Endothelin Receptor Subtype Distribution Predisposes Coronary Arteries to Damage

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Several vasoactive drugs that lower blood pressure and increase heart rate induce regional cardiotoxicity in the dog, most frequently of right coronary arteries and right atrium. The basis for this selective damage is thought to result from local changes in vascular tone and blood flow. Administration of an endothelin receptor antagonist (ETRA, SB 209670) to dogs induced damage most frequent and severe in the right coronary artery and right atrium. Because site predisposition may correlate with distribution of vasoactive receptors, the objectives of this study were to map endothelin (ET) receptor distribution and density within regions of dog heart using both gene (mRNA) and protein expression endpoints for dog ET_A and ET_B receptors, and, additionally, correlate ET receptor subtype density with regional cardiac blood flow. A 10- to 15-mmHg reduction in mean arterial pressure with a concomitant increase in heart rate (10–20%), a six- and twofold increase in regional blood flow to the right and left atrium, respectively, and acute hemorrhage, medial necrosis, and inflammation were observed in the right coronary arteries and arteries of the right atrium after ETRA infusion for 5 days. Radioligand protein binding to quantify both ET receptors in normal dog heart indicated a twofold greater density of ET receptors in atrial regions *versus* ventricular regions. Importantly, ET receptor density in coronary arteries was markedly (about five- to six-fold) increased above that in atrial or ventricular tissues. ET receptor subtype characterization indicated ET_B receptors were three times more prevalent in right coronary arteries compared to left coronary arteries and *in situ* hybridization confirmed localization of ET_B in vascular smooth muscle. ET_A receptor density was comparable in right and left coronary arteries. Quantitative real-time polymerase chain reaction for ET_A and ET_B receptor mRNA transcripts supported the site prevalence for message distribution. Consequently, the composite of protein and message expression profiles for ET_A and ET_B recep-

tors indicated a disproportionate distribution of ET_B receptors within right coronary artery of dog and this, along with functional measures of blood flow after ETRA infusion indicated a predisposition for exaggerated pharmacological responses and subsequent damage to right coronary arteries by ET and/or ETAs. (Am J Pathol 2000, 157:123–134)

Cardiovascular toxicity frequently occurs at specific anatomical sites and generally has characteristic histopathological features. Lesion location and the combination of vascular and myocardial damage is an indicator of vasopressor or vasodilator activity of an insulting agent.¹ Various vasodilators, when given to dogs induce hemorrhage, medial necrosis, and acute inflammation predominantly of the right coronary arteries and/or right atrium.^{1–5} For example, when endothelin receptor antagonists (ETRA) are given to dogs, characteristic lesions occur in the atrium and coronary arteries, even in the absence of meaningful clinical signs.^{6,7} Because endothelin (ET) is a potent vasoconstrictor,^{8,9} blockade of ET receptors should mediate vasodilation. However, the final biological outcome of ET receptor blockade is variable and influenced by a several factors, including (i) ET receptor subtypes involved, (ii) association of ET subtypes with specific cells (endothelium or vascular smooth muscle), (iii) ratios of ET receptor subtypes at affected sites, and (iv) specific signal transduction systems conducting responses in particular species or tissue types. Two major ET receptors, ET_A and ET_B, are recognized; however, pharmacological evidence suggests additional subtypes are present but not well characterized.^{10–15} Classically, vascular smooth muscle cells express ET receptors ET_A and ET_B,^{12,16} whereas endothelial cells express only ET_B.¹⁵ In general, ET_A mediates vasoconstriction and ET_B receptors on endothelium, through release of nitric oxide, indirectly mediate vasorelaxation.^{17,18} However, an ET_B receptor subtype of vascular smooth muscle directly mediates smooth muscle contraction.^{14,19–23} In the dog, ET_B receptors of coronary arteries are potent vasoconstrictors.^{22,24,25}

SB 209670, a novel nonpeptide ETRA, has high affinity for human cloned ET_A and ET_B receptors (K_{iS} of 0.2 and

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Table 1. Study Design, Toxicokinetic Data, and Frequency of Coronary Arterial Lesions

Length of ETRA infusion	Total dose (mg/kg/day)	Infusion rate (μ g/kg/min)	Mean C _{ss} (μ g/mL)	Arterial lesion
5 days	72	50	5.0	6/6*
24 hours	72	50	5.0	2/6
12 hours	36	50	5.0	1/6
12 hours	7.2	10	1.0	0/6

*Each group consisted of 3 males and 3 females.
 C_{ss}, concentration at steady state.

18 nmol/L, respectively) which, in a variety of model systems, competitively inhibits both ET_A- and ET_B-mediated arterial contractions.²⁶ SB 209670, when given to dogs as an intravenous infusion for 5 days (50 μ g/kg/minute), increased heart rate (HR), decreased mean arterial pressure (MAP), and caused atrial hemorrhage and acute coronary arterial injury (medial hemorrhage, necrosis, and perivascular inflammation), predominantly of the right heart. These right heart lesions after administration of selective ETRA indicated that ET receptors predispose specific locations to damage. The objectives of this study were, first, to quantify ET receptor subtypes in specific anatomical regions of the normal dog heart; second, to quantify regional blood flow in dog heart during periods of ET receptor blockade by SB 209670 as a measure of vascular function; and third, to correlate ET receptor density with regional blood flow and severity and frequency of cardiovascular damage. ET receptor mRNA and protein expression were quantified in selected areas of dog heart including right and left atria, ventricles, and coronary arteries. Quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR) and radiolabeled ligands were used to measure ET_A and ET_B mRNA, and ET_A and ET_B receptor proteins, respectively. Dog-specific radiolabeled ET receptor riboprobes in conjunction with *in situ* hybridization were used to anatomically characterize receptor distribution within dog coronary arteries. Right and left atria and ventricular blood flow was determined both before and after administration of SB 209670 using fluorescent microspheres.

