32), a product in which the terminal 6 amino acids of big ET-1 has been cleaved. ET-1 (1–32) functions as a vasoconstrictor in rat mesenteric arteries, and a potential vasodilator in rat renal arteries *via* activation of ET_B receptors. Thus, the endogenous metabolism of big ET-1 *via* MMP leads to the possibility of production of an additional vasoactive ET peptide [4,5,10, 20,23,24,28]. Similarly, the enzyme chymase can metabolize big ET-1 to ET-1 (1–31) [7,12, 13,14,15,21,25,37]. ET-1 (1–31) has recently received attention as an endogenous agonist of the ET_A receptors, stimulation of which has been almost exclusively associated with vasoconstriction [3,17,18,21,25].

We used an integrative set of techniques that allowed us to ask the questions of whether these three separate processing enzymes – ECE, MMPs and chymase—existed, whether the products of these 3 pathways produced substances that could influence smooth muscle tone in arteries and veins, and whether the pathways participated in the contraction of isolated vessels to big ET-1. Our model of vein and artery, the isolated thoracic vena cava and thoracic aorta, allowed us to carry out pharmacological, biochemical and immunohistochemical studies in concert.

2. Methods

2.1 Isometric contraction Protocols

All animal procedures were approved by the Institutional Animal Care University Committee of Michigan State University. Male Sprague Dawley rats (Charles River) were deeply anesthetized with pentobarbital (50 mg kg⁻¹, i.p.) to the point of a loss of eyelid reflex and lack of withdrawal from painful stimuli. Thoracic aorta and vena cava were placed in physiologic salt solution consisting of (in mM) NaCl, 130; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄·7H₂O, 1.17; CaCl₂·2H₂O, 1.6; NaHCO₃, 14.9; dextrose, 5.5; and CaNa₂EDTA, 0.03. Aorta and vena cava were cleaned of fat and connective tissue, left with an intact endothelium, mounted as rings (3–4 mm long) on stainless steel hooks and placed on stainless steel holders in tissue baths (30 mL) for isometric tension recordings using Grass polygraphs and transducers as previously described [32]. Aorta were initially challenged with a maximal concentration of the α -adrenergic agonist phenylephrine (10⁻⁵ M). A maximal concentration of norepinephrine (10⁻⁵ M) was used for vena cava as phenylephrine does not produce a reproducible contraction in this tissue. In preliminary experiments, maximal contractions to norepinephrine and phenylephrine in aorta were statistically similar (~2.2 grams), and thus use of these two different agonists in normalizing venous *vs* arterial contraction is reasonable. Response to NE provides a reference for past and future experiments, and a validation that the tissues are alive using an agonist that washes out from the tissues easily.

Tissues were washed, and the functional integrity of the endothelial cells was evaluated by testing relaxation caused by acetylcholine (10⁻⁶ M) in tissues contracted with PGF_{2 α} (20 μ M). Tissues were washed and then taken through one of the following two protocols.

2.1.1 Protocol 1: Examination of ET peptide agonists—When evaluating the contractile or relaxant effect of an ET-based peptide, tissues were exposed to only one agonist. This is because ET peptides all washed out poorly from the isolated tissue bath, and thus every tissue was naïve when originally challenged with ET peptides. A cumulative concentration response curve to ET peptide (10^{-11} – 10^{-7} M) was constructed, waiting a minimum of 5 minutes after every addition. In testing relaxation, tissues were contracted first with PGF_{2α} (20 μM) and then peptides added in a cumulative fashion.

2.1.2 Protocol 2: Examination of enzyme inhibition on big ET-1-induced contraction—Vessels were incubated with either vehicle (0.1 % dimethylsulfoxide or 0.1% ethanol) or enzyme inhibitor (phosphoramidon, CGS-26393, minocycline, GM6001, chymostatin) for one hour without washing. A cumulative concentration response curve to big ET-1 or KCl (6–100 mM) was then constructed.

2.2 Histochemical Protocols

2.2.1 DAB Immunohistochemistry—Sections of formalin-fixed paraffin-embedded thoracic aorta and vena cava (8 micron) were cut, air dried overnight, deparaffinized, and taken through standard protocol using a Vector kit. Slides for smooth muscle α-actin were unmasked through Vector Laboratories Antigen Unmasking protocol, modified to use a microwave instead of a pressure cooker. After blocking with 1.5% serum in phosphate buffered serum (PBS), sections were incubated overnight at 4°C with an antibody raised against ET-1 (1:50; clone ET-1/58, Sigma Chemical Co, St. Louis, MO, USA), ECE (1:50; human ECE, Zymed laboratories, San Francisco, CA, USA), MMP-2 (1:40; MAB3308, Chemicon, Temecula, CA, USA), human chymase (2 ug/ml; ab2377, ABCAM, Cambridge, MA, USA) or no antibody (1.5% blocking serum in PBS). In the case of ECE and ET-1 antibody, primary antibodies were incubated for two hours with a 5x excess (weight/weight) of competing peptide (N terminus sequence of ECE for ECE-1 antibody; 5x excess of ET-1 for ET-1 antibody) prior to incubation with sections overnight. Separate, parallel sections were taken along with experiments in which tissues incubated without primary antibody.

