Tumor-Evoked Sensitization of C Nociceptors: A Role for Endothelin

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Hamamoto DT, Khasabov SG, Cain DM, Simone DA. Tumor-evoked sensitization of C nociceptors: a role for endothelin. J Neurophysiol 100: 2300–2311, 2008. First published August 6, 2008; doi:10.1152/jn.01337.2007. Primary and metastatic cancers that effect bone are frequently associated with pain. Sensitization of primary afferent C nociceptors innervating tissue near the tumor likely contributes to the chronic pain and hyperalgesia accompanying this condition. This study focused on the role of the endogenous peptide endothelin-1 (ET-1) as a potential peripheral algogen implicated in the process of cancer pain. Electrophysiological response properties, including ongoing activity and responses evoked by heat stimuli, of C nociceptors were recorded in vivo from the tibial nerve in anesthetized control mice and mice exhibiting mechanical hyperalgesia following implantation of fibrosarcoma cells into and around the calcaneous bone. ET-1 (100 μM) injected into the receptive fields of C nociceptors innervating the plantar surface of the hind paw evoked an increase in ongoing activity in both control and tumor-bearing mice. Moreover, the selective ET<sub>A</sub> receptor antagonist, BQ-123 (3 mM), attenuated tumor-evoked ongoing activity in tumor-bearing mice. Whereas ET-1 produced sensitization of C nociceptors to heat stimuli in control mice, C nociceptors in tumor-bearing mice were sensitized to heat, and their responses were not further increased by ET-1. Importantly, administration of BQ-123 attenuated tumor-evoked sensitization of C nociceptors to heat. We conclude that ET-1 at the tumor site contributes to tumor-evoked excitation and sensitization of C nociceptors through an ET<sub>A</sub> receptor mediated mechanism.

INTRODUCTION

Primary bone cancers and cancers that metastasize to bone often cause severe pain in humans (Mercadante 1997; Portenoy et al. 1999). Although the mechanisms that contribute to pain associated with cancer in bone are not well understood, significant progress has been made following the development of rodent models of cancer pain (Medhurst et al. 2002; Menendez et al. 2003a; Schwei et al. 1999; Wacnik et al. 2003).

We used a murine model of cancer pain in which fibrosarcoma cells were implanted into and around the calcaneous bone (Wacnik et al. 2003). This model permits direct quantification of tumor-related changes in response properties and morphology of primary afferent fibers (Cain et al. 2001; Gilchrist et al. 2005). Tumor growth in this model concurrently evokes mechanical and thermal hyperalgesia (Hamamoto et al. 2007; Khasabov et al. 2007; Wacnik et al. 2001), sensitization of nociceptive dorsal horn neurons (Khasabov et al. 2007), sensitization of C nociceptors that innervate skin overlying the tumor (Cain et al. 2001), and development of peripheral neuropathy as indicated by decreased innervation of the epidermis (Cain et al. 2001; Gilchrist et al. 2005).

Studies using rodent models of cancer pain have revealed a number of mediators that contribute to tumor-evoked nociceptive behaviors and hyperalgesia (Peters et al. 2004; Sevcik et al. 2005a,b; Wacnik et al. 2001). One such mediator is endothelin-1 (ET-1), a 21 amino acid peptide that was first isolated from endothelial cells and defined functionally as a vasoconstrictor (Yanagisawa et al. 1988). ET-1 is a member of the endothelin family, which also includes ET-2, ET-3, and the sarafotoxins, and is synthesized by a variety of cells including neurons and glial cells in the central and peripheral nervous systems (Giaid et al. 1989; MacCumber et al. 1990), macrophages (Ehrenreich et al. 1990), endothelial cells (Yanagisawa et al. 1988), and keratinocytes (Yohn et al. 1993). Members of the endothelin family bind to two distinct G-protein-coupled receptors, ET<sub>A</sub> and ET<sub>B</sub> (Vane 1990). The ET<sub>A</sub> receptor preferentially binds ET-1 whereas the ET<sub>B</sub> has similar affinities for ET-1, ET-2, and ET-3 (Arai et al. 1990; Sakurai et al. 1990).

ET-1 is expressed in several human cancer types, including prostate, lung, breast, and colorectal, that have a high incidence of metastasis to bone (Kusuhara et al. 1990; Nelson 2005; Nelson and Carducci 2000). Increased expression of the ET<sub>A</sub> receptor has been associated with progression of some cancers in humans (Gohji et al. 2001), and ET-1 stimulates proliferation of some cancer cell lines (Shichiri et al. 1991) through a ET<sub>A</sub> receptor-mediated mechanism (Nelson et al. 1996). Furthermore, ET-1 is involved in tumor mitogenesis, apoptosis, invasion, and metastasis (Grant et al. 2003). Thus ET-1 appears to play a major role in the tumorgenesis of some cancers.

In addition to a potential role in tumorgenesis, ET-1 may also serve as an algogen to produce nociception. Numerous studies have demonstrated that injection of ET-1 into the knee or hind paw evokes nocifensive behaviors (Baamonde et al. 2004; Balo et al. 2000; Sevcik et al. 2005a,b; Wacnik et al. 2001). Injection of ET-1 into the knee or hind paw evokes nocifensive behaviors (Baamonde et al. 2004; Balo et al. 2000; Sevcik et al. 2005a,b; Wacnik et al. 2001). Injection of ET-1 into the skin excites C nociceptors (Gokin et al. 2001; McKelvy et al. 2007; Menendez et al. 2003b; Piovezan et al. 1998, 2004; Verri et al. 2004, 2005), tactile allodynia (Balenov et al. 2006; McKelvy et al. 2007), and mechanical (da Cunha et al. 2004; Ferreira et al. 1989) and heat (Menendez et al. 2003b) hyperalgesia through a mechanism involving the ET<sub>A</sub> receptors located in peripheral tissues (Baamonde et al. 2004; Dabar et al. 1998; De-Melo et al. 1998a; Fareed et al. 2000; Gokin et al. 2001; Menendez et al. 2003b; Piovezan et al. 1998, 2004). Injection of ET-1 into the skin excites C nociceptors (Gokin et al. 2001; Khodorova et al. 2002; Namer et al. 2007) and likely contributes to ET-1-evoked nocifensive behaviors.

