Endothelin-1-induced venous contraction is maintained in DOCA-salt hypertension; studies with receptor agonists

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1 Deoxycorticosterone acetate (DOCA) salt hypertension is associated with an endothelin-1 (ET-1)-dependent increase in arterial resistance and mean circulatory filling pressure. Contraction of endothelium-intact arteries and veins from sham and DOCA-salt hypertensive rats to agonists of the ET_A (ET-$1_{(1-31)}$) and ET_B receptor (sarafotoxin 6c; S6c) was investigated in tissue baths as was expression of mRNA for ET-1 and mRNA and protein for the ET_A and ET_B receptor.

2 ET-$1_{(1-31)}$ contracted aorta and vena cava from sham rats with a 30 fold lower potency than ET-1. Contraction was not altered by the ET_B receptor antagonist BQ788 (100 nM) but was abolished by the ET_A receptor antagonist ABT-627 (30 nM).

3 In DOCA-salt thoracic aorta, maximum contraction to ET-1 and ET-$1_{(1-31)}$ was reduced (36.6 ± 6.3 and $13.3 \pm 4.4\%$ of sham response, respectively); aorta did not contract to S6c.

4 In vena cava from DOCA-salt rats, contraction to ET-1 and ET-$1_{(1-31)}$ was not reduced compared to sham contraction; vena cava from sham and DOCA-salt rats contracted to S6c with a similar potency.

5 Real time RT–PCR revealed that prepro ET-1 mRNA was increased 6.6 ± 3.3 fold and 8.7 ± 3.9 fold greater in DOCA-salt aorta and vena cava, respectively, compared to sham. Vena cava expressed a higher content of ET_A and ET_B receptor mRNA than aorta ($P < 0.05$), but no differences were observed between sham and DOCA-salt tissues. ET_A and ET_B receptor protein was identified in all tissues. Immunoreactive ET_A receptor, observed as a 65, 30 and 28 kDa bands, was expressed 400% greater in DOCA-salt aorta compared to sham, but was not altered in vena cava. Immunoreactive ET_B receptor, observed as 120, 45 and 30 kDa bands, tended to be higher in vena cava compared to aorta, but was not different in sham and DOCA-salt vena cava.

6 These results suggest that ET_A receptor function is impaired in aorta but not vena cava of DOCA-salt rats. The ET_B receptor was present in the aorta but, unlike in veins, does not mediate contraction directly. A sustained response to ET-1 in the venous circulation may contribute to the elevated blood pressure in the DOCA-salt model.

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Introduction

The DOCA-salt model of hypertension is associated with elevated arterial levels of the peptide endothelin (ET-1) (Lariviere *et al.*, 1993). ET-1 has been implicated in multiple cardiovascular functions and diseases, but recent evidence suggests a significant involvement in hypertension (Li *et al.*, 1994; Verhagen *et al.*, 1998; Matsumura *et al.*, 1999; Schiffrin, 2001). The endothelins combine with two receptor subtypes to exert their physiological effect, the ET_A and ET_B receptors. The ET_B receptor can be activated selectively by sarafotoxin 6c (S6c) and antagonized by BQ788. Several receptor antagonists, including ABT-627 and BQ610, are selective for the ET_A receptor and recently the chymase-derived peptide ET-$1_{(1-31)}$ has been described as a relatively selective agonist of ET_A receptors (Hanson *et al.*, 1997; Nagata *et al.*, 2000; Maguire *et al.*, 2001).

We are interested in changes in venous function that may contribute to the elevated blood pressure observed in the DOCA-salt model. We and others have demonstrated that endothelin receptor antagonists reduce total peripheral resistance in the DOCA-salt rat and have recently observed that endothelin receptor antagonists also reduce mean circulatory filling pressure, indicating a possible contribution of increased venous smooth muscle contractility to determination of blood pressure in the DOCA-salt model (Fink *et al.*, 2000; Johnson *et al.*, 2001). Notably, veins are more sensitive to contraction induced by ET-1 than are arteries. This increased sensitivity, observed as a lower threshold concentration of ET-1 and higher potency, has been observed by several groups; the contractile potency of ET-1 in veins has been reported as anywhere from 3–10 times more than in arteries (Cocks *et al.*, 1989; D'Orleans-Juste *et al.*, 1989). Importantly, small unpressurized mesenteric veins from the DOCA-salt rat maintain their responsiveness to ET-1 whereas arteries from the same site have a depressed

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contraction to ET-1 (Nguyen *et al.*, 1992; Fujita *et al.*, 1995; Laurant & Berthelot, 1996; Zhao *et al.*, 2000; Johnson *et al.*, 2002).

Thus, we currently test the hypothesis that veins maintain endothelin responsiveness in the face of overexpression of ET-1. We used large arteries and veins for two reasons. First, this was done to test the principle that the maintained responsiveness is occurring in large veins as it is in small veins. Second, arteries and veins of this size allow us to perform biochemical experiments to measure mRNA and protein for the ET_A receptor and ET_B receptor.

Methods

Model of hypertension

Male Sprague-Dawley rats (0.225–0.250 kg; Charles River, Portage, MI, U.S.A.) were uninephrectomized and deoxycorticosterone acetate (DOCA, 200 mg kg^{-1} in silicone rubber) implanted subcutaneously. Postoperatively, the rats were given a solution of 1% NaCl and 0.2% KCl for drinking. Sham normotensive rats were uninephrectomized, received no DOCA and drank normal tap water. All rats were given free access to standard pelleted rat chow (Harlan/ Teklad 8640 rodent diet). Animals remained on this regimen for 4 weeks prior to use.

Blood pressure measurement

Systolic blood pressures of rats were determined in the conscious state by the tail cuff method (pneumatic transducer, Narco, TX, U.S.A.).

Isolated smooth muscle contractility measurement

Rats were deeply anaesthetized with pentobarbitone (50 mg kg^{-1}, i.p.) to the point of a loss of eyelid reflex and lack of withdrawal from painful stimuli. Aorta and vena cava were placed in physiologic salt solution consisting of (in mM) NaCl 130; KCl 4.7; KH_2PO_4 1.18; $MgSO_4$-$7H_2O$ 1.17; $CaCl_2$-$2H_2O$ 1.6; $NaHCO_3$ 14.9; dextrose, 5.5; and Ca-Na_2EDTA, 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 (Astro-Med, West Warwick, RI, U.S.A.) or PowerLab® for the Macintosh (ADInstruments, Dover, NH, U.S.A.). Tissues were placed under optimum resting tension (4000 mg for aorta, 1000 mg for vena cava; determined in preliminary experiments). Tissues from sham and DOCA-salt rats were placed in the same bath, controlling for experimental variations. Muscle baths were filled with warmed (37°C), aerated (95% O_2, 5% CO_2) physiological salt solution. Tissues were challenged with a maximal concentration of α adrenergic agonist (noradrenaline for vein, phenylephrine for artery). Phenylephrine was not used in the vena cava as it could not stimulate a reproducible contraction in our hands. Phenylephrine was, however, used for aorta so as to be able to relate these findings to those generated from our laboratory in the past. Functional integrity of the endothelial cells was evaluated by testing relaxation caused by acetylcholine (1 uM) in strips contracted with α adrenergic agonist (10–100 nM). Cumulative concentration response curves to agonists were generated. Endothelin receptor agonists contract tissues slowly, so tissues were exposed to each concentration of endothelin receptor agonist (ET-1, S6c, ET-1$_{(1-31)}$) for a minimum of 5 min prior to adding a higher concentration of agonist. Antagonists, inhibitors or vehicle incubated with vessels for 1 h prior to addition of agonists.

