Developmental Expression of Endothelin Receptors in Postnatal Swine Mesenteric Artery

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ABSTRACT

Studies were conducted to determine whether endothelin (ET) ET_A and ET_B receptor protein and mRNA expression is developmentally regulated in the postnatal swine mesenteric circulation. To this end, Western blotting and real-time reverse PCR were performed on protein and total RNA isolated from the mesenteric artery harvested from 3-, 10-, and 30-d-old swine. Western blot analysis revealed that ET_A and ET_B receptor protein expression in the swine mesenteric artery decreased over the age range studied; thus, ET_A and ET_B receptor protein expression was significantly greater in the 3-d-old group then progressively declined over the first postnatal month. Similar to the Western data, real-time PCR analysis revealed that ET_A and ET_B receptor mRNA expression also decreased over the age range studied; thus, ET_A and ET_B receptor mRNA expression was significantly greater in the 3-d-old group then progressively declined over the first postnatal month. Immunohistochemistry localized the ET_A receptor to the vascular smooth muscle and the ET_B receptor to the endothelial cell layer. Additionally, we report a partial cDNA sequence for the swine ET_B receptor. We conclude that ET_A and ET_B receptor protein and mRNA expression is developmentally regulated in the postnatal swine mesenteric artery, being expressed to a greater degree in younger animals. (*Pediatr Res* 56: 359–365, 2004)

Abbreviations
ET, endothelin
ET_A, endothelin A receptor
ET_B, endothelin B receptor

ET-1 is a potent vasoactive 21-amino acid peptide first isolated by Yanagisawa *et al.* (1) from the conditioned medium of cultured porcine aortic endothelial cells. ET-1 exerts its effects *via* the activation of at least two receptor subtypes, designated ET_A and ET_B (2–5). Both receptor subtypes are members of the seven-transmembrane-spanning, G protein–coupled superfamily of receptors whose activation leads to phospholipase C–mediated production of inositol triphosphate and diacylglycerol (6, 7). The ET_A receptor is localized on vascular smooth muscle and its activation leads to vasoconstriction (8, 9). The ET_B receptor is located on both endothelial cells and vascular smooth muscle. Activation of the endothelial cell ET_B receptor leads to vasodilation *via* the production of nitric oxide (NO), whereas activation of the vascular smooth muscle ET_B receptor leads to vasoconstriction (5, 10, 11). Thus, the overall effect of ET-1 on vascular tone is dependent on the proportion and location of ET_A and ET_B receptors within the vascular bed in question (12).

Previous work in this laboratory has demonstrated that the expression of ET_A and ET_B receptors may be developmentally regulated in the postnatal swine intestinal circulation. This possibility was first raised by a series of studies that used pharmacological probes to selectively block these receptors. Blockade of ET_A receptors with BQ610 increased intestinal capillary perfusion and oxygen extraction in gut loops from 3-d-old but not 35-d-old swine, suggesting that ET_A receptors are more abundant within the intestinal microcirculation of younger subjects (13). Blockade of ET_B receptors with BQ788 caused a leftward shift in the contractile dose-response curve for ET-1 in quiescent mesenteric artery rings from 3-d-old but not from 35-d-old subjects; also, stimulation of the ET_B receptor with the selective agonist sarafotoxin 6c caused greater relaxation of precontracted mesenteric artery rings in 3-d-old but not 35-d-old subjects (14, 15). These observations suggest that endothelial ET_B receptors may also be more abundant in the mesenteric artery from younger subjects. Further evidence suggesting that expression of the ET_A and endothelial ET_B receptors in the postnatal intestinal circulation is developmentally regulated was provided by competitive binding assays, which demonstrated that both the ET_A and the endothelial ET_B receptor are expressed to a greater degree in younger animals compared with their older counterparts (16).

Early postnatal life represents a critical stage in intestinal development. During this period, the intestine grows dramatically as it transitions from relative dormancy *in utero* to become the sole means of water and nutrient assimilation after parturition (17). Regulation of ET_A and ET_B receptor expression in the attendant intestinal circulation might play a critical role during this transition. Little is known about the expression pattern of these receptors in the intestinal circulation during early postnatal life. The goal of this study therefore was to determine the expression of ET_A and ET_B receptor protein and mRNA in postnatal swine mesenteric artery during early postnatal life. Based on our previous observations, we hypothesized that ET_A and ET_B receptor protein and mRNA expression are developmentally regulated; specifically, that the newborn swine superior mesenteric artery expresses ET_A and ET_B receptor protein and mRNA to a greater degree than that noted in their older counterparts. To test this hypothesis, Western blot analysis and real-time PCR for the ET_A and ET_B receptor were carried out on protein and total RNA isolated from the mesenteric arteries of 3-, 10-, and 30-d-old swine. Immunohistochemistry was performed on sections of whole mesenteric artery from the same age groups to localize the ET_A and ET_B receptors. Additionally, we report a partial cDNA sequence for the swine ET_B receptor.