The following day, sections were washed 3x with PBS, incubated 30 minutes with appropriate secondary antibody, washed again and incubated 30 minutes with Vector ABC Elite reagent. Antibody binding was detected by incubating sections 1 minute with a DAB developing solution (Vector Laboratories, Burlingame, CA, USA). Primary antibody binding was observed by a dark brown/black precipitate. A loss of primary antibody staining, observed in those sections incubated with primary antibody and competing peptide, was considered qualitatively specific binding of the antibody. All sections were counterstained with Vector Hematoxylin. Sections were photographed using an inverted Nikon T2000 microscope connected to a SPOT Insight color camera using MetaMorph® software. Images were processed using Adobe® Photoshop.

2.2.2. Histological stains—Hematoxylin, Modified Trichrome and May Grunwald stains were performed by the Investigative Histopathology Laboratory at Michigan State University. The modified Trichrome lacked the staining element for collagen, so collagen remains colorless rather than the normal blue in a trichrome stain.

2.3 Zymography Protocol

Gel-based zymography was performed using 10% gelatin gels from Bio Rad Laboratories (Hercules, CA, USA) and running, renaturing and developing buffers from Invitrogen (Carlsbad, CA, USA). Fifty (50) micrograms of total protein were loaded in each lane; a positive control of an active MMP-2/MMP-9 mix was loaded on every gel (Chemicon, Temecula, CA, USA). The ability of minocycline (5×10^{-5} M) to inhibit MMP activity was

verified by incubating the aorta in normal PSS with minocycline for 30 minutes at 37 °C, and then processing for zymography in buffer that also contained minocycline.

Standard electrophoresis was run using zymography running buffer. The gel was renatured for 30 minutes with rocking, and then developed with an initial step of 30 minutes at room temperature with rocking, then at 37 °C in a water bath with gentle rocking for 16–24 hours. Gels were stained for 5 minutes with 0.5% Coomassie Blue and then destained using 10% methanol/10% acetic acid/water solution for at least 4 hours with multiple solution changes. Final destaining used a 50% methanol/1% acetic acid/water solution for at least 4 hours with multiple solution changes. Gels were scanned on a Bio-Rad® Fluor-S Imager.

2.4 Western Protocol

Tissues were isolated directly from the animal, cleaned and placed directly into liquid nitrogen. In liquid nitrogen, tissues were ground to a powder and ice-cold homogenation buffer added [1 ml for one-half an aorta; 0.2 ml for one vena cava; 125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin]. Homogenates were vortexed, sonicated briefly and transferred to a plastic centrifuge tube and spun at 4°C to pellet debris. Supernatant was separated from the pellet and analyzed for protein concentration (BCA protein kit, Sigma Chemical Co., St. Louis, USA). Equivalent amounts of total protein [fifty (50) micrograms] were boiled for 5 minutes with standard 4:1 sample buffer and loaded onto separate lanes. Proteins were separated on 1 mm-thick, 10% SDS polyacrylamide gels using a Mini Bio-Rad® III or Criterion apparatus. A positive control of active MMP-2/MMP-9 (Chemicon, Temecula, CA, USA) was run in some experiments. After transfer, membranes were blocked overnight in 5% milk (4 °C, phosphate buffered saline + 0.025% NaN₃). Primary antibodies [MMP-2 (MAB3308, Chemicon International, Temecula, CA, USA), chymase (human mast cell chymase, ab2377; ABCAM, Cambridge, MA, USA)] were incubated with blots for 24 hours. Blots were then rinsed thrice in Tris-buffered saline (TBS) + Tween (0.1%) with a final rinse in TBS and incubated with the appropriate horseradish peroxidase-linked secondary antibody (1:2000; Cell Signaling Technology, Beverly, MA, USA) for 1 hour at 4 °C (rocking). ECL® reagents (Amersham Life Sciences, Arlington Heights, IL, USA) were used to visualize bands. Gels were stained with Gel Code Blue® (Pierce, Rockford, IL, USA) to verify protein loading and blots were reprobed with smooth muscle α -actin primary antibody (1:1000; Oncogene Research Products, Boston, MA, USA) to ensure equal protein loading and for normalization of reported data.

2.5 Materials

Endothelin peptides were solubilized in water and stored at –20 °C until use. The following are the sources of material used: ET-1 (1–21) (Bachem, King of Prussia, PA, USA), big ET-1 (Phoenix Pharmaceuticals, Belmont, CA USA), ET-1 (1–31) (Peptides International, Louisville, KY, USA), MMP-2/9, GM6001 (Chemicon International, Temecula, CA, USA). ET-1 (1–32) was purified using solid state chromatography after reacting big ET with purified recombinant human MMP-2 (Chemicon International, USA). Purity of ET-1 (1–32) was determined through high pressure liquid chromatography followed by mass spectrometry at the Macromolecular Facility at Michigan State University. Chymostatin (dimethylsulfoxide, 0.1%), minocycline, norepinephrine hydrochloride, phenylephrine hydrochloride, phosphoramidon (ethanol), thiorphan (Sigma Chemical Co, St. Louis, MO, USA). CGS-26393 was a generous gift from Ciba-Geigy and solubilized in dimethylsulfoxide.