Real time RT–PCR

Two step RT–PCR was performed using a GeneAMP 5700 Real Time PCR machine (Applied Biosystems, Foster City, CA, U.S.A.). Total RNA was isolated using standard TRIzol® procedures (GIBCO Life Technologies, Rockville, MD, U.S.A.). Vena cava and thoracic aorta from both sham and DOCA-salt animals were isolated concurrently. Vena cava from three animals were pooled so that sufficient RNA could be isolated. Concentration/purity/integrity of RNA was ascertained spectrophotometrically (A_{260}/A_{280}) and by running a qualitative 1% agarose gel to visualize samples with ethidium bromide (18S/28S check). Two micrograms of total RNA were reverse transcribed using a Taqman® reverse transcriptase kit (Applied Biosystems, Foster City, CA, U.S.A.; buffer, $MgCl_2$ 5.5 mM, dNTP 500 uM, of each random hexamer 2.5 uM, RNase inhibitor 0.4 u ul^{-1} and MultiScribe Reverse Transcriptase 1.25 u ul^{-1}; 10 min hold at 25°C, 30 min hold at 48°C, 5 min hold at 95°C). Samples were always paired with ones in which reverse transcriptase was not included. One-tenth of this cDNA was taken through polymerase chain reaction using a SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, U.S.A.), forward primer and reverse primer for 40 cycles [AmpliTAQ® activation, 10 min hold at 95°C (hot start); cycle = 15 s 95°C, 60 s 60°C]. Primer concentration (25 pM – 500 nM) was optimized and primers designed using Primer Express® 1.5 (Applied Biosystems, Foster City, CA, U.S.A.) and checked for selectivity using BLAST® within the program MacVector® 1.5 (Accelrys, San Diego, CA, U.S.A.). Samples from sham and DOCA-salt tissues were run in parallel as well as samples containing primers for GAPDH to use as a calibrator reference. Samples without cDNA were also taken along as no template controls. A dissociation protocol (60– 95°C melt) was performed at the end of the experiment to verify that one product was present and melted at the appropriate temperature. Final PCR product was examined on a 2% agarose gel stained with ethidium bromide (0.5 ug ml^{-1}) to determine that the appropriately sized amplicon was amplified. Gels were visualized and captured using a Bio-Rad® Fluor-S imaging station (Hercules, CA, U.S.A.). Rodent-derived primers that were used are GAPDH forward, reverse (purchased from Applied Biosystems, Foster City, CA, U.S.A.) with a T_m of 86°C and 228 base pairs (bp). All other primers were synthesized by the Macromolecular Structures and Synthesis Facility at Michigan State University; ET-1 forward = TCT GGG TCA ACA CTC CCG A, ET-1 reverse = AGG ATC GCT TAG ACC TAG AAG GG (T_m = 83°C, 69 bp); ET_A forward = GGA ATG GGA GCT TGC GG; ET_A reverse = TTT GCC ACC TCT CGA CGC (T_m = 84°C, 61 bp); ET_B forward = CAA AGG AGG GAG

GGT GGC, ET_B reverse = CAA TTT TTC GTT GGC ACG G ($T_m = 84°C$, 65 bp).

Western analysis

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 phenylmethylsulphonyl fluoride, 1 mM orthovanadate, 10 ug ml^{-1} aprotinin, 10 ug ml^{-1} 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 analysed for protein concentration (BCA protein kit, Sigma Chemical Co., St. Louis, U.S.A.). Eighty micrograms of total protein for measurement of the ET receptors were heated at 37°C for 30 min with standard 4:1 sample buffer. This approach was taken to minimize aggregation of the heptahelical receptors. Proteins were separated on 1 mm-thick, 12% SDS poly-acrylamide gels using a Mini Bio-Rad III apparatus. Membranes were blocked overnight in 5% milk (4°C, phosphate buffered saline + 0.025% NaN_3). Primary antibodies (1:200 for ET_A and 1:1000 for ET_B receptor from Alomone Laboratories, Jerusalem, Israel) incubated with blots from 48 h. In some experiments, the antibodies were quenched with a competing peptide (microgram/microgram; CP) so as to visualize those bands identified specifically by the antibody. Blots were then rinsed three times in Tris-buffered saline (TBS) + Tween (0.1%) with a final rinse in TBS and incubated with an horseradish peroxidase-linked antirabbit secondary antibody (1:2000; Cell Signaling Technology, Beverly, MA, U.S.A.) for 1 h at 4°C (rocking). ECL® reagents (Amersham Life Sciences, Arlington Heights, IL, U.S.A.) were used to visualize bands. Gels were stained with Gel Code Blue® (Pierce, Rockford, IL, U.S.A.) to verify protein loading and blots were reprobed with smooth muscle α-actin primary antibody (1:1000; Oncogene Research Products, Boston, MA, U.S.A.) to ensure equal protein loading.

Data analysis

Data are presented as means ± standard error of the mean for the number of animals. Contraction is reported as force (milligrams) or as a percentage of response to maximum contraction or phenylephrine/noradrenaline (10 uM). pD_2 values (negative logarithm of the agonist concentration necessary to produce a half-maximal response [M]) were determined using non-linear regression analysis in GraphPad Prism® (San Diego, CA, U.S.A.). When comparing two groups, the appropriate Student's *t*-test was used or, in concentration response curves, 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. Band density was quantified using the public domain program NIH Image (v 1.62). For RT–PCR, an *n* of 1 for vena cava actually represent three animals whose tissues were pooled. C_T values were derived as the threshold cycle at which product was first detected and are reported as cycle numbers.

