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Contralateral Paw Sensitization Following Injection of Endothelin-1: *Effects of local anesthetics differentiate peripheral and central processes*

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Abstract

Subcutaneous injection of the peptide endothelin-1 (ET-1) into the rat's footpad is known to cause rapid, transient ipsilateral mechanical and thermal sensitization and nocifensive hind paw flinching. Here we report that local injection of ET-1 (2 nmoles) into one hind paw slowly sensitizes the contralateral paw to chemical and mechanical stimulation. There was a 1.5–2-fold increase in the hind paw flinching response, over that from the first injection, to a second injection of the same dose of ET-1 delivered 24 h later into the contralateral paw. A similar increase in the number of flinches during the second phase of the response to formalin also occurred in the contralateral paw 24 h after ET-1. The contralateral paw withdrawal threshold to von Frey hairs was lowered by ~ 55% at 24 h after ipsilateral ET-1 injection. ET-1 injected subcutaneously at a segmentally unrelated location, the nuchal midline, caused no sensitization of the paws, obviating a systemic route of action. Local anesthetic block of the ipsilateral sciatic nerve during the period of initial response to ipsilateral ET-1 prevented contralateral sensitization, indicating the importance of local afferent transmission, although ipsilateral desensitization was not changed. These findings suggest that peripheral ET-1 actions lead to central sensitization that alters responses to selected stimuli.

Keywords

contralateral hypersensitivity; nociception; hyperalgesia; local anesthesia

Introduction

Endothelin-1 is a potent algogenic peptide that produces acute nocifensive behavior (hind paw flinching), via activation of ET_A receptors, when applied at high concentrations (200–600 μ M) to the sciatic nerve (Davar et al., 1998; Fareed et al., 2001) or injected into the rat footpad (Gokin et al., 2001). Lower concentrations of ET-1 (300 nM–10 μ M) evoke sensitizing actions, producing hyperalgesia and allodynia via both ET_A and ET_B receptors (Menendez et al. 2003; da Cunha et al. 2004; Balonov et al. 2006; Khodorova et al., 2009a, b). Along with this sensitization to mechanical stimulation, first shown by Ferreira for the human forearm and rat

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hind paw (Ferreira et al. 1989), ET-1 induces local “chemical sensitization”, to injections of formalin and capsaicin in mice (Piovezan et al., 1997, 1998).

Recently we found that ET-1 is able to enhance basal and capsaicin-stimulated release of glutamate and CGRP from primary rat DRG neurons (Khodorova et al. 2009b). This study also reported that the rapidly developing mechanical allodynia from paw injection of ET-1 was effectively suppressed throughout its course by local pre-treatment with NMDA receptor antagonists, and although not diminished in extent, it was shortened in duration by a CGRP1 receptor antagonist delivered 30 min before at the ET-1 injection site. This latter effect was the same as that of a TRPV1 antagonist (Balonov et al., 2006).

Local responses to ET-1 are profoundly desensitized when the peptide is applied onto the sciatic nerve, as shown by a significantly lowered flinching response to a second dose of ET-1 (200 μ M), administered ipsilaterally as late as 24 h after the first one (Fareed et al., 2001). Very similar results occur with repeated paw injections of ET-1 (see Results, below). In contrast, preliminary findings show that the response to a second intraplantar injection of ET-1, given contralaterally 24 h after the initial ipsilateral one, is greater than the initial response.

We hypothesized that peripheral and, possibly, central mechanisms underlie ET-1’s sensitizing actions. Along with the demonstrated direct excitation of afferents (Gokin et al., 2001a), ET-1 injected into the paw may evoke ipsilateral local changes in skin that sensitize nociceptive primary afferent fibers, lowering threshold for impulse generation and amplifying their discharge, thus generating a larger nociceptive input to the CNS. These afferent barrages may be sufficient to enhance central pain processing, via mechanisms of “central sensitization”, as has been shown for electrical and physiological peripheral stimulation (Woolf, 1983; Campbell et al., 1988).