2.6 Data analysis

Data are presented as means $\pm$ standard error of the mean for the number of animals. Band density (Western) was quantified using the public domain program NIH Image (v 1.62). When comparing two groups, the appropriate Student's t test was used or ANOVA with repeated

measures. ANOVA followed by Student Newman Keuls post hoc test was performed when comparing three or more groups. In all cases, a P value less than or equal to 0.05 was considered statistically significant.

3. Results

3.1 ECE and big ET-1 in Aorta and Vena Cava

The smooth muscle in the aorta is significantly more abundant than in the vena cava as demonstrated by the significantly darker and homogenous pink staining using a Modified Trichrome stain (Figure 1A), consistent with the greater magnitude of force generated in the aorta observed to agonists (see keys in figure 1). Collagen staining (blue) was omitted from the Trichrome stain so the pink of the smooth muscle layer was more readily observed. The smooth muscle cell layer of the vena cava appears to be one cell layer that underlies the endothelium. Aorta and vena cava had to respond to an adrenergic stimulus prior to proceeding in the experiment. Response to an adrenergic stimulus is an excellent measure of the viability of vascular tissue, as both veins and arteries are innervated by the sympathetic nervous system. All tissues included in experiments reporting contractile data responded to adrenergic stimuli. In the experiments presented within this study, aorta contracted to PE (10^{-5} M) with an average of 2340 ± 280 milligrams, and vena cava contracted to NE (10^{-5} M) with an average of 96 ± 16 milligrams.

Figure 1B compares the isometric contraction of aorta and vena cava to the product of ECE, ET-1 (1–21) and the substrate of ECE, big ET-1 [ET-1 (1–38)]. ET-1 (1–21) contracted aorta and vena cava in a concentration-dependent fashion and with a slightly greater potency in the vena cava as compared to the aorta ($-\log EC_{50}$ [M]; aorta = 8.1 ± 0.1 , vena cava = 8.3 ± 0.1). The maximum contraction (in milligrams) to ET peptide is in the figure legend of each figure for figure 1. The threshold for ET-1 (1–21)-induced contraction was also lower in the vena cava (3×10^{-11} M) compared to the aorta (3×10^{-9} M). Compared to the contraction elicited by maximum adrenergic stimulus (PE/NE), a maximal concentration of ET-1 (1–21) caused a significantly greater contraction in the vena cava ($553 \pm 69\%$ NE) compared to aorta ($134 \pm 11\%$ PE). Qualitatively comparable results were observed when big ET-1 was used as the agonist. Big ET-1, in both aorta and vena cava, was considerably less potent than ET-1 (1–21) (compare top and bottom of figure 1B). Because of foaming issues, concerns as to big ET-1 staying in solution in the tissue bath, and prohibitive expense, we did not use a concentration of big ET-1 higher than 10^{-7} M and did not achieve an absolute maximal response. Thus EC_{50} values could not be calculated exactly and thus are estimated. Notably, vena cava possessed a lower threshold for big ET-1-induced contraction (1×10^{-8} M) compared to aorta (1×10^{-7} M).

Immunohistochemical experiments using an ECE antibody directed against the N terminal sequence of human ECE and an antibody against ET-1 (1–21) demonstrated the presence of ECE and its product in both aorta and vena cava (figure 2). Specific binding is indicated by the reduction in DAB staining (black/brown precipitate) in the tissues in which the antibody was quenched with a competing peptide (CP), and the arrows point to these sites (including the endothelial cell layer and adventitia). The dual neutral endopeptidase/ECE inhibitor phosphoramidon and ECE inhibitor CGS-26393, but not the neutral endopeptidase inhibitor thiorphan, inhibited contraction in aorta and vena cava (figure 3). In the vena cava, CGS-26393-induced inhibition of big ET-1 induced contraction was overcome at the highest concentration of big ET-1 tested (10^{-7} M). At the same concentration that inhibited big ET-1-induced contraction, neither phosphoramidon nor CGS 26393 inhibited KCl-induced contraction (6–100 mM), suggesting that the inhibition of big ET-1-induced contraction was selective. We fully recognize that quantification of ECE expression using Western analyses would have supported these findings, but we have been unable to obtain an antibody that is useful in Western analyses.

