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Endothelin B Receptor Modulates Inflammatory Pain and Cutaneous Inflammation

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ABSTRACT

The role of endothelin B (ET_B) receptors in inflammation and nociception was examined using ET_B receptor knockout mice. Genotyping studies were used with tissues from $ET_B^{(+/+)}$, $ET_B^{(+/-)}$, and $ET_B^{(-/-)}$ mice to confirm the loss of ET_B receptors. Algesia induced by phenylbenzoquinone was evident in the (+/+) mice, reduced by ~80% in the (+/−) mice, and absent in the (−/−) mice. Phenylbenzoquinone-induced algesia in (+/+) mice was inhibited 74% by the ET_B receptor-selective antagonist A192621 (25 mg/kg p.o.), but unaffected by the ET_A receptor-selective antagonist SB 234551 (25 mg/kg p.o.). Noninflammatory pain, induced by hotplate, was equivalent between (+/+) and (−/−) mice. The cutaneous inflammatory response to topical arachidonic acid (AA) also was evaluated. Whereas (+/+) mice had a marked inflammatory response to AA, the (+/−), and (−/−) mice had significantly reduced fluid phase responses (37 and 65% inhibition, respectively). Neutrophil infiltration also was reduced in the (+/−) and (−/−) mice (51 and 65% reduction, respectively). Topical administration of A192621 (500 μg/ear) in (+/+) mice inhibited AA-induced swelling (39%), whereas SB 234551 (500 μg/ear) was without effect. Collectively, these results implicate the ET_B receptor in mediation of inflammatory pain and cutaneous inflammatory responses in mice.

The endothelin isopeptides (ET-1, ET-2, and ET-3) are a family of distinct gene products with a broad distribution in both the central and peripheral nervous systems that possess a multiplicity of biological actions (for review, see Masaki et al., 1992; Rubanyi and Polokoff, 1994). The most widely studied isoform, ET-1, was the first member of the family identified, in 1988 (Yanagisawa et al., 1988). Although recognized initially for its potent vasoconstrictor activity, subsequent extensive research revealed an array of effects of ET-1, and related ligands, in a variety of cells and tissues, and a pathophysiological role for ET-1 has been proposed for several diseases (Masaki et al., 1992; Rubanyi and Polokoff, 1994; Michael and Markewitz, 1996).

The effects of ET-1 are mediated by G protein-coupled, seven-transmembrane-spanning receptors of which two major subtypes (ET_A and ET_B) have been characterized pharmacologically and by molecular biologic techniques (Hosoda et al., 1992; Arai et al., 1993). Many peptide and nonpeptide antagonists for ET receptors have been identified, including the two compounds used in the present study: SB 234551, a nonpeptide ET_A receptor-selective antagonist (Ohlstein et al., 1998), and A192621, a nonpeptide ET_B receptor-selective antagonist (Douglas, 1997).

Several studies have provided evidence that ET-1 modulates inflammatory processes (Rubanyi and Polokoff, 1994; Michael and Markewitz, 1996). For example, ET-1 stimulates tumor necrosis factor-α, granulocyte monocyte colony-stimulating factor, interleukin (IL)-1, and IL-8 synthesis and release from monocytes (McMillen and Sumpio, 1995); enhances β-integrin expression; and activates neutrophils (Lopez-Farre et al., 1993; Elferink and de Koster, 1994; Helset et al., 1994; Filep et al., 1995). In addition, recent data suggest a role for ET-1 in nociception. Thus, ET-1 and sarafatoxins 6c, the ET_B receptor-selective agonist (Williams et al., 1991), induced algesia in the mouse by a mechanism apparently distinct from those used by acetic acid and phenylbenzoquinone (PBQ) (Raffa et al., 1996a,b). Furthermore, ET-1 has been suggested to be involved in mediating formaldehyde (Formalin)-induced pain and inflammation in mice (Piovezan et al., 1997). It is unclear, however, whether these algesic and inflammatory effects are mediated through actions at ET_A and/or ET_B receptors. The current study addressed this issue by investigating inflammation and nociception in ET_B receptor knockout mice (Hosoda et al., 1994). The results suggest a significant role for the ET_B receptor in mediating inflammation and inflammatory pain in the mouse.