Because administration of ET-1 into peripheral tissues produces nocifensive behaviors in animals and pain in humans...
ET-1 in tumor-evoked sensitization of C nociceptors

Ongoing activity of nociceptors may contribute to spontaneous pain (Djouhri et al. 2006). Therefore persistent release of ET-1 from tumor cells could produce ongoing excitation of C nociceptors and contribute to spontaneous pain in cancer patients. Indeed we have shown that C nociceptors in mice with tumor-evoked hyperalgesia exhibited ongoing activity and sensitization to heat (Cain et al. 2001). Therefore the aim of this study was to examine the role of ET-1 in sensitization of C nociceptors in mice with tumor-evoked hyperalgesia.

Methods

Subjects

A total of 76 adult, male C3H mice (36 control and 40 tumor-bearing mice) weighing 20–30 g obtained from the National Institutes of Health were used in the current study. Animals were housed on a 12-h light/dark schedule and given ad libitum access to food and water. All procedures were approved by the Animal Care Committee of the University of Minnesota. Experiments were conducted according to the guidelines set forth by the International Association for the Study of Pain (Zimmermann 1983).

Implantation of fibrosarcoma cells

NCTC clone 2472 fibrosarcoma cells were obtained from American Type Cell Culture (Manassas, VA) and maintained as described previously (Cain et al. 2001). These cells were initially derived from a spontaneous connective tissue tumor in C3H mice. Briefly, fibrosarcoma cells were grown to confluence in 75 cm² flasks in NCTC 135 medium (Sigma, St Louis, MO) with 10% horse serum at pH 7.35 and passaged weekly (1:4–6 split ratio). Fibrosarcoma cells were trypsinized to create a cell suspension then counted with a hemacytometer, pelleted, and resuspended in phosphate-buffered saline (PBS) for implantation in a concentration of 2 × 10⁴ cell/μl.

Mice were anesthetized with 1–2% halothane then fibrosarcoma cells (2 × 10⁴ cell in 10 μl) were injected unilaterally into and around the calcaneus bone using a 0.3-ml insulin syringe with a 29.5-gauge needle as described previously (Cain et al. 2001). None of the mice showed signs of permanent motor dysfunction after implantation. Mice without implantation of fibrosarcoma cells served as the control group because a previous study has shown that a sham implantation of PBS into and around the calcaneus bone did not produce significant hyperalgesia (Wacnik et al. 2001).

Behavioral measures of nociception

Mice were placed on a wire mesh platform, covered with a hand-sized container, and allowed to acclimate to their surroundings for a minimum of 30 min before testing. A von Frey filament (bending force of 3.4 mN) was applied 10 times for 1–2 s each time at 5-s intervals to random locations of the plantar surface of the hind paw. Vigorous paw withdrawals were counted, and a withdrawal response frequency was calculated for each hind paw. Withdrawal response frequencies were obtained for 3 days preceding implantation of fibrosarcoma cells and on every second day thereafter until day 16. Only tumor-bearing mice exhibiting a paw withdrawal frequency of ≥70% for two consecutive days were used in the electrophysiological experiments. Approximately 90% of mice implanted with fibrosarcoma cells exhibited sufficient mechanical hyperalgesia and were used in the electrophysiological studies. Electrophysiology studies of C nociceptors in tumor-bearing mice were performed 9–16 days after implantation as mechanical hyperalgesia is maximal during this period (Hamamoto et al. 2007; Wacnik et al. 2001).

Electrophysiological recording from primary afferent fibers

The method of recording electrophysiological responses of single primary afferent fibers from the tibia nerve has been described previously (Cain et al. 2001). Mice were anesthetized with acepromazine maleate (20 mg/kg ip) and sodium pentobarbital (Nembutal, 48 mg/kg ip). Supplemental doses of sodium pentobarbital (15 mg/kg) were given as needed to maintain areflexia. Hair was removed from one hind limb, and an incision was made through the skin of the dorsal aspect of the lower leg. The gastrocnemius muscle was surgically removed to expose the tibial nerve. The skin was sutured to a metal ring (1.3 cm ID) to form a basin, a rubber-based polysulfide impression material (Co-Flex, Sullivan Schein, Melville, NY) was applied to the skin around the ring to prevent leakage, and the basin was filled with warm mineral oil. The tibial nerve was dissected from connective tissue and placed on a small, mirrored platform. The epineurium of the nerve was opened using a microtome scalpel, and small fascicles were cut to allow the proximal ends to be spread out on the platform for separation with fine jeweler’s forceps. Nerve fascicles were teased apart, and fine filaments were placed on a silver-wire recording electrode to obtain an extracellular recording from a single fiber that could be easily discriminated according to amplitude. Action potentials were amplified, audio-monitored, displayed on an oscilloscope, discriminated and stored in a PC computer for off-line data analysis using a customized program (LabVIEW, version 5.1, National Instruments, Austin, TX).

Electrophysiological characteristics and response properties of primary afferent fibers

Identification of primary afferent fibers. Only fibers in control and cancer mice that had mechanical receptive fields (RFs) on the plantar surface of the hind paw were studied. Once a fiber was isolated, the location of its RF was identified using a small glass probe (1 mm diam tip), and the location was then marked on the skin with a felt-tip pen. The mechanical threshold of the fiber was obtained by using a series of von Frey filaments with increasing bending forces and was expressed as the minimum force needed to evoke a response in ≥50% of the trials. To ensure that recordings were obtained exclusively from fibers innervating the skin rather than from fibers innervating deep tissues, the skin surrounding the RF was gently grasped with dull forceps and lifted. Only fibers that discharged primarily while the skin containing the RF was lifted and lightly squeezed were considered to be cutaneous units. In addition, toes and
joints were manipulated to identify proprioceptive units, which were not studied further.

CONDUCTION VELOCITY. Once the location of the RF on the plantar surface of the hind paw was identified, two fine needle electrodes (30 gauge) were inserted into the skin on opposite sides adjacent to the RF. Square-wave pulses (duration: 0.2 ms, 0.5 Hz) were delivered at a stimulating intensity of 1.5 times the minimal voltage required to evoke an action potential. The conduction latency of action potentials and distance between the RF and the recording electrode were determined for calculation of conduction velocity (CV). Primary afferent fibers were classified as Aβ (CV > 13.6 m/s), Aδ (13.6 ≥ CV ≥ 1.3 m/s) or C (CV < 1.3 m/s) fibers (Cain et al. 2001).