Materials and Methods

Animals

Purebred beagle dogs 12 to 14 months old (Marshall Farms, Inc., North Rose, NY) were used. Dogs were housed individually in stainless steel cages and fed Purina Certified Canine Diet 5007 (Purina Mills Inc., Richmond, IN) and water *ad libitum*. Animal care, husbandry, and cage specifications conformed to the current guidelines of ILAR/AALAC and the "Guide for the Care and Use of Laboratory Animals." In preparation for intravenous infusion, dogs received a jugular catheter and were jacketed for protection.

Test Article, Vehicle, and Dose Regimen

Dogs (3 males and 3 females/group) were given 50 μ g/kg/minute SB 209670 (5 mg/ml) or 0.9% sodium chloride

(saline) as a continuous intravenous infusion for up to 5 days. The solutions were sterile-filtered through a 0.22- μ m Sterivex-GV filter into clear PVC Viaflex infusion bags and an ambulatory peristaltic pump (CADD plus, Model 5400, Sims Deltec, St. Paul, MN) was used for delivery through polyvinyl catheters. The study design is shown in Table 1.

Measurement of Blood Pressure and Heart Rate

A series of MAP and HR plots were obtained before, during, and after dosing using Dataquest (Data Sciences International, St. Paul, MN) from surgically implanted radiotelemetry transmitters. Plots were constructed from averaged intervals. Average values, standard deviations, number of observations, and minimum and maximum values were summarized for 24-hour time periods.

Determination of Regional Myocardial Blood Flow

Regional myocardial blood flow (RBF) was measured using color-coated fluorescent microspheres²⁷⁻²⁹ before and after infusion of ETRA (SB 209670). Briefly, multiple sets of NuFlow microspheres (15 μ m in diameter, Triton Technology, San Diego, CA), each having a distinct fluorescent color emission, were supplied in sterile saline containing 0.05% Tween 80 and 0.01% Thimersal. Microsphere suspensions were diluted in 0.5% (w/v) dextrose and microspheres/100 μ l quantified. Before ETRA infusion, dogs were pretreated with 0.01% Tween 80 to preclude Tween-related hemodynamic effects during study.³⁰ Dogs were also acclimated to stabilize MAP and HR before microsphere injection. Vascular access ports (VAP) to the left atrium were used for microsphere injection and reference blood samples obtained from an aortic VAP immediately after microsphere administration. One color-set of microspheres was given on day 1, before ETRA infusion, and another color-set was given on day 5 during completion of ETRA infusion, allowing each dog to serve as its own control, thereby reducing interanimal variability. Regional myocardial tissues were collected at necropsy, weighed, homogenized, and analyzed by flow cytometry for quantification of each set of microspheres per gram of tissue.

Toxicokinetics of SB 209670

Serial jugular vein blood samples were collected at 0, 2, 4, and 8 hours of ETRA infusion and at the end of days 1,

Table 2. Primers and Fluorogenic Probes

Reagent	Sequence	Size
Quantitative RT-PCR		
ET _A		101 bp
Forward Primer	5'-TCG AGA AGT GGC AAA AAC AGT TT-3'	
Reverse Primer	5'-CAT CGT ACA CGG TTT TCT TCA A-3'	
Probe*	5'-FAM-TGG TTC CCG CTT CAC TTA AGC CGT-TAMRA-3'	
ET _B [†]		107 bp
Forward Primer	5'-TCA GAA TGA TCC CAA TAG ATG TGA A-3'	
Reverse Primer	5'-ACA GAG CTA TAG GAT TAA TGC AGG AAT-3'	
Probe*	5'-FAM-TGC CGA TAT AAT CCA ACA CCA ACA AAA AGC T-TAMRA-3'	
β-Actin [‡]		86 bp
Forward Primer	5'-GAT GAG GCC CAG AGC AAG AG-3'	
Reverse Primer	5'-TTC TCC ATG TCG TCC CAG TTG-3'	
Probe	5'-FAM-TGA CCC TGA AGT ACC CCA TTG AGC ACG GCA T-TAMRA-3'	
<i>In Situ</i> Hybridization		
ET _A		389 bp
Forward Primer	5'-AAG GAC TGG TGG CT-3' (sense)	
Reverse Primer	5'-GGC ATG ACT GGA AA-3' (antisense)	
ET _B [†]		565 bp
Forward Primer	5'-TAA TGA CGC CAC CCA CTA AGA CCT-3' (sense)	
Reverse Primer	5'-GCC AGA ACC ACG GAG ACC A-3' (antisense)	

*FAM (6-carboxy-fluorescein) and TAMRA (6-carboxytetramethyl-rhodamine) are the detection and quencher dyes, respectively.

