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ENDOTHELIN RECEPTORS AND PAIN

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INTRODUCTION

The endogenous endothelin (ET) peptides participate in a remarkable variety of pain-related processes. Pain that is elevated by inflammation, by skin incision, by cancer, during a Sickle Cell Disease crisis and by treatments that mimic neuropathic and inflammatory pain are all reduced by local administration of antagonists of endothelin receptors. Many acute effects of endogenously released endothelin can be simulated by local administration of endothelin, which at high concentrations causes pain and at lower concentrations sensitizes the nociceptive reactions to mechanical, thermal and chemical stimuli.

This review covers the topic of ET-1 and pain in five sections. The first section describes the biochemistry, second messenger pathways and receptor coupling that are activated by ET receptors. The second section summarizes the cellular physiological responses to ET receptor activation, emphasizing those neuronal responses that might account for nociceptor activation or sensitization. The third section begins with a description of the pharmacological analysis of receptors involved in pain caused by activation of the different ET receptors by exogenous ET-1, and then segues to a description of the neurophysiological (and vascular) effects in vivo. The final two sections review the evidence for the involvement of endogenous ET-1 and its receptors in pain arising from peripheral inflammation, injury and diseases, including cancer, and finally, the modulation of pain by ET receptors in the CNS. Our goal is to broadly frame the subject of endothelin and pain and to present the generally accepted as well as the disputed concepts, including important unanswered questions.

1. BIOCHEMICAL PROPERTIES

1.A. Structure of endothelins and their receptors

Endothelins are a family of naturally occurring peptides 261, with growth-promoting, vasoactive and nociceptive properties affecting the function of a number of tissues and systems, including somatosensory, respiratory, circulatory, endocrine, urogenital, visual, digestive and central nervous systems 204. To date, there are three known, 21-aminoacid long endothelins

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in humans (i.e. ET-1, ET-2 and ET-3) produced from as many distinct genes located on chromosomes 6, 1 and 20, respectively 24. Genes for ET-1, ET-2, ET-3 are also present in bovine and porcine tissues, suggesting that endothelins are produced in all mammalian species 111,208 Endothelins have overlapping tissue distribution with ET-1 being the most widely expressed. 148,224 Endothelin-1 is synthesized and released by epithelia, 1 and also produced by cardiomyocytes, leukocytes and macrophages, 143 endothelial cells 149.

Endothelin-1's actions are primarily local, in a paracrine (ET-1 secreted by one cell acts on neighboring cells) or autocrine (ET-1 released acts on the cell producing it) manner. The immediate actions are usually brief, since all endothelins are rapidly degraded by extracellular proteases, the endothelin converting enzymes (ECE's).53,109 Endothelins share high homology with sarafotoxins, a family of cardiotoxic peptides from snake. 225

All endothelins and sarafotoxins act via two subtypes of G protein-coupled receptors, termed ET_A 5 and ET_B,209 which are encoded by two distinct genes found on chromosomes 4 (4q31.2) and 13 (13q22), respectively. 210 The human ET_A and ET_B receptor share ~51% amino acid sequence identity, with higher homology in the transmembrane regions 64. In particular, transmembrane domains 2 and 5 have been shown to play a crucial role in subtype-selective ligand binding and peptide docking. 25,136,174,203,241 Several splice receptors have been variants of ET_A26,96,158 as well as ET_B65,165,222 described for which physiological roles remain unconfirmed. The ET_A receptor is abundant in vascular smooth muscle cells where its activation elicits an increase of intracellular calcium, leading to a long-lasting contraction. 152 The ET_B receptor is predominantly found in endothelial cells where it stimulates the release of nitric oxide (NO) thereby causing the relaxation of vascular smooth muscle cells. 242 However, in cardiac tissue ET_A receptors are also found in endothelial cells, where they mediate ET-1's elevation of intracellular Ca²⁺ 114 while in the stomach ET_B receptors are located on smooth muscle cells and may mediate contraction. 160 Despite these often separate and distinct tissue distributions, 105 ET_A and ET_B receptors also appear to be co-expressed in a number of cells, including smooth muscle cells, 99 astrocytes, 93 epithelial cells of the choroid plexus 4 and the anterior pituitary 92 and cardiac endothelial cells. 114

ET_A and ET_B receptors display different ligand selectivity. ET_B binds ET-1, ET-2 and ET-3 with about equal affinities whereas ET_A binds ET-1>ET-2>>ET-3 210. Specific competitive peptide or non-peptide antagonists of ET_A as well as antagonists and agonists of ET_B receptors have proven to be important tools to study the function of each receptor type 54.

