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LOCAL ANTINOCICEPTION INDUCED BY ENDOTHELIN-1 IN THE HAIRY SKIN OF THE RAT'S BACK

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Abstract

Subcutaneous injection of endothelin-1 (ET-1) into the glabrous skin of the rat's hind paw is known to produce impulses in nociceptors and acute nocifensive behavioral responses, such as hind paw flinching, and to sensitize the skin to mechanical and thermal stimulation. Here we show that, in contrast to the responses in glabrous skin, ET-1 injected subcutaneously into rat hairy skin causes transient antinociception. Concentrations of 1-50 μ M ET-1 (in 0.05 mL) depress the local nocifensive response to noxious tactile probing at the injection site with von Frey filaments, for 30 - 180 mins.; distant injections have no effect at this site, showing that the response is local. Selective inhibition of ET_A, but not of ET_B receptors inhibits this antinociception, as does co-injection with nimodipine (40 μ M), a blocker of L-type Ca²⁺ channels. Local subcutaneous injection of epinephrine (45 μ M) also causes antinociception, through α -1 adrenoreceptors, but such receptors are not involved in the ET-1-induced effect. Both epinephrine and ET-1, at antinociceptive concentrations, reduce blood flow in the skin; the effect from ET-1 is largely prevented by subcutaneous nimodipine. These data suggest that ET-1-induced antinociception in the hairy skin of the rat involves cutaneous vasoconstriction, presumably through neural ischemia, resulting in conduction block.

Perspective—The pain-inducing effects of endothelin-1 have been well-documented in glabrous skin of the rat, a frequently used test site. The opposite behavioral effect, antinociception, occurs from endothelin-1 in hairy skin, and is correlated with a reduction in blood flow. Vasoactive effects are important in assessing mechanisms of peripherally acting agents.

Keywords

endothelin-1; analgesia; anesthesia; antinociception; blood flow; Ca channels

INTRODUCTION

Endothelin-1 (ET-1) is an endogenous peptide of 21 amino acids 31,67, whose effects are mediated by two G-protein-coupled receptors, ET_A and ET_B49,40,41. ET-1, and ET_A and

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ET_B receptors are localized in the epidermis and subcutaneous vasculature¹, as well as in dorsal root ganglia and peripheral nerve, where, respectively, ET_A receptors have been identified on neuronal cell bodies and axons and ET_B receptors on satellite cells and Schwann cells⁵⁵.

The most prominent physiological action of ET-1 is transient vasoconstriction, triggered by activation of ET_A receptors on vascular smooth muscle and a corresponding increase in intracellular Ca²⁺, ⁵⁷. This vasoconstriction is reversed by an activation of ET_B receptors on the vascular endothelium that leads to the release of nitric oxide ⁶⁵. More recent work suggests that ET-1 is an important mediator of hyper-nociceptive responses to injury and disease⁴¹. The use of selective antagonists of ET_A and ET_B receptors has revealed that ET-1 contributes importantly to elevated pain from nerve injury⁴⁶, from inflammation ^{14,19,30}, from cancer ²² and from incision of the skin ⁵⁰.

Because ET-1 was known to be involved in a variety of inflammatory events, the pain associated with its elevation at sites of injury was initially believed to result from its vascular actions. More recently it has been shown that ET-1 applied directly to nerve^{12,18} or injected into the plantar hindpaw²³, at >100μM, leads rapidly to pain in the affected limb. Lower ET-1 concentrations, 2-20μM, injected into the paw result in sensitization of the skin to mechanical and thermal stimulation^{2,19}. These local, acute actions of ET-1 occur within a few minutes, can persist for several hours, and often are followed by desensitization which lasts for 24h or longer¹⁸.

High concentrations of ET-1 injected into glabrous paw skin activate not only ET_A receptors, causing pain behavior, but also ET_B receptors, resulting in anti-hyperalgesia⁴². Such anti-hyperalgesia is mediated in part through the release of the opioidergic peptide β-endorphin from keratinocytes and its subsequent binding to μ-opiate receptors (MORs) on nociceptor endings in the epidermis⁴².

Despite the well-documented vasoconstrictive effects of ET-1, a role for such constriction, and the likely occurrence of local ischemia, has not been established in the pain-related effects of peripherally applied ET-1. It now appears that such events may occur, and even dominate, the overall sensory response to ET-1, although they are not dominant in the glabrous paw skin. In the present study, we report the results of experiments to determine the role of ET-1-induced vasoconstriction and related behavior in the hairy skin of the rat.