THERMAL STIMULATION. Heat stimuli were delivered by a Peltier-type thermode controlled by the LabVIEW software program. Stimuli (35 to 51°C) were applied to the plantar surface of the paw for a duration of 5 s. Stimuli were delivered in ascending steps of 2°C from a base temperature of 32°C with a ramp rate of 20°C/s and an duration of 5 s. Stimuli were obtained. ET-1, BQ-123, or vehicle was injected into the RF of a nociceptor, vehicle was injected first and ET-1 (or ET-1 (Phoenix Pharmaceuticals, Mountain View, CA) and an ET receptor selective antagonist, BQ-123 (American Peptide, Sunnyvale, CA), were dissolved in PBS at a concentration of 100 μM and 3 mM, respectively. ET-1, BQ-123, or vehicle (10 μl PBS) was injected subcutaneously into the RF using a 0.3-ml syringe with 30-gauge needle.

For each C nociceptor, the baseline level of ongoing activity was recorded over a 2-min period, and then responses to the series of heat stimuli were obtained. ET-1, BQ-123, or vehicle was injected into the RF, and ongoing activity and responses to heat were again determined. Ongoing activity and responses to heat stimuli were determined at 5 and 20 min after injection of ET-1 and at 10 and 20 min after injection for BQ-123. Most nociceptors were exposed to only one injection. When both vehicle and ET-1 (or BQ-123) were injected into the RF of a nociceptor, vehicle was injected first and ET-1 (or BQ-123) was injected ≥60 min after injection of vehicle.

Data analyses

Conduction velocity (m/s) of single C nociceptors, mechanical response thresholds (mN), levels of ongoing activity (imp/s), heat-response thresholds (°C), and responses (number of impulses) to the series of heat stimuli are presented as means ± SE. For the responses to the heat stimuli, the level of ongoing activity over the 10 s prior to delivery of the heat stimulus was subtracted from the response. Conduction velocities, mechanical thresholds, intra-burst frequencies, burst durations, and intervals between bursts for C nociceptors were compared between control and tumor-bearing mice using t-tests. Levels of ongoing activity, heat-response thresholds, and responses to heat before (baseline) and after injection of vehicle, ET-1, or BQ-123 were compared by ANOVAs. Pairwise comparisons between groups were made using Newman-Kuels post hoc analyses or t-test with the Bonferroni correction for multiple comparisons. For all comparisons, P < 0.05 was considered statistically significant.

RESULTS

General characteristics of C nociceptors

Electrophysiological recordings were made from 46 C nociceptors in control mice (n = 36) and 58 C nociceptors in tumor-bearing mice (n = 40). The mean conduction velocity of C nociceptors in tumor-bearing mice (0.38 ± 0.02 m/s) was slightly slower than that in control mice (0.51 ± 0.03 m/s; P < 0.001). Only 2 of 46 (4%) C nociceptors in control mice exhibited ongoing activity, and the discharge frequencies for these two fibers were 1.5 and 1.6 imp/s. In contrast, 49 of 58 (84%) C nociceptors studied in tumor-bearing mice exhibited ongoing activity that ranged in discharge rates from 0.1 to 4.5 imp/s (1.0 ± 0.2 imp/s). Mean mechanical thresholds for C nociceptors were determined using von Frey filaments and did not differ between tumor-bearing (29.1 ± 3.6 mN) and control (25.7 ± 5.3 mN) mice.

Effect of ET-1 on ongoing activity of C nociceptors

Figure 1A shows an example of the electrophysiological responses of a typical C nociceptor in a control mouse. This C nociceptor did not exhibit ongoing activity either at baseline or 5 min after injection of vehicle (10 μl of PBS) into its RF. Sixty minutes later, 100 μM of ET-1 (in 10 μl) was injected into the RF. Five minutes after injection of ET-1, this C nociceptor exhibited ongoing activity.

The mean discharge rate of ongoing activity for C nociceptors (n = 35) recorded in 28 control mice was 0.1 ± 0.1 imp/s at baseline. Thirty C nociceptors (in 25 mice) were injected with vehicle (10 μl PBS) into their RFs. Five minutes after the injection, the level of ongoing activity was unchanged (0.1 ± 0.1 imp/s). Ten C nociceptors (in 10 mice) were injected with vehicle and then 60 min later received 100 μM of ET-1 (in 10 μl) into their RFs. Another five nociceptors (in 5 mice) only received 100 μM of ET-1. Because no differences were observed in responses to ET-1 between the C nociceptors that had been previously injected with vehicle and those that had not, the data were pooled. Injection of ET-1 rapidly evoked responses of C nociceptors. The mean discharge rate of ongoing activity of C nociceptors (n = 15) increased from 0.1 ± 0.1 to 1.6 ± 0.6 imp/s by 5 min after injection of ET-1 (Fig. 1C). This mean discharge rate was greater than that of C nociceptors at baseline and than the rate produced by injection of vehicle (P < 0.001).

Similar responses to ET-1 were observed in tumor-bearing mice (Fig. 1D). This C nociceptor exhibited ongoing activity at a discharge rate of 1.4 imp/s at baseline, and 5 min after injection of vehicle (10 μl of PBS) into its RF, the discharge rate was 1.3 imp/s. Sixty minutes later, 100 μM of ET-1 (in 10 μl) was injected into the RF, and 5 min after the injection the discharge rate increased to 4.2 imp/s. The response of this C nociceptor to ET-1 was one of the most robust responses of C nociceptors in tumor-bearing mice.

When considering all C nociceptors studied, the mean discharge rate of ongoing activity of C nociceptors (n = 58) in tumor-bearing mice (1.0 ± 0.2 imp/s) was higher than that of C nociceptors (n = 46) in control mice (0.1 ± 0.1 imp/s; P < 0.001). Nineteen C nociceptors in 19 tumor-bearing mice were examined for the effect of injection of ET-1 on ongoing activity. At baseline, these C nociceptors exhibited a mean discharge rate of 0.7 ± 0.2 imp/s (Fig. 1F). Injection of vehicle did not alter the mean discharge rate (0.9 ± 0.3 imp/s) of C nociceptors (n = 11). However, as in control mice, injection of ET-1 into the RF of C nociceptors (n = 11) increased the mean discharge rate to 1.9 ± 0.4 imp/s (P < 0.05). Three C nociceptors received both vehicle and ET-1, but their responses did not differ from C nociceptors that had received either vehicle or ET-1 alone. Thus the data were pooled. The mean
discharge rates of C nociceptors after injection of ET-1 were similar in control and tumor-bearing mice.