To address these hypothetical mechanisms, we have further characterized the effects of repeat injections of ET-1 into ipsilateral and contralateral paws. Specifically, we examined the effect of ipsilateral hind paw ET-1 injection on, 1. the contralateral hind paw’s responsiveness to ET-1, and 2. to mechanical and thermal stimulation and to formalin injection, and 3. the effect of ipsilateral nerve blockade (with local anesthetics) on the CLP’s sensitization to ET-1 and the ipsilateral paw’s desensitization.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on adult male Sprague-Dawley rats (220–250 g, Charles River, USA). Rats were housed 2 per cage under a 12:12 h dark-light cycle and were provided with food and water *ad libitum*. Animals were experimentally treated and cared for according to the ethical standards and guidelines for investigations of experimental pain in animals prescribed by the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann 1983), and using policies and procedures approved by the Harvard Committee on Animals.

In vivo experiments

Acute pain response evaluation—Behavioral assessments were made with individual animals freely moving on a flat surface enclosed by an inverted (24 cm $\times$ 46 cm) Plexiglas® cage. Animals were allowed to recover for at least 5 min after the injection of ET-1 before the onset of measurements. Spontaneous hind paw flinches were recorded every 5 min for 65–75 min after the ET-1 injection (in several series with multiple ET-1 injections the observation time was prolonged until 2 h after injection, see results).

Mechanical testing—Unrestrained rats were placed on an elevated plastic mesh floor (28 × 17.5 cm; 9.5×9.5 mm openings) and allowed to habituate for 30 min before initial testing. Withdrawal threshold to mechanical stimulation was determined using calibrated von Frey hairs applied to the plantar surface of a hind paw.

The animals were tested over 5 days before each experiment to obtain a stable baseline of the withdrawal threshold to mechanical stimulation. On the treatment day, baseline was assessed 30, 20 and 10 minutes before the first injection; the lowest value of these 3 measurements was considered as the baseline level against which changes were compared (cf. Balonov et al., 2006 and Khodorova et al., 2009b for a justification of this approach). Each von Frey hair was applied once starting with a force of 0.07 g (1 g, in naïve rats) and continuing until a withdrawal response occurred (or until 60 g, the cutoff value in naïve rats, was reached). This paw withdrawal threshold was verified by testing with the next thicker von Frey hair, which always caused paw withdrawal (Chaplan et al., 1995; Brennan et al. 1996; Balonov et al. 2006). Withdrawal thresholds were measured at 20, 30, 40, 50, 60, 75, 90 min after injection of 10 µM ET-1, or at 2.5 h and 24 h after injection of 200 µM ET-1.

Thermal testing—The sensitivity to noxious heating was determined by the method of Hargreaves et al. (1988). The animals were habituated and tested on a raised glass platform over 5–6 days before each experiment to achieve a consistent paw withdrawal latency (PWL). The heat source was adjusted to consistently produce ~10 sec PWL in normal rats, and a cut-off time of 22 sec was enforced to minimize thermal sensitization. A series of 4–5 paw withdrawal latencies was determined alternately on left and right paws by tests applied with 2–4 min intervals. The first paw tested was assigned as left or right randomly. Measurements of PWL for each hind paw performed over the 3 days before the test (including the day of the experiment) were averaged and the mean value taken as the *baseline thermal nociceptive latency*.

Rats were habituated on the platform for 20 min prior to the start of the experiment and 10 min post injection. Testing started 18 min and 2.5 h after injection of 10 µM or 200 µM ET-1, respectively. Latency measurements were carried out alternately on the injected and contralateral paw, as indicated above, starting with the injected one.

Injection procedures—ET-1 (10 µl) or its vehicle, PBS, was injected subcutaneously (s.c.) into the mid-plantar hind paw, 1 cm distal from the heel using a 30-G needle attached to a 10 µl Hamilton microsyringe (Hamilton Co., Reno, NV, USA). Injection occurred under brief general anesthesia (0.5–1 min), which was accomplished with the rapidly reversible agent sevoflurane (Abbott Labs, N. Chicago, IL, USA). In this procedure ca. 0.3 ml of sevoflurane liquid was placed on cotton gauze in the bottom of a 50 ml conical centrifuge tube, and the rat was gently restrained as the open end of the tube was placed over its muzzle. Anesthesia was evident by the animal's flaccid paralysis, usually occurring within 10–15 sec, and recovery of the righting reflex occurred in <30 sec after the anesthetic-containing tube was removed. A second injection of ET-1, of the same volume (10 µl), was made into the same plantar area of the ipsilateral or the contralateral hind paw. Formalin (Sigma-Aldrich, St. Louis, MO; 0.5% in PBS; 10 µl) was injected identically to the second ET-1 injection.