Materials

ET-1 and sarafotoxin 6c were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.). ET-1$_{(1-31)}$ was obtained from Peptide International (Lexington, KY, U.S.A.). These peptides were solubilized in deionized water or 0.1% acetic acid. BQ788 (solubilized in dimethylsulphoxide) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.) and ABT-627 (dimethylsulphoxide) was a gift from Abbott Laboratories. Acetylcholine chloride, deoxycorticosterone acetate, noradrenaline hydrochloride and phenylephrine hydrochloride were solubilized in water and purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Blood pressures

For all studies, rats were on DOCA-salt for 4 weeks prior to experimentation. Systolic blood pressures were 120 ± 2 mmHg in sham rats ($n = 48$) and DOCA-salt rats were hypertensive ($n = 48$; 192 ± 9 mmHg; $P < 0.05$). We validated that the endothelium was intact in these preparations as the muscarinic agonist acetylcholine (1 uM) relaxed sham vena cava ($60.1 \pm 8.7\%$ relaxation) and DOCA-salt vena cava (59.1 ± 9.6; $P > 0.05$) which were contracted with a half-maximal concentration of α adrenoreceptor agonist. As expected, acetylcholine relaxed the DOCA-aorta ($29.1 \pm 12\%$) significantly less than in sham aorta ($62.2 \pm 6.2\%$; $P < 0.05$) as endothelium-dependent arterial relaxation is reduced in this model (Lockette *et al*., 1986).

Effect of ET-1

Figure 1a depicts vessel contraction to ET-1 as a percentage of initial contraction to phenylephrine (PE) or noradrenaline (NA) (left) and as a percentage of maximum contraction (right) for aorta and vena cava. ET-1 contracted all vessels but a few distinct and important differences were observed. First, the vena cava of sham and DOCA-salt animals had a lower threshold for ET-1 than in aorta ($-$log threshold [M] aorta = 9.25 ± 0.20, vena cava = 10.43 ± 0.22; $P < 0.05$ using grouped data). Second, ET-1 was 2.5 times more potent in vena cava (pD_2 [M] = 8.29 ± 0.03) than in aorta (7.99 ± 0.08; $P < 0.05$). Significantly, the maximum response of the aorta to ET-1 was markedly diminished in DOCA-salt tissue as compared to sham tissues (upper left). This decrease was not due to a change in the affinity of ET receptors present as the potency of ET in aorta from DOCA-salt and sham rats was not different (upper right). By contrast, the efficacy of ET-1 was not diminished in the vena cava of the DOCA-salt rat when compared to the sham (lower left), nor did the potency of ET-1 change (lower right). Thus, these data suggest that ET receptors or contractile elements used by ET receptors are desensitized in the aorta but not in vena cava.
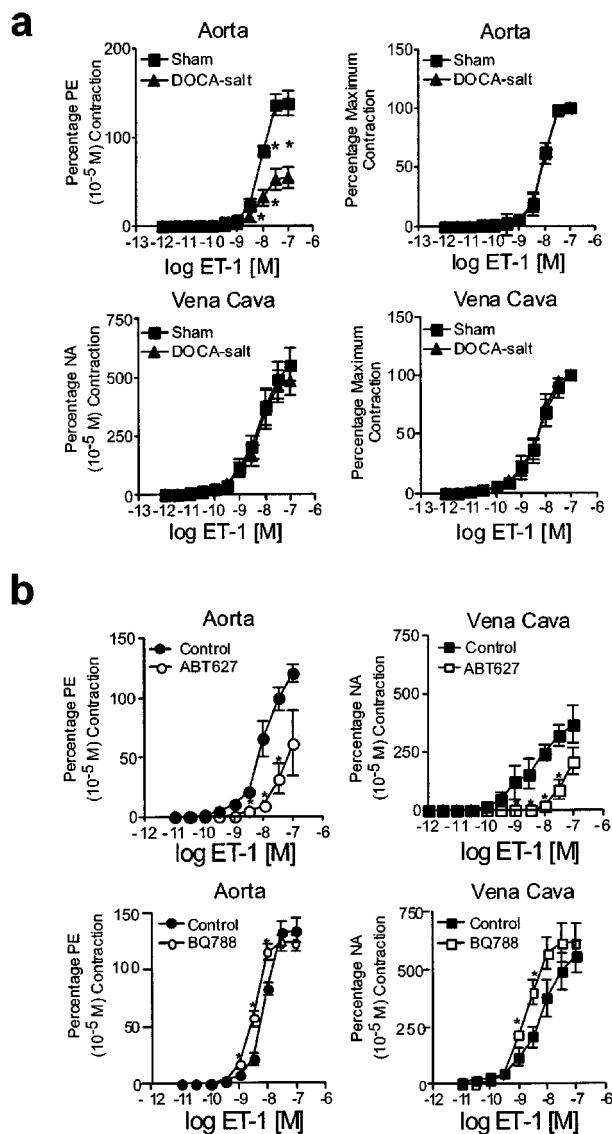
a

b

Figure 1 (a) Effect of endothelin-1 (ET-1) on aorta and vena cava. Data are presented as means ± s.e.mean and as a percentage of an initial phenylephrine (PE) or noradrenaline (NA) response (left) or as a percentage maximal response (right) for an n of 5–6 animals. (b) Effect of ET_A receptor antagonist ABT-627 (30 nM; top) and ET_B receptor antagonist BQ788 (100 nM; bottom) on ET-1-induced contraction in aorta (left) and vena cava (right) isolated from sham normotensive rats. Data are presented as means ± s.e.mean and as a percentage of an initial phenylephrine (PE) or noradrenaline (NA) response (left) for an n of 5–6 animals. * $P < 0.05$, ANOVA repeated measures.

Effect of ET receptor antagonists on ET-1-induced contraction

To establish the subtype of receptor primarily responsible for mediating ET-1-induced contraction in the normotensive state, we performed experiments examining the ability of the ET_A receptor antagonist ABT-627 (30 nM) and ET_B receptor antagonist BQ788 (100 nM) to alter ET-1-induced contraction. ABT-627 antagonized contraction to ET-1 in both normal aorta and vena cava (Figure 1b). By contrast, the ET_B receptor antagonist BQ788 did not reduce but

significantly potentiated contraction to ET-1 in both aorta (Figure 1b, left) and vena cava (Figure 1b, right). This was reflected by a significant change in the pD_2 value for ET-1 [aorta control: 8.00 ± 0.12; aorta BQ788: 8.45 ± 0.12, $P < 0.05$; vena cava control: 8.23 ± 0.03; vena cava BQ788 8.72 ± 0.06, $P < 0.05$]. These studies indicate that in both aorta and vena cava, the ET_A receptor is the predominant contactile receptor and the ET_B receptor modestly depresses contraction to ET-1.