ABBREVIATIONS: ET, endothelin; ET_A; endothelin A receptor; ET_B, endothelin B receptor; IL, interleukin-8; PBQ, phenylbenzoquinone; AA, arachidonic acid; PCR, polymerase chain reaction; MPO, myeloperoxidase.

Materials and Methods

Compounds and Reagents. Arachidonic acid (AA) was obtained from Sigma Chemical Co (St. Louis, MO); PBQ from Eastman Kodak Co. (Rochester, NY); and IRL-1620, sarafatoxin S6c, and ET-1 from American Peptide (Sunnyvale, CA). ^{125}I-ET-1 (specific activity, 2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA). A192621 [(+/−)-*trans*, *trans*-2-(4-*n*-propoxyphenyl)-4-(1,3-benzodioxol-5-yl)-1-[(2,6-dienthylphenyl) aminocarbonylmethyl]pyrrolidine-3-carboxylic acid and SB 234551 (E)-α-[[1-butyl-5-[2-[(2-carboxyphenyl)methoxy]-4-methoxyphenyl]-1H-pyrazol-4-yl]methylene]-6-methyoxy-1,3-benzodioxole-5-propanoic were synthesized in the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals (King of Prussia, PA).

Animals. BALB/c mice (18–20 g) were obtained from Charles River Breeding Laboratories (Raleigh, NC). ET_B receptor knockout mice were obtained from an in-house colony originally derived from heterozygous *129/Sv-Ednrb*^{tm1Ywa} breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME) and initially described by Hosada et al. (1994) and Puffenberger et al. (1994). All animals were housed in a barrier facility and fed water and pellet food ad libitum. Wild-type ET_B receptor knockout mice (+/+) and heterozygotes (+/−) appeared healthy and of normal lifespan and were used in a weight range of 18 to 20 g except for a study comparing weanlings (+/+) with adults, in which both sets of animals were 6 to 14 g. The (−/−) mice were used at 6 to 14 g and had a healthy appearance at the time of use. The (−/−) mice, however, became ill with megacolon within several days after weaning. All procedures used were approved by the Animal Care and Use Committee and met or exceeded standards of the American Association for the Accreditation of Laboratory Animal Care, the United States Department of Health and Human services, and all local and federal animal welfare laws. All of the experiments described were terminal.

Genotyping and Characterization of ET_B Receptor Knockout Mice. Individual genotype analysis was performed by polymerase chain reaction (PCR) using genomic DNA (0.2 μg) isolated from tail snip samples, as described by Moore (1994). Amplification was performed in 50-μl aliquots (50 mM KCl, 10 mM Tris-HCl, 0.001% gelatin, 17 mM $MgCl_2$, 200 nM diethylnitrophenyl thiophosphate, 10% dimethylsulfoxide, 1.25 U *Taq* DNA polymerase, pH 8.3; Perkin Elmer, Norwalk, CT) using neomycin-(280 base pair (bp) amplicon; 0.75 μM 5′-CTT GGG TGG AGA GGC TAT TC-3′) and 5′-AGG TGA GAT GAC AGG AGA TC-3′) and ET_B receptor-(400 bp amplicon; 0.15 μM 5′-TTG CTC GCA GAG GAC TGG CCA-3′ and 5′-AAG CAT GCA GAC CCT TAG GGG C-3′) specific primers. Amplification used a "two-step" protocol consisting of primary high-stringency amplification (11 cycles; annealing: 64°C, 45 s; extension: 72°C, 45 s; denaturation: 95°C, 35 s), followed by secondary low-stringency PCR (21 cycles; annealing: 58°C). Amplification of a single species of PCR product of 280 bp (neomycin insert) or 400 bp (ET_B receptor) corresponded to a (−/−) or (+/+) genotype. Samples that generated a (280/400 bp) doublet corresponded to a (+/−) genotype. Amplification fidelity was confirmed by subcloning (pCR2.1 TA vector; Invitrogen, San Diego, CA) and sequencing selected PCR products and by comparison with coat color phenotype.