[†]GenBank Accession number AF034530.

[‡]GenBank Accession number Z700440.

2, 3, 4, and 5 to analyze SB 209670 concentration using a high pressure liquid chromatography method.

Tissue Collection and Histopathology

Heart and coronary arteries were examined at necropsy. Atria, ventricles, and coronary arteries were collected and identified as right or left chambers; this identity was maintained throughout processing and examination. Light microscopic assessment was completed after paraffin embedding and staining with hematoxylin and eosin. Expression of ET_A and ET_B receptor mRNA was localized using isotopic *in situ* hybridization procedures.³¹ [³²P]-UTP-labeled riboprobes were synthesized from PCR-generated cDNA templates containing flanking T3 and T7 promoter sequences for sense and antisense riboprobes, respectively. Primer sequences are illustrated in Table 2.

ET Receptor Binding Studies

Membrane Preparation and Radioligand Binding

Regions of femoral, right (RCA) and left (LCA) coronary arteries, and heart (right and left atria and ventricles) were collected from untreated dogs ($n = 4$). Cell membranes were prepared following the procedure of Brooks et al³² for radioligand binding studies. Briefly, dissected tissues were homogenized in buffer containing 20 mmol/L Tris-HCl pH 7.5, 5 mmol/L EDTA, 0.25 mmol/L sucrose, 100 μg/ml phenylmethyl sulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After low-speed centrifugation (1000 × g) for 10 minutes at 4°C, supernatants were collected and centrifuged again for 30 minutes at 45,000 × g at 4°C. Resulting pellets were re-suspended in buffer containing 50 mmol/L Tris-HCl, pH 7.5, and 20 mmol/L MgCl₂ and frozen.

[¹²⁵I]-ET-1 (specific activity 2200 Ci/mmol) and [¹²⁵I]-ET-3 (specific activity 2200 Ci/mmol) were obtained from New England Nuclear (Boston, MA); unlabeled ET-1 and ET-3 were obtained from American Peptides (Sunnyvale, CA). For radioligand binding, 0.3 nmol/L of [¹²⁵I]-ET-1 or [¹²⁵I]-ET-3 was added to membrane preparations (5–10 μg/tube) and incubated at 30°C for 60 minutes. Reactions were quenched with cold buffer. Bound and free ligands were separated by filtering and counted using a γ counter. Nonspecific binding was determined in the presence of 100 nmol/L unlabeled ET-1 or ET-3. Because ET-1 binds to both ET_A and ET_B receptors and ET-3 binds only to ET_B, ET_B receptor density was measured directly and ET_A receptor density was calculated.

Tissue Collection and RNA Isolation

Using normal dog tissues, total RNA was isolated from right coronary artery (RCA) and left coronary artery (LCA), atrial and ventricular myocardium, kidney, and femoral artery. RNA was treated with 0.8 units RQ1 RNase-free DNase (Promega, Madison, WI) before extraction with phenol/chloroform to reduce DNA contamination.³³ RNA integrity was verified by fractionation on 1% agarose-formaldehyde gel containing 0.5 μg/ml of ethidium bromide followed by ultraviolet visualization.

Cloning of Partial Coding Sequence for Canine ET_A Receptor

To partially clone the dog ET_A receptor gene, PCR primers were designed from conserved regions (GenBank Accession numbers U20577, S67127, X57765, M60786, AF039892, U06633) using the Basic Local Alignment Search Tool (National Center for Biotechnology