3.2 MMPs and big ET-1 in Aorta and Vena Cava

MMPs can process big ET-1 into the primary product ET-1(1–32). We first examined whether aorta and vena cava responded to ET-1(1–32). Figure 4 demonstrates that the vena cava and aorta contracted to ET-1(1–32) in a concentration-dependent fashion. ET-1(1–32) had a significantly greater relative efficacy in the vena cava ($252 \pm 88\%$ NE contraction) than in aorta ($26 \pm 9\%$ PE contraction). ET-1 (1–32), however, was significantly less potent than ET-1(1–21) (figure 4B and 4C), and EC_{50} values for ET-1(1–32) could not be determined as a maximum response was not obtained because of a limited amount of available ET-1(1–32). Because of reports of relaxation of the rat renal artery to ET-1 (1–32), we also investigated the ability of vena cava and aorta to relax to ET-1(1–32). In $PGF_{2\alpha}$ -contracted aorta and vena cava, ET-1 (1–32) caused a minimal change in vessel tone, with the aorta having a modest relaxation that was statistically similar to the vena cava to 100 nM ET-1(1–32) (% contraction remaining: aorta = 83.5 ± 6.4 ; vena cava = 96.2 ± 3.7 ; $p > 0.05$). By contrast, $PGF_{2\alpha}$ -contracted aorta and vena cava readily relaxed over 50% to acetylcholine (1×10^{-6} M), validating the ability of these blood vessels to relax.

Zymographic analyses of an equivalent amount of total protein from the aorta and vena cava demonstrated that the aorta qualitatively possessed a greater amount of latent and active MMPs (MMP2 and MMP9) compared to vena cava (figure 5A, top gel). Densitometry of zymography is not adequately reflected in inverted images, and thus we present these data only qualitatively. This qualitative finding was supported quantitatively by Western analyses of these same homogenates in which the aorta expressed over 250% as much MMP-2 compared to vena cava (figure 5A, middle blot). Smooth muscle α -actin is shown for comparison (figure 5A). The signal for MMP-9 was significantly weaker and was difficult to detect in Western analyses. While active MMPs were present in both aorta and vena cava, pharmacological inhibition by the tetracycline antibiotic minocycline (5×10^{-5} M) (figure 5B) and GM6001 (5×10^{-6} M) did not statistically alter big ET-1 induced contraction in either vessel type. These concentrations of inhibitors were chosen based on previous demonstration of inhibition of MMP2 in arterial tissue (26). Moreover, zymography of aortic homogenates incubated with minocycline prior to and during processing showed a reduction in MMP activity as evidenced by the reduction in intensity of white bands (Figure 5A, bottom panel). Thus, these data suggest that neither the aorta nor vena cava rely on MMP2/9 for generation of vasoactive ET peptides.

3.3 Chymase and big ET-1 in Aorta and Vena Cava

The final system we investigated was production of the putative ET_A receptor agonist, ET-1 (1–31), a product of chymase activity. Figure 6 demonstrates that both aorta and vena cava contracted to ET-1(1–31), though with a significantly lower potency and efficacy than to ET-1 (1–21). Big ET-1-induced contraction was significantly reduced by the chymase inhibitor chymostatin (1×10^{-4} M) in aorta (% PE contraction; control = $21.2 \pm 3.8\%$, chymostatin = $4.3 \pm 1.0\%$) but not in the vena cava ($339.7 \pm 74.3\%$; chymostatin $360.0 \pm 82.7\%$) (figure 7). Notably, big ET-1 induced contraction was reduced by the vehicle for chymostatin, 0.1% dimethylsulfoxide. Chymostatin did not reduce KCl (6–100 mM) contraction in aorta (figure 7, insert), suggesting the inhibition exerted by chymostatin was not non-selective. Immunohistochemical and western analyses validated the presence of chymase in arterial and, to a significantly lesser extent, venous tissue. Anti-chymase antibody recognized one band in homogenates of aorta, was weakly expressed in the vena cava and where the positive control was liver homogenate (figure 8A). Densitometry of these data revealed an expression in aorta (normalized to total protein) that was over 300% of that in the vena cava. Importantly, chymase, also recognized immunohistochemically in figure 8B, is likely not mast cell chymase as no mast cells could be detected in aortic sections using a May-Grunwald stain (figure 8B) and only a few distinct, round punctate mast cells were detected in sections of vena cava (arrows on lower left hand side image). A faint, diffuse tissue staining with the chymase antibody was

detected in the arterial sections (middle panel of figure 8B) and to a lesser extent in vena cava sections; this is also marked with an arrow.

4.0. Discussion

This work was undertaken to understand how big ET-1 could be processed in an isolated artery and vein to form vasoactive peptide(s). ET-1(1–21), the product of ECE, was one of the peptides considered but other peptides were also investigated. Our main finding was a significant difference in the apparent expression and function of the enzyme chymase in arteries when compared to veins, with arteries possessing higher chymase function.

Since its discovery in 1988, ET-1 (1–21) has received intense scrutiny as an endogenously produced substance with profound vasoconstrictive properties. Both arteries and veins are sources of ET-1 (1–21) and are targets for ET peptides [29]. We currently understand that veins and arteries differ in a few basic responses to ET peptides. For example, ET-1 (1–21) is typically more potent in veins than in arteries, veins contract faster to ET-1 (1–21) than do arteries, and veins do not desensitize to ET-1 (1–21) to the magnitude experienced by arteries [32]. Collectively, this work suggests the interaction of ET peptides and veins is worth investigating because of the potential role played in hypertension. This project serves to identify tissue specific differences in both big ET-1 processing and functional responsiveness to ET peptides. The idea and knowledge of alternative processing for endothelin related peptides falls considerably behind that of angiotensin related peptides. A recent study suggests that the processing of prepro ET-1 to pro ET-1 and then big ET-1 may be more complex than anticipated [31]. Our study addresses this complexity only from the standpoint of processing of big ET-1.