In Vitro Characterization of A19261 and SB 234511. Male BALB/c mice, 18 to 20 g (Charles River), were killed and exsanguinated. First- and second-generation pulmonary artery and trachea of each animal were removed and cleaned of adherent tissue. Two rings (~1 mm in diameter, 3–4 mm in length) were cut from the second-generation pulmonary artery and two rings (~2 mm diameter, 4 cartilage rings in length) were cut from the trachea. The endothelium of the pulmonary artery and the epithelium of the trachea were left intact. Tissues were put into modified Krebs–Henseleit solution (composition of the solution was 113.0 mM NaCl , 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, and 11.0 dextrose), which was gassed with 95% O_2/5% CO_2 and maintained at 37°C. Experiments were run in the presence of 1 μM meclofenamic acid. Individual tissues were suspended via stainless steel hooks and silk suture in 10-ml water-jacketed organ baths containing Krebs–Henseleit solution and connected to Grass FTO3C force-displacement transducers. Mechanical responses were recorded isometrically by MP100WS/Acknowledge data acquisition system (BIOPAC Systems, Santa Barbara, CA) run on Macintosh computers. The tissues were equilibrated under a resting tension of 0.35 g and washed with Krebs–Henseleit solution every 15 min for 1 h. After the equilibration period, pulmonary tissues were contracted with 10 μM phenylephrine and trachea with 10 μM carbachol until reaching plateau. Tissues then were rinsed every 15 min over 1 h until reaching baseline tone. The preparations then were left for at least 30 min before the start of the experiment.

ET-1 and S6c concentration-response curves were obtained by a cumulative addition of the agonist in half-log increments. Each concentration was left in contact with the preparation until the response plateaued before the addition of the subsequent agonist concentration. At the end of the experiment, tissues were exposed again to 10 μM phenylephrine or 10 μM carbachol, which served as a reference contraction for data analysis. Paired tissues were exposed to SB 234551 or A192621 (10 or 100 nM) or vehicle for 30 min before ET-1 cumulative concentration–response curves were generated.

In Vivo Characterization

PBQ-Induced Hyperalgesia. Mice were given PBQ (2 mg/kg, i.p., at a dose volume of 0.01 ml/g) and placed in 4-liter beakers for observation. After a 5-min pretreatment time, the number of abdominal constrictions were recorded over a 10-min period. In studies examining the effects of antagonists, SB 234551 or A192621 or vehicle was administered p.o. 15 min before challenge with PBQ.

"Hotplate" Response. A hotplate method for determination of thermal pain threshold was modified from the procedure described previously by Rubat and coworkers (1997). Each mouse was placed into a 4-liter beaker maintained at 54°C in a water bath and timed for their response, which included hopping and/or paw-licking.

AA-Induced Pruritus. A topical dose of AA (2.0 mg/20 μl in cold acetone) was administered to the left ear of the mouse. Each mouse was placed in a 4-liter beaker for observation. After a 2-min pretreatment time, the number of ear rubs and head shakes were recorded over 10 min.

Cutaneous Inflammation Induced by AA. Mice were administered AA (2.0 mg of in 20 μl of cold acetone) to the left ear, and the difference in ear thickness of the left ear versus right ear was taken with an ear thickness gauge 1 h after AA administration. Mice then were killed using carbon dioxide and the left ears harvested for myeloperoxidase (MPO) analysis as described previously (Bradley et al., 1982). Results were recorded in cm $\times$ 10^{-3} and were analyzed using P57 software and linear regression.

Histology. Normal and AA-exposed ear pinnae of (+/+), (+/−), and (−/−) mice were fixed in 10% phosphate-buffered formaldehyde (formalin), and the samples processed to paraffin blocks. Five micrometer-thick sections, cut from the base to apex of the pinnae, were stained with Harris's hematoxylin and eosin Y (Sigma Chemical Co.) and examined by light microscopy. Digital images were captured with a Sony DKC-5000 digital photo camera (Sony Corp., Tokyo, Japan) using Image-Pro Plus analysis (Media Cybernetics, Silver Spring, MD).

Statistical Analysis. Where appropriate, results are expressed as the mean ± S.E. Statistical evaluation was conducted using Student's *t* test or ANOVA where appropriate, with a probability value, $p < .05$, considered statistically significant.

Results

Characterization of Knockout Mice. Three different genotypes of mice derived originally from the ET_B receptor

gene targeted disruption mice described by Hosada et al. (1994) were used for these studies.