ET-1 was dissolved in PBS (0.1 mg/0.2 ml) and kept in aliquots for up to 1 week at –80°C. Stock solution was diluted in PBS to its final concentration. During all experiments, the working solutions were kept on ice.

Neural blockade of afferent impulses: To provide a fast-developing and long-lasting block of the sciatic nerve that would overlap the duration of robust overt nociception (Houck et al., 2004), local anesthetics (LA) lidocaine and bupivacaine were injected together (in a volume

of 0.1 ml) percutaneously at the greater trochanter level into the sciatic notch. Duration of block was defined as the duration of full functional deficit (no response to deep pinch, presence of abnormal posture and gait) of the injected limb. LA solution contained 1% lidocaine (Hospira Inc., Lake Forest, IL, USA) + 0.5% bupivacaine (Chiroscience R&D Limited, Hertfordshire, UK) (2% lidocaine mixed V:V with 1% bupivacaine in saline; pH=6.3). ET-1 was injected into the plantar hind paw 6 min after LA injection into sciatic notch. In control experiments, ET-1 was injected initially after full recovery from LA-induced impairment of nociception and gait, i.e. at the time when rats responded with a brisk paw withdrawal with vocalization to deep pinch of the 5th toe, had normal posture of the toes and the limb, and performed a normal gait.

Data analysis

Data are presented as means $\pm$ S.E.M and evaluated using GraphPadInStat version 3.0 (GraphPadSoftware, CA, USA). Statistical analysis applied a two-tailed Mann-Whitney U-test to compare the acute nocifensive flinching response of the contralateral paw to that of the ipsilateral paw, and also to compare scratching after nuchal mid-line injection of ET-1 with scratching in un-injected rats. A two-tailed Mann-Whitney U-test or Kruskal-Wallis Test followed by Dunn's Multiple Comparisons Test were used to compare flinching of, respectively, one or several "treated" to "untreated" groups. Friedman's test with Dunn's post-hoc correction for repeated measures was applied to compare ET-1-affected responses with baseline responses using mechanical or thermal stimulation in the same paw over time after the injection. For all these tests $P < 0.05$ was considered significant.

RESULTS

Desensitization in the ipsilateral hind paw versus sensitization in the contralateral hind paw

Subcutaneous injection of ET-1 (200 μ M, 2 nmoles/paw) rapidly stimulates hind paw flinching, behavior previously shown to be an indicator of pain (Davar, et al., 1998; Fareed et al., 2001; Gokin et al., 2001a). A strong desensitization of this acute nocifensive response was apparent when a second, identical dose of ET-1 was injected into the ipsilateral paw 24 h after the first one (Fig. 1). The number of flinches of the second response was reduced with higher concentrations of initially administered ET-1 (2, 20 and 200 μ M ET-1), and ET-1 at concentrations that induced low hind paw flinching themselves, e.g. 20 μ M (total flinches, TF= 51 ± 7 , $n=18$) were able to halve the flinching response to the second, higher dose (200 μ M). The time course of the flinching response was also altered by a preceding injection, such that the later responses were suppressed and the earlier ones were not, shifting the peak response to shorter times with greater desensitization (Figure 1A). The reduction in response is probably not due to a restriction in access of ET-1 to the receptors, because when flinch behavior was examined previously as a function of ET-1 concentration, the lower doses produced a smaller initial response but virtually equal later responses (Gokin et al., 2001a), opposite the pattern seen here. Whether this change is accounted for exclusively by peripheral, receptor-related changes, by accommodations in blood flow secondary to the initial vasoconstriction caused by ET-1, or by modification of the central responsiveness to ET-1 cannot be determined by these data alone.