METHODS

Care and handling of experimental animals. Studies were conducted using farm-bred swine of either sex obtained on the day of use (18, 19). Three age groups were studied: 3, 10, and 30 d old, and all subjects were studied on that postnatal day; age ranges were not used. Subjects from each age group were allowed access to the age-appropriate diet (sow's milk for the 3- and 10-d-old group, chow for the 30-d-old group) until transport to the laboratory and were studied within 2 h of their arrival. Anesthesia was induced with telezol (6 mg/kg i.m.) and xylazine (7.5 mg/kg i.m.) and maintained with sodium pentobarbital (5 mg/kg/h i.v.). After tissue removal, animals were killed with an overdose of pentobarbital (50 mg/kg i.v.) while still anesthetized. All animal care was provided in accordance with *The Guide for the Care and Use of Laboratory Animals* [DHHS Publication No. (National Institutes of Health) 85–23, Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892]. All experimental protocols were approved and monitored by the Institutional Animal Care and Use Committee of the Children's Research Institute. All work involving live animals was carried out in the Children's Research Institute Vivarium, an Association for Assessment and Accreditation of Laboratory Animal Care International–approved facility under the supervision of a full-time veterinarian.

Protein isolation for Western blot analysis. The entire mesenteric artery was removed *en bloc*, snap frozen in liquid nitrogen, and stored at $-80°C$ until use. The frozen vessel segments were pulverized to a fine powder using a mortar and pestle on dry ice and were suspended in homogenization buffer (20 mM MOPS, 1% Triton, 4% SDS, 10% glycerol, 5.5 mM leupeptin, 5.5 mM pepstatin, 200 KIU aprotinin, 1 mM Na_3VO_4, 10 mM NaF, 100 μM $ZnCl_2$, 20 mM B-glycerophos-

phate, an 20 μM phenylmethylsulfonyl fluoride). The samples were mixed by repeated pipetting, placed on ice for 10 min, and finally centrifuged at 10,000 rpm for 5 min at room temperature to pellet any debris. The supernatant was collected and a small aliquot was used to determine protein concentration using the bicinchoninic acid (BCA) protein assay (Pierce Chemical, Rockford, IL, U.S.A.).

Western blot analysis. For Western blot analysis, 10 μg of protein was resolved on a 10% denaturing polyacrylamide gel (SDS-PAGE) and then electrophoretically transferred overnight to polyvinylidene difluoride membranes (Immuno-Blot PVDF Membrane, Bio-Rad, Hercules, CA, U.S.A.). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20. The membranes were then incubated for 1 h at room temperature in a shaker with a 1:200 dilution of either rabbit anti-ET_A receptor antibody or rabbit anti-ET_B receptor antibody (Alomone Labs, Jerusalem, Israel). After incubation with primary antibody, the membranes were washed with Tris-buffered saline containing 0.1% Tween-20 and then incubated with a 1:2000 dilution of anti-rabbit IgG-horseradish peroxidase conjugate. The membranes were washed with Tris-buffered saline containing 0.1% Tween-20 and the protein bands of interest were detected *via* chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). All samples from each age group were resolved simultaneously on a single gel. Each blot was then stripped and incubated with 1° antibody to β-actin to correct for sample loading. Previous studies in our laboratory revealed that β-actin expression within mesenteric artery does not change over the age range examined in this study. The bands of interest were quantified using scanning densitometry (Model GS-700 Imaging Densitometer, Bio-Rad), normalized to the 3-d-old group, and the relative expression of ET_A, ET_B and β-actin protein were then calculated.

RNA isolation for partial cloning of the swine ET_B receptor and real-time PCR. The tissue of interest was removed, cut into small segments (<0.5 cm in any dimension), and immediately placed in RNA storage solution (RNAlater, Ambion, Austin, TX, U.S.A.). The tissue segments were placed at 4°C overnight and then stored at $-80°C$ until final preparation per the manufacturer's instructions. The segments were removed from the storage solution, weighed, and total RNA extracted using a commercially available kit (RNAwiz, Ambion) before spectrophotometric determination of total RNA concentration.