Genotyping of Mice by PCR and Southern Blot Analysis. PCR amplification of genomic DNA samples resulted in the generation of a single reaction product of either 400 bp (wild-type ET_B receptor) or a 280 bp (neomycin insert) corresponded to wild-type (+/+) and homozygous (−/−) knockout genotypes, respectively (Fig. 1). In contrast, samples that resulted in the simultaneous amplification of a 280 bp/400 bp doublet genotyped as heterozygous (+/−) mice. These results were consistent with animal phenotype, i.e., agouti (+/+) and (+/−) genotypes or piebald (−/−) genotype, coat color.

In addition, radioligand binding studies supported the genotyping results in that membrane preparations from brain tissue of (+/+), (+/−), and (−/−) indicated that ET_B receptor-specific ligands (sarafatoxin S6c, IRL-1620, and A192621) displaced ^{125}I-ET-1 in (+/+)- and (+/−)-derived tissue but not in the (−/−)-derived membranes (data not shown).

Pharmacology of A192621 and SB 234511. To confirm that A192621 and SB 234511 antagonized ET_B receptor- and ET_A receptor-mediated responses, respectively, in mouse tissues, the effects of the compounds against ET-1-induced response in mouse pulmonary artery (ET_A receptor-mediated) and sarafotoxin S6c-induced response in mouse trachea (ET_B receptor-mediated) were investigated. SB 234551 (10 nM) potently inhibited ET-1-induced responses in mouse pulmonary artery with a pK_B of 8.4, whereas A192621 (100 nM) was without effect. SB 234551 (100 nM) was without effect on sarafotoxin S6c-induced responses in mouse trachea. In contrast, A192621 (100 nM) produced a marked shift to the right in sarafotoxin S6c concentration–response curves with a pK_B of 8.3 (data not shown).

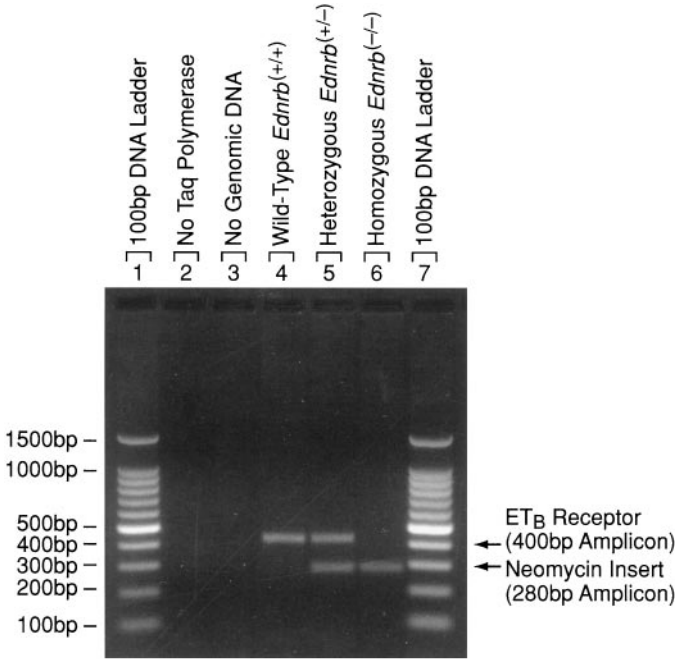

Fig. 1. Genotyping of ET_B knockout mice by PCR. After PCR amplification, samples were size-fractionated in an ethidium bromide stained 2% agarose gel (DNA ladder is shown in lanes 1 and 7). Amplification of either a single 400 bp (wild-type ET_B receptor) or a 280 bp (neomycin insert) PCR product corresponded to a wild-type ET_B (+/+) (lane 4) or homozygous ET_B (−/−) knockout (lane 6) genotype, respectively. Simultaneous amplification of a 280 bp/400 bp doublet corresponded to a heterozygous ET_B (+/−) genotype (lane 5). Specificity of amplification was supported by sequence analysis and by the observation that under identical conditions, no PCR products were generated if either *Taq* DNA polymerase (lane 2) or genomic DNA template (lane 3) was omitted from the amplification reaction.

PBQ-Induced Hyperalgesia. As seen in Fig. 2, the algesic response to PBQ (2 mg/kg i.p.) was clearly quantifiable in the wild-type controls, markedly reduced (~80%) in the (+/−) mice, and absent in the (−/−) mice; the responses among all groups were statistically significantly different from each other, as demonstrated by $p < .05$.