MATERIALS AND METHODS

Experimental Animals

All procedures were approved by the Standing Committee on Animals of Harvard Medical School and the Animal Care and Use Committee at Indiana University School of Medicine, Indianapolis, IN. Male, Sprague-Dawley rats (Charles River Laboratories, Cambridge, MA) weighing 225-320g were used for all experiments and were housed in pairs on a 12-hour light:dark cycle with food and water *ad libitum*. For studies measuring nociceptive behaviors, rats were handled for 5-7 days until they were accustomed to sitting still on the bench top for up to 5 minutes at a time, and their local skin responses to innocuous touching had become extinguished (see next passage)⁶³. Rats had the hair clipped on the dorsum of the trunk prior to the day of the experiment.

Graded Response Assessment

Responses were evaluated by *cutaneous trunci muscle reflexes* (CTMR) of subcutaneous muscles responding to stimulation with von Frey filaments, near the dorsal medio-thoracic site of drug injection, and on the contralateral dorsum^{16,50}. The CTMR is a response to noxious tactile stimulation involving the local contraction of skeletal muscle beneath the skin with

concurrent movement of the nearby skin over the rat dorsum⁶⁴. The sensory input for this reflex, after the rats have been handled for several days to accommodate the reactions to light touch, is conducted by fine myelinated and non-myelinated nociceptor afferents⁶⁴. An absence of the response by locally applied agents is considered an indication of local *anti-nociception*, unless the same agent has systemic actions, in which case *analgesia*, mediated by the CNS, cannot be ruled out. The rats were probed with von Frey hairs (VFHs) at points located 0.5, 1 and 2 cm ipsilateral and 2 cm contralateral from the center of the injection site, along a line that is perpendicular to the spinal midline. A “graded response” was assessed by probing the back skin with VFHs of varying stiffness/force, each one applied four times at any given distance with an interstimulus interval of ~2s¹⁶. Each filament was applied perpendicularly to the skin until the tip bent, at which point the force exerted is maximum. Each response is graded **1**, **0.5** or **0**, with **1** defined by a brisk, large amplitude and short latency CTMR response, **0.5** by a slower, smaller response and a grade of **0** indicating no detectable response. The response for 4 applications of any one force/VFH, at a given distance, was then summed and divided by 4 to get the normalized “graded response” (range = 0 - 1).

Measurements using VFHs were made every 15-minute for the first hour after injection and every 30-minute thereafter until the rat’s responses recovered to baseline. Baseline responses were taken for 3 days before any s.c. injections to get an *averaged baseline response* with which to compare the results of injection. Clipping the back on the day before the experiment, to facilitate visualization of CTMR, did not change this baseline responsiveness.

Drugs and injection protocols—All solutions were adjusted to pH 7.4 and the experimenter was blinded to the subcutaneous treatments received by rats. ET-1 (Axxora, San Diego, CA) was freshly prepared by dissolving it in phosphate buffered saline (PBS). ET_A and ET_B receptor antagonists, BQ-123 and BQ-788, respectively, (both purchased from American Peptide, Sunnyvale, CA), were dissolved, respectively, in PBS only and in PBS with 40% DMSO (v:v) at stock concentrations of 6mM. These two antagonists have The final concentration of DMSO, used in the VEHICLE control solutions, had no effect on the behavioral response.

Rats were anesthetized briefly (~1min) with sevoflurane (Abbott Laboratories, North Chicago, IL) delivered by nose inhalation and the pre-shaved back skin was swiped with alcohol. Subcutaneous injections were made on the medial region of the dorsum of the rat’s back, 1 cm from the midline. Circles of radii 1 and 2 cm were drawn around the injection site, for locating test sites (see above) and animals were returned to their cage for recovery.

ET-1 was administered at concentrations of 1, 5, 10, 50 and 100uM (n=4), in s.c. injections of 50 µL. Two forces were applied, a weak force giving ~0.3 baseline score on the graded response scale (1.0 is the maximum value,) and a strong force giving ~0.7 on this scale; sensitivity was followed for up to 5 h, or until the behavioral changes had returned to baseline. To further quantify the effects of drugs, the area-under-the-curve (AUC) was calculated. Area-under-the-curve was determined as the area between the graded response after ET-1, and the baseline graded response value extended over the full time that behavior was tested, usually 3-4 h. Since the graded response is a dimensionless parameter, the AUC has the value of time (hours).