Previous studies have reported that injection of ET-1 into the receptive field of C nociceptors evoked impulses in bursting patterns (Gokin et al. 2001; Khordova et al. 2002). In control mice, neither of the two C nociceptors that exhibited ongoing activity displayed a bursting pattern. Of the 15 C nociceptors in control mice that received injection of ET-1 into their receptive fields, only 3 (20%) exhibited a bursting pattern to the ongoing activity after injection of ET-1. Mean intra-burst frequency was 2.6 \pm 1.5 imp/s (range: 1.1 \pm 0.3 to 5.6 \pm 1.3 imp/s) and bursting periods lasted 5.1 \pm 2.0 s (range: 3.1 \pm 0.2 to 7.1 \pm 2.2 s). Mean time between bursts was 8.8 \pm 2.5 s (range: 5.9 \pm 0.6 to 13.8 \pm 5.6 s).

Eight of 58 (14%) C-nociceptors in tumor-bearing mice exhibited ongoing activity with periods of bursting. Mean intra-burst frequency was 1.1 \pm 0.4 imp/s (range: 0.5 \pm 0.1 to 1.5 \pm 0.5 imp/s), and bursting periods lasted 11.5 \pm 3.1 s (range: 2.3 \pm 0.7 to 21.7 \pm 1.3 s). Mean time between bursts was 19.7 \pm 5.5 s (range: 3.8 \pm 0.1 to 38.2 \pm 11.1 s). There were no differences in the proportion of C nociceptors exhibiting bursting ongoing activity, mean intraburst frequencies, duration of bursting periods, or time between bursts between tumor-bearing mice and control mice following injection of ET-1 into the RFs.

The effect of the ET\textsubscript{A} receptor antagonist, BQ-123, on ongoing activity was examined for 15 spontaneously active C nociceptors in 13 tumor-bearing mice. At baseline, their mean discharge rate was 1.4 \pm 0.4 imp/s. Ten minutes after injection of BQ-123 (3 mM in 10 \mu l), mean discharge rate decreased to 0.6 \pm 0.2 imp/s (P < 0.01, Fig. 2). Ongoing activity did not change further at 20 min after injection of BQ-123.
shown). In contrast, ongoing activity did not change following injection of vehicle. Thus injection of an ET$_A$ receptor selective antagonist into the RF reduced but did not completely abolish ongoing activity of C nociceptors in tumor-bearing mice.

**Effect of ET-1 on responses of C nociceptors to heat**

In control mice (n = 32), 34 of 41 (83%) C nociceptors were excited by heat stimuli (35 to 51°C) at baseline with a mean heat-response threshold of 43.8 ± 0.7°C (Fig. 3). Injection of vehicle into the RFs of 18 C nociceptors in 14 control mice did not significantly change the heat-response thresholds (44.1 ± 0.8°C). In contrast, injection of ET-1 (100 µM) into the RFs of 19 C nociceptors in 16 control mice reduced their heat-response threshold to 39.1 ± 0.8°C (P < 0.001).

In 34 tumor-bearing mice, 24 of 34 (71%) C nociceptors were sensitive to heat at baseline, and their heat-response threshold (38.4 ± 0.8°C) was lower than that of C nociceptors in control mice (P < 0.001, Fig. 3). Injection of vehicle into the RFs of C nociceptors (n = 8) in eight tumor-bearing mice did not alter their heat-response threshold (38.1 ± 1.0°C). In contrast to its effect in control mice, injection of ET-1 into the RFs of C nociceptors (n = 8) in eight tumor-bearing mice did not further reduce their heat-response threshold (38.6 ± 0.9°C) compared with baseline levels. Furthermore, the heat-response thresholds of C nociceptors in tumor-bearing mice at baseline were similar to the heat-response thresholds of C nociceptors in control mice after injection of ET-1 suggesting that ET-1 at the tumor site may contribute to the reduction in heat-response threshold of C nociceptors in tumor-bearing mice.

For C nociceptors (n = 34) in 32 control mice, the number of impulses evoked by heat increased as stimulus temperatures increased (P < 0.01, Fig. 4A). The mean cumulative number of impulses evoked by all heat stimuli at baseline was 50.3 ± 9.5 impulses (Fig. 4B). Injection of vehicle into the RF of 18 C nociceptors in 14 control mice did not alter the cumulative number of impulses evoked by the heat stimuli (40.9 ± 11.0). In contrast, injection of ET-1 into the RFs of 19 C nociceptors in 16 control mice increased the number of impulses evoked by heat stimuli at ≥39°C (P < 0.05). The mean cumulative number of impulses after injection of ET-1 (122.4 ± 26.1) was greater than that at baseline or after injection of vehicle (P < 0.01, Fig. 4B). Thus injection of ET-1 into the RF increased responses of C nociceptors in control mice to heat stimuli.

For C nociceptors (n = 24) in 24 tumor-bearing mice, the number of impulses evoked by heat also increased as stimulus temperatures increased (Fig. 4C). At baseline, each heat stimulus evoked a greater number of impulses from C nociceptors in tumor-bearing compared with those in control mice (P < 0.05). Interestingly, the number of impulses evoked by the heat stimuli in tumor-bearing mice were similar to the enhanced responses to heat stimuli in control mice after injection of ET-1. Furthermore, the cumulative number of impulses (164.8 ± 38.1) evoked by the heat stimuli in tumor-bearing mice at baseline was similar to that of C nociceptors in control mice (122.4 ± 26.1) following injection of ET-1. These findings suggest that injection of ET-1 into the RFs of C nociceptors in control mice produced a level of sensitization to heat that was similar to that exhibited by C nociceptors in tumor-bearing mice.

Injection of vehicle into the RFs of 8 C nociceptors in eight tumor-bearing mice did not change responses evoked by each
of $\geq 39^\circ$C compared with baseline values ($P < 0.05$; Fig. 4C). The cumulative number of impulses evoked by the series of heat stimuli decreased from 158.9 ± 44.3 to 33.3 ± 8.0 impulses following injection of BQ-123 and was lower than baseline values (Fig. 4D, $P < 0.005$). These results suggest that ET-1 may contribute to tumor-evoked sensitization of nociceptors to heat through $E_{\text{T}}\alpha$ receptors in the periphery.