ET-1 and ECE

Predictably, ECE and ET-1 (1–21) were localized immunohistochemically to the endothelial cells of both vena cava and aorta. We have been unable to quantify ECE in Western analysis for comparing expression between veins and arteries because of difficulties using the available antibodies against ECE. The antibody used to detect ET-1 (1–21) does not cross react with big ET-1, has limited cross reactivity with ET-2 and ET-3, and it is unknown whether this particular antibody has affinity for ET-1 (1–31) and ET-1 (1–32). Nonetheless, the ability of contraction to big ET-1 to be inhibited by both phosphoramidon and CGS-26393 (but not thiorphan) suggests that part of the contraction to big ET-1 is likely mediated by ET-1 (1–21). The negative results with thiorphan are especially important in the face of data suggesting that CGS-26393 might also possess NEP activity (33). In the aorta, maximum contraction to big ET-1 was reduced in a similar quantitative fashion by the two ECE inhibitors examined. By contrast, the maximum contraction to big ET-1 was not similarly reduced by ECE inhibitors in the vena cava. We do not have a good explanation for this, and can speculate that this could potentially be because of different expression of ECE isoforms in vein *vs* artery. We are unaware, though, of reports that suggest different affinities of CGS-26393 and phosphoramidon for ECE isoforms. Another explanation may be a reduction in the vehicle response (DMSO) in tissues paired with CGS-exposed tissues, thereby minimizing the reduction caused by CGS-26393. This is somewhat unsatisfying given the goal of this experiment. We state here an added piece of published data that suggests that ECE is likely functional in aorta and vena cava as ET-1(1–21) could be detected at similar concentrations in an ELISA assay (35). This study measured content, which would not discriminate between ET-1 bound externally to the tissue *vs* that made by the tissue. These data, collectively, provide modest support for a functional role for ECE in both aorta and vena cava.

A number of studies demonstrate that use of phosphoramidon or inhibition of ECE can reduce elevated blood pressure in genetic and experimental rodent hypertension [1,2,22,27,28,34].

Thus, the role of this pathway in both arterial and venous function is relevant for understanding vascular abnormalities contributing to hypertension.

ET-1 and MMPs

Matrix metalloproteases are capable of processing big ET-1 into the vasoactive peptide ET-1 (1–32). This discovery is important because of the potential of this peptide to be produced *in vivo*, given the proximity of MMPs to the endothelial cells. We examined the ability of purified ET-1(1–32) to contract and/or relax isolated aorta and vena cava. The reason for investigating the relaxation to ET-1 (1–32) was because of the discovery that this peptide was produced through MMP-2 activation and was vasorelaxant at ET_B receptors in small renal arteries [10]. ET-1 (1–32) only weakly contracted and relaxed both vessels. Vena cava and aorta both possess the mechanisms to produce ET-1(1–32), namely expression of functional MMP-2. However, pharmacological inhibition of MMPs by either minocycline [26] or GM6001 did not inhibit big ET-1 induced contraction. These findings suggest that ET-1 (1–32) may not be produced within these blood vessels and have *in vivo* effects. Inhibitors of MMP have not been examined for their ability to inhibit blood pressure elevation, and thus the contribution of MMP-derived ET like peptides to blood pressure regulation, normal or elevated, is unknown.

ET-1 and chymase

Chymase has received considerable attention as an angiotensin converting enzyme-independent means to produce angiotensin II. The source of chymase is largely attributed to mast cells, but there is at least one chymase—vascular chymase-- that is mast cell-independent [7,12]. Both forms of chymase can be inhibited by chymostatin, and chymostatin is the only identified inhibitor that can inhibit vascular chymase in a concentration-dependent manner [12]. Chymase, as detected immunohistochemically, was present in the aorta and less obviously in the vena cava. Western analyses demonstrated a robust signal from the aortic homogenates but not from the vena cava. The source of arterial chymase is likely not mast cell derived, as May-Grunwald stain revealed only a sparse population of mast cells in blood vessels, more readily detectable in the vena cava than in the aorta. Interestingly, the chymase inhibitor chymostatin reduced contraction to big ET-1 in the aorta, but not the vena cava. Tissues incubated with chymostatin had a normal contraction to exogenous ET-1 (1–21) (over 130% PE contraction), indicating that chymostatin did not affect aortic sensitivity to ET peptides, but specifically inhibited big ET-1 processing. These data are consistent with the finding that the aorta contracted to chymase product of big ET-1, ET-1 (1–31). We have been unable to locate a specific inhibitor for vascular chymase or other inhibitors known to inhibit vascular chymase, and thus chymostatin represents the best tool available at the present time for investigating vascular contractility. Collectively, these findings suggest that chymase may be important in producing ET peptides in arterial but not venous tissue. Chymase was recognized as an enzyme that converted big ET-1 to constrictor endothelin peptides in human umbilical venous smooth muscle [17]. Thus, processing of big ET-1 is likely tissue specific, and does not follow along strictly definitive lines in a venous *vs* arterial manner. We are aware of two reports investigating the ability of chymase inhibition to modify blood pressure [15,16]. The outcome of these reports suggests that the role of chymase is far from being understood, and warrants more direct approaches.