For the experiments testing ET receptor antagonists, rats were co-injected with ET-1 (10uM, 50uL) with or without BQ123 (0.1-3 mM), or BQ788 (3 mM). Control experiments followed the graded response after injection of 50 uL of PBS, with or without DMSO (control for BQ788). These antagonists have very similar affinities for their respective receptors^{35,36} so that comparable blockade of cutaneous receptors probably occurs from the equal, highest dosing used here.

Naloxone was purchased from Sigma Aldrich, as the hydrochloride salt, and was dissolved in PBS to give a stock concentration of 27.5mM, and then equal volumes of 5.5 mM naloxone and 20 μ M ET-1 were mixed to give a final injectate concentration of 2.75mM and 10 μ M, respectively.

Prazosin (Sigma Aldrich) was dissolved in slightly acidified buffer (pH = 6.8) and heated to 60°C to accelerate dissolution, to a stock concentration of 120 μ M then cooled to room temperature for final dilution and injection. Yohimbine (Sigma Aldrich) was dissolved directly in PBS to a stock concentration of 130 μ M and was co injected with ET-1 at 20 μ M.

Local analgesia from ET-1, applied epineurially in vivo, diminishes endoneurial blood flow on rat sciatic nerve⁶⁸, an effect that is inhibited by systemically administered (i.p.) nimodipine, a blocker of L-type Ca^{2+} channels. A stock solution of nimodipine (Sigma Aldrich), for co-injection with ET-1, was made to 24mM by dissolving in 100% ethanol (Fisher Scientific), and then sequentially diluted in PBS to give 40uM, containing <0.2% ethanol.

Cutaneous blood flow measurements

For experiments on cutaneous blood flow measurement by laser Doppler flowmetry, male Sprague Dawley rats weighing 225 to 250 gms were utilized. They were housed in a light controlled room (light from 6:00 to 19:00) at a constant temperature of 22°C. Food and water were available *ad libitum*. Endothelin-1 (ET1) was purchased from Alexis Biochemicals, and nimodipine was purchased from Sigma Chemical Company (St. Louis, MO). The ET-1 was reconstituted in PBS to a concentration of 60 μ M and was stored at -20°C and was diluted to 30 μ M before the experiment. The nimodipine was initially dissolved in 1-methyl, 2-pyrrolidinone (MPL; Aldrich Chemical Co., Milwaukee, WI) to a concentration of 400 mM, and then diluted to 40 μ M in PBS. Blood flow was measured with a BLF21D laser Doppler flowmeter (Transonic systems Inc.). The rats were anaesthetized with sodium thiopental (100mg/kg) throughout the experiment. The hair from the paw was shaved with a trimmer and the animals were placed on a heated platform to maintain body temperature at 37° C. The laser flowprobe was placed on the hairy skin of the back of the paw of the rat. After an initial baseline recording for 15 minutes to establish baseline, 10 μ L of 40 μ M nimodipine or vehicle (0.01% MPL in PBS) was injected intradermally, followed in 5 min by a second 10 μ L injection of 30 μ M ET-1. The blood flow response was recorded for a subsequent 30 min.

Analysis

Behavioral studies—Results were expressed as graded response $\pm$ standard error of the mean (S.E.M.) We used t-test (independent) for comparing groups of two, and 1-way ANOVA for multiple group comparisons. All statistical analyses were performed using Origin 7 (OriginLab Corp., Northampton, MA). A P value <.05 was considered to be significant.

Blood flow measurements—All data are presented as mean response (measured over 3 min) $\pm$ S.E.M. Blood flow was recorded as tissue perfusion units (TPU). To compare the effects of the different drugs on blood flow, the average response over 15 minutes to injection of each drug/combination of drugs was subtracted from the average baseline with needle over 15 minutes to give the average evoked response over 15 minutes. One way ANOVA and Tukey's multiple comparison post hoc test was used to compare the average evoked responses between different treatment groups.