Despite the ipsilateral paw (IPSI) desensitization, on the contralateral side a sensitization to ET-1 occurred. This was shown by an increase in hind paw flinching when the second injection of ET-1 (200 μ M, dose 2 nmoles/paw) was made into the *contralateral* paw (CLP) (total flinches (TF)= 178 ± 24 in CLP vs. 111 ± 14 in IPSI, $n=8$, $P < 0.05$; TF increased in 7 out of 8 rats) (Fig. 2, column A). The enhanced nocifensive flinching in the CLP also occurred in response to a third ET-1 injection at 48 h following two ipsilateral injections, at 0 h and 24 h (223 ± 37 CLP flinches compared to 103 ± 12 flinches from the initial ipsilateral injection, $n=5$, $P < 0.05$), and this occurred despite the very strong desensitization for the second ipsilateral

injection (30 ± 5 flinches, $n=5$, vs. 103 ± 12 flinches for the first injection; Fig. 2, column B). Vehicle (PBS) injection did not induce acute nociception and did not sensitize the contralateral paw.

Sensitization was also indicated by an increased response to lower doses of ET-1. Endothelin-1 injected contralaterally at $2 \mu\text{M}$ (20 picomoles/paw) following two separate sequential ipsilateral injections of $200 \mu\text{M}$ ET-1 (at 0 h and 24 h) also caused an enhanced number of flinches (59 ± 9 flinches, $n=7$, compared to the response to $2 \mu\text{M}$ ET-1 in naïve rats, 29 ± 14 flinches, $n=12$, different group of rats, $P<0.05$) (Fig. 2, column C).

Delayed contralateral sensitization to mechanical stimulation in response to ET-1

At lower concentrations ($10 \mu\text{M}$, dose 0.1 nmole/paw), ET-1 sensitizes the ipsilateral paw to mechanical stimulation by von Frey hairs (Balonov et al., 2006; Motta, et al., 2006). Only the injected paw's tactile withdrawal threshold (PWT) decreased from baseline at 2.5 h after low dose ET-1 injection ($n=5$), with no significant change in the contralateral paw's threshold at that time (data not shown). At a high concentration, ET-1 ($200 \mu\text{M}$; 2 nmoles/paw) sensitized both hind paws to mechanical stimulation by von Frey hairs by 24h. The ipsilateral paw withdrawal threshold, measured 2.5 h post-injection, when robust hind paw flinching was over, was maximally decreased then and had begun to rise at 24 h post injection. The contralateral paw's threshold fell by 40% below the baseline level at 2.5 hours after ET-1's injection, but this was insignificant: a significant decline, of about 50%, was measured at 24 h after ET-1 injection (Fig. 3).

Unilateral sensitization to thermal stimulation induced by ET-1

Plantar subcutaneous injection of endothelin-1 ($10 \mu\text{M}$, 0.1 nmoles/paw) also induced ipsilateral thermal hyperalgesia, i.e. reduction of PWL by $\sim 80\%$, that reached its maximum at 20 min after injection and then slowly decreased over the next 1–1.5 h ($n=6$) (Fig. 4A). Thermal hyperalgesia fully recovered to baseline by 4 h (data not shown); thermal hyperalgesia had also disappeared 3 h after injection of a 20–25-fold higher ET-1 concentration (200 and $250 \mu\text{M}$, 2 and 2.5 nmoles/paw) ($n=6$ and $n=8$, respectively) (Fig. 4B). The *contralateral hind paw* showed a moderate decrease, by $\sim 35\%$ from control PWL, and only at 42–80 min after ET-1 ($10 \mu\text{M}$) injection (Fig. 4A). No signs of thermal hyperalgesia were observed 24 h post-ET-1 (200 – $250 \mu\text{M}$) injection ($n=14$) (Fig. 4B), a time when contralateral mechanical sensitization was well developed (Fig 3).

Potentiation of the nocifensive response to formalin in the contralateral paw

Injection of formalin (0.5% , $50 \mu\text{l}$) into the contralateral hind paw 24 h after ET-1 ($400 \mu\text{M}$, 4 nmoles) induced about twice as many flinches during the second stage (267 ± 42 flinches over 10–60 min, $n=10$) than the number of second stage flinches in control rats, not treated with ET-1 (114 ± 11 flinches, $n=10$, $P<0.05$; different group of rats) (Fig. 5). Formalin's effect in naïve rats, $n=5$, was the same as that in rats injected contralaterally with vehicle 24 h before formalin ($n=5$, $P>0.05$), therefore, these groups were merged to compose a single control group. (Because of the lingering effect of the general anesthesia that was used during the formalin injection it was not possible to accurately count the flinches during the first phase.) Formalin also caused an occasional repeated flinch, marked by rapid multiple up and down motions of the hind paw. When these were analyzed separately, the number of triple flinches and total repeated flinches (double + triple) induced by formalin in the contralateral paw increased after ET-1 even more than the single flinches, ca. 5-fold and 6-fold, respectively (see Fig. 5).