Partial clone of the swine ET_B receptor. To design primers and a probe for real-time PCR for the ET_B receptor, it was necessary to clone this sequence inasmuch as the cDNA sequence for the swine ET_B receptor has not been reported. To this end, total RNA was isolated from swine liver and was used to obtain poly (A) RNA using a Poly (A) Pure mRNA purification kit (Ambion). A pool of enriched full-length double-stranded cDNA was generated using the SMART technique from the SMART cDNA Library Construction Kit (CLONTECH, Palo Alto, CA, U.S.A.). Briefly, the purified poly (A) RNA was used in the first-strand synthesis using a modified oligo (dT) primer (CDSIII/3'PCR Primer). During the reverse transcription reaction, the PowerScript reverse transcriptase adds a few additional nucleotides, primarily deoxycytidine, to

the 3' end of the cDNA, which compliment to the oligo (G)s at the 3' end SMART IV oligonucleotide. Enriched double-stranded cDNA were then generated by a follow-up PCR step using these two oligonucleotides (CDSIII and SMART IV). For partial cloning of the swine ET_B cDNA sequence, two PCR primers (ET_B sense: 5'-CAAAATGGACAGCAGTAGAAA-3' and ET_B antisense 5'-GACTTAAAGCAGTTTTTGAATCT-3') were designed based on the consensus sequences of cDNA homologues of other species (20). These primers generated a 552-bp fragment. The amplified PCR product was subsequently cloned into a pCR4-TOPO plasmid vector and the insert of one positive clone was verified by sequencing in the Children's Research Institute core laboratory using an automated DNA sequencer. Sequence information is available in GenBank (accession number AY583500).

Real-time PCR. To quantify expression of ET_A and ET_B mRNA, we chose real-time PCR methodology, as this technique provides the most precise means of mRNA quantification when compared with Northern blotting or RNA protection assays. Two micrograms of total RNA isolated from the swine mesenteric artery was treated with DNase I before reverse transcription to cDNA using a commercially available kit (SuperScript First-Strand Synthesis System, Invitrogen, Carlsbad, CA, U.S.A.). The Primer 3 software package (Steve Rozen and Helen J. Skaletsky, Whitehand Institute, MIT Center for Genome Research) was used to generate real-time PCR primers and fluorescence-tagged probes for the ET_A and ET_B receptor. The ET_A primers and probe were designed based on the published cDNA sequence for the swine ET_A receptor (GenBank accession number S80652), whereas those for the ET_B receptor were designed based on the 552-bp partial cDNA sequence for the swine ET_B receptor generated in our laboratory. The real-time PCR primers and probe for the ET_A receptor flanked a 130-bp fragment from bp 608 to bp 737. The real-time PCR primers and probe for the ET_B receptor were nested within the PCR primers used for the partial cloning of the ET_B receptor and amplified a 76-bp fragment within this 552-bp partial sequence. The sequences for the real-time PCR primers and probe for the ET_A and ET_B receptor are presented in Table 1. As it has been used as a loading standard in other studies dealing with the developmental expression of other proteins, 18S ribosomal RNA served as an internal control. Preliminary work in our laboratory has indicated stability of the 18S ribosomal RNA signal during the postnatal time period under study. The real-time PCR primers and fluorescence-tagged probe for 18S ribosomal RNA controls are commercially available and were purchased directly from the manufacturer (PerkinElmer Life Science, Boston, MA, U.S.A.). The different fluorescent labeling of the probes allows simultaneous determination of a target gene (ET_A or ET_B) and the internal control (18S ribosomal RNA) mRNA abundance in a real-time fashion. A typical 25-μL real-time reaction for the ET_A receptor contained 1/20 of RT reaction cDNA, 200 nM and 100 nM for ET_A primers and probe, 100 nM and 50 nM for 18S ribosomal RNA primers and probe, and real-time PCR master buffer. The real-time reaction for the ET_B receptor was identical to that for the ET_A receptor except that ET_B primers and probe were used. PCR reactions were carried out in an ABI Prism 7700 Detection System (PerkinElmer Life Science) under the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All PCR reactions were run in duplicate and the results were analyzed using the Sequence detector 1.6 program (PerkinElmer Life Science). The ABI Prism 7700 provides an amplification curve constructed by relating the fluorescence signal intensity (ΔRn) to the PCR cycle number. The threshold cycle value (C_t) corresponds to the cycle at which an increase in ΔRn above background occurs and is thus a direct marker of the abundance of the mRNA in question. A lower C_t value indicates a greater the abundance of the mRNA under study. The C_t values obtained for the internal control (18S ribosomal RNA) were subtracted from the C_t values obtained for the target gene mRNA in question (ET_A or ET_B), normalized to 3-d-old group, and the relative expression of ET_A and ET_B mRNA then calculated.