**DISCUSSION**

As we have shown previously, C nociceptors in mice with tumor-evoked hyperalgesia exhibited ongoing activity, decreased heat-response thresholds, and increased responses to heat stimuli compared with C nociceptors in control mice (Cain et al. 2001). Injection of ET-1 into the RFs of C nociceptors in control mice evoked ongoing activity, decreased heat-response thresholds, and increased responses to the heat stimuli to levels similar to those exhibited by C nociceptors in tumor-bearing mice. In tumor-bearing mice, injection of ET-1 into the RFs of C nociceptors increased the level of ongoing activity but did not further reduce heat-response thresholds or further increase responses to heat stimuli. Injection of an $E_{\text{T}}\alpha$ receptor selective antagonist, BQ-123, into the RFs of C nociceptors in tumor-bearing mice attenuated the ongoing activity and returned their responses to heat stimuli to levels similar to those of C nociceptors in control mice. These results suggest that tumor-evoked hyperalgesia to heat is mediated, at least in part, by endothelin-evoked sensitization of C nociceptors that terminate in the skin overlying the tumor in this murine model of cancer pain. Furthermore, this sensitizing effect of endothelin appears to be mediated through the $E_{\text{T}}\alpha$ receptor subtype.

In the present study, tumor-bearing mice were monitored for the development of mechanical but not heat hyperalgesia even though sensitization of C nociceptors occurred to heat but not mechanical stimuli. As we have shown previously, mechanical response thresholds of C nociceptors in tumor-bearing mice were not reduced compared with those in non-tumor-bearing mice (Cain et al. 2001). Indeed tumor-evoked mechanical hyperalgesia was mediated by sensitization of nociceptive dorsal horn neurons (Khasabov et al. 2007). However, C nociceptors were studied 9–16 days after implantation of fibrosarcoma cells, and during this period, both mechanical and heat hyperalgesia are present in this murine model of cancer pain (Khasabov et al. 2007). Thus although the mice in the present study were not examined for their behavioral responses to heat stimuli, they likely would have exhibited heat hyperalgesia as both tumor-evoked mechanical and heat hyperalgesia develop simultaneously.

BQ-123 was used to determine if the sensitization of C nociceptors in tumor-bearing mice was due to ET-1 acting on $E_{\text{T}}\alpha$ receptors. BQ-123 was one of the first ET-1 receptor antagonists discovered and is highly selective for the $E_{\text{T}}\alpha$ receptor subtype (Ihara et al. 1992a,b). Newer $E_{\text{T}}\alpha$-selective antagonists that have greater bioavailability when given orally have been developed and some (e.g., ABT-627 or Atrasentan and BMS-207940) are more potent and more selective than BQ-123 (Murugesan et al. 2003; Opgenorth et al. 1996; Verhaar et al. 2000). However, BQ-123 has been shown to effectively attenuate nocifensive behaviors and hyperalgesia when applied locally to the sciatic nerve (Davar et al. 1998), injected intra-articularly into the knee (Daher et al. 2004; De-Melo et al.

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**FIG. 3.** Mean ± SE heat-response thresholds of C nociceptors in nontumor-bearing (control) and in tumor-bearing (cancer) mice before (baseline) and after injection of vehicle (10 μl of PBS) or ET-1 (100 μM in 10 μl). C nociceptors in tumor-bearing mice exhibited lower heat-response thresholds than those in control mice at baseline and after injection of vehicle. Injection of ET-1 decreased the heat-response thresholds of C nociceptors in control mice to a level that was similar to heat-response thresholds of C nociceptors in tumor-bearing mice. *, significant difference from baseline ($P < 0.001$); #, significant differences from control mice ($P < 0.001$).

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heat stimulus (Fig. 4C) or by the series of heat stimuli (Fig. 4D). The cumulative number of impulses (147.6 ± 24.4) after injection of vehicle remained similar to baseline values (164.8 ± 38.1). In contrast to its effects on C nociceptors in control mice, injection of ET-1 into the RFs of eight C nociceptors in eight tumor-bearing mice did not increase their responses to the heat stimuli (Fig. 4, B and D). The cumulative number of impulses evoked by the series of heat stimuli after injection of ET-1 (185.3 ± 47.2) was not different from that at baseline or after injection of vehicle.

Thus C nociceptors in tumor-bearing mice exhibited lower heat-response thresholds and increased responses to suprathreshold heat stimuli as compared with C nociceptors in control mice. Interestingly, the level of sensitization of C nociceptors in tumor-bearing mice was similar to that of C nociceptors in control mice after injection of ET-1 into the RF. These data suggest that release of ET-1 from the tumor or surrounding tissues could contribute to tumor-evoked sensitization of C nociceptors.

To investigate whether $E_{\text{T}}\alpha$ receptors contributed to tumor-evoked sensitization of C nociceptors to heat, responses of 10 C nociceptors in 10 tumor-bearing mice to the heat stimuli were determined before and at 10 min following injection of BQ-123 into the RF. In 8 of the 10 C nociceptors, BQ-123 reduced the mean number of impulses evoked by heat stimuli after injection of vehicle (10 μl of PBS) or ET-1 (100 μM in 10 μl). C nociceptors in tumor-bearing mice exhibited lower heat-response thresholds than those in control mice at baseline and after injection of vehicle. Injection of ET-1 decreased the heat-response thresholds of C nociceptors in control mice to a level that was similar to heat-response thresholds of C nociceptors in tumor-bearing mice. *, significant difference from baseline ($P < 0.001$); #, significant differences from control mice ($P < 0.001$).
injected subcutaneously into the plantar surface of the hind paw, (Baamonde et al. 2004; Balonov et al. 2006; Gokin et al. 2001; Houck et al. 2004; Menendez et al. 2003b; Piovezan et al. 1998, 2004; Verri et al. 2004, 2005) or skin of the back (Mujenda et al. 2007), and injected into tumor-bearing tissues (Pickering et al. 2007; Schmidt et al. 2007; Wacnik et al. 2001). In contrast, the newer ETA receptor-selective antagonists have been administered systemically (Chichorro et al. 2006a; Klass et al. 2005; Matwyshyn et al. 2006; Piovezan et al. 2000; Trentin et al. 2006). Because we were interested in the role of ETA receptors near the tumor, presumably on the terminals of C nociceptors, we chose BQ-123 as the ETA receptor-selective antagonist as it has been shown to be effective when injected into peripheral tissues.