Contralateral sensitization to ET-1 is inhibited by ipsilateral sciatic nerve block during the initial ET-1 injection

Local anesthetic injected into the sciatic notch abolishes mechano-nocifensive responses to ipsilateral toe pinch (Nakamura et al. 2003, Houck et al., 2004) and also reduces the ipsilateral flinching response induced by ET-1 administered either directly onto sciatic nerve or injected into the plantar hind paw (Houck et al., 2004). Local anesthetic injected percutaneously into the sciatic notch here resulted in full sensory and motor block of these hind paw functions that developed within 5 min after injection, at doses that are known to block impulses in virtually all afferent sciatic nerve fibers (Gokin et al., 2001b). The initial flinching pain response to ET-1 during this sciatic nerve block was strongly attenuated (by ~88%) compared to controls (Fig. 6); the total number of flinches equaled 9 ± 1 ($n=9$) over 70 min. The flinching response to a second injection of 200 μ M ET-1, given 24 h later into the CLP, did not show signs of sensitization, as it was not different from the initial response to ET-1 in naïve rat/paw (102 ± 14 flinches, $n=8$, vs. 107 ± 19 flinches, $n=9$, respectively, $P>0.05$). In experiments where an initial ET-1 injection was performed shortly after full recovery from the LA block of the pinch response, a second injection of 200 μ M ET-1 into CLP resulted in ca. 1.5-fold increase in total flinches (197 ± 32 , $n=9$, $P<0.05$), the same behavior as occurred when no LA had been used (cf. above and Figure 2). This suggests a correlation between the block of afferent impulses and the prevention of contralateral sensitization.

In contrast to this contralateral effect, blockade of the ipsilateral sciatic nerve by local anesthetics did not affect the ipsilateral desensitization. The same smaller number of hind paw flinches to a second injection of ET-1 was observed at 24 h after a first injection made during sciatic nerve block, despite the fact that virtually no flinches occurred in the ipsilateral leg during this blocked episode (data not shown).

Interestingly, the duration of sciatic block by local anesthetics in the presence of ET-1 injected in the plantar paw was shorter than in control, 73.0 ± 2.1 min ($n=9$) vs 97.0 ± 6.6 min ($n=8$), respectively ($P<0.01$).

The absence of potentiation of the contralateral behavioral response when the initial ipsilateral ET-1 injection occurred during local anesthesia indicates the importance of afferent impulse conduction from the ET-1-injected ipsilateral hind paw. However, subcutaneous injection of ET-1 might also lead to a systemic distribution that could effectively engage processes in the CNS, such as central sensitization, independent of any enhanced afferent input. To test for such possible systemic effects, ET-1 was initially administered subcutaneously into the nuchal midline, then 24 h later, and at the same concentration (200 μ M, dose 2 nmoles) it was injected subcutaneously into one plantar hind paw. The initial (nuchal midline) injection of ET-1 did not induce any flinching response, but provoked enhanced scratching behavior in rats, 18 ± 4 , $n=9$, scratching bouts over 70 min compared to 5 ± 2 , $n=4$, in non-injected rats; $P<0.02$). Such behavior has been reported previously for ET-1 injected in mice (Trentin et al., 2006; McQueen et al., 2007; Imamachi et al., 2009) and is evidence for the systemic distribution after injection at the nuchal midline. The subsequent plantar injection of ET-1 at 24 h evoked the same flinching response as that from ET-1 given into the plantar paw of untreated rats. The total flinches over 75 min equaled 103 ± 14 ($n=9$) after systemic ET-1, compared to 100 ± 8 flinches in control naïve rats ($n=9$); over 90 min. the TF count was 113 ± 12 flinches vs. 103 ± 10 flinches in control (for both times $P>0.05$). The injection of ET-1 into the contralateral paw (the third dose of ET-1 per rat) 24 h after this second injection, however, did induce elevated nocifensive flinching [in 7 out of 9 rats, compared to 8 out of 9 rats in control] with TF= 192 ± 11 over 75 min or 232 ± 19 over 90 min ($n=7$) ($P<0.05$). The above response was almost equal to that in the CLP responding to a second injection of ET-1: 163 ± 9 flinches over 75 min, $n=8$, or 209 ± 43 flinches over 90 min ($n=5$), ($P>0.05$). Thus, subcutaneous administration of ET-1 to effect a systemic distribution, at a segmentally unrelated location, neither changed

the immediate response to paw-injected ET-1 nor modified its ability to enhance a subsequent response to a contralateral ET-1 injection.