Because of the need to use the (−/−) mice as weanlings, because of their short life-expectancy, a comparison was made between the response to PBQ of (+/+) pups with that of the adult (+/+) mice. The number of PBQ-induced abdominal constrictions in the (+/+) weanling mice (16.4 ± 1.7; $n = 5$) was not different from that of the adult (+/+) mice (17.2 ± 3.5, $n = 5$; $p > .05$). These results indicate that there are no age-related differences in the response to PBQ in (+/+) mice.

Noninflammatory Pain. The profound deficit in the PBQ-induced response in the ET_B receptor gene-targeted knockouts led to investigation of the possibility that nociceptors and nerves carrying the pain signals might be developmentally and functionally immature. This was conducted by evaluating the pain threshold using a hotplate methodology. As seen in Table 1, the threshold response of the (−/−) mice was not statistically different from that of the wild-type (+/+) control animals.

Pharmacology of PBQ Response. The role of the ET_B receptor in inflammatory pain in wild-type (+/+) mice also was explored pharmacologically using A192621. A192621 (25 mg/kg p.o.; 15 min pretreatment) strongly inhibited (74%) the response to PBQ (2 mg/kg i.p.), compared with vehicle-treated mice. In contrast, SB 234551 (25 mg/kg p.o.; 15 min pretreatment) was without significant effect on the PBQ-induced response (Fig. 3).

Cutaneous Inflammatory Response to AA. To explore further the inflammatory deficit in ET_B receptor gene-targeted knockout mice, the pruritic and inflammatory responses to topical application of AA were investigated. The

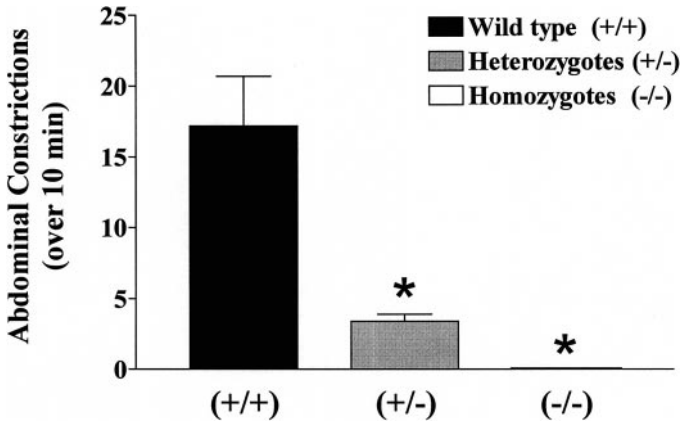

Fig. 2. The effect of PBQ in wild-type (+/+), heterozygous (−/+) and homozygous (−/−) ET_B receptor knockout mice. Mice (n = 5–6/group) were administered PBQ (2 mg/kg, i.p.) at a dose volume of 0.01 ml/g. Five minutes after injection of PBQ, the number of abdominal constrictions were counted over 10 min. Results are presented as the mean ± S.E. *, indicates statistically significant versus the (+/+) mice at a $p < .001$. All groups are statistically significantly different from each other by ANOVA ($p < .05$).

pruritic response to topical AA (2 mg/ear) was similar in the wild-type (+/+) and homozygous (−/−) mice with equivalent episodes of scratching and rubbing (Table 2).

In contrast to the pruritic component, the inflammatory response to AA was clearly attenuated in ET_B receptor gene-targeted knockout mice (Table 3). Thus, both the fluid and cellular phases of the inflammatory response were reduced in (+/−) mice (37 and 51%, respectively) and (−/−) mice (~65% inhibition of both endpoints), compared with wild-type animals. This inflammatory deficit also was demonstrable histologically (Fig. 4). The wild-type controls had appreciable tissue swelling and neutrophil infiltration of the dermis (Fig. 4A), the (+/−) mice had reduced swelling and modest inflammatory cell infiltration (Fig. 4B). In the case of the (−/−) mice, tissue swelling was markedly reduced and neutrophils were seen only occasionally and then only in the intravascular compartment (Fig. 4C).

Topical A192621 (500 μg/ear) significantly reduced tissue swelling induced by AA in wild-type mice (39%, $n = 5$; $p < .001$); there was no significant effect on AA-induced neutrophil infiltration (16.0% inhibition, $n = 5$; $p > .05$ (data not shown). Application of SB 234551 (500 μg/ear) did not alter significantly either tissue swelling or inflammatory cell infiltration elicited by AA (data not shown).