900	K	D	W	W	L	F	G	F	Y	F	C	M	P	L	V	C	T	A	I	F	HUMAN
	AAG	GAC	TGG	TGG	CTC	TTC	GGG	TTC	TAT	TTC	TGT	ATG	CCC	TTG	GTG	TGC	ACT	GCG	ATC	TTC	
	K	D	W	W	L	F	G	F	Y	F	C	M	P	L	V	C	T	A	I	F	CANINE
	AAG	GAC	TGG	TGG	CTC	TTT	GGG	TTC	TAT	TTC	TGT	ATG	CCT	TTG	GTG	TGC	ACT	GCA	ATC	TTC	
	Y	T	L	M	T	C	E	M	L	N	R	R	N	G	S	L	R	I	A	L	HUMAN
	TAC	ACC	CTC	ATG	ACT	TGT	GAG	ATG	TTG	AAC	AGA	AGG	AAT	GGC	AGC	TTG	AGA	ATT	GCC	CTC	
	Y	T	L	M	T	C	E	M	L	N	R	R	N	G	S	L	R	I	A	L	CANINE
	TAT	ACC	CTT	ATG	ACC	TGT	GAG	ATG	TTA	AAT	AGA	AGA	AAT	GGC	AGC	TTG	AGA	ATT	GCA	CTC	
	S	E	H	L	K	Q	R	R	E	V	A	K	T	V	F	C	L	V	V	I	HUMAN
	AGT	GAA	CAT	CTT	AAG	CAG	CGT	CGA	GAA	GTG	GCA	AAA	ACA	GTT	TTC	TGC	TTG	GTT	GTA	ATT	
	S	E	H	L	K	Q	R	R	E	V	A	K	T	V	F	C	L	V	V	I	CANINE
	AGT	GAA	CAC	CTT	AAA	CAG	CGT	CGA	GAA	GTG	GCA	AAA	ACA	GTT	TTC	TGC	TTG	GTT	GTA	ATT	
	F	A	L	C	W	F	P	L	H	L	S	R	I	L	K	K	T	V	Y	N	HUMAN
	TTT	GCT	CTT	TGC	TGG	TTC	CCT	CTT	CAT	TTA	AGC	CGT	ATA	TTG	AAG	AAA	ACT	GTG	TAT	AAC	
	F	A	L	C	W	F	P	L	H	L	S	R	I	L	K	K	T	V	Y	D	CANINE
	TTT	GCT	CTT	TGC	TGG	TTC	CCG	CTT	CAC	TTA	AGC	CGT	ATT	TTG	AAG	AAA	ACC	GTG	TAC	GAT	
	E	M	D	K	N	R	C	E	L	L	S	F	L	L	L	M	D	Y	I	G	HUMAN
	GAG	ATG	GAC	AAG	AAC	CGA	TGT	GAA	TTA	CTT	AGT	TTC	TTA	CTG	CTC	ATG	GAT	TAC	ATC	GGT	
	E	M	D	K	N	R	C	E	L	L	S	F	L	L	L	M	D	Y	I	G	CANINE
	GAG	ATG	GAC	AAG	AAC	CGA	TGT	GAA	TTA	CTT	AGT	TTC	TTG	CGT	CTC	ATG	GAT	TAC	ATC	GGT	
	I	N	L	A	T	M	N	S	C	I	N	P	I	A	L	Y	F	V	S	K	HUMAN
	ATT	AAC	TTG	GCA	ACC	ATG	AAT	TCA	TGT	ATA	AAC	CCC	ATA	GCT	CTG	TAT	TTT	GTG	AGC	AAG	
	I	N	L	A	T	M	N	S	C	I	N	P	I	A	L	Y	F	V	S	K	CANINE
	ATT	AAC	TTG	GCA	ACC	ATG	AAT	TCA	TGT	ATA	AAC	CCA	ATA	GCT	CTC	TAC	TTT	GTA	AGC	AAG	
	K	F	K	N	C	F	Q	S	C	Q											HUMAN
	AAA	TTT	AAA	AAT	TGT	TTC	CAG	TCA	TGC	C											
	K	F	K	N	C	F	Q	S	C												CANINE
	AAA	TTT	AAA	AAC	TGT	TTC	CAG	TCA	TGC	C											

Figure 1. Partial nucleotide and amino acid sequences for the dog ET_A receptor is shown aligned with homologous region of the human ET_A receptor, the non-identical region at the amino acid level are shaded.

ical Information, Washington, D.C.). Sequences of forward and reverse primers were 5'-AAGGACTGGTGGCT-3' and 5'-GGCATGACTGGAAA-3', respectively. Based on previous reports describing ET_A receptor localization in the dog,³² RT-PCR was performed using total RNA isolated from the dog kidney. Single-stranded cDNA synthesis was accomplished using the Superscript pre-amplification system for first strand cDNA synthesis (Life Technologies, Gaithersburg, MD); random hexamers were used to prime cDNA synthesis. PCR was performed in a total volume of 50 μ l consisting of 1.5 mmol/L MgCl₂, 200 μ mol/L each ATP, CTP, GTP, and TTP, 200 nmol/L of each primer, 2.5 U of AmpliTaq DNA polymerase (PE Applied Biosystems, Foster City, CA) and 1 $\times$ PCR buffer containing 20 mmol/L Tris-HCl, pH 8.4, and 50 mmol/L KCl. All reactions were performed in a PE Applied Biosystems GeneAmp PCR System 2400. PCR products were analyzed by gel electrophoresis and subsequently recovered using the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). Purified PCR products were ligated into a bidirectional TA cloning vector, pCRII, and transferred into INV α F' cells (Invitrogen, San Diego, CA). Minipreps of 20 clones were prepared using the Wizard Plus Minipreps DNA Purification System (Promega). Clones containing inserts were identified by restriction enzyme digestion with EcoRI (Promega) and subsequent agarose gel analysis. Sequencing and analysis of clones were performed using PE Applied Biosystems 373A Au-

tomated DNA Sequencer and Basic Local Alignment Search Tool and Lasergene biocomputing software for Windows (DNASTAR Inc., Madison, WI). The nucleic acid sequence obtained had a 93% homology to the human ET_A receptor gene (Figure 1).³⁴ This cloned ET_A sequence was subsequently used in the quantitative real-time PCR (Taqman) and *in situ* hybridization analysis. Primer and probe sequences for dog ET_A and ET_B receptor genes and β -actin genes are shown in Table 2.