DISCUSSION

Two major observations are reported here. First, injection of ET-1 into one paw of the rat sensitizes the other paw to chemical and mechanical, but not thermal, stimulation. This contralateral sensitization occurs over several hours, persists for at least one day and is present at a time when sensitization of the ipsilateral paw is waning. Second, the contralateral sensitization depends on afferent transmission from the first injected paw, since it is largely prevented by local anesthetic blockade of impulses in the ipsilateral sciatic nerve innervating that paw. In contrast to these changes in the contralateral paw, the ipsilateral paw quickly becomes profoundly insensitive to ET-1 injections subsequent to the first one, and remains in this less sensitive state for at least 24 h. This insensitivity may not be due to ET_A receptor desensitization, since that phenomenon examined at a cellular level *in vitro*, is correlated with receptor internalization, a process that is completely reversed in a few hours (Cyr and Kris, 1993; Marsault et al., 1993; Bremnes et al., 2000). Other, physiological indications of desensitization to ET-1 *in vitro* have reversed fully by 20 h (Oles et al., 1997), showing how exceptional the long duration of insensitivity to ET-1 is *in vivo*.

Despite its insensitivity to ET-1, the ipsilateral paw develops a significant tactile allodynia and a shorter lasting thermal hyperalgesia. Such differential sensitivity has been reported previously for capsaicin injected into human hairy skin (Ali et al., 1996) and for ligation-induced injury in rat peripheral nerve (Ossipov et al., 1999). Earlier work has shown that tactile allodynia from ET-1 is abbreviated by the local presence of antagonists to NMDA, CGRP1 and TRPV1, (Khodorova et al., 2009b; Balonov et al., 2006) suggesting that acute ipsilateral sensitization is most strongly effected by cutaneous chemical processes and that the insensitivity to ET-1 is not a reflection of some generalized desensitization. At this time the mechanism for ET-1 –induced local insensitivity to ET-1 remains unexplained.

Numerous reports document the existence of bilateral hyperesthesia after unilateral injury or inflammation (Kidd et al., 1995; Amann et al., 1996; Koltzenburg et al., 1999; Sinnott et al., 1999; Oaklander and Brown, 2004; Shenker et al., 2008). Unilateral injection of CFA into the rodent paw results in elevated pre-protachykinin mRNA in the contralateral DRG as early as 6 h (Leslie et al., 1995) and ipsilateral paw injection with NGF increases CGRP-like immunoreactivity in contralateral DRG and sciatic nerve (Amann et al., 1996). Likewise, injection of carrageenan into one rat hind paw results in contralateral thermal and mechanical sensitization 6 hr (but not 3h) later (Bileviciute-Ljungar and Lundeberg, 2000). As we found with ET-1, these changes were not caused by some systemically distributed factors but were clearly dependent on activity transmitted through the ipsilateral peripheral nervous system.

The findings on contralateral sensitization, including the increased contralateral second phase response to formalin, whence spinal cord facilitation is known to be a component (Puig and Sorkin, 1996), imply the involvement of central effects, e.g. involving cells in the spinal cord. It seems that afferent impulses initiated by ET-1's actions in the injected paw (Gokin et al., 2001a) cause a segmental sensitization of the spinal cord which results in relatively long-lasting changes. Inflammation results in bilateral hyperalgesia that requires transmission through efferent aspects of the ipsilateral and contralateral peripheral nervous system (Levine et al., 1985). In the spinal cord, elevated glutamate may activate presynaptic NMDA receptors that are coupled to C-fibers with TTX-resistant impulses, like those activated by ET-1 (Houck et al., 2004), to cause retrograde mechanical sensitization of nociceptors (Parada et al., 2003). Inflammation is also known to induce the release of neuropeptides like CGRP that correlates with hyperalgesia (Ambalavanar et al., 2006; Li et al., 2008), analogous to the release of CGRP