Immunohistochemistry. The entire mesenteric artery was removed *en bloc* and immediately immersed in 10% neutral buffered formalin then processed for paraffin sectioning by standard methods. Paraffin sections were mounted on glass slides and refrigerated until use. Slides were deparaffinized in xylene for 10 min then rehydrated in descending concentrations of ethanol (100%, 90%, 70%, 5 min in each) and transferred to distilled water. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide for 15 min at room temperature. The sections were rinsed with distilled water for 2 min and digested with trypsin for 10 min at 37°C followed by distilled water rinses. The sections were incubated with Super Block (ScyTek, Logan, UT, U.S.A.) for 5 min and rinsed twice for 5 min each in buffer (Optimax buffer, Biogenex, San Ramon, CA, U.S.A.). The sections were incubated at 4°C overnight in the primary antiserum (1:50). Sections were rinsed with buffer and incubated with UltraTek Anti-Polyvalent secondary antibody (ScyTek) for 20 min. The sections were rinsed twice for 5 min each in buffer and incubated with UltraTek horseradish peroxidase (ScyTek) for 20 min at room temperature. The sections were rinsed twice for 10 min in buffer and incubated in chromagen (Dako High Sensitivity Chromagen System, DAKO Corporation, Carpinte-

Table 1. *Oligonucleotide primer and probe sequences used for real-time PCR for the ET_A and ET_B receptor*

Gene	Oligonucleotide	Sequence
ET_A receptor	Forward primer	5'-GGTACAGAGCAGTTGCCTCCT-3'
	Reverse primer	5'-ATGACAAAGCCGATTGCTTC-3'
	Probe	5'-TCGTGTTCAGGGAATCGGGAT-3'
ET_B receptor	Forward primer	5'-AAGCCTTGGTTTTGACATGAT-3'
	Reverse primer	5'-CTGAGTAGGATGGAGCAAGC-3'
	Probe	5'-ACCACTGACTATAAAGGAAATCGCCTG-3'

ria, CA, U.S.A.) for 5 min at room temperature followed by counterstaining with hematoxylin for 30 s. The sections were rinsed in tap water and placed in 0.2% ammonia for 15 s. The sections were rinsed in tap water followed by polymerization with Crystal Mount (Biomeda, Foster City, CA, U.S.A.), coverslipped with Permount (Fisher Scientific, Fairlawn, NJ, U.S.A.), and then visualized using standard light microscopy.

Data analysis. In statistical analysis and data presentation, *n* refers to the number of subjects in which observations were made. Data are expressed as means $\pm$ SEM. Statistical comparisons were made by a one-way ANOVA. Tukey-Kramer multiple comparison tests were carried out on data sets in which the *F* statistic was significant ($p < 0.01$) to determine the sites of significant differences between variables. A *p* value of <0.05 was considered statistically significant.

RESULTS

Western blot analysis. Western blot analysis was performed to determine whether the degree of protein expression of the ET_A and ET_B receptor differed between age groups. ET_A and ET_B receptor protein expression in the swine mesenteric artery decreased over the age range studied; thus, ET_A and ET_B receptor protein expression was significantly greater in the 3-d-old group then progressively declined over the first post-

natal month (Fig. 1). β-actin expression did not change over the age range studied (Fig. 1).

Partial clone of the swine ET_B receptor. Because the cDNA sequence for the swine ET_B receptor has not been reported, it was necessary to clone this sequence to design primers and a probe for real-time PCR for the ET_B receptor. Thus, a 552-bp partial clone of the swine ET_B receptor cDNA was cloned and sequenced (Fig. 2). Based on the reported cDNA sequences from other mammalian species, this sequence corresponds to exons 3–6 for the ET_B receptor and shares 91%, 87%, and 87% sequence homology with the published human, rat, and mouse ET_B cDNA sequences, respectively. The deduced amino acid sequence from the partial swine ET_B cDNA showed 94%, 91%, and 91% sequence homology with the published human, rat, and mouse ET_B amino acid sequences, respectively (Fig. 3).