Discussion

The results of the present study strongly implicate the ET_B receptor as playing a role in inflammatory pain and cutaneous inflammation in the mouse. The major findings in support of this conclusion are:

1. PBQ-induced algesia was reduced markedly (80%) in heterozygous (+/−) ET_B receptor knockout mice and absent in homozygous (−/−) animals, compared with wild-type (+/+) controls.
2. The selective ET_B receptor antagonist A192621 inhibited PBQ-induced algesia, whereas SB 234551, a selective ET_A receptor antagonist, was without effect.
3. In heterozygous (+/−) and homozygous (−/−) mice, there was significant inhibition of topical AA-induced cutaneous inflammation and neutrophil infiltration, compared with wild-type animals.
4. A192621, but not SB 234551, inhibited topical AA-induced inflammatory responses.

The findings of Raffa and coworkers (1996a,b) have suggested that PBQ-induced writhing may involve, at least in part, ET-1 release. In addition, ET-1-induced writhing may be mediated by both ET_A and ET_B receptors. Involvement of the ET_A receptor was suggested by the results showing that ET_B receptor-selective ligands were less potent than were ET-1 in causing algesia. However, in the presence of the ET_A antagonist BQ-123, the ET_B receptor selective ligand ET-3 still induced algesia, suggesting a role for the ET_B receptor. In the current study, alternative approaches were used to explore the relative roles of ET_A and ET_B receptors, namely, gene-targeted knockout mice and ET_B receptor-selective antagonism.

A PCR genotyping unequivocally identified all mice. It was clear that the (+/+) mice had only the ET_B receptor amplicon, the (+/−) mice had both the ET_B receptor and the neomycin insert amplicons, and the (−/−) mice had only the neomycin insert amplicon. These results are consistent with the conclusion that the (+/+) mice have two alleles encoding for ET_B receptors, the (+/−) only one allele, and the (−/−) mice have no alleles encoding for ET_B receptors.

Radioligand-binding studies with ET-1 (which interacts with both ET_A and ET_B receptors) using brain tissue from (+/+), (+/−), and (−/−) also support the conclusions from genotyping. Use of the ET_B receptor selective agonists sarafatoxin S6c and IRL-1620 as well as of the ET_B receptor selective antagonist A192621 clearly indicated a lack of ^{125}I-ET-1 competitive binding (presumably to the loss of the ET_B receptor binding component) in the (−/−) mice, compared with the (+/+) mice. Recent results indicate that the relative ET receptor density in trachea from (+/+), (+/−), and (−/−) mice were 100:84:59, and that the ET-1 binding in the (−/−) mice was exclusively through the ET_A receptor (R. Goldie, personal communication). Thus, these data support the contention that the heterozygotes have reduced ET_B number and that the ET_B receptor is absent in the (−/−) mice.

Pharmacological assessment of the involvement of ET receptors in the responses measured in this study was conducted using A192621 and SB 234511 as selective antagonists of the ET_B and ET_A receptors, respectively. Although A192621 and SB 234511 have been suggested to be potent and selective antagonists for ET_B and ET_A receptors (Douglas, 1997; Ohlstein et al., 1998), to our knowledge there is no published information on their binding and functional properties against mouse ET receptors. It was determined that A192621 and SB 234511 potently antagonized ET_B receptor-mediated and ET_A receptor-mediated responses, respec-

TABLE 1

Response of wild-type (+/+) and homozygous (−/−) ET_B receptor knockout mice to thermal hyperalgesia

Mice were placed into a beaker held at a constant temperature of 54°C. The results are given as the mean ± S.E.

Genotype	Threshold Response
	sec
Wild type	13.5 ± 2.7 ($n = 6$)
Homozygous	10.6 ± 0.7 ($n = 8$)