induced by ET-1 and its contribution to ET-1-induced tactile allodynia (Khodorova et al., 2009a). Injury and inflammation also elicit the activation of spinal microglia, which have been implicated in contralateral allodynia (Schreiber et al., 2008). The observation that mechanical and chemical stimuli are strongly sensitized contralaterally by ET-1, whereas thermal responses are smaller and only briefly changed, is consistent with others' findings on distinct subsets of peripheral fibers that subserve these different modalities (Ossipov et al., 1999; Cavanaugh et al., 2009). With respect to the role of the CNS, it is noteworthy that secondary hyperalgesia after s.c. capsaicin injection occurs in response to mechanical but not to heat stimuli (Ali et al., 1996).

Local anesthetic that was applied to the ipsilateral sciatic nerve has been shown to effectively block all afferent impulses in all fiber types for about 30–40 minutes (Gokin et al., 2001b). Contralateral changes from peripheral nerve injury also require activity in peripheral C-fiber afferents (Scott et al., 1995). The large reduction in hind paw flinching from ET-1 under such local anesthesia is consistent with this block, since afferent discharge of C-nociceptors induced by 16–20 nmoles, 10-times the dose of ET-1 used here, lasts for about 30 min after hind paw injection (Gokin et al., 2001a). Interestingly, we observed an approximately 25% decrease in the duration of the functional, analgesic effect from local anesthetic when ET-1 was injected into the paw. Such an effect is consistent with central sensitization, whereby the elevated sensitivity of the dorsal horn nociceptive circuits allows an earlier return of functional response for the same time-course of peripheral nerve block (Lee et al., 1994; Wilder et al., 1996), a sensitization that is proposed in this paper to result from ET-1-evoked impulses. This effect is opposite to the prolongation of lidocaine's conduction block when ET-1 was applied onto the sciatic nerve (Houck et al. 2004), an effect that may result from pharmacokinetic factors (Choi et al., 1997), particularly epineurial vasoconstriction from ET-1 (Zochodne et al. 1992), a process that can result in cutaneous analgesia from ET-1 alone (Shrestha et al., 2009).

In summary, the results of the present study show that an initial injection of ET-1 into a rats' plantar hind paw induces an increased pain responsiveness in the contralateral rat hind paw, an effect dependent on the initial ipsilateral afferent transmission and, secondarily, on central sensitization. We speculate that ongoing peripheral nociceptive input from persistently released endogenous ET-1 under conditions of injury or disease (e.g., cancer) contributes to both central and peripheral sensitization, producing hypersensitivity of different tissues to various chemical and mechanical stimuli.

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List of Abbreviations

CGRP	calcitonin gene related peptide
CNS	central nervous system
DRG	dorsal root ganglion
ET-1	endothelin-1
ET _A	endothelin receptor-A
ET _B	endothelin receptor-B
NGF	nerve growth factor
NMDA	N-methyl-D-aspartic acid

PBS	phosphate buffered saline
TF	total number of flinches
PWT	paw withdrawal threshold
PWL	paw withdrawal latency
LA	local anesthetics
CFA	Freund's complete adjuvant
CLP	contralateral hind paw
IPSI	ipsilateral hind paw

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Fig 1.

Ipsilateral hind paw desensitization of the acute nocifensive flinching response to a repeat ipsilateral ET-1. **A.** The time-course as well as the peak value of hind paw flinch frequency in response to a second, standard dose of ET-1 (200 μ M) is progressively changed by increasing concentrations of ET-1 (2–200 μ M, 20 pmoles -2 nmoles/paw) given in a first injection. **B.** Total flinches in response to the local repeat injection of ET-1 (200 μ M, 2 nmoles/paw) into the same plantar site 24 h after the initial one (range 2–200 μ M ET-1, 20 pmoles -2 nmoles/paw), are fewer than the number in naïve rats receiving the same test dose. * P < 0.05, ** P < 0.001 compared to control (Kruskal-Wallis test followed by Dunn's *post hoc* test applied to raw data).

Fig 2.