Real-time PCR. Real-time PCR for the ET_A and ET_B receptors was performed to determine whether the increased level of ET_A and ET_B protein expression noted in younger subjects with Western blotting was associated with an increased rate of mRNA transcription. ET_A and ET_B receptor mRNA expression in the swine mesenteric artery mirrored the Western data; thus, ET_A and ET_B receptor mRNA expression was significantly greater in the 3-d-old group then progressively declined over
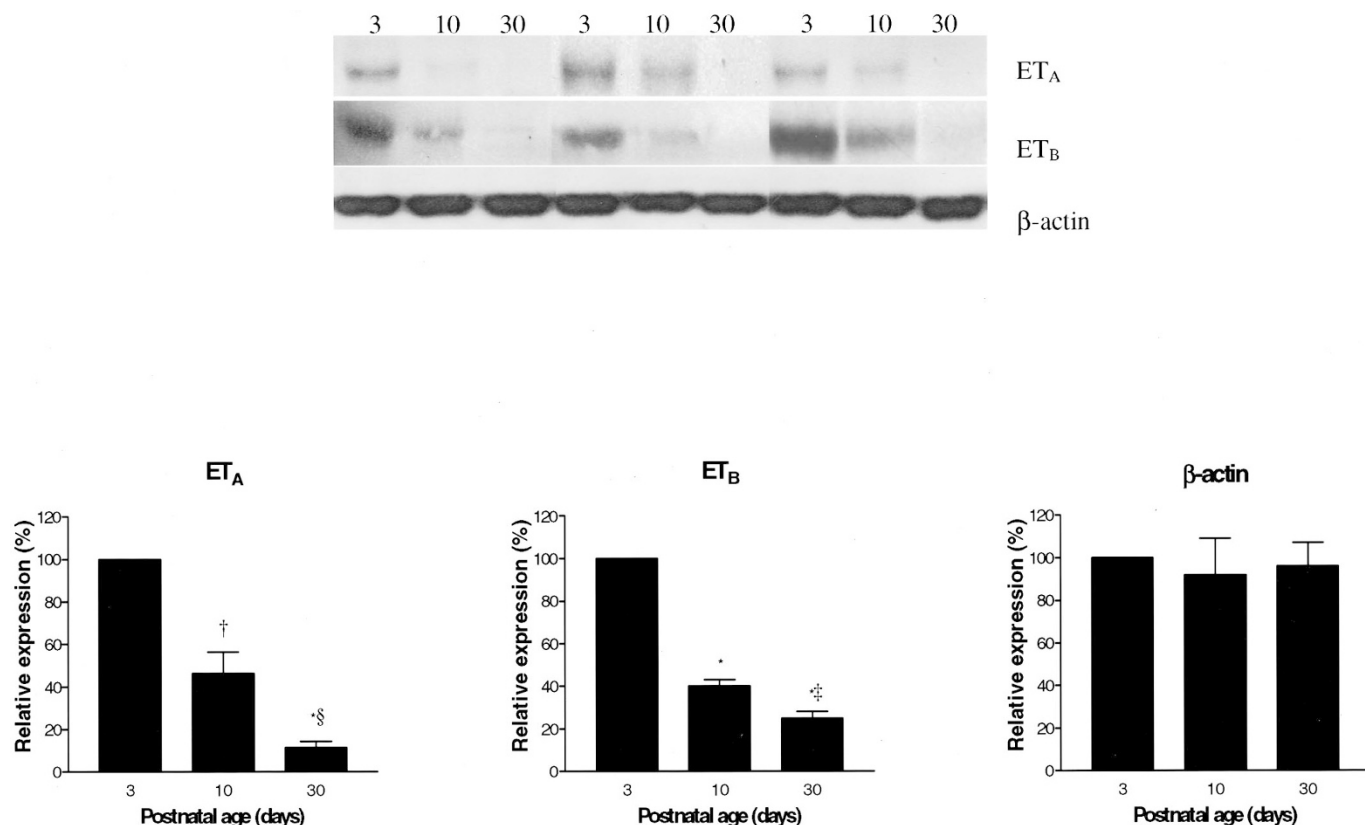

Figure 1. Western blots and densitometry data for ET_A receptor, ET_B receptor, and β-actin expression in 3-, 10-, and 30-d-old subjects. Densitometry was carried out on the individual blots generated for each age group for the ET_A receptor and ET_B receptor. Expression of ET_A receptor and ET_B receptor was normalized to the 3-d-old group and the relative expression then calculated. The membrane was first incubated with 1° antibody to either the ET_A receptor or ET_B receptor, then stripped and incubated with 1° antibody to β-actin to correct for sample loading. Data are given as means $\pm$ SEM, $n = 4$ for each experimental group; *$p < 0.001$ *vs* 3-d-old, †$p < 0.01$ *vs* 3-d-old, ‡$p < 0.01$ *vs* 10-d-old, §$p < 0.05$ *vs* 10-d-old.