ET-1-evoked nocifensive behaviors and hyperalgesia

Injection of ET-1 into the knee or hind paw evokes nocifensive behaviors in animals (Baamonde et al. 2004; Balonov et al. 2006; De-Melo et al. 1998a; Ferreira et al. 1989; McKelvy et al. 2007; Piovezan et al. 1998, 2004; Verri et al. 2004, 2005). For example, injection of ET-1 into the plantar surface of the hind paw evoked dose-dependent hind paw licking (Gokin et al. 2001; Menendez et al. 2003b; Piovezan et al. 2000), and application of ET-1 directly to the sciatic nerve evoked robust hind paw flinching (Davar et al. 1998; Fareed et al. 2000). Injection of ET-1 into the hind paw also produced tactile allodynia (Balonov et al. 2006; McKelvy et al. 2007), and mechanical (da Cunha et al. 2004; Ferreira et al. 1989) and heat (Menendez et al. 2003b) hyperalgesia. Furthermore, local administration of ET-1 potentiated nocifensive behaviors evoked by intraplantar injection of capsaicin (Piovezan et al. 1998) or formalin (Piovezan et al. 1997; Yuyama et al. 2004a) and intra-articular injection of an inflammatory evoking substance, carrageenan (Daher et al. 2004; De-Melo et al. 1998b), or prostaglandin E2 (Ferreira et al. 1989).

In humans, early studies reported that intradermal injection of ET-1 evoked mechanical hyperalgesia (Ferreira et al. 1989), whereas intra-arterial injection of ET-1 evoked deep muscular pain that was intensified by touch (Dahlof et al. 1990). More recently, it has been reported that intradermal injection of ET-1 produced a short-lasting spontaneous pain and longer-lasting mechanical and cold hyperalgesia (Hans et al. 2007; Namer et al. 2007).
The mechanisms by which injection of ET-1 into peripheral tissues evokes nocifensive behaviors and hyperalgesia to heat appears to be mediated through the ET<sub>A</sub> receptor as pretreatment with systemic (i.e., atrasentan or A-127722-5) or local (i.e., BQ-123) ET<sub>A</sub> receptor-selective antagonists or co-application of BQ-123 with ET-1, attenuated these effects (Baamonde et al. 2004; Davar et al. 1998; De-Melo et al. 1998a; Fareed et al. 2000; Gokin et al. 2001; Menendez et al. 2003b; Piovezan et al. 2000). In contrast, pretreatment with or co-application of ET<sub>B</sub> receptor-selective antagonists (e.g., BQ-788) did not attenuate the ET-1-evoked nocifensive behaviors or heat hyperalgesia (Baamonde et al. 2004; Davar et al. 1998; De-Melo et al. 1998a; Fareed et al. 2000; Menendez et al. 2003b; Piovezan et al. 2000). The potentiating effect of ET-1 on nocifensive behaviors evoked by capsaicin, formalin, or inflammation was also mediated through the ET<sub>A</sub>, but not ET<sub>B</sub>, receptor subtype (Daher et al. 2004; Piovezan et al. 1998; Yuyama et al. 2004a).

ET<sub>B</sub> receptors appear to contribute to ET-1-evoked mechanical hyperalgesia (Baamonde et al. 2004; da Cunha et al. 2004). However, the contribution of ET<sub>A</sub> receptors to ET-1-evoked mechanical hyperalgesia is unclear as pretreatment with BQ-123 has been reported to either block (Baamonde et al. 2004) or fail to block (da Cunha et al. 2004) ET-1-evoked mechanical hyperalgesia.

Thus injection of ET-1 into peripheral tissues evokes nocifensive behaviors and mechanical and heat hyperalgesia in animals and evokes pain, mechanical, and cold hyperalgesia in humans. ET-1 also potentiates subsequent nocifensive behaviors evoked by capsaicin, formalin, or inflammation. These effects are for the most part mediated by ET<sub>A</sub>, but not ET<sub>B</sub>, receptors in the peripheral tissues. However, ET<sub>B</sub> receptors appear to contribute to ET-1 evoked mechanical hyperalgesia. These effects of ET-1 could contribute to tumor-evoked spontaneous pain and mechanical, heat, and cold hyperalgesia observed in the murine model of cancer pain used in the present study (Hamamoto et al. 2007; Khasabov et al. 2007; Wacnik et al. 2001).

Role of ET-1 in inflammatory, neuropathic, and tumor-evoked hyperalgesia

Activation of endothelin receptors contributes to inflammatory, neuropathic, and tumor-evoked hyperalgesia. For example, intraplantar injection of BQ-123 attenuated mechanical and heat hyperalgesia evoked by inflammatory agents, such as carrageenan or complete Freund’s adjuvant (Baamonde et al. 2004). In contrast, an ET<sub>B</sub> receptor-selective antagonist, BQ-788, attenuated inflammation-evoked mechanical but not heat hyperalgesia. Similarly BQ-788, but not BQ-123, blocked mechanical hyperalgesia produced by intraplantar injection of the proinflammatory cytokines interleukin 12 (Verri et al. 2005) and interleukin 18 (Verri et al. 2004). Postincisional pain may be due, at least in part, to inflammation and pretreatment of the skin with BQ-123 prior to the incision attenuates mechanical allodynia (Mujenda et al. 2007). Thus ET<sub>A</sub> receptors contribute to mechanical allodynia and mechanical and heat hyperalgesia, whereas ET<sub>B</sub> receptors contribute only to mechanical hyperalgesia in these models of inflammation.

In models of neuropathic pain, the endothelin receptor subtype that is involved depends on the model and the type of hyperalgesia (i.e., mechanical or thermal). For example, systemic administration of an ET<sub>A</sub> receptor-selective antagonist attenuated mechanical allodynia in the streptozotocin-induced diabetic rat model of neuropathic pain (Jarvis et al. 2000) and heat and mechanical hyperalgesia produced by chronic constriction injury of the sciatic nerve (Klass et al. 2005). However, a similar constriction injury to the infraorbital nerve produced mechanical allodynia that was attenuated by systemic administration of an ET<sub>B</sub> receptor-selective antagonist (Chichorro et al. 2006a). Interestingly, this same model of neuropathic pain produced cold hyperalgesia that was attenuated by administration of either ET<sub>A</sub> or ET<sub>B</sub> receptor-selective antagonists into the lip (Chichorro et al. 2006b).