Effect of noradrenaline

We next examined contraction to the α adrenoceptor agonist noradrenaline in contractile experiments as a comparative agonist to ET-1. The adrenoceptor and endothelin receptors use similar signalling elements. Aortae from the DOCA-salt hypertensive rat were more sensitive to NA than aorta from sham rats (pD_2 [M] sham = 7.42 ± 0.07; DOCA-salt = 7.9 ± 0.03; $P < 0.05$) (Figure 2a). The maximum aortic response to NA was not altered in DOCA-salt hypertension. The efficacy of NA was not altered in DOCA-salt hypertension in vena cava, nor was the potency of NA in the vena cava altered (pD_2 [M] sham = 6.3 ± 0.05; DOCA-salt = 6.3 ± 0.12; $P > 0.05$). These data support that the decrease in response to ET-1 in the aorta was not because of general hyporeactivity of aorta from DOCA-salt hypertensive rats.

Effect of sarafotoxin 6c

Figure 2b demonstrates that S6c was without activity in aorta of either sham or DOCA-salt rats, indicating that the ET_B receptor does not play a direct role in modifying contraction. However, S6c caused a concentration-dependent contraction in the vena cava of both sham and DOCA-salt rats; in small unpressurized veins, we have demonstrated the ability of BQ788 to block S6c-induced contraction (Fink *et al.*, 2000; Johnson *et al.*, 2001). The maximum contraction to S6c was variable, and was approximately 10–15% that elicited by ET-1. Neither the potency (pD_2 [M] sham = 9.14 ± 0.14; DOCA-salt = 8.76 ± 0.09) or maximum contraction to S6c in the vena cava was different under conditions of DOCA-salt hypertension. Thus, these findings are consistent with veins but not arteries possessing ET_B receptors which directly mediate contraction and venous smooth muscle ET_B receptor being maintained in DOCA-salt hypertension.

Effect of ET_A receptor agonist ET-1$_{(1-31)}$

In related experiments, we examined arterial and venous contraction to the peptide ET-1$_{(1-31)}$ as a selective agonist of ET_A receptors. Preliminary experiments in vena cava and aorta from animals with normal blood pressure were performed to validate the selectivity of this peptide. Both vena cava and thoracic aorta from normotensive rats contracted to ET-1$_{(1-31)}$ (100 nM) to approximately 50–60% of the maximal response to ET-1 (Figure 3a). ET-1$_{(1-31)}$-induced contraction in both vessels could not be reduced by the ET_B receptor antagonist BQ788 but was abolished by the ET_A receptor antagonist ABT-627 (Figure 3a,b). These data support ET-1$_{(1-31)}$ as a selective agonist for the ET_A receptor.

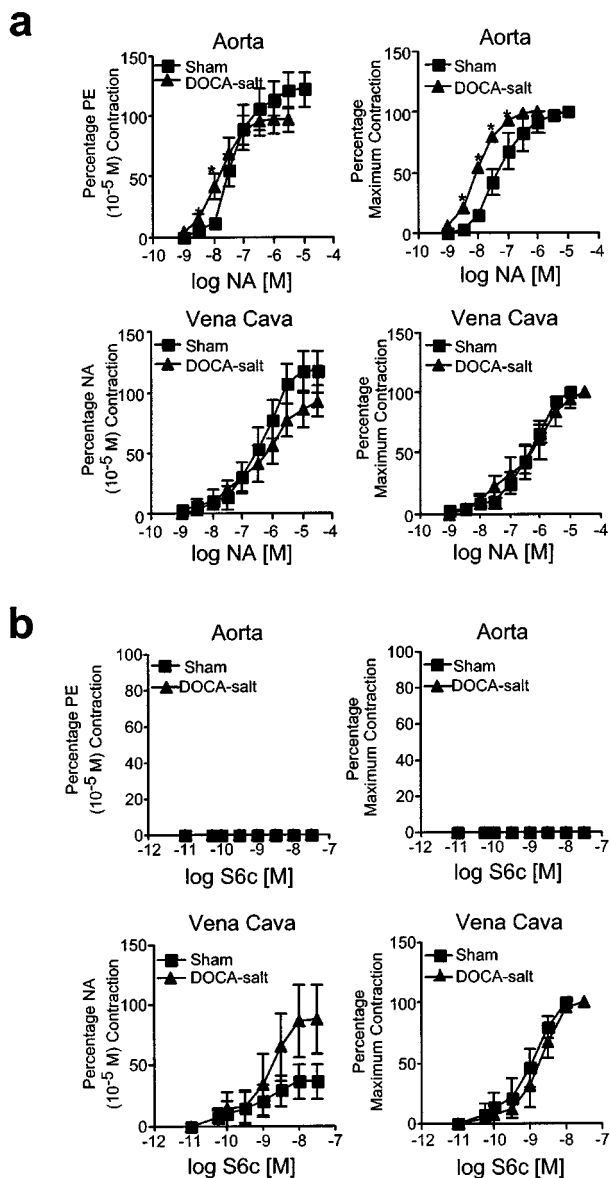**Figure 2** (a) Effect of noradrenaline (NA) on aorta and vena cava. Data are presented as means±s.e.mean and as a percentage of an initial phenylephrine (PE), or noradrenaline (NA) response (left) or as a percentage maximal response (right) for an *n* of 5–6 animals. (b) Effect of sarafotoxin 6c (S6c) on aorta and vena cava. Data are presented as means±s.e.mean and as a percentage of an initial phenylephrine (PE) or noradrenaline (NA) response (left) or as a percentage maximal response (right) for an *n* of 5–6 animals. *$P<0.05$, ANOVA repeated measures.

We next compared the response of tissues from normotensive and hypertensive animals. Figure 3c, left shows that aorta from DOCA-salt rats were virtually unresponsive to ET-1$_{(1-31)}$. These tissues are clearly functional as they contracted to phenylephrine (10 uM) to a magnitude similar to that of all other aorta from DOCA-salt rats. By contrast, the concentration-dependent contraction elicited by ET-1$_{(1-31)}$ in vena cava from DOCA-salt rats was similar to that observed in vena cava from normotensive sham rats (Figure 3c). Thus, it is clear that ET$_A$ receptor function in aorta but not vena cava is severely depressed in DOCA-salt hyperten-

sion. This depressed functional response has been proposed to occur through desensitization of the ET$_A$ receptor because of overexpression of ET-1 (Nguyen *et al.*, 1992; Schiffrin, 2001). To determine whether ET-1 overexpression occurs in the vena cava, we performed real time RT–PCR to measure prepro ET-1.