RESULTS

Subcutaneous ET-1 causes local antinociception

Subcutaneous injection of ET-1 resulted in tactile antinociception, measured as reduced or absent responses to von Frey hair stimulation. Antinociception was detected at the first measured time, 15 minutes after injection of 5, 10 and 50 μ M (Fig. 1A, B). Partial antinociception, which lasted for less than 1h, followed injection of 1 μ M or 5 μ M ET-1 and was significantly greater than the response to vehicle only at the 15 min time point (Figure 1A, B). Ten micromolar ET-1 produced significant antinociception which lasted for 3 hours, whereas the maximum, reversible duration of antinociception was seen with 50 μ M ET-1, with antinociception lasting for 4 hours when tested at the weaker force (Figure 1A) and 3 hours at the stronger one (Figure 1B). Although in one study we attempted using 100 μ M ET-1, 3 of the 4 rats injected became hyperactive, constantly licked at the site of injection, defecated more frequently, and died within 2 hours.

Considering the responses to the strong force only, and correcting for the brief antinociception from vehicle, the ET-1 concentration that reduced the CTMR response by half at its peak effect, i.e., that caused 50% antinociception, is $\sim$ 1 μ M. Analysis of area-under-the-curve for these anti-nociceptive actions shows that $\sim$ 5 μ M ET-1 gave about 50% of the maximum area (Figure 1C).

ET-1's antinociceptive effect was also seen at distances of 1 and 2 cm from the injection site, although the durations were shorter at these locations. There was less than 0.25 reduction in the graded response seen at 2 cm from the site of injection at 15-min after 5 μ M ET-1 ($p>0.05$ compared to vehicle), compared to the drop of 0.6, constituting complete antinociception, seen within the injection-raised wheal (0.5 cm).

Tachyphylaxis of ET-1-induced antinociception

A decline of the antinociceptive effect after one injection of ET-1 could be due to cellular uptake of the peptide, 15,53 or to proteolytic degradation¹³, or to desensitization of the active receptors⁸ or their downstream pathways. We tested this second possibility by injecting a second, higher dose of ET-1 (5 μ M, 50 μ L) at 4h after the initial dose of 1 μ M ET-1, when the antinociceptive effect had just returned to baseline. Injecting the first dose gives a robust response similar to the responses observed in figure 1. In contrast, with the second dose there was a much more rapid return to baseline compared to the first dose. The AUC from the second injection of ET-1 was reduced to $75\pm 14\%$, for the strong force, and to $53\pm 35\%$, for the weak force, when compared to responses to a single injection of 5 μ M ET-1 (Figure 2).

ET-1 induced antinociception is mediated by ET_A receptors

The receptor specificity for antinociception was determined by co-injecting animals with ET-1 plus selective ET receptor antagonists. Treating rats with an ET_A receptor antagonist, BQ-123, effectively abolished ET-1-induced analgesia in a concentration-dependent manner (Figures 3A, 3B, and 3C) with peak antinociception blocked by more than 70% for the weak force, and more than 90% for the strong force (Figure 3E). Analysis of maximum possible effect (%MPE) for this inhibition gave an IC₅₀ of $\sim$ 0.3mM. In contrast, blockade of ET_B receptors with the selective antagonist BQ-788, at 3mM, had no effect on ET-1-induced analgesia (Figure 3D), showing the exclusive involvement of ET_A receptors in this phenomenon.

Opiate receptors are not involved in ET-1's antinociceptive effect

Previous studies have shown that an antinociceptive action of ET-1, mediated through ET_B receptors, in the rat's glabrous skin involves the direct participation of β -endorphin and is blocked by antagonists of μ -opiate receptors⁴⁰. We tested for such a mechanism in the

antinociceptive actions of ET-1 in the hairy skin. Co-injection of the non-selective opiate receptor antagonist naloxone (2.75 mM) with 10 μ M ET-1 did not alter antinociception (data not shown), showing that opiate receptors are not involved in the hairy skin.

A role for L-type calcium channels in ET-1-induced antinociception

Earlier studies had shown that ET-1 applied to the rat sciatic nerve *in vivo* caused analgesia and conduction block, accompanied by a transient reduction in endoneurial blood flow⁶⁸. These effects were prevented by systemic (i.p.) administration of nimodipine, a selective inhibitor of L-type voltage-gated Ca²⁺ channels. To determine if local antinociception caused by s.c. ET-1 also involved such Ca²⁺ channels, we co-injected nimodipine (40 μ M) with ET-1 (10 μ M). This concentration of the channel inhibitor strongly attenuated the effects of ET-1 (Figure 4), although lower concentrations, of 5 μ M or 10 μ M, were ineffective (data not shown).