Release of ET-1 from tumor cells may contribute to tumor-evoked pain and hyperalgesia (Davar 2001). In animal models of tumor-evoked hyperalgesia, including the model used in the present study, expression of ET-1 was increased in tumor-bearing tissues (Peters et al. 2004; Schmidt et al. 2007; Wacnik et al. 2001) and administration ET<sub>A</sub> receptor-selective antagonists into tumor-bearing tissues attenuated tumor-evoked hyperalgesia (Pickering et al. 2007; Schmidt et al. 2007; Wacnik et al. 2001). Administration of ET-1 into tumor-bearing hind paws of mice potentiated tumor-evoked nocifensive behaviors, and this potentiation was attenuated by blockade of ET<sub>A</sub> receptors (Yuyama et al. 2004a,b). In humans, systemic administration of ET<sub>A</sub> receptor-selective antagonists attenuated pain associated with prostate cancer (Carducci et al. 2002). Thus release of ET-1 from tumor cells could contribute to tumor-evoked hyperalgesia perhaps via excitation and sensitization of C nociceptors.

ET-1-evoked excitation of C nociceptors

We confirmed our previous findings demonstrating that a subpopulation of C nociceptors exhibit ongoing activity, decreased response thresholds to heat, and increased responses to suprathreshold heat stimuli (Cain et al. 2001). In the present study, a greater proportion (84%) of C nociceptors in tumor-bearing mice exhibited ongoing activity compared with our previous report (34%). This difference arose because in the present study we searched primarily for sensitized C nociceptors as indicated by ongoing activity. However, the discharge rate of ongoing activity, mechanical and heat-response thresholds, and responses to the heat stimuli were similar in the two studies, suggesting that the C nociceptors in the present study were from the same subpopulation of C nociceptors that we originally described.

Injection of ET-1 into the RFSs of C nociceptors in nontumor-bearing mice evoked ongoing activity. This finding is consistent with previous studies in which C nociceptors in rats were excited by injection of ET-1 (Gokin et al. 2001; Khodorova et al. 2002) and likely contributes to ET-1-evoked nocifensive behaviors. The latency from the time of injection of ET-1 to excitation of C nociceptors in rats ranged from 1.2 to 5.6 min with a mean of 3.16 ± 0.31 (SE) min (Gokin et al. 2001; Khodorova et al. 2002). We also observed that the latency to excitation varied between C nociceptors. Thus we determined the level of ongoing activity 5 min after injection of ET-1 to allow sufficient time for the majority of C nociceptors to respond. Recording ongoing activity of C nociceptors at 5 min after injection of ET-1 coincides well with a recent report that
pain evoked by ET-1 in humans was maximal between 4 and 5 min after intracutaneous injection (Namer et al. 2007). Because the duration of responses to ET-1 of C nociceptors ranged from 15 to 40 min (Gokin et al. 2001; Khodorova et al. 2002), it is unlikely that many C nociceptors responded to the ET-1 earlier but did not exhibit ongoing activity at 5 min.

A subpopulation (14%) of C nociceptors in tumor-bearing mice exhibited bursting patterns of ongoing activity. A similar proportion (20%) of C nociceptors in nontumor-bearing mice exhibited bursting patterns of ongoing activity after injection of ET-1 into their RFs. The patterns of bursting were similar in mean intra-burst frequencies, durations, and intervals between bursting periods in C nociceptors in tumor-bearing mice and in nontumor-bearing mice after injection of ET-1. Administration of bradykinin or tumor necrosis factor alpha (TNFα) to the RF produces bursts of impulses in a proportion of C nociceptors in rats (Banik et al. 2001; Sorkin et al. 1997). Injection of ET-1 into the RFs of C nociceptors in rats evoked ongoing activity that exhibited bursting patterns with peak intra-burst frequencies of ~3 imp/s, periods of bursting that lasted 10–15 s, and intervals between bursts of ~50 s following injection of ET-1 (Gokin et al. 2001; Khodorova et al. 2002). In the present study, nontumor-bearing mice exhibited bursting periods (5.1 ± 2.0 s) and time between bursts (8.8 ± 2.5 s) that were shorter than those reported for rats. However, the dose of ET-1 (16–20 nmol) administered to the rats was higher that the dose (100 μM in 10 μl = 1 nmol) administered to the nontumor-bearing mice in the present study. Thus ET-1 released at the tumor site may contribute to excitation of C nociceptors, some of which exhibit a bursting pattern of ongoing activity.

In healthy human volunteers, electrophysiological responses of mechanosensitive and -insensitive C nociceptors to intracutaneous injection of ET-1 into hairy skin were examined using microneurography (Namer et al. 2007). Injection of ET-1 into their RFs excited 65% of mechanosensitive C nociceptors but none of the mechanosensitive C nociceptors. Interestingly, the latency to excitation [31.4 ± 4.8 (SE) s] and duration of the excitation (8.3 ± 1.5 min) were shorter for C nociceptors in humans than for C nociceptors in rats (3.16 ± 0.31 and 30 ± 3 min, respectively) (Gokin et al. 2001; Khodorova et al. 2002) even though the dose of ET-1 was lower (10 μl of 1 μM) for humans than for the rats (50 μl of 400 μM). This may represent differences in sensitivity to or activation by ET-1 between nociceptors in humans and in rats or differences between nociceptors located in hairy and glabrous skin.

In the present study, the level of ongoing activity evoked by ET-1 in nontumor-bearing mice was similar to that of C nociceptors in tumor-bearing mice. Because ongoing activity of nociceptors may contribute to persistent pain (Djouhri et al. 2006), and persistent pain often occurs with bone cancer in humans (Banning et al. 1991; Mercadante 1997; Sarlak et al. 2000), ongoing activity in C nociceptors evoked by ET-1 may contribute to the tumor-evoked nociceptive behaviors reported previously (Wacnik et al. 2001). In support of this idea, injection of BQ-123 into the RF of C nociceptors attenuated ongoing activity and nociceptive behaviors in tumor-bearing mice (Wacnik et al. 2001).

Intraplantar injection of ET-1 into the RFs of C nociceptors in tumor-bearing mice further increased their ongoing activity indicating that sensitivity of C nociceptors to ET-1 is maintained in tumor-bearing tissues. This ET-1 evoked increase in ongoing activity of C nociceptors could contribute the increase in nocifensive behaviors produced by ET-1 in this murine model of cancer pain (Wacnik et al. 2001). The ET-1 evoked increase in ongoing activity of C nociceptors was attenuated by injection of BQ-123 (Gokin et al. 2001). Thus the ongoing activity of C nociceptors in tumor-bearing mice could be due to the effects of ET-1 on ETα receptors in the tumor-bearing tissues and contribute to tumor-evoked nocifensive behaviors in this model and other models of cancer pain (Honore et al. 2000; Schwei et al. 1999; Wacnik et al. 2001).