Quantitative Real-Time RT-PCR Analysis

Single-stranded cDNA templates were made from the selected tissues and Taqman analysis used to determine relative differences in PCR products.^{35,36} Briefly, fluorogenic probes designed to hybridize to the amplicon had a reporter dye (6-FAM) conjugated to the 5' end and a quencher dye (TAMRA) conjugated to the 3' end. Fluorescence for each cycle was quantitatively analyzed on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Each reaction consisted of 1 $\times$ Taqman Buffer A, 200 μ mol/L each of dATP, dCTP, and dGTP, 400 μ mol/L dUTP, 100 nmol/L fluorogenic probe, 200 nmol/L each of forward and reverse primers, 0.01 U/ μ l AmpErase uracil-N-glycosylase, 0.025 U/ μ l Taq Gold (PE Applied Biosystems), and either 0.5, 1, 10, 100, or 200 ng of template. Standard curves were generated from dog femoral artery cDNA. β -Actin was used as an

Figure 2. Mean arterial pressure (MAP; **A**) and heart rate (HR; **B**) from control and ETRA infused dogs. * $P < 0.05$ (statistically significant increase).

endogenous control and active reference to normalize quantities of cDNA. Target quantities were normalized to β -actin and all quantities expressed as a fold difference from the femoral artery value.

Statistical Methods and Analyses

Statistical analyses of RBF, HR, and MAP were completed. Baseline (predosing) and post-dosing values from control and drug-treated dogs were analyzed using a repeated measures analysis of variance and pairwise comparisons to determine drug effect. Differences were considered significant if the P value was less than 0.05.

Results

Hemodynamic Parameters in Dogs Given ETRA

Intravenous infusion of SB 209670 induced a slight but persistent decrease in MAP (10–15 mmHg), which was apparent 1 to 3 hours after start of infusion. This decrease in MAP persisted for 24 hours (last time point measured) after infusion termination (Figure 2), and the magnitude of change was considered within normal physiological range for MAP in the dog. During the 5-day infusion period, HR increased over time and was maximally increased (10 to 20% over pre-infusion values) on day 4

Figure 3. Regional blood flow from vehicle- and ETRA-infused dogs. Note marked increase in right atrial blood flow after ETRA infusion for 5 days. * $P < 0.05$ (statistically significant increase). No change was observed in vehicle-infused dogs (inset).

(Figure 2). A statistically significant increase in HR ($P < 0.05$) above baseline values occurred on days 2–5. HR returned to near pre-infusion values 24 hours after termination of infusion.

Regional Blood Flow (RBF)

Quantification of RBF in right and left atria and ventricles is summarized in Figure 3. Baseline values represent flow measured on day 1, just before the start of ETRA infusion, and was compared to flow measure after 5 days of infusion. Infusion of vehicle for 5 days did not alter flow characteristics from those of day 1 (Figure 3, insert). Baseline flow in ventricular chambers was approximately three- to fourfold greater than baseline flow in atria. After ETRA infusion for 5 days, right atrial blood flow increased ($P < 0.05$) approximately sixfold when compared to pre-infusion values (Figure 3). In contrast, there were only marginal increases in blood flow to other cardiac chambers. The duration of ETRA infusion also appeared to influence myocardial flow rates, since flow was increased only 0.5- to 1.8-fold after only 3 hours of SB 207960 infusion (data not shown) but increased up to sixfold by day 5.

Pathology

All dogs given 50 μ g/kg/minute SB 209670 for 5 days had heart lesions readily recognized at necropsy. The lesions consisted of petechial hemorrhages in the right atrium and atrial appendage and/or coronary groove. Changes were infrequent in the left heart (Figure 4, A and B) and there were no lesions in the septum or ventricles. There were no macroscopic observations in hearts of dogs given 10 or 50 μ g/kg/minutes SB 209670 for either 12 or 24 hours. Changes were most prevalent in large extra-mural coronary arteries, both right and left branches. Subepicardial and intramural branches of the RCA were also damaged, but not branches of the LCA. Lesions were characterized histologically by multifocal hemorrhage, segmental medial necrosis, acute inflammation,

Figure 4. Heart from ETRA-infused dog. Note areas of hemorrhage in right atria (**A**) and right atrial appendage (**B**). Periarterial inflammation and fibrinoid necrosis of right extramural coronary arteries with inflammation extending into pericardial adipose tissue of the coronary groove (**C** and **D**). Multifocal areas of hemorrhage in tunica media of right coronary artery (**E** and **F**). H&E stain; original magnifications, $\times 24$ (**C**), $\times 120$ (**D** and **E**), $\times 240$ (**F**).

Figure 5. ET_A and ET_B receptor density in different regions of the normal dog heart. RCA, right coronary artery; LCA, left coronary artery; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.

and perivascular edema (Figure 4, C and D). Medial necrosis of severely affected arteries was generally associated with hemorrhage into the vascular wall and perivascular tissues and accumulations of neutrophils. The more extensive vascular lesions were associated with perivascular hemorrhage, which extended into adjacent atrial tissue and widely separated atrial cardiomyocytes. Additionally, varying severity of adventitial edema, chronic inflammation, and fibrosis were observed. Focal to circumferential intimal thickening, enlarged endothelial cells, disruption of the internal elastic lamina with red blood cells, and inflammatory cells in subendothelial sites were observed in some affected arteries (Figure 4, E and F). In studies of shorter duration (data not shown), microscopic coronary arterial lesions occurred in dogs given 50 $\mu\text{g/kg/minute}$ for 12 or 24 hours, but lesions were not observed in dogs given 10 $\mu\text{g/kg/minute}$ for 12 hours. Early lesions (12 and 24 hours) were similar in location and microscopic character but less severe. In general, atrial cardiomyocytes were morphologically unaltered, and there was no evidence of atrial infarction.