Sensitization of the acute nocifensive flinching response to ET-1 in the contralateral rat hind paw. An increase in the Total number of Flinches (TF) is observed when ET-1 (200 μM, dose 2 nmoles/paw) is injected into the contralateral paw (CLP) (A) after one injection ($n=8$), or (B) after two ipsilateral injections ($n=5$). (C) ET-1 at 2 μM (dose 20 picomoles/paw) injected contralaterally after two sequential ipsilateral (IPSI) injections of 200 μM ET-1 also shows an enhanced number of flinches ($n=7$), as compared to the response in naïve rats (different group of rats, $n=12$). * $P < 0.05$ for the repeat, contralateral 200 μM ET-1 (CLP, 24 h), 200 μM ET-1 (CLP, 48 h), or 2 μM ET-1 (CLP, 48 h) compared to the initial (1st, IPSI) flinching response.

Fig 3.

Delayed contralateral sensitization to mechanical stimulation by von Frey hairs in response to unilateral s.c. ET-1 (200 μ M, 2 nmoles/paw). *Ipsilaterally*, the PWT measured when robust nocifensive behavior is over, at 2.5 h after injection, is significantly decreased compared to baseline values and remains so at 24 h post injection. *Contralaterally*, the changes in mechanical sensitivity develop more slowly, with PWT decreased at 24 h after ET-1 injection (two separate groups of rats, $n=6$ each). The mean is the average of 3–4 measurements during a test episode of 10 min. * $P < 0.05$ for ipsilateral PWT compared to ipsilateral baseline values; # $P < 0.05$ for contralateral PWT compared to contralateral baseline values.

Fig. 4.

Unilateral sensitization to thermal stimulation induced by ET-1. **(A)** Thermal hyperalgesia in the *ipsilateral hind paw* is apparent at 20 min after injection of ET-1 (10 μ M, 0.1 nmoles/paw) and persists at this low level for ca. 1–1.5 h ($n=6$). *Contralateral hind paw* shows a moderate brief decrease in PWL only at 42–80 min after ET-1 injection. **(B)** Thermal hyperalgesia in the treated paw has almost disappeared by 3 h, even when a 25-fold higher ET-1 concentration (250 μ M, 2.5 nmoles/paw) was injected ($n=8$). No signs of sensitization were observed 24 h post ET-1 injection in both ipsi- and contralateral paws. * $P < 0.05$ for PWT compared to baseline PWT values in the same paw.

Fig 5.

Potentiation of the nocifensive response to formalin in the contralateral paw. Sensitization of the nocifensive flinching response to formalin in the contralateral rat hind paw after ipsilateral injection of ET-1. Formalin (0.5%, 50 μ l) injected into the *contralateral* rat plantar hind paw 24 h after ET-1 (400 μ M, 4 nmoles/paw) induced more flinches at 10–60 min a.i. ($n=10$), than the number in rats not treated with ET-1 (different group of rats, $n=10$). Both ET-1 and formalin were injected subcutaneously. *Control* ($n=10$): formalin effect in naïve rats ($n=5$) was the same as that in rats injected contralaterally with 10 μ l PBS 24 h before formalin ($n=5$). * $P < 0.05$ for flinching induced by formalin injected 24 h after ET-1 compared with flinching response to formalin in control (naïve and vehicle injected) rats.

Fig 6.

Contralateral sensitization to ET-1 is inhibited by ipsilateral sciatic nerve block during the initial ET-1 injection. Increase in the nocifensive response to a second (contralateral) ET-1 injection into rat hind paw is fully abolished if the initial hind paw ET-1 injection is performed under ipsilateral sciatic nerve block. *Control condition:* The nocifensive flinching response to a second plantar injection of 200 μ M ET-1 (2 nmoles/paw), into the contralateral paw (CLP), was greater ($P < 0.05$) than the response to a first 200 μ M ET-1 injection given 24 h previously into the ipsilateral hind paw (after full recovery from ipsilateral sciatic nerve block by local anesthetics, LA, injected percutaneously into sciatic notch). *Sciatic n. block condition:* Initial flinching “pain” response to ET-1 (injected 5 min after LA) under the condition of sciatic nerve block induced by local anesthetics was highly attenuated (by ~88%). The flinching response to a second injection of 200 μ M ET-1, given 24 h later into CLP ($n = 9$), did not show signs of sensitization, as it was not different from the first control response to ET-1 ($n = 9$, $P > 0.05$).