Epinephrine's antinociceptive effect

In order to determine if vasoconstriction and the associated reduction in blood flow could, in general, produce antinociception, we examined the effects of the classic vasoconstrictor epinephrine³. Subcutaneous injection of epinephrine (45 μ M) in the back resulted in transient antinociception, detectable at 15 min and lasting for about 2h (Figure 5 A,B), as previously reported⁴³. When epinephrine was co-co-injected with the α_1 - adrenoreceptor antagonist prazosin (10 μ M) the antinociceptive effect was reduced by more than 90% (Figures 5 A-C). Antinociception from epinephrine selectively involved α_1 - adrenoreceptors since yohimbine, an α_2 -adrenoreceptor antagonist, did not significantly affect analgesia induced by epinephrine (Figure 5C).

Endothelin receptors are present on many types of cells in the skin, including those in vascular tissue and in keratinocytes themselves (see review⁴¹,) and it is possible that a series of linked stimulus-release reactions could couple exogenous ET-1 to the release of endogenous epinephrine and subsequent activation of α_1 -adrenoreceptors, and thus cause antinociception. This possibility was eliminated by results that showed that co-injection of ET-1 with prazosin, at the same inhibitor concentration that almost abolished epinephrine-induced analgesia (10 μ M), had no effect on ET-1-induced antinociception (Figure 5C).

The concordance between cutaneous analgesia and reduced blood flow

The question remains whether a common vasoconstrictive effect, caused by either ET-1 acting through activation of ET_A receptors or by epinephrine acting through α_1 - adrenoreceptors, might account for the antinociceptive effect of these agents. To initially test this notion, we confirmed that ET-1 at 30 μ M, a concentration within those that produced antinociception, caused local vasoconstriction. Laser-Doppler measurements of blood flow in the hairy skin showed a rapidly developing, constant reduction of flow for at least 50 mins after injection of 30 μ M ET-1, from a baseline value of 18.3 ± 1.6 TPU to 6.9 ± 0.6 TPU (Figures 6A and 7). Injection of nimodipine (40 μ M) 5 min prior to the injection of ET-1 almost abolished the reduction in blood flow caused by ET-1; the blood flow decreased from 19.6 ± 1.2 TPU to 15.7 ± 2.4 TPU (Figures 6B, 7). Nimodipine by itself had no effect on blood flow (Figure 6C), nor did the injection of vehicle (Figures 6D, 7).

Discussion

This paper reports that: 1) subcutaneous injection of ET-1 causes a local, transient antinociception to punctate mechanical stimulation of the hairy back skin of the rat that is concentration-dependent, with complete, reversible absence of response lasting for ~3 h with 50 μ M ET-1; 2) antinociception correlates pharmacologically with reduced blood flow, being mediated exclusively through ET_A receptors and involving L-type Ca²⁺ channels. ET-1 is

known to have many diverse biological actions, including its traditional vasoactive effects, positive inotropic actions on the heart, and stimulation of gene expression^{49,32}. However, this is the first report of *antinociception* resulting from ET-1. In contrast, it was previously reported that ET-1's injection into the glabrous skin of the rat plantar hindpaw, or application directly to sciatic nerve, causes ET_AR-dependent flinching behavior^{12,18,23}, which is inhibited by morphine, testifying to the pain-like nature of this response. Injection of ET-1 into the joints causes motor incapacitation, apparently due to elevated pain¹⁹ and tissue levels of ET-1 are elevated in arthritic conditions, suggesting a role for endogenous ET-1 in arthritic pain¹⁴.

Although both ET-1 and epinephrine appear to cause antinociception in hairy skin primarily through vasoconstriction, they operate via different pathways. Epinephrine is a less potent vasoconstrictor⁴⁴, and, consistent with this, antinociception resulting from epinephrine (45uM) develops more slowly over time, with an initial 10-20min latency before detectable functional loss after injection, and has a smaller effect, a maximum loss of response to pinprick, of ~80%, occurring 30-40 min after injection with full recovery by 90 min⁴³. In contrast, ET-1 (50uM) produces a more rapid onset, causing complete antinociception at 15 min after injection that lasts for several hours. Such a rapidly developing block is consistent with the very rapid drop in blood flow that occurs after ET-1 injection (Figure 6). Interestingly, epinephrine caused observable blanching of the injected hairy skin but ET-1 caused no local pallor or flare. Perhaps the locations of the ET_A receptors that cause vasoconstriction in the skin are different from those for the α_1 -adrenoreceptors, even though they result in the same gross vascular response.