Real time RT–PCR for prepro ET-1, ET$_A$ and ET$_B$ receptor mRNA

Figure 4a depicts ethidium bromide-stained products of real time RT–PCR for prepro ET-1 mRNA. We observed one product of the expected size (69 bp) and melting temperature ($T_M = 83$) and product was observed in all samples. GAPDH was used as a calibrator control. We confirm previous findings in the arteries that preproET-1 mRNA was increased in aorta from DOCA-salt rats as compared to sham, and demonstrate that prepro ET-1 mRNA was elevated in the vena cava from DOCA-salt rats as compared to sham. When normalized to the C_T value of GAPDH, DOCA-aorta expressed 6.6 ± 3.3 and DOCA-vena cava 8.7 ± 3.9 times greater prepro ET-1 mRNA than their corresponding sham tissues ($P<0.05$). Thus, while the cellular site of increased prepro ET-1 mRNA cannot be pinpointed in this experiment, prepro ET-1 mRNA levels were increased on both the arterial and venous side.

With a decreased arterial contraction to ET-1 and ET-1$_{(1-31)}$, one explanation for the decrease in arterial response to ET-1 in the arteries as compared to veins is that ET receptors may be downregulated under conditions of DOCA-salt hypertension in the arteries only. Using the same cDNA as used in experiments displayed in Figure 4a, we performed real time RT–PCR to measure expression of ET$_A$ and ET$_B$ receptor mRNA in arteries and veins. Figure 4b displays a visualized ethidium bromide-stained gel for the ET$_A$ receptor, ET$_B$ receptor and GAPDH. Messenger RNA for the ET$_A$ and ET$_B$ receptor was observed in all tissues and the C_T values for GAPDH were statistically similar ($P>0.05$) between all four groups. When comparing sham vena cava to sham aorta and DOCA-salt vena cava to DOCA-salt aorta, vena cava from either the sham or DOCA-salt rat possesses a significantly higher density of the ET$_A$ and ET$_B$ receptor mRNA, as indicated by the lower C_T values (Figure 4c). However, there was no significant difference in ET$_A$ and ET$_B$ receptor mRNA density in DOCA vs sham when comparing between tissue type.

Western analyses for ET$_A$ and ET$_B$ receptor protein

Western analyses were performed to determine if there were differences in protein density and Figures 5 and 6 show these results. Preliminary experiments using a competing peptide for either the ET$_A$ or ET$_B$ receptor were first performed to determine the bands of interest. Only those proteins that were reproducibly competed against were considered as specific for the antibody. For the ET$_A$ receptor, three bands of approximately 65, 30 and 28 kDa were eliminated reproducibly in the presence of competing peptide (Figure 5a, bands that are boxed); a 37–39 kDa protein was also observed, but was not reproducibly competed against. For the ET$_B$ receptor, bands at 120, 45 and faint triplets with the highest
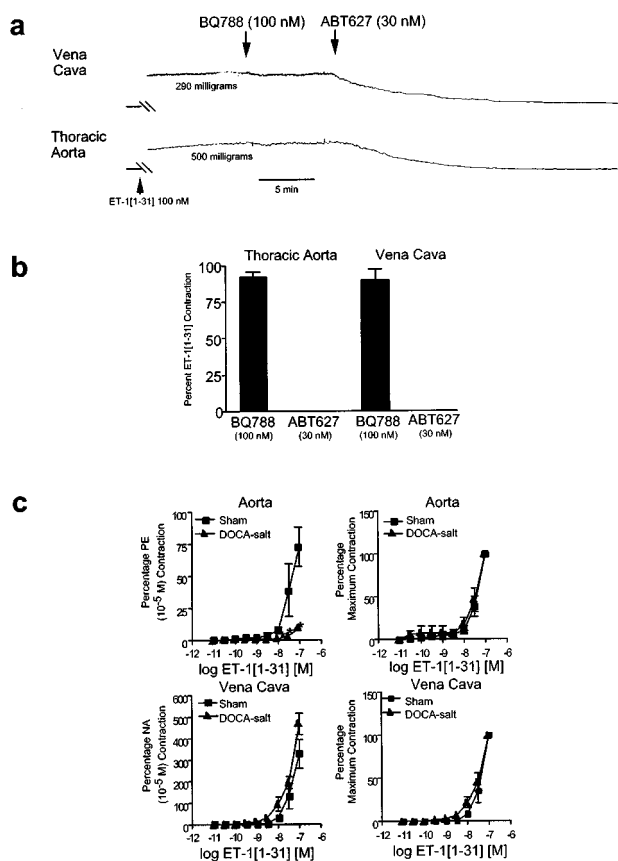
Figure 3 (a) Tracing of vena cava and aortic response to ET-1$_{(1-31)}$ in the presence of the ET$_B$ receptor antagonist BQ788 (100 nM) or ET$_A$ receptor antagonist ABT-627 (30 nM). (b) Quantitation of effect of receptor antagonists on contraction induced by ET-1$_{(1-31)}$ (100 nM). Bars represent ± s.e.mean for $n = 4-6$ animals. (c) Effect of ET-1$_{(1-31)}$ on aorta and vena cava. Data are presented as means ± s.e.mean and as a percentage of an initial phenylephrine (PE) or noradrenaline (NA) response (left) or as a percentage maximal response (right) for an *n* of six animals. *$P < 0.05$, ANOVA repeated measures.

Figure 4 (a) Final product of real time RT–PCR performed for prepro ET-1 (40 cycles, 200 nM primers). Half of the final PCR product was used for this gel (2% agarose). RT = reverse transcriptase. Representative of three separate isolations performed in duplicate. (b) Final product of real time RT–PCR performed for ET$_A$, ET$_B$ and GAPDH mRNA in sham and DOCA-salt aorta and vena cava run on 2% agarose and stained with ethidium bromide (40 cycles, 200 nM primers). Half of the final PCR product was used for this gel. Representative of four separate isolations performed in duplicate. (c) Quantitation of C_T values for GAPDH, ET$_A$ and ET$_B$ receptor mRNA in experimental broups. Bars represent ± s.e.mean for $n = 4$ performed in duplicate. *Indicates statistically significant differences ($P < 0.05$) vs aortic comparison within experimental group.