Toxicokinetics

Steady state plasma concentration of SB 209670 was achieved within 2 hours after initiation of infusion, was dose-proportional, and remained unchanged during the 5-day infusion period. Toxicokinetic data suggest that a steady state plasma concentration of $\sim 5 \mu\text{g/ml}$ for 12 hours represents a threshold concentration, at or above which arterial lesions occurred (Table 1).

ET Receptor Protein Expression

Dog atrial tissue had up to a 2.5-fold greater density of ET receptors as compared to the ventricles (Figure 5). Within myocardial tissue, ET_A receptor density was highest in right atria and second highest in left atria; ventricular tissue contained fewer ET_A receptors and density in right and left ventricles was similar. Density of ET_B receptors in right and left atria were similar but greater than that observed in right and left ventricles. Ratios of ET_A:ET_B receptors were $\sim 1.2:1$ in right atrium, $0.7:1$ in the left atrium, and approximately $1:1$ in the right and left ventricle (Table 3). In comparison, values for ET receptors in coronary arteries were markedly increased above that measured for myocardial tissue (Figure 5). Density of ET_A receptors in LCA was 1.5-fold higher than values for RCA; ET_B receptor density in RCA was threefold increased above that observed in LCA. The ratio of ET_A:ET_B in RCA was $\sim 1:1$, whereas this ratio in LCA was $\sim 3.2:1$.

ET Receptor mRNA Expression

Quantitative real-time PCR analysis of ET_A and ET_B receptor mRNA expression in heart regions and coronary arteries is shown in Figure 6. All values are relative to the expression measured in normal femoral artery as a control; absolute quantification of copy number was not done. ET_A mRNA expression was greatest in right and left atria; values in RCA were higher than LCA but considerably lower than that expressed within atrial tissue. Values for ET_A message from LCA and left ventricle were lower than those for femoral artery. ET_B mRNA expression in RCA and right atria was 60- to 80-fold increased, respectively, and expression in left atria was about 40-fold above that of femoral artery. Expression in LCA and ventricular tissue was essentially comparable to control.

The relative distribution of ET receptor mRNA in coronary arteries was confirmed by *in situ* hybridization. Hybridization signal for ET_A receptor mRNA was weak and difficult to detect in both RCA and LCA. In contrast, an intense hybridization signal for ET_B receptor mRNA was evident in RCA and of only limited expression in LCA, illustrated in Figure 7. Hybridization signal for both ET receptors was localized predominantly to vascular smooth muscle of the media and intima of these arteries. No hybridization signal was evident using sense-strand riboprobes for either receptor.

Table 3. Summary of Endothelin Receptor Radioligand Binding Using [¹²⁵I]ET-1 and 3

Site	ET _A receptor density (fmol/mg protein)	ET _B receptor density (fmol/mg protein)	Ratio ET _A :ET _B
Right coronary artery	943 $\pm$ 250	1138 $\pm$ 148	0.8:1
Left coronary artery	1356 $\pm$ 319	399 $\pm$ 108	3.4:1
Right atrium	260 $\pm$ 32	215 $\pm$ 10	1.2:1
Left atrium	155 $\pm$ 40	220 $\pm$ 70	0.7:1
Right ventricular	106 $\pm$ 20	115 $\pm$ 10	0.9:1
Left ventricular	90 $\pm$ 8	100 $\pm$ 3	0.9:1

Results express as mean $\pm$ SE. Competition binding experiments using radiolabeled ligands were done as explained in Materials and Methods.

Figure 6. Quantitative analysis of ET_A (A) and ET_B (B) receptors mRNA by RT-PCR (Taqman) in normal dog heart and coronary arteries ($n \geq 3$). The normal dog femoral artery (FA) was use as a reference base and values for all sites are expressed relative to ET_A or ET_B receptor mRNA abundance in FA.

Discussion

Data from this series of studies provide the first demonstration that regional cardiotoxicity is strongly associated with distribution of cellular receptors regulating vascular tone; specifically, distribution and frequency of ET_A and ET_B receptors within dog coronary arteries and immedi-

ate intramural branches predispose myocardial regions to damage. Regional receptor density and/or shifts in receptor subtype ratios can elicit an exaggerated physiological response to vasoactive agents. The right coronary vasculature and intramural atrial branches are sites of the dog heart predisposed to damage by vasodilator agents.

Figure 7. Localization of ET_B receptor mRNA expression in extramural coronary arteries of the dog. ET_B anti-sense hybridization signal was localized predominantly to the vascular intima and media of the RCA (A). Hybridization signal was weak or absent in the LCA (B) as compared to the RCA (A), suggesting a lower density of ET_B receptors in the LCA. Using ET_B sense probes, hybridization signal was not observed in either LCA (B) or RCA (not shown). Dark field illumination; original magnifications, $\times 130$.