5.0 Limitations

There are several limitations to this study that need to be recognized. First, we have used large arteries and veins because of their ease of use in biochemical experiments, and thus this work may not necessarily apply to small arteries and veins. Our laboratories have, however, observed a similar relative response of small mesenteric veins and arteries with respect to ET-1 and adrenergic stimuli, where the ET peptides are amongst the most potent and efficacious

venoconstrictors we have studied. Second, we have not addressed the possibility of different isoforms of ECE being expressed in arteries vs veins – phosphoramidon does not distinguish between the forms recognized, ECE-1, 2 and 3. Third, the endothelial cell layer was intact in all experiments, and thus endogenous big ET-1 was present in these experiments. Lastly, it needs to be confirmed that some of these ET peptides are actually made within the aorta and vena cava, though HPLC separation of the four peptides of interest [big ET-1, ET-1 (1–21), ET-1 (1–31), ET-1 (1–32)] has proven difficult.

6.0 Conclusions

- These results suggest an overall similarity in the ability of large arteries and veins to convert big ET-1 into vasoactive peptides.
- ET-1 (1–21) and likely ECE are present and functional in both arteries and veins.
- The machinery for converting big ET-1 into two relatively understudied peptides – ET-1 (1–31) and ET-1 (1–32)—is present in both arteries and veins.
- Arteries but not veins have the ability to utilize chymase as an ET processing enzyme.
- Thus, there are differences in the *in vitro* processing of ET peptides in arteries and veins.

Acknowledgments

Supported by NIH PO1HL70687

References

1. Battistini B, Ayach B, Molez S, Blouin A, Jeng AY. Effects of benazepril, an angiotensin converting enzyme inhibitor, combined with CGS 35066, a selective endothelin converting enzyme inhibitor, on arterial blood pressure in normotensive and spontaneously hypertensive rats. *Clin Sci* 2002;103(S48): 363S–366S. [PubMed: 12193123]
2. Battistini B, Daull P, Jeng AY. CGS 3601, a triple inhibitor of angiotensin converting enzyme, neutral endopeptidase and endothelin converting enzyme. *Cardiovasc Drug Rev* 2005;23:317–330. [PubMed: 16614731]
3. Fecteau MH, Honore JC, Plante M, Labonte J, Rae GA, D'Orleans-Juste P. Endothelin-1 (1–31) is an intermediate in the production of endothelin-1 after big endothelin-1 administration in vivo. *Hypertension* 2005;46:87–92. [PubMed: 15956117]
4. Fernandez-Patron C, Radomski MW, Davidge ST. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circ Res* 1999;85:906–911. [PubMed: 10559137]
5. Fernandez-Patron C, Radomski MW, Davidge ST. Role of matrix metalloproteinase-2 in thrombin-induced vasorelaxation of rat mesenteric arteries. *Am J Physiol* 2000;278:H1473–H1479.
6. Gonzalez-Santiago L, Lopez-Ongil S, Grier M, Rodriguez-Puyol M, Rodriguez-Puyol D. Regulation of endothelin synthesis by extracellular matrix in human endothelial cells. *Kid Int* 2002;62:537–543.
7. Guo C, Ju H, Leung D, Massaeli H, Shi M, Rabinovitch M. A novel vascular smooth muscle chymase is upregulated in hypertensive rats. *J Clin Invest* 2001;107:703–715. [PubMed: 11254670]
8. Harrison VJ, Barnes K, Turner AJ. Identification of endothelin 1 and big endothelin 1 in secretory vesicles isolated from bovine aortic endothelial cells. *Proc Natl Aca Sci USA* 1995;92:6344–6348.
9. Hisaki K, Matsumura Y, Ikegawa R, Nishiguchi S, Hayashi K, Takaoka M, Morimoto S. Evidence for phosphoramidon-sensitive conversion of big endothelin-1 to endothelin-1 in isolated rat mesenteric artery. *Biochem Biophys Res Commun* 1991;177:1127–1132. [PubMed: 2059203]
10. Jeyabalan A, Novak J, Danielson LA, Kerchner LJ, Opett SL, Conrad KP. Essential role for vascular gelatinase activity in relaxin-induced renal vasodilation, hyperfiltration, and reduced myogenic reactivity of small arteries. *Circ Res* 2003;93:1249–1257. [PubMed: 14593002]