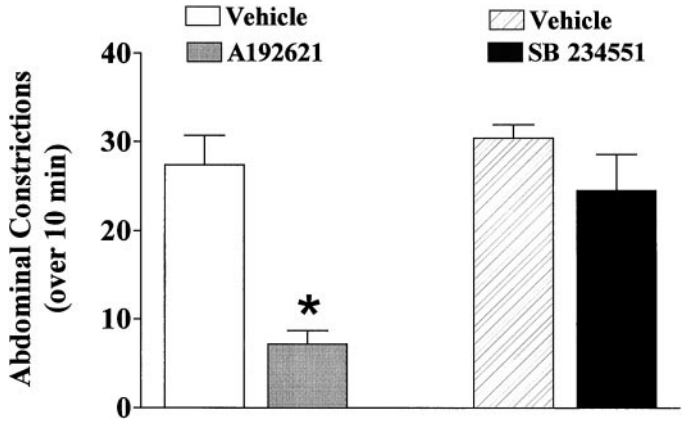

Fig. 3. Effect of A, A192621 (ET_B receptor-selective antagonist) and B, SB 234551 (ET_A receptor-selective antagonist) on PBQ-induced hyperalgesia in wild-type (+/+) control mice. Wild-type mice ($n = 4$–5/group) were administered test compound 15 min before injection with PBQ; the abdominal constrictions were counted over a 10 min period immediately after administration of PBQ. The results are presented as the mean ± S.E. *, indicates statistically significant versus the vehicle-treated mice at a $p < .001$.

tively, in mouse pulmonary tissues, inhibiting responses induced by sarafotoxin S6c in trachea (ET_B receptor-induced) and ET-1 in pulmonary artery (ET_A receptor-induced). These data support the use of A192621 and SB 234551 as appropriate tool compounds in this species and suggest that any effects observed with these molecules in mice in vivo can be attributed to antagonism of ET_B and ET_A receptors, respectively.

The responsiveness of gene-targeted knockout mice was examined in different models. The PBQ test is a classical model of inflammatory pain, which has been used widely to study the analgesic properties of nonsteroidal anti-inflammatory drugs (Griswold et al., 1991). It was found that the (−/−) mice were unresponsive to PBQ, suggesting that the ET_B receptor is essential for this response. In addition, the ability of A192621, but not of SB 234511, to inhibit PBQ-induced algesia provides additional evidence that the ET_B receptor is involved exclusively in the inflammatory pain induced by this particular stimulus, with no participation of ET_A receptor activation. However, these results do not rule out a role for the ET_A receptor in other nociceptive processes and pathways activated by other stimuli. The involvement of prostanoids, a widely recognized component of the PBQ response (Griswold et al., 1991) is unlikely to be important with respect to the ET-associated response since Raffa and coworkers (1996a) demonstrated that ET-1 induced writhing is primarily insensitive to nonsteroidal anti-inflammatory agents.

The possibility exists that the diminution in the PBQ response in nonwild-type mice is not the result of a reduction in the inflammation per se. Thus, although in the current study thermal pain was not reduced in the (+/−) or (−/−) mice, it is known that the pathways for mediation of this pain would likely be distinct from the visceral pain induced in the PBQ model. It is possible that distinct pain pathways may be altered differentially in the knockout mice, which could be manifest as differences in responsiveness to different stimuli.

It was of interest that the inflammatory deficits observed in ET_B receptor gene knockout extended to the skin where the response to AA was clearly reduced. Expression of ET-1- and ET-binding sites in skin predominantly occur in the dermis and are associated with the microvasculature (Bull et al., 1991). Inhibition of the inflammatory response was supported by the histological findings, in which the (+/−) and (−/−) animals had reduced neutrophil infiltration and tissue swelling. The AA-induced inflammatory response is primarily driven by eicosanoids; more specifically, cysteinyl leukotrienes and leukotrienes B_4, with a minimal contribution from prostanoids (Carlson et al., 1985). Although some studies have indicated that ET-1 is not able to stimulate leukotriene production (Hay et al., 1993a,b), mouse bone marrow-derived mast cells produce leukotrienes C_4 on stimulation with ET-1 (Uchida et al., 1992); the latter effect appeared to be mediated through the ET_A receptor (Egger et al., 1995). ET-1 also has been demonstrated to enhance neutrophil endothelial cell adhesion (Hayasaki et al., 1996). It remains to be clarified whether ET_B receptors mediate ET-1-induced neutrophil activation and adhesion. As in the case of PBQ-induced inflammatory pain, A192621 reduced the cutaneous inflammatory response (edema) to AA, whereas SB 234551 was not effective. Although there was a trend toward an effect of A192621 on AA-induced neutrophil infiltration, a significant effect was not demonstrated. These results suggest that tissue swelling may represent the more sensitive endpoint to ET_B receptor antagonism, and higher doses of A192621 may be required to see marked inhibition of neutrophil infiltration. Furthermore, the possibility exists that both ET_A and ET_B receptors may be involved in neutrophil-induced influx, and also activation, and that antagonism of

TABLE 2

AA-induced pruritus in wild-type (+/+) and homozygous (−/−) ET_B receptor knockout mice.