Regional damage by vasodilators has been best characterized in the dog because this species is frequently used in preclinical testing of novel pharmaceuticals.³⁷ However, recognition of regional sensitivity to vasodilator-induced cardiotoxicity is not confined to the dog; similar observations have been made in monkeys³⁸ and pigs.³⁹ Interestingly, species difference of both region and size of vessel damaged have been reported after administration of minoxidil; in the pig, left atrial vessels with ≤ 3 layers of medial smooth muscle are damaged, but in dogs, right atrial vessels with 3 to 10 layers of medial smooth muscle are most sensitive.³⁹ Reasons for these differences in various species are unknown, but presumably relate to site-specific functional responsiveness. Previous reports from studies in dogs suggested that extreme increases in regional blood flow preceded atrial and coronary arterial damage. Humphrey et al⁴⁰ and Mesfin et al^{5,41,42} reported that minoxidil, a long-acting vasodilator, when given to dogs at cardiotoxic doses induced a 6- to 10-fold increase in regional cardiac blood flow and that this sustained increase in flow resulted in damage to coronary vasculature. In a preliminary report we showed that the morphological observations in dogs given an ETRA (SB 207960) were very similar to those in dogs given minoxidil and to those in monkeys given another ETRA.^{6,7} The sixfold increase in right atrial flow, we reported in dogs given an ETRA were comparable to those of minoxidil. Consequently, it appears that vasodilators, when given to dogs, induced marked increases in regional blood flow which tended to precede evidence of cardiotoxicity.

Interestingly, in addition to markedly increasing regional myocardial blood flow, minoxidil when given to dogs also lowered MAP sufficiently to induce concomitant severe reflex tachycardia. This tachycardial event has been associated with additional pathological changes in heart, including subendocardial hemorrhage and papillary muscle necrosis.⁴¹ Since systemic hypotension and tachycardia were not observed in dogs given ETRA, it was not unusual that endocardial or papillary muscle lesions were not observed after ETRA exposure. In dogs given SB 207960, only minimal reductions in MAP (10–15 mmHg) and slight increases in HR (10–20%) were observed; these minimal changes were considered within the physiological range for the dog and consequently not expected to be associated with the ischemic type of myocardial damage associated with minoxidil administration.

Because cardiovascular lesions predominated in the RCA and right atrium of dogs given ETRAs, our working hypothesis was that ET receptors were most prevalent at these sites, thereby predisposing RCA and right atrium to damage during pharmacological manipulation of vascular tone. Comparison of blood flow between right atria, left atria and ventricular tissue indicated a six-, two-, and less than onefold increase, respectively, after infusion with ETRA; this reflects a marked difference in regional pharmacological responsiveness. Several investigators have demonstrated that the dog is pharmacologically responsive to endothelin and reported decreased coronary

blood flow, increased coronary vascular resistance, and subsequent myocardial ischemia after endothelin challenge.^{16,20,24,32,43} Teerlink and coworkers,²² using anesthetized dogs, demonstrated after challenge with a selective ET_B agonist that a transient period of vasodilation was followed by pronounced and sustained vasoconstriction of the left coronary artery; unfortunately, their observations were confined to the left coronary vascular bed and therefore comparison to the right vascular bed was not possible. Importantly, their observations indicated a potent ET_B-mediated vasoconstrictor response in dog coronary artery, an observation which our findings, after ET receptor blockade, strongly support. Teerlink and coworkers used anesthetized dogs, a system which might have resulted in periods of unregulated cardiovascular responses; however, our results from unanesthetized dogs correlated well with those of Teerlink et al, suggesting minimal experimental consequences of anesthesia.

Morphological localization of ET_B receptors in coronary arteries using *in situ* hybridization demonstrated an abundance of ET_B receptor mRNA, predominantly in medial smooth muscle cells of coronary arteries, supporting findings that ET_B receptors have a broader distribution than just within endothelium. Our observations are the first to emphasize the prevalence of ET_B receptors in RCA versus LCA and consequently the potential difference in the role of ET_B receptors in regulating blood flow to various myocardial regions. The prevalence of ET_B receptors within RCA suggests ET_B regulated vascular tone may be of more physiological importance in right heart vasculature than left. Our observations, as well as others, on blood flow alterations after challenge with either ET or ETRA certainly emphasized regional differences in pharmacological responsiveness, most likely mediated by a predominance of ET_B receptors in these regions.

Characterization of ET receptor subtypes in dog coronary arteries was technically challenging since only the ET_B receptor gene had been sequenced for dog. This report is the first to provide a partial nucleotide sequence for dog ET_A receptor subtype, of sufficient specificity to allow production of ET_A receptor signature probes for dog tissues (see Figure 1).⁴⁴ This partial coding sequence was highly homologous to human ET_A receptor sequence with 93 and 99% identity at the nucleotide and amino acid levels, respectively. Because G protein-coupled receptors (which include ET receptors) are highly regulated at the mRNA level and correlation of message expression with protein expression is unpredictable, it was our objective, first, to quantify both ET_A and ET_B receptor protein as the best measure of receptor abundance within selected sites and, second, to quantify message (mRNA) as a confirmatory indicator of a tissue's ability to express selected receptors. Protein quantitation by immunoreactivity would have been the preferred approach; however, extensive alternative splicing and post-translation modification of ET receptors across species required the use of dog specific reagents, which were not available. Therefore, our approach was to quantify receptors using competitive radioligand binding; this approach

technically compromised our ability to detect receptors pre-bound to endogenous ligand. Our results indicated, except for the abundance of message within atrial tissues, that protein receptor expression paralleled mRNA levels; this suggested ET receptor abundance was tightly coupled to message expression in the dog heart. The mismatch between protein and mRNA expression in atrial tissue may reflect limitation of the radioligand assay approach, in that an abundance of ET-1 production with subsequent endogenous ligand-receptor binding in atrial tissue probably resulted in an underestimation of receptor density and discordance between protein and message.