Discussion

These studies were undertaken to determine whether arteries and veins display similar changes in responsiveness to ET-1 in DOCA-salt hypertension, with the long term goal of understanding the potential role of the venous circulation in contributing to the elevation of blood pressure. The DOCA-salt model was the model of choice as it has been well established as ET-1-dependent (Lariviere *et al.*, 1993; Matsumura *et al.*, 1999; Schiffrin, 2001). We found aortic contraction to ET-1 significantly depressed in DOCA-salt hypertension. This is in agreement with previous work demonstrating a decreased arterial contraction to ET-1 (Nguyen *et al.*, 1992; Fujita *et al.*, 1995; Laurant & Berthelot, 1996; Zhao *et al.*, 2000; Johnson *et al.*, 2002). This was in marked contrast to the unchanged ET-1-induced contraction in vena cava. A majority of the experiments performed were done to determine whether the pharmacology of the receptor

at 30 kDa were eliminated in the presence of competing peptide (Figure 6a, bands that are boxed). The pattern of each antibody was distinctive and the ET$_B$ receptor competing peptide did not block the ET$_A$ receptor antibody function and *vice versa* (data not shown). Western analyses revealed the presence of the ET$_A$ and ET$_B$ receptor in all tissues (Figures 5b, 6b) but with several notable differences. First, the ET$_A$ receptor was, for the same micrograms of total protein, observed as three bands in aorta but only one in vena cava. ET$_A$ receptor density, whether quantified using the 65, 30 or 28 kDa band, was approximately 300–400% greater in the aorta from DOCA-salt as compared to aorta from sham (Figure 5c); such a difference was not observed for the single band detected by the ET$_A$ receptor in vena cava from sham and DOCA-salt rats (Figure 5c). Second, for the same micrograms of total protein, the ET$_B$ receptor tended to be more densely expressed in vena cava when compared to aorta but was expressed in both vessel types (Figure 6b,c). When comparing sham to DOCA-salt, aortic expression of the ET$_B$ receptor was similar except for a small increase of the 45 kDa band (Figure 6c).
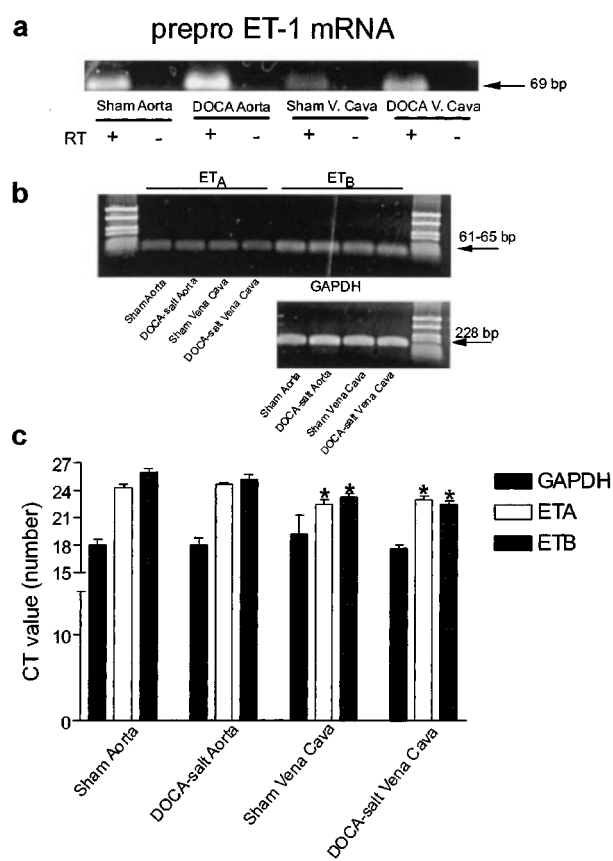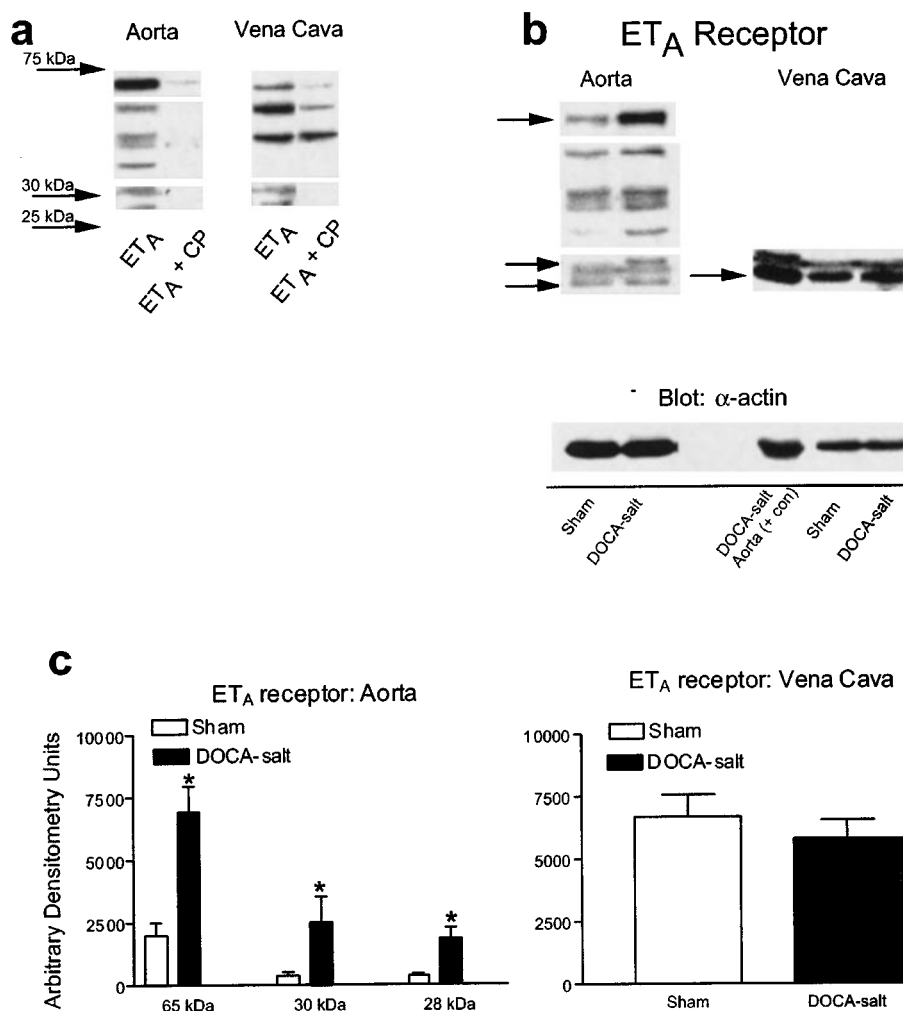
Figure 5 (a) Visualized Western blot showing immunoreactive bands that drop out in the presence of a competing peptide for the ET_A receptor. (b) Representative blots for the ET_A receptor in aorta and vena cava of sham and DOCA-salt rats. (c) Densitometry results for ET_A receptor immunoreactive bands in aorta and vena cava. Bars represent means $\pm$ s.e.mean for seven to eight animals. *Indicates statistically significant differences ($P < 0.05$) in sham vs DOCA-salt comparison.

response between artery and vein, sham and DOCA, was similar and to begin to investigate potential mechanisms for a maintained venous response to ET-1.