11. Johnson RJ, Fink GD, Watts SW, Galligan JJ. Endothelin receptor function in mesenteric veins from deoxycorticosterone acetate salt hypertensive rats. *J Hypertension* 2002;20:665–676.
12. Ju H, Gros R, You X, Tsang S, Husain M, Rabinovitch M. Conditional and targeted overexpression of vascular chymase causes hypertension in transgenic mice. *Proc Natl Acad Sci USA* 2001;98:7469–7474. [PubMed: 11416217]
13. Kido H, Nakano A, Okishima N, Wakabayashi H, Kishi F, Nakaya Y, Yoshizumi M, Tamaki T. Human chymase, an enzyme forming novel bioactive 31-amino acid length endothelins. *Biol Chem* 1998;379:885–891. [PubMed: 9705152]
14. Kido T, Sawamura T, Masaki T. The processing pathway of endothelin-1 production. *J Cardiovasc Pharmacol* 1998;31:S1, S13–S15. [PubMed: 9595383]
15. Kirimura K, Takai S, Jin D, Muramatsu M, Kishi K, Yoshikaw K, Nakabayashi M, Mino Y, Miyazaki M. Role of chymase-dependent angiotensin II formation in regulating blood pressure in spontaneously hypertensive rats. *Hypertens Res* 2005;28:457–464. [PubMed: 16156510]
16. Li M, Liu K, Michalick J, Angus JA, Hunt JE, Dell'Italia LJ, Feneley MP, Graham RM, Husain A. Involvement of chymase-mediated angiotensin II generation in blood pressure regulation. *J Clin Invest* 2004;114:112–120. [PubMed: 15232618]
17. Maguire J, Davenport AP. Alternative pathway to endothelin-converting enzyme for the synthesis of endothelin in human blood vessels. *J Cardiovasc Pharmacol* 2004;44(S1):S27–29. [PubMed: 15838298]
18. Maguire JJ, Johnson CM, Mockridge JW, et al. Endothelin converting enzyme (ECE) activity in human vascular smooth muscle. *Br J Pharmacol* 1997;122:1647–1654. [PubMed: 9422810]
19. Maguire JJ, Kuc RE, Davenport AP. Vasoconstrictor activity of novel endothelin peptide, ET-1(1–31), in human mammary and coronary arteries in vitro. *Br J Pharmacol* 2001;134:1360–1366. [PubMed: 11704658]
20. Matsumura Y, Ikegawa R, Tsukahara Y, Takaoka M, Morimoto S. Conversion of big endothelin-1 to endothelin-1 by two types of metalloproteinases of cultured porcine vascular smooth muscle cells. *Biochem Biophys Res Commun* 1991;178:899–905. [PubMed: 1872872]
21. Mazzocchi G, Rossi GP, Malendowicz LK, Champion HC, Nussdorfer GG. Endothelin-1[1–31], acting as an ET_A receptor selective agonist, stimulates proliferation of cultured rat zona glomerulosa cells. *FESB Lett* 2000;487:194–198.
22. McMahon EG, Palomo MA, Brown MA, Bertenshaw SR, Carter JS. Effect of phosphoramidon (endothelin converting enzyme inhibitor) and BQ-123 (endothelin receptor subtype A antagonist) on blood pressure in hypertensive rats. *Am J Hypertens* 1993;6:667–673. [PubMed: 8217029]
23. Merchant SJ, Davidge ST. The role of matrix metalloproteinases in vascular function: implications for normal pregnancy and preeclampsia. *BJOG* 2004;111:931–939. [PubMed: 15327607]
24. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPS. *Cardiovasc Res* 2006;69:562–573. [PubMed: 16405877]
25. Nagata N, Niwa Y, Nakaya T. A novel 31-amino acid length endothelin, ET-1(1–31), can act as a biologically active peptide for vascular smooth muscle cells. *Biochem Biophys Res Commun* 2000;275:595–600. [PubMed: 10964709]
26. Pinney SP, Chen HJ, Liang D, Wang X, Schwartz A, Rabbani LE. Minocycline inhibits smooth muscle cell proliferation, migration and neointima formation after arterial injury. *J Cardiovasc Pharmacol* 2003;42:469–476. [PubMed: 14508231]
27. Quaschnig T. Vasopeptidase inhibition for blood pressure control: emerging experience. *Curr Pharm Des* 2005;11:3293–3299. [PubMed: 16250856]
28. Roques BP. Cell surface metalloproteinases involved in blood pressure regulation: structure, inhibition and clinical perspectives. *Pathol Biol* 1998;46:191–200. [PubMed: 9769915]
29. Schiffrin EL. Role of endothelin-1 in hypertension and vascular disease. *Am J Hypertens* 2001;14:83S–89S. [PubMed: 11411770]
30. Schweizer A, Valdenaire O, Nelbock P, Deuschle U, Dumas Milne Edwards JB, Stumpf JG, Löffler BM. Human endothelin-converting enzyme (ECE-1): three isoforms with distinct subcellular localizations. *Biochem J* 1997;328:871–877. [PubMed: 9396733]
31. Struck J, Morgenthaler NG, Bergmann A. Proteolytic processing patterns of the endothelin-1 precursor in vivo. *Peptides* 2005;26:2482–2486. [PubMed: 15978702]