CAAAATGGACAGCAGTAGAAATTGTTTTAATTTGGGTGGTCTCCGTGGTCCTGGCCGTCC
CGGAAGCCTTGGTTTTGACATGATTACCACTGACTATAAAGGAAATCGCCTGCGAATCT
AGCTTGCTCCATCCTACTCAGAAAACAGCCTTCATGCAGTTTTACAAGACAGCTAAAGTTGG
TGGCTATTCAGTTTCTATTTCTGCTTGCCACTAGCCATCACTGCATTTTTTTATACCCCGATGA
CCTGTGAAATGCTGAGAAAGAAGAGTGGCATGCAAATTGCTTTAAATGATCACTTAAAGCAG
AGACGGGAAGTGGCCAGAACCGTATTTTGCCTGGTCCTTGTCTTTGCCCTGTGTTGGCTTCC
CCTTCATCTCAGCAGGATTTTGAAGCTCACTCTGTATGATCAAAATGATTCGAATAGATGTGA
ACTTTTGAGCTTTTTGTTGGTATTGGATTACATTGGCATCAAACATGGCGTCCCTGAATTCCTG
TATTAATCCAATAGCTCTGTATTTGGTGAGCAAAAGATTCAAAAACTGCTTTAAGTC

Figure 2. Partial cDNA sequence for the swine ET_B receptor. A 552-bp cDNA fragment corresponding to exons 3–6 of the ET_B receptor was cloned and sequenced as described in the text (GenBank accession number AY583500). The underlined sequences represent the primers and probe used for real-time PCR.

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Swine   FDMITTDYKGNRLRICLLHPTQKTAFMQFYKTAKDWWLFSFYFCLPLAITAFFYTPMTCE
Human   - - I - -M- - - - - SY - - I- LLH -V- - - - - - - - - - - - - - - - - - - - - - - - - - - - F - - -L - - - -
Rat     - - V - -S- - - - KP - -V-M-N - F- - - - - - - - - - - - - - - - - - - - - - - - - - - I - - -L - - - -
Mouse   - - M- -S - - - - KP - -V-M-N - F- - - - - - - - - - - - - - - - - - - - - - - - - - V- - -L - - - -
                                                              TM V
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Swine   MLRKKSGMQIALNDHLKQRREVARTVFCLVLVFALCWLPLHLSRILKLTLYDQNDSNRC
Human   - - - - - - - - - - - - - - - - - - - - - - - -R- - - - - - - - - - - - - - - - - - - - N- NDPN - -
Rat     - - - - - - - - - - - - - - - - - - - - - - K- - - - - - - - - - - - - - - - - - - - - - D - SNPQ - -
Mouse   - - - - - - - - - - - - - - - - - - - - - K- - - - - - - - - - - - - - - - - - - - - D - SNPH - -
                                                  TM VI
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Swine   ELLSFLLVLDYIGINMASLNSCINPIALYLVSKRFKNCFK
Human   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Rat     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Mouse   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                          TM VII
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Figure 3. Comparison of the amino acid sequences between the swine, human, rat, and mouse ET_B receptor. The deduced amino acid sequence from the partial cDNA for the swine ET_B receptor was compared with the published amino acid sequences for the human, rat, and mouse ET_B receptor. The sequence contains three transmembrane domains, V–VII (underlined), and the third cytoplasmic loop between TM V and VI.

the first postnatal month (Fig. 4). 18S ribosomal RNA expression did not change over the age range studied (data not shown).

Immunohistochemistry. Immunohistochemistry was performed to localize the ET_A and ET_B receptors within the superior mesenteric artery in 3-, 10-, and 30-d old subjects. The ET_A receptor was localized to the vascular smooth muscle, whereas the ET_B receptor was localized in the endothelial layer in a 3-d-old subject (Fig. 5). A similar pattern was noted in superior mesenteric arteries harvested from 10- and 30-d-old subjects (data not shown).

DISCUSSION

The novel observation made in this study is that expression of endothelin ET_A and ET_B receptors within the swine mesenteric artery is developmentally regulated during early postnatal life. This developmental regulation occurred at the level of gene transcription inasmuch as the changes in protein expression noted on Western analysis paralleled the changes in mRNA expression noted by real-time PCR. Immunohistochemistry localized the ET_A receptor to the vascular smooth muscle and the ET_B receptor to the endothelial cell layer. Additionally, we report a partial cDNA sequence for the swine ET_B receptor.