AA (2.0 mg/20 μl) in cold acetone was applied to the left ear. The mice were then placed in 4-liter beakers. After 2 min, the scratching, ear rubs, and head-shaking events were counted for 10 min. The results are given as the mean ± S.E.

Genotype	Response (scratching and head rub)
Wild type	34.0 ± 1.1 (n = 3)
Homozygous	37.5 ± 5.0 (n = 7)

TABLE 3

Inflammatory response to AA in wild-type (+/+) heterozygous (+/−) and homozygous (−/−) ET_B receptor knockout mice.

Mice were administered 2 mg/ear of AA to the left ear. The difference in ear thickness of the left versus right ear was measured with an ear thickness gauge 1 h after exposure to AA. The left ear was then taken for MPO analysis.

Genotype	Inflammatory Response	
	Ear swelling	MPO
	cm^{-3}	*OD units*
Wild type	21.6 ± 1.1 (n = 12)	0.136 ± 0.025 (n = 5)
Heterozygous	13.6 ± 0.7** (n = 10)	0.067 ± 0.004* (n = 5)
Homozygous	7.6 ± 0.9** (n = 14)	0.048 ± 0.010* (n = 5)

*, statistically significant at $p < 0.01$ compared with wild type. All groups are statistically significantly different from each other using ANOVA ($p < 0.05$). **, statistically significant at $p < 0.001$ compared with wild type.

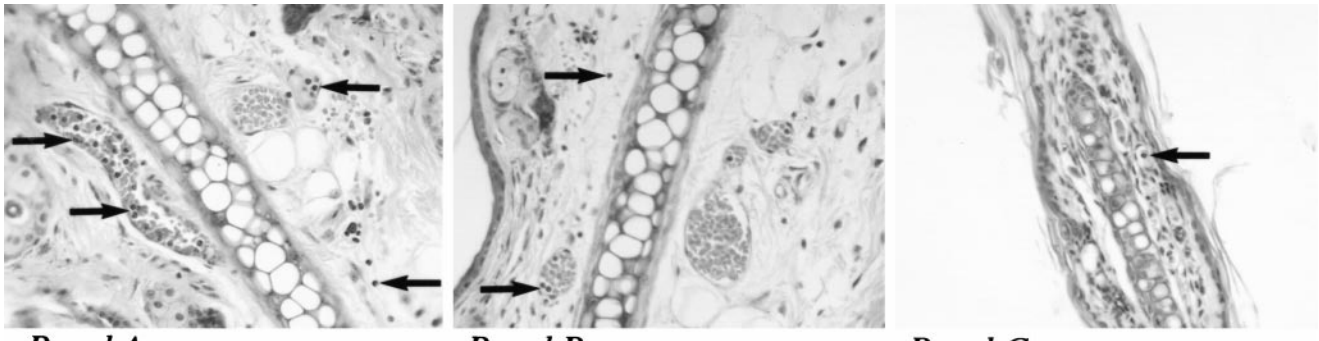

Fig. 4. Photomicrographs of AA-treated (+/+), (+/−), and (−/−) mouse pinnae. The tissue was taken 1 h after the application of AA (2 mg/ear). Histograph of ear pinnae from A, (+/+); B, (+/−); and C, (−/−) mice taken 1 h after treatment with AA. Arrow indicates the presence of neutrophils. Histographs were taken at 400× magnification, under immersion oil.

both receptor subtypes is required for a significant effect on this parameter to be observed. A requirement for antagonism of both receptor populations to produce significant inhibition of ET-1-induced responses has been demonstrated in human bronchus (Fukuroda et al., 1996).

In summary, the current results suggest that ET_B receptors are involved in inflammatory processes and cutaneous inflammation in the mouse. Thus, inflammatory pain, tissue swelling, and inflammatory cell infiltration all appear to involve ET_B receptor activation. These data raise the possibility that strategies to interrupt this pathway, including ET_B receptor antagonists, may provide novel therapeutics for the treatment of diseases involving inflammation and inflammatory pain. However, it will be important to confirm these results in mice in other species, particularly, in humans.

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