Nimodipine attenuated ET-1 induced antinociception through its selective blockade of L-type channels. ET-1 is known to activate a phosphoinositide cascade, which in turn produces IP₃ that triggers Ca²⁺ release by mobilizing intracellular stores and extracellular Ca²⁺⁵⁹. Our finding lends further support to the role of ET-1 in a biphasic elevation of cytosolic Ca²⁺⁵⁸ and acute vasoconstriction of microvessels induced by epineurial ET-1, resulting in endoneurial ischemia and axonal conduction block⁶⁸.

A second injection of ET-1, delivered after recovery to baseline of the antinociception from the first injection, produced an attenuated effect, demonstrating tachyphylaxis, as seen with nociception induced by direct application of ET-1 to sciatic nerve¹⁸. Such tachyphylaxis is consistent with the known desensitization of the ET_A receptor^{8,21}.

The question remains whether there is an anatomic or physiologic basis for the opposite effects on pain from ET-1 injected into hairy versus glabrous skin. The answer is complex, because the behavioral effects of ET-1 result from the local effects on cutaneous neurons, on skin cells, e.g., keratinocytes, that release neuroactive substances^{42,61}, and on vascular tissue essential for supporting local metabolism as well as for delivering bioactive agents. Some differentiating features between glabrous and hairy skin are listed in Table 1. In the glabrous skin, pain from s.c. exogenous ET-1 is suppressed by local co-injection of the ET_B receptor agonist, IRL-1620, in a naloxone-reversible manner, a reflection of a local opioidergic mechanism for ET-1-induced anti-hyperalgesia^{40,42}. This mechanism was not involved in the antinociception observed here, since naloxone had no effect on the reduced response to tactile stimulation caused by ET-1. There are several possible explanations for this difference between skin types, including an absence of ET_B receptors on keratinocytes in hairy skin, a lack of coupling between such ET_B receptors and release of β -endorphin, or an absence of μ -opiate receptors on nociceptor endings in hairy skin. Although data are not available for rat skin, human hairy skin is known to express both β -endorphin in keratinocytes and μ -opiate receptors, on both keratinocytes and non-myelinated nerve endings⁴. However, the presence of ET_B receptors on keratinocytes and their coupling to release β -endorphin has not been shown in hairy skin.

In glabrous skin epinephrine, like ET-1, exerts the opposite behavioral effect that it does in hairy skin.³⁹ Acting through β -adrenoreceptors, epinephrine induces tactile hyperalgesia in the glabrous skin, contrasting with the α -adrenoreceptor-induced anti-nociception in hairy skin. Although the authors of this study proposed from in vitro experiments that epinephrine is acting directly on cutaneous afferents to induce hyperalgesia³⁹, others have presented in vivo results that suggest that epinephrine (released from sympathetic nerve endings) only modulates cutaneous afferents indirectly, through vascular control.¹⁷ It seems apparent that interactions between nociceptive afferents and blood vessels in the skin are reciprocal and involve several potential feedback loops^{17,27-29} and that ET-1 may influence these two systems in a number of different, dynamically responding ways.

We hypothesize that the pro-algesic actions of ET-1, initiating nociceptor firing via ET_A receptors, occur in both glabrous (paw) and hairy (back) skin, but that the anti-hyperalgesic, vasoconstrictive actions dominate on the back and are less prominent on the paw. Both vasoconstriction and vasodilatation have been observed after ET-1 administration, the former occurring at higher concentrations of ET-1 than the latter^{10,44}. Whereas vasoconstriction was inhibited by Ca²⁺ channel blockers^{44,45}, as observed in the present study, vasodilatation from ET-1 was blocked by local H₁ antagonists and also by s.c. local anesthetics, suggesting a mechanism involving the local release of histamine¹⁰. We did not see any evidence of increased blood flow after ET-1 in the Laser-Doppler flowmetry records from the hairy skin, but we have observed a delayed rubor in the plantar paw following an initial blanching after ET-1 injection there (initial blanching of the paw develops at ~1 min and lasts for 15-24 min after subcutaneous injection of 200 μ M ET-1⁻¹; 10 μ M ET-1 also induces blanching, that appears no sooner than 5-6 min after injection. A. Khodorova, personal observations). Activation of ET_A receptors known to be present on smooth muscle cells of the neural microvessels^{9,11} causes a temporary, local reduction in blood flow^{6,7,10}. Studies using radionuclide (¹³³Xe) clearance techniques to measure skin blood flow⁶⁰ have shown a > 60% reduction in flow caused by 10 pmol ET-1 injection^{7,10}, consistent with our own finding (albeit after 250 pmol of ET-1), but using Laser Doppler flowmetry⁶². It is noteworthy, however, that ¹³³Xe clearance measures the blood flow through all layers of the skin, whereas Laser Doppler flowmetry selectively captures the flow from the more superficial layers³⁴. The overall effect on blood flow by ET-1's sub-dermal injection is likely to result from the difference between vasoconstrictive and vasodilatory actions, perhaps with the former dominating in the back and causing anti-nociception, and the latter occurring in the paw. Vasoconstriction may also result from a stronger effect of ET-1 on sub-dermal vessels, located in the deeper levels that are closer to the locus of ET-1's delivery. A differential control of superficial compared to the deeper cutaneous vasculature in glabrous skin is known to result from the differential innervation by primary afferent and sympathetic fibers²⁷, with the former accounting for local vasodilation and the latter for vasoconstriction.²⁹