Arterial responsiveness

The predominant receptor present in arterial tissue appears to be the ET_A receptor. This is supported by functional studies in multiple different arteries from several species (rat thoracic aorta (White *et al.*, 1993), rat mesenteric artery, rabbit carotid artery (Calo *et al.*, 1996), human coronary artery, human pulmonary artery, rabbit femoral artery (Eguchi *et al.*, 1997) and human pial artery (Pierre & Davenport, 1998). ET-1 causes a contraction with greater potency than ET-3 and the ET_B receptor agonist S6c is virtually inactive in these arteries. By contrast, in coronary and pulmonary arteries, the ET_B receptor appears to play a direct role in modulating contractility (Teerlink *et al.*, 1994; Calo *et al.*, 1996; White *et al.*, 1993) and some arteries show a mixed response (Bax *et al.*, 1994; Lodge *et al.*, 1995; Mickley *et al.*, 1997). In our experiments, it is clear that under normal conditions the ET_A

receptor is important to ET-1-induced contraction in both aorta and vena cava. This is supported by the significant blockade of ET-1-induced contraction by the ET_A receptor antagonist ABT-627 and the concentration-dependent contraction elicited by the ET_A receptor agonist ET-1$_{(1-31)}$.

A recent elegant study by Maguire suggests that ET-1$_{(1-31)}$ may have affinity for the ET_B receptor in the human heart (Maguire *et al.*, 2001). Our functional studies and those of others were not able to find a significant interaction of ET-1$_{(1-31)}$ with the ET_B receptor, and thus we used this peptide as a relatively selective agonist of the ET_A receptor. As such, it is important that contraction to ET-1$_{(1-31)}$ was reduced in aorta from DOCA-salt hypertensive rats. Because the aorta has a significant ET_A receptor component, the reduced contraction to ET-1$_{(1-31)}$ suggests a downregulation in the ET_A receptor. However, real time RT–PCR demonstrated the presence of a similar amount of ET_A receptor mRNA in sham and DOCA-salt aorta, and Western analyses revealed that the ET_A receptor was more densely expressed in aorta from DOCA-salt as compared to sham arteries, regardless of whether putative monomers (28 and 30 kDa) or potential
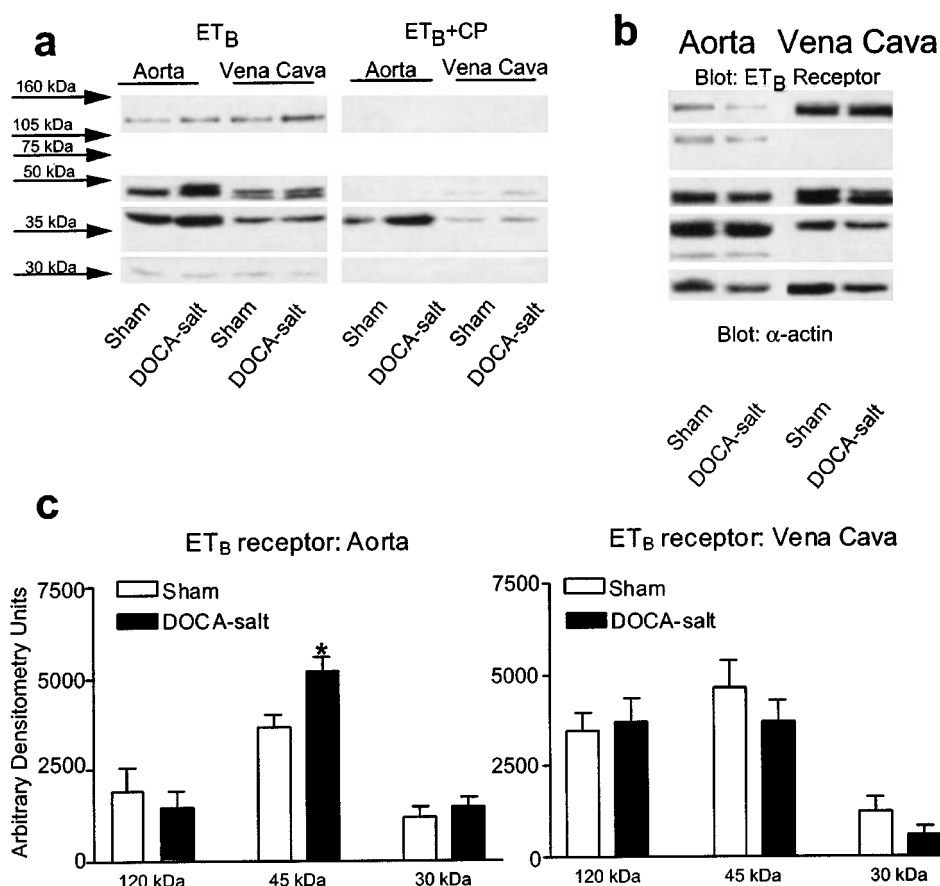
Figure 6 (a) Visualized Western blot showing immunoreactive bands that drop out in the presence of a competing peptide for the ET$_B$ receptor. (b) Representative blots for the ET$_B$ receptor in aorta and vena cava of sham and DOCA-salt rats. (c) Densitometry results for ET$_B$ receptor immunoreactive bands in aorta and vena cava. Bars represent means $\pm$ s.e.mean for seven to eight animals. *Indicates statistically significant differences ($P < 0.05$) in sham vs DOCA-salt comparison.

oligomeric bands were measured. These findings indicate that the ET$_A$ receptor, while present, is uncoupled from contraction in the aorta in DOCA-salt hypertension. Reduced arterial contraction to ET-1 and increased or normal receptor binding has been observed in the coronary artery of the DOCA-salt rat (Giulumian *et al.*, 2002). Moreover, in ET$_B$ receptor deficient rats, plasma ET-1 is increased as it is in the DOCA-salt rat but ET$_A$ receptor-mediated contraction in small mesenteric arteries was reduced compared to normal rats, but the ET$_A$ receptor membran protein density was increased (Perry *et al.*, 2001). Thus, our findings are consistent with those of others. It is important, however, to understand the limitations of the Western experiments herein, and these limitations are true for experiments measuring both ET$_A$ and ET$_B$ receptor density. First, we have used a whole tissues preparation as opposed to a membrane preparation. Thus, we may have detected proteins located intracellularly in the process of degradation and thus observed proteins smaller (28, 30 kDa) than the size reported for use with this antibody (39 kDa and approximately 58–60 kDa). Second, we did not boil the homogenates prior to loading on the gel but instead warmed the samples at 37°C for 30 min so as to minimize oligomeric formation. It is possible that full receptors were degraded during this time. Thus, the measures of protein we have done

represent a picture of the ET proteins in all aspects, not just that at the membrane level. It may be useful to perform radioligand binding studies to measure the membrane and thus functional ET$_A$ receptor population, but there are inherent difficulties with such an experiment. It is difficult to choose a receptor antagonist, which is ideal for such studies, with which to label the receptors because evidence suggests that the ET$_A$ and ET$_B$ receptor may interact physically and alter the pharmacological sensitivity of the ET$_A$ receptor to blockade by ET$_A$ receptor antagonists (Lodge *et al.*, 1995). While agonists could certainly be used as radioligands, they are fraught with their own difficulties. Thus, these studies represent an initial step in measuring protein density.