32. Thakali K, Fink GD, Watts SW. Arteries and veins desensitize differently to endothelin-1. *J Cardiovasc Pharmacol* 2004;43:387–393. [PubMed: 15076222]
33. Trapani AJ, De Lombaert S, Kuzmich S, Jeng AY. Inhibition of big ET-1-induced pressor response by an orally active dual inhibitor of endothelin-converting enzyme and neutral endopeptidase 24.11. *J Cardiovasc Pharmacol* 1995;26(S3):S69–S71. [PubMed: 8587471]
34. Vemulapalli S, Watkins RW, Brown A, Cook J, Bernardino V, Chiu PJ. Disparate effects of phosphoramidon on blood pressure in SHR and DOCA-salt hypertensive rats. *Life Sci* 1993;53:783–793. [PubMed: 8355566]
35. Wang H, Chen AF, Watts SW, Galligan JJ, Fink GD. Endothelin in the splanchnic vascular bed of DOCA-salt hypertensive rats. *Am J Physiol Heart Circ Physiol* 2005;288:H729–H736. [PubMed: 15471983]
36. Watts SW, Fink GD, Northcott CA, Galligan JJ. Endothelin -1-induced venous contraction is maintained in DOCA-salt hypertension; studies with receptor agonists. *Br J Pharmacol* 2002;137:69–79. [PubMed: 12183332]
37. Wypij DM, Nichols JS, Novak PJ, Stacy DL, Berman J, Wiseman JS. Role of mast cell chymase in the extracellular processing of big endothelin-1 to endothelin-1 in the perfused rat lung. *Biochem Pharmacol* 1992;43:845–853. [PubMed: 1540238]
38. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;332:411–415. [PubMed: 2451132]

A. Modified Trichrome

B.

Figure 1.

A. Modified trichrome staining (no collagen, blue stain) that demonstrates the significantly greater smooth muscle mass (pink) in the aorta (top) compared to the vena cava (bottom) (arrows)

B. Concentration dependent contraction elicited by ET-1 (top) and big ET-1 (bottom) in rat thoracic aorta and vena cava. Values in key is the maximum response in milligrams. Points represent means±SEM for number of animals in parentheses. PE = phenylephrine, NE = norepinephrine, L = lumen.

Figure 2.

Comparison of immunohistochemical staining for ECE and ET-1 in rat thoracic aorta (left) and vena cava (right). Sections were incubated with primary antibody (ECE or ET-1), primary antibody quenched with a 5x (weight/weight) excess of competing peptide (+ CP), or no primary antibody (no primary). Arrows indicate immunohistochemical staining and sections to be compared with CP. Representative of five (5) separate animals. L = lumen.

Figure 3.

Effect of the ECE/neutral endopeptidase inhibitor phosphoramidon (A), ECE inhibitor CGS-26393 (B) and neutral endopeptidase inhibitor thiorphan (C) on contraction stimulated by big ET-1 in the rat thoracic aorta (top) and vena cava (bottom). Points represent means $\pm$ SEM for number of animals in parentheses. * indicate significant differences from vehicle ($p < 0.05$). PE = phenylephrine, NE = norepinephrine.

Figure 4.

Comparison of contraction stimulated by ET-1(1–21) with the MMP derived endothelin, ET-1 (1–32) in the rat thoracic aorta (A) and vena cava (B). Panel C depicts results when ET-1(1–32) was examined as a relaxant agonist in PGF_{2α}-contracted aorta and vena cava. Points represent means±SEM for number of animals in parentheses. PE = phenylephrine, NE = norepinephrine.

Figure 5.

A. Zymographic (top) and Western (bottom) demonstration of the existence of MMP-2 in homogenates of rat thoracic aorta and vena cava. α -actin was also run as a control for amount of muscle. The bottom zymogram shows the effects of minocycline preincubation on MMP activity in aortic homogenates. Representative of eight separate rats. B. Effect of the non-specific MMP-1 inhibitor minocycline on big ET-1 induced contraction in rat thoracic aorta (top) and vena cava (bottom). Points represent means $\pm$ SEM for number of animals in parentheses. PE = phenylephrine, NE = norepinephrine.

Figure 6.

Contraction of rat thoracic aorta and vena cava to the chymase derived endothelin peptide ET-1 (1–31). Points represent means $\pm$ SEM for number of animals in parentheses. PE = phenylephrine, NE = norepinephrine.

A.

B.

Figure 7.

Comparison of the effect of the chymase inhibitor chymostatin on big ET-1-induced contraction in rat thoracic aorta (top) and vena cava (bottom). Points represent means $\pm$ SEM for number of animals in parentheses. * indicate significant differences from vehicle ($p < 0.05$). PE = phenylephrine, NE = norepinephrine. In panel A, the insert depicts the effects of the same concentration of chymostatin on KCl-induced contraction in aorta.

Figure 8.

A. Western analyses of chymase expression homogenates of rat thoracic aorta and vena cava. + control = liver homogenate. B. May Grunwald staining for mast cells in aorta (top panel) and vena cava (bottom panel). Arrows point to the dark blue/black rounded shape of mast cells. The middle panel is immunohistochemical results of staining for human chymase, followed by hematoxylin staining in the last panels to the right. Representative of six (6) separate rats. L = lumen.