The developmental changes noted in ET_A and ET_B expression are consistent with prior observations reported from this laboratory. Studies within blood-perfused, innervated *in situ* gut loops (13), in mesenteric artery rings suspended for tension measurement (14), and in buffer-perfused terminal mesenteric
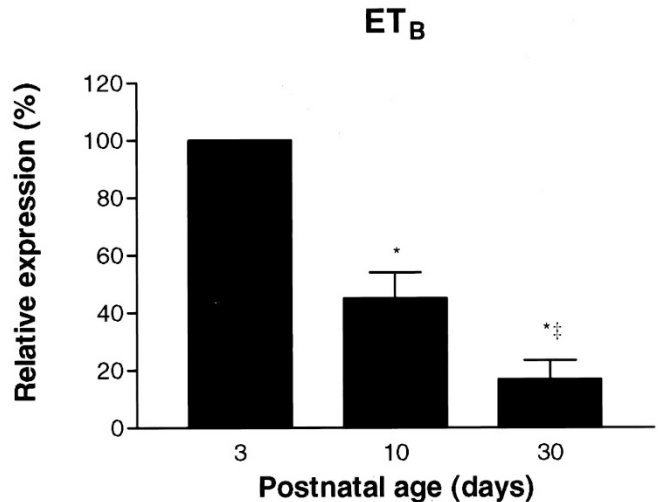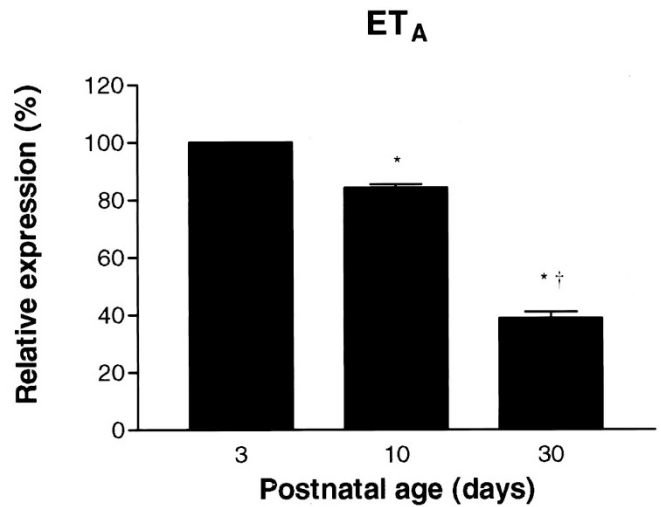

ET$_A$

ET$_B$

Figure 4. Real-time PCR detection of ET_A receptor and ET_B receptor expression levels in 3-, 10-, and 30-d-old subjects. The C_t values obtained for the internal control (18S ribosomal RNA) were subtracted from the C_t values obtained for the target gene mRNA in question (ET_A or ET_B), normalized to the 3-d-old group, and the relative expression was then calculated. Data are given as means $\pm$ SEM, $n = 4$–6 for each experimental group; $*p < 0.01$ *vs* 3-d-old, $\dagger p < 0.001$ *vs* 10-d-old, $\ddagger p < 0.05$ *vs* 10-d-old.

arterioles mounted within a microvascular chamber (21) have all provided physiologic evidence that ET_B receptors are present in greater abundance in newborn (*i.e.*, 3-d-old) intestine. Thus, selective blockade of ET_B receptors with BQ788 resulted in a leftward shift in the contractile dose-response curve for ET-1 in intestine or mesenteric artery from 3-d-old but not 35-d-old subjects, whereas the administration of the selective ET_B receptor agonist sarafotoxin 6c to precontracted preparations caused vasodilation only in preparations from 3-d-old subjects (14, 21). These observations are each consistent with the presence of an abundant endothelial ET_B receptor population within the newborn intestinal circulation whose activation leads to vasodilation. Physiologic evidence for an
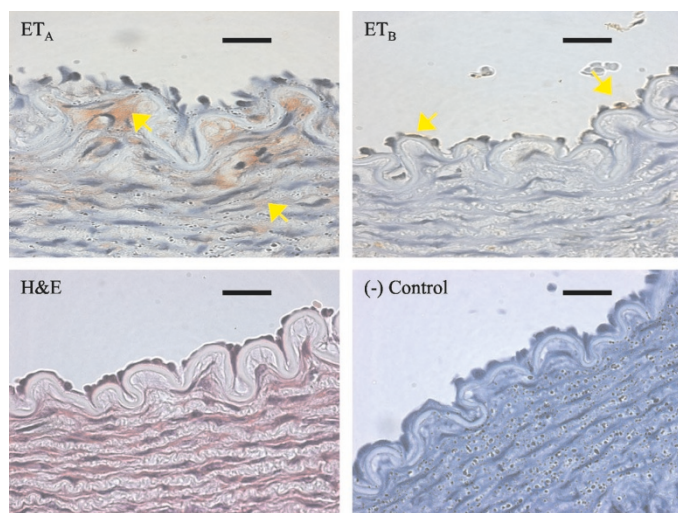
Figure 5. Immunohistochemical localization of ET_A receptors and ET_B receptors in a 3-d-old subject. Representative photomicrograph (1000×) of immunohistochemical staining for the ET_A and ET_B receptors in the superior mesenteric artery from a 3-d-old subject. Note the localization of the ET_A receptor to the vascular smooth muscle (*upper left panel*), and the ET_B receptor to the endothelial cell layer (*upper right panel*). A hematoxylin-and-eosin–stained control section is presented for comparison (*lower left panel*). Additionally, a negative control section (without primary antibody) is presented to verify the specificity of the ET_A and ET_B antisera used (*lower right panel*). Immunohistochemical staining in 10- and 30-d-old subjects demonstrated a similar pattern for the ET_A and ET_B receptors. Scale bars in each photomicrograph represent 20 μm.