Another important finding is that mRNA and protein for the ET$_B$ receptor is present in the aorta. The ET$_B$ receptor may be located in the endothelial cell or smooth muscle cell because endothelial-cell intact homogenates were made for isolation of mRNA and protein; future immunohistochemical studies will enable this distinction. Evidence supporting a direct function of the ET$_B$ receptor in arteries is limited. The ET$_B$ receptor, in an endothelium-dependent manner, may mediate ET-1 and/or ET-3-induced relaxation in several arteries (Karaki *et al.*, 1993; Magazine & Srivastava, 1996). The aorta of either the sham or DOCA-salt rat does not

possess a contractile ET_B receptor as S6c was inactive. However, blockade of the ET_B receptor with BQ788 resulted in a small but significant leftward shift in ET-1-induced contraction in arteries, suggesting that the ET_B receptor can modulate contraction, either through blocking ET_B receptor-mediated vasorelaxation or by clearing ET-1 from the extracellular fluid (Sato *et al.*, 1995; Pollock, 2001). It is difficult to determine which of these functions of the ET_B receptor is responsible. These two possibilities could not be distinguished even with removal of the endothelium since this cell type mediates both ET-1-induced relaxation and clearance of ET-1. Given the fact that endothelial cell mass is small compared to smooth muscle mass in the aorta, it is likely the ET_B receptor is expressed in aortic smooth muscle. While the ET_B receptor does not appear to mediate contraction directly, the receptor may serve other functions in the aorta.

Venous responsiveness

Both the ET_A and ET_B receptor mediate contraction in venous smooth muscle. This mixed receptor population has been reported for the rabbit saphenous vein (Douglas *et al.*, 1995; Sudjarwo & Karaki, 1995), rabbit portal vein (Wang *et al.*, 1997), rabbit jugular vein and human saphenous vein (Wang *et al.*, 1997). There are exceptions such as the dog pial vein in which the predominant receptor appears to be the ET_A receptor (Fernandez *et al.*, 1995). Presumably, ET_B receptors are present in both smooth muscle and endothelial cells. In the vena cava, both ET_A and ET_B receptors directly modulate contraction as evidenced by concentration-dependent contraction to the ET_A receptor agonist $ET-1_{(1-31)}$ and the ET_B receptor agonist S6c. Interestingly, while the ET_A receptor antagonist ABT-627 antagonized ET-1-induced contraction in vena cava, the ET_B receptor antagonist BQ788 did not; in fact, BQ788 enhanced contraction to ET-1 in vena cava. One interpretation of these data is that the clearance function of the ET_B receptor may overwhelm the contractile function of the ET_B receptor such that the net effect observed is a potentiation of contraction to ET-1 rather than a reduction. There is also the possibility that ET_A and ET_B receptors may influence the function of one another (Lodge *et al.*, 1995; Fukuroda *et al.*, 1996; Adner *et al.*, 2001). The ET_A receptor, as evidenced by contractile and immunoblot analysis, is expressed and functional in mediating contraction in the vena cava of the DOCA-salt rat. By contrast, the ET_A receptor in the aorta of the DOCA-salt rat is highly expressed but dysfunctional in mediating contraction. One explanation for this difference may be that the aorta is exposed to a higher level of ET-1 than the vein, causing desensitization in the aorta but not in the vein. The relative amount of prepro ET-1 mRNA was elevated in the DOCA-salt vena cava compared to the sham, indicating that both vena cava and aorta have the potential of higher than normal concentrations of ET-1. Recent work by Wang *et al.* (2002) demonstrated that while aortic ET-1 content was increased in DOCA-salt hypertension, vena cava ET-1 was

not. However, basal levels of ET-1 in vena cava were higher than in the aorta such that levels of ET-1 in vena cava and DOCA-salt aorta are similar. One can speculate that ET-1 may actually be secreted at a higher rate in veins compared to arteries, and this would not be reflected when measuring ET-1 content.

Speculation

The question remains as to why veins and arteries respond to ET-1 so differently in DOCA-salt hypertension. We speculate that the ET_B receptor, present and functional in the smooth muscle of the vena cava but not in the aorta, may protect the tissue from desensitization to ET-1, as the ET_B receptor serves as a receptor of clearance and is degraded rapidly within lysosomes (Bremnes *et al.*, 2000). It is clear that the ET_B receptor is present in both tissue types, but the coupling or processing of the ET_B receptor must be significantly different in a vein and artery for the differences we have observed to occur. Moreeover, the recycling/handling of the ET_A receptor in arteries and veins is necessarily different in the condition of DOCA-salt hypertension given the observed results.

A maintained response to ET-1 is important not only to contraction elicited by ET-1, but in the ability of ET-1 to facilitate or potentiate contraction to important modulators of venomotor tone such as noradrenaline (Shimamoto *et al.*, 1992; Kita *et al.*, 1998). We speculate that the ET_B receptor may be important in hypertension as S6c, given chronically, induces a hypertension that cannot be reduced by the ET_A receptor antagonist ABT-627 (Fink *et al.*, 2001). These studies suggest that activation of the ET_B receptor can result in hypertension, but we have yet to prove whether the ET_B receptor is necessary for DOCA-salt hypertension. Another interesting finding was the observation of potential multimers or high molecular weight complexes of the ET receptors and this will be the focus of future studies.

In summary, the ET_A receptor is the primary receptor mediating ET-1-induced contraction in aorta and vena cava from normotensive rats. Additionally, the vena cava has a ET_B receptor component relevant directly to contraction as the ET_B receptor agonist S6c contracts veins but not arteries. Under the condition of DOCA-salt hypertension, contraction elicited by either ET-1 or the ET_A receptor agonist $ET-1_{(1-31)}$ was profoundly depressed in the aorta but not in the vena cava; ET_A receptor density was increased in DOCA-salt aorta but not in DOCA-salt vena cava. Both vessel types appear to have increased prepro ET-1 mRNA levels in DOCA-salt hypertension and express the ET_B receptor. The presence of a functional ET receptor in veins, in the face of dysfunctional receptors in arteries, may be permissive for maintaining responsiveness to ET-1 in DOCA-salt hypertension.

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