**ET-1-evoked sensitization of C nociceptors**

In nontumor-bearing mice, injection of ET-1 into the RFs of C nociceptors decreased heat-response thresholds and increased responses to suprathreshold heat stimuli. This is a novel finding because although hyperalgesia to heat produced by ET-1 has been reported (Menendez et al. 2003b), previous studies in rats did not examine the effect of ET-1 on the electrophysiological responses of C nociceptors to heat (Gokin et al. 2001; Khodorova et al. 2002). In humans, injection of ET-1 into the RF sensitized 60% of mechanosensitive C nociceptors to heat as response thresholds decreased from 40.1 to 38.7°C (Namer et al. 2007). In the present study, heat-response thresholds in nontumor-bearing mice decreased from 43.8 ± 0.7 to 39.1 ± 0.8°C. Thus although heat-response thresholds were higher for C nociceptors in mice than in humans, injection of ET-1 in the RFs decreased heat-response thresholds to a similar level. Thus sensitization of C nociceptors to heat may be the neuropathological mechanism by which ET-1 induces heat hyperalgesia. Interestingly, the decrease in heat-response thresholds and increase in responses to suprathreshold heat stimuli produced by ET-1 in nontumor-bearing mice was similar to that observed for C nociceptors in tumor-bearing mice in the present study and to those that we reported previously (Cain et al. 2001), suggesting that ET-1 in tumor-bearing tissue contributes to sensitization of C nociceptors to heat in tumor-bearing mice.

Injection of BQ-123 into the RF of sensitized C nociceptors in tumor-bearing mice returned their responses to suprathreshold heat stimuli back to levels exhibited by C nociceptors in nontumor-bearing mice. Peripheral administration of ET-1 into the tumor-bearing hind paw did not further sensitize C nociceptors to heat as heat-response thresholds, and responses to suprathreshold heat stimuli did not change. This finding suggests that the tumor-evoked sensitization of C nociceptors to heat may have been maximal. Whether injection of ET-1 into tumor-bearing tissues increases the level of heat hyperalgesia has not been reported. Thus tonic release of ET-1 in tumor-bearing tissues may act via ETα receptors to induce sensitization of C nociceptors to heat stimuli and contribute to the heat hyperalgesia observed in this model of cancer pain (Khasabov et al. 2007). BQ-123 returned the responses of C nociceptors to heat stimuli in tumor-bearing mice back to levels exhibited by nontumor-bearing mice but only partially attenuated tumor-evoked ongoing activity. It is possible that tumor-evoked heat hyperalgesia is mainly due to ET-1 induced sensitization of C nociceptors to heat but that tumor-evoked ongoing nocifensive behaviors are only partially mediated by ET-1 evoked excitation of C nociceptors. However, higher doses of BQ-123 were not evaluated in the present study leaving open the possibility...
that our dose of BQ-123 was not high enough to fully block ET-A receptors.

**Mechanisms of ET-1-evoked excitation and sensitization of C nociceptors**

Excitation of C nociceptors in tumor-bearing mice may be due to a direct effect of ET-1 as application of ET-1 to the sciatic nerve produced nocifensive behaviors in an ETA receptor-dependent manner (Davar et al. 1998). ET_A, but not ET_B, receptors are expressed on small-diameter CGRP-immunoreactive dorsal root ganglion neurons and axons (Peters et al. 2004; Pomonis et al. 2001) that likely detect nociceptive stimuli (Lawson et al. 2002). Further evidence for direct excitation of C nociceptors by ET-1 is that ET-1 induced dose and ET_A receptor-dependent release of intracellular Ca²⁺ in nociceptor-like neurons (Zhou et al. 2001). Also ET-1 produced an ETA receptor-dependent hyperpolarizing shift in the voltage-dependent activation of tetrodotoxin-resistant (TTX-R) sodium channels in isolated dorsal root ganglion neurons (Zhou et al. 2002). Such a shift could increase membrane excitability and render the neuron more susceptible to generating action potentials. TTX-R sodium channels are found exclusively in small-diameter dorsal root ganglion neurons associated with nociceptive properties (Amaya et al. 2000; Elliott and Elliott 1993; Fjell et al. 2000), and hyperpolarizing shifts in TTX-R sodium channel activation are associated with treatments that sensitize nociceptors and produce hyperalgesia (Gold et al. 1996; Kral et al. 1999). Application of TTX to the sciatic nerve before application of ET-1 at a more distal site of the nerve did not attenuate ET-1-evoked nocifensive behaviors, suggesting that ET-1-evoked nociception is transmitted to the CNS via primary afferent fibers using TTX-R sodium channels (Houck et al. 2004).

The mechanisms by which ET-1 produces sensitization of C nociceptors to heat are unclear. One possibility is that ET-1 modulates function of the capsaicin receptor, TRPV1, which is a transducer for noxious heat (Caterina and Julius 1999; Caterina et al. 1997; Tominaga et al. 1998). ET-1 enhanced capsaicin-evoked release of intracellular Ca²⁺ (Yamamoto et al. 2006) and neuropeptides [calcitonin gene-related peptide (CGRP) and substance P] (Dymshitz and Vasko 1994) in cultured dorsal root ganglion neurons and potentiated capsaicin-evoked hyperalgesia (Pievozan et al. 2000), suggesting that ET-1 might enhance activity at TRPV1 receptors.

**Conclusions**

The present study confirms our earlier findings that a subset of C nociceptors that innervate the skin overlaying a tumor develop ongoing activity and sensitization to heat (Cain et al. 2001). We now provide electrophysiological evidence suggesting that ET-1 contributes to tumor-evoked ongoing activity and sensitization of C nociceptors to heat and that these effects of ET-1 occur through ETA receptors. Because ET-1 is expressed by several human cancer cell lines and stimulates proliferation of some cancer cell lines via an ETA receptor-mediated mechanism and because blockade of ETA receptors attenuates tumor-evoked nocifensive behaviors and sensitization of C nociceptors in animals, targeting of ETA receptors may be a beneficial therapeutic approach for the management of pain associated with certain cancers in humans.

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