The ratio of ET_A to ET_B receptors at specific sites was initially considered a factor in predisposing sites to damage; however, the ratio of ET_A to ET_B within RCA was essentially 1:1 and quite similar ratios were observed in atrial and ventricular regions. In fact the only site with a markedly differing ratio of 3.4:1 was the LCA, which indicated a prevalence of ET_A receptors in LCA. Our conclusion from assessment of ET_A to ET_B receptor ratios was that the ratio appeared less important than the overall abundance of ET_B receptor at a particular site; the role of ET_B receptors in regulating vascular tone appeared crucial in setting sensitivity of a site to vasodilator damage.

Radioligand receptor binding data indicated ET_B receptors to be most prevalent in RCA, in fact, 3 times more prevalent than in LCA. The frequency of ET_B receptors within atrial tissue was somewhat less than that observed in LCA, but twofold more frequent than observed in ventricular tissue; the ranking of cardiovascular sites evaluated in this study by prevalence of ET_B receptor protein was RCA, LCA, atrial tissue, and ventricular tissue. Right versus left side differences were only observed for coronary arteries. Comparison of coronary arterial tissue for prevalence of message indicated RCA contained considerably more message for ET_B receptors than LCA, and this corresponded to prevalence of protein. Regional frequency of cardiotoxic lesions also correlated closely with abundance of ET_B receptor protein expression. ET_B receptor mRNA was most prevalent in right heart (about similar in RCA and right atrium) followed by left atria, LCA, and ventricular tissue. The rank order of lesion occurrence and ET_B receptor abundance correlated well and was RCA, LCA, and atrial tissue.

This is the first report in which ET receptor mRNA expression has been localized specifically to vascular smooth muscle of the dog coronary artery. These findings support and extend observations by others of an ET receptor subtype in the dog that mediates vasoconstriction and is expressed primarily in smooth muscle of the coronary vasculature.²⁵ Based on gene homology and mRNA expression data, it is plausible that this ET_B receptor subtype, which mediates vasoconstriction in the dog, is a homologue of the cloned human ET_B receptor,^{15,45} a receptor previously described as the ET₁ clearance receptor primarily localized to endothelium.^{12,18} Evidence now suggests obvious species differences in functionality of ET_B receptors. *In vitro* studies in dog²⁵ and pig⁴⁶ coronary artery, rabbit veins,²¹ and rat systemic circulation³² indicate that ET_B-like receptors do mediate vasoconstriction, but some organ variability in function oc-

curs; the role as an ET₁ clearance mechanism in various species is poorly defined. Hence the physiological effect of ET/ET_B receptor signaling (ie, vasoconstriction, vasodilation, and/or ET₁ clearance) can clearly differ between species and location of vascular bed. Consequently, one could predict that toxicological outcomes would also differ between sites and species.

It was not unexpected to find that ET_B receptors mediate different functions or that differences occur between organ systems or animal species, since G protein-coupled receptors, which include the ET receptors, are generally known to have multiple, even dichotomous, functional roles.^{47,48} Receptors encoded by a single gene product but modified by post-transcriptional (mRNA splicing) and/or post-translational (glycosylation or phosphorylation) events can, as a consequence of these modifications, display multiple or disparate responses.^{19,47,49} Multiplicity of receptor functionality or variations between species can result from stimulation of differing G protein-coupled signal transduction systems⁵⁰ or, alternatively, as recently proposed by McLatchie and coworkers, through involvement of varying receptor activity modifying proteins.⁵¹ Hence, as a result of G-protein subunit expression, receptor activity modifying proteins and the coordinated regulation of receptor expression, the biological response to receptor activation may vary widely between cells and/or species, as with, eg, ET_B receptor stimulation mediating vasopressor, vasorelaxant, and/or ET clearance functionality. It is, therefore, important to balance gene protein and message expression data with measurements of biological response, because various and widely differing outcomes can result. The mere presence of message or protein will not consistently predict the responsiveness of a tissue or species to receptor ligand binding.

In conclusion, this series of studies quantifies ET_A and ET_B receptor protein and mRNA message at regional levels within dog heart and coronary arteries, ET_B receptor protein was localized by *in situ* hybridization to vascular smooth muscle cells and demonstrated a marked prevalence of ET_B receptor protein in the right coronary vascular bed. ET receptor subtype density was correlated with changes in regional blood flow during ETRA infusion and ultimately to a toxicological response. These studies provide a balanced data set of gene and protein expression in concert with regulatory effects on blood flow to ultimately explain a biological outcome. These data emphasize the importance of ET_B receptor distribution in regulating vascular tone of the dog right coronary vasculature and the predisposition of this anatomical site to damage because of ET_B receptor frequency.

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