With respect to differences in vasculature, back skin is known to contain predominantly nutritive, capillary vessels with high resistance to flow while paw skin has a high density of arterioles and venules, with low resistance, high flow vessels, at a 3-fold higher density than in the back⁵⁶. ET-1 is reported to contract isolated human resistance vessels in vitro³³, consistent with a stronger vasoconstriction on the back than on the paw skin. Skin vascular anatomy and physiology alone, therefore, might explain the difference in pain reactions to ET-1.

The innervation of hairy and glabrous skin also differs, with a substantially higher density of epidermal neurites in the glabrous skin^{32,38,51}. Physiological responses to thermal stimuli differ between these skin types in humans, with a lower heat detection threshold but a higher heat pain threshold in the palm than on hairy skin²⁴, correlated with the abundance of heat-sensitive C-fibers in the glabrous skin. Glabrous skin also contains cold-activated fibers which

are virtually absent in hairy skin 54, as well as generally smaller receptive fields for mechano-receptive afferents that are sensitive to punctate stimuli. 54 However, there are no general rules for differences in sensory innervation between these skin types, exemplified by similar densities of Merkel cells 20 and of CGRP-expressing neurites 47 in both skin types but differences in the acid-sensing ion channel expression between them 37. It is possible that the generally lower density innervation of the hairy skin accounts in part for its greater behavioral susceptibility to ischemia, since equal degrees of nerve block in glabrous and hairy skin might leave the latter with a subliminal afferent output when the former was still able to evoke sensation.

Although we have no direct evidence of conduction block from ischemia in these studies, others have shown that ischemia of peripheral nerve leads to increased neuronal accommodation⁵ and slowing of conduction and lengthening of the refractory period²⁶, all three being indicators of reduced axonal excitability. These changes in conduction parameters were made in large myelinated axons, and it was determined that prolonged membrane depolarization could not alone account for the conduction defects, suggesting that some neuroactive substance or metabolic shift had a direct effect on Na⁺ channel inactivation⁴⁸. If such a substance also is elevated by ischemia of small, cutaneous afferents then, given the much larger surface:volume ratio in these fibers, the putative blocking substance could reach even higher concentrations. But regardless of the mechanism, there is ample published evidence of ischemia-induced peripheral nerve block that would couple reduced blood flow to sensory deficits.

In conclusion, sensory losses, exemplified by the antinociceptive effects of ET-1, can result from local vascular actions of many types of neuroactive agents. Such deficits will affect physiological and behavioral assays of drug actions, and can thereby confound analyses when only targets on neuronal cells are considered. A broad accounting of the several factors that can alter neuronal activity in vivo is necessary in understanding drug actions.

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Figure 1.

ET-1 injection under the rat's hairy back skin produces a dose-dependent antinociception. Testing at 0.5cm from the center of the wheal formed by injection, at a medio-dorsal location at time = 0, of ET-1 (50uL) of different concentrations, using von Frey filaments with force, (A) 66mN and (B) 172mN. (▼, 1uM n=4), (●, 5uM n=6), (▲, 10uM n=8), (■, 50uM n=6) or vehicle (○, PBS n=6). ($P < 0.05$ when compared to PBS for [ET-1]: 1uM(##), 5uM(**), 10uM(#) and 50uM(*); (C) Area under curve (AUC) vs. [ET-1]. Curve is an empirical fit to data.