increased level of ET_A receptor expression in newborn intestine also exists; thus, selective blockade of the ET_A receptor within *in situ* newborn intestine leads to an increase in the perfused capillary density and enhanced oxygen extraction in 3-d-old but not 35-d-old intestine, suggesting that the precapillary sphincters that govern capillary perfusion (22) are under tonic control of ET-1 in newborn intestine (13). The endothelial localization of this age-specific ET_B receptor expression was demonstrated by studies in which the endothelium was mechanically removed (14, 16). We have further shown that ligand binding to these endothelial ET_B receptors caused an NO-mediated vasodilation (14). Thus, the newborn intestine has a relative abundance of contractile ET_A receptors located on vascular smooth muscle, but also has a large population of vasodilatory ET_B receptors located on the vascular endothelium.

The present data do not provide insight into the reason for the increased expression of ET_A and ET_B receptors in newborn intestine; however, some speculation that is based on the established roles for the ET-1 peptide might be relevant. ET-1 is the most potent vasoconstrictor substance discovered to date (1) and there is a substantial body of evidence that the contractile capacity of this peptide plays an important role in cardiovascular regulation, especially on a systemic scale (23, 24). ET-1 is also an exceptionally potent mitogenic factor and has been shown to stimulate DNA synthesis and cell proliferation in a variety of cells including fibroblasts and endothelial and vascular smooth muscle cells largely *via* stimulation of ET_A receptors (25–30). ET-1 may also play an important role

in the process of angiogenesis by interacting with known angiogenic factors such as vascular endothelial growth factor and platelet-derived growth factor to regulate endothelial cell activity, again *via* interaction with the ET_A receptor (31, 32). The newborn intestine is the site of intense growth during early postnatal life (17) as it transitions from relative dormancy *in utero* to become the sole means of water and nutrient assimilation after birth. Part of this growth process involves angiogenesis; thus, it is not surprising that the ET_A receptor is substantially expressed in newborn intestine or that the concentration of ET-1 in the mesenteric venous effluent is greater in newborn subjects (13). It seems logical that an enhanced expression of endothelial ET_B receptors is also present at this time to offset the potential vasoconstrictor effect of an abundance of ET_A receptors and ET-1. Thus, we speculate that this developmental expression of the ET receptor system is designed to facilitate the role of ET-1 in intestinal growth and angiogenesis in the postnatal newborn intestine without causing undue vasoconstriction.

The ET_B receptor, like the ET_A receptor, is a member of the seven-transmembrane-spanning, G protein–coupled superfamily of receptors whose stimulation leads to the activation of multiple effector pathways (33). The cDNA sequence for the ET_B receptor has been reported in several mammalian species including the human, rat, and mouse (34–36); however, the cDNA sequence for the swine ET_B receptor has not been previously reported. The partial cDNA sequence and deduced amino acid sequence for the swine ET_B receptor reported here shares significant sequence homology with other mammalian species and codes for transmembrane domains V–VII, which contains the highly conserved third cytoplasmic loop between transmembrane domains V and VI.

We conclude that, within the swine mesenteric artery, expression of endothelin ET_A and ET_B receptors is developmentally regulated during early postnatal life. This developmental regulation of ET_A and ET_B receptor expression occurs at the level of gene transcription, although the mechanisms involved were not established by the current work. However, we speculate that these mechanisms may be closely coupled with the maturation of the intestine and its attendant vasculature, and may play important roles in gut development during early postnatal life.

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