Figure 2.

Comparison of responses to a dose of ET-1 (5uM, 50uL), delivered by a second injection, between rats who received either PBS (■) or ET-1 (□, 1uM) for the first injection. The second injection was delivered 4 h after the first one, a time when the initial antinociceptive effect from 1 μ M ET-1 returned to baseline. Tests made using two forces, (A) 66mN and (B) 172mN. (n=4 for both groups). * indicates a significant difference between animals receiving a first injection of ET-1 and animals first injected with PBS.

Figure 3.

Effects of ET receptor antagonists on ET-1-induced antinociception. (A-C) The ET_A receptor antagonist, BQ-123, coinjected with ET-1 (10μM) at **A**(0.1mM), **B**(0.3mM), and **C**(3 mM), and **D**, the ET_B receptor antagonist BQ-788 (3mM) coinjected with 10 μM ET-1. Tests using a 172 mN von Frey filament for rats injected with antagonists plus ET-1 (■) or vehicle plus ET-1 (●) ET-1 (n=6, **P<.001). (**E**) BQ-123's dose-dependent suppression of ET-1 induced antinociception. Graded response measured at 30 min after ET-1 injection; using 66 mN (open bars) or 172 mN (filled bars), n=6; *, ** P<0.01 for PBS vs. ET-1; #, ## P<0.01 for BQ-123 + ET-1 vs. ET-1.

Figure 4.

Nimodipine's (40uM) effect on ET-1 (10uM) induced antinociception at the primary site of injection using two forces, (A) 66mN and (B) 172mN. ET-1 (□, n=6), Nimodipine + ET-1 (■, n=6, **P<.001).

Figure 5.

Epinephrine causes antinociception on the rat's back skin. Responses to tactile stimulation at the injection site with epinephrine alone (□, 45μM n=6) or with epinephrine (45 μM) plus prazosin (●, 10μM n=4), using two forces, (A) 66mN and (B) 172mN. C. Averaged changes in peak antinociceptive responses to 66mN (open columns) and 172 mN (filled columns) von Frey filaments after injections of epinephrine alone (45 μM) or epinephrine with prazosin (PRA, 10μM) or yohimbine (YOH, 20μM) and ET-1 alone (10μM) or with prazosin (10μM). Differences between response at baseline and responses at 45 min post-injection for each treatment; n=6; only the addition of prazosin caused a significant attenuation of epinephrine's action: *, ** P<.01.

Figure 6.

ET-1 induced vasoconstriction is attenuated by nimodipine. Measurements of blood flow in the hairy skin using Laser Doppler Flowmetry, showed a rapidly developing, constant reduction of flow for at least 50 mins after injection of 30 μ M ET-1(A). This effect was significantly attenuated by pretreatment with 40 μ M nimodipine (B), whereas vehicle injection (D) or nimodipine alone (C) had no effect on blood flow.

Figure 7.

Average flow in baseline period (left panel) and during the 30 min after ET-1's injection (middle panel) with various treatments. The averaged differences between these two conditions are graphed in the right-hand panel. Flow reduction by 30 μ M ET-1 was significant at about 60%. Co-injection of nimodipine (40 μ M) with ET-1 strongly suppressed the reduction in blood flow caused by ET-1, whereas nimodipine alone had no effect on blood flow. * indicates a significant difference in blood flow compared to vehicle alone and # indicates significant difference in animals treated with nimodipine and ET-1 versus ET-1 alone.

Table 1
Comparison of Responses and Properties of Glabrous and Hairy Skin

Treatment/Parameter	Glabrous Skin	Hairy Skin
ET _A activation	pain, sensitization vasoconstriction	anti-nociception vasoconstriction
ET _B activation	anti-hyperalgesia	no effect
Epinephrine	hyperalgesia, via β -adrenoreceptors 39	anti-nociception, via α -adrenoreceptors
Naloxone	blocks ET _B 's anti-hyperalgesic effect	no effect
cold receptors ⁵⁴	present	absent
size of punctate RF for penetrating stimuli ⁵⁴	Smaller	Larger
temperature-induced sympathetic vaso-response	decreased vasoconstriction when T increased	Increased vasoconstriction when T increased
antidromic vasodilatation ²⁸	Neurokinins + CGRP	CGRP
dynamic auto-regulation of blood flow ⁶⁶	evident	weak

Superscripts refer to citations in the reference section. If no superscript, then observations are in this paper.