Some evidence suggests an interaction between separate ET_A and ET_B receptors expressed in the same cells. In ET_A receptor-transfected human Girardi heart cells, which intrinsically express ET_B receptors, the stimulation of ET_A receptors with ET-1 results in a lowering of the affinity for ET_B receptors for both the agonist BQ-3020 and the antagonist BQ-788, indicating intracellular cross-talk between ET_A and ET_B receptors. 176 We hypothesize that such interactions between ET receptor subtypes could be of particular importance in pathological conditions and may contribute to determining which of the dual roles of ET_B receptors, pro- or anti-algesic, is the dominant one (see 3.D., below).

1.B. G Protein coupling and intracellular pathways

The coupling of ET receptors to heterotrimeric G proteins has been extensively studied, mostly using heterologous expression systems, in an attempt to clarify the cellular pathways underlying the different actions of ETs (e.g., rapid intracellular signaling, mitogenesis, cell-growth, etc.). A survey of the literature indicates that both ET_A and ET_B receptors are able to couple to pertussis toxin-sensitive (G_{α_i}) as well as insensitive (G_{α_{q/11}}) families of G proteins, although the particular G_α-subunit depends on the cell type. 6,106,237 These findings are corroborated by a heterologous expression study of ET_A or ET_B in Chinese hamster lung

fibroblasts (CCL39), reporting that the affinity for various G proteins varies between the ET_A and ET_B receptors, with G_{ai}3 interacting to a greater extent with ET_A while G_{ai}1, G_{ai}2 and G_{aq}/11 are more likely to interact with ET_B.²²¹ Both receptors appear to couple to IP₃ production through G_{aq}, ostensibly activating PLC and PLD 3, as well as to cAMP, through adenylyl cyclase inhibition,²²⁶ or to cGMP^{219,220} through guanylyl cyclase activation by NO.¹⁰¹ Moreover, ET-1 has been linked to the modulation of a plethora of signaling pathways, including the increase in cytosolic [Ca²⁺],¹³³ activation of phospholipase A₂,⁶ regulation of Na⁺/H⁺ exchange,²⁵⁰ production of NO²³², extracellular-signal regulated kinase^{1,2} (ERK1/2) activation, protein kinase C or phosphatidylinositol-3-kinase stimulation^{97,118,171} and activation of the ras/raf/mitogen-activated protein kinase pathway.^{125,264} The pathways mediating the mitogenic activities of ET_A receptors are being unraveled in relation to their role in cancer.¹⁰ They involve protein kinase C as well as phosphatidylinositol-3-kinase,²¹⁸ while ET_B receptor mediated cell proliferation appears to be mediated by a PKC-independent pathway.²³¹

Although heterologous expression systems have contributed much to our knowledge of receptor coupling, the association of endothelin receptors with different G proteins and the activation of signaling pathways in the various native cells, in normal and pathological conditions, remains largely unknown. One particular area concerns the ability of G_{βγ} subunits released by the activation of ET receptors to modulate the activity of effectors such as PLC β , ACs, ion channels, kinases and components of the synaptic vesicle release machinery, all effects which occur with other GPCRs.^{140,157,239}

1.C. Desensitization, internalization and recycling

Both ET receptor types are desensitized by phosphorylation through the G protein-coupled receptor kinase type 2 (GRK2),⁷³ subsequently uncoupling the receptor from its G protein. Both ET_A and ET_B receptors are internalized via clathrin-dependent pathways with very different outcomes. ET_A receptors are recycled back to the surface^{29,177,178} whereas ET_B receptors are sorted to the lysosome and degraded.^{84,172} This is seemingly linked, in neuronal cells, with the presence of a postsynaptic density-95/disc-large/zona occludens (PDZ) binding motif in the cytoplasmic carboxyl-terminal region of the ET_A receptor that is lacking in the ET_B receptor.¹⁷⁸

Clearance of ET-1 from the circulation is mediated largely through ET_B receptors expressed on endothelial, lung and kidney cells by a mechanism involving uptake and subsequent degradation by neutral endopeptidase.^{29,59,75,119,173} This is also the case in the central nervous system where the astrocytic ET_B receptor is responsible for the elimination of accumulated extracellular ET-1,⁹⁴ a feature linked to the ability of ET_B to form a much more stable complex with ET-1 than does ET_A.²³⁶

2. CELL PHYSIOLOGICAL RESPONSES TO ENDOTHELINS

2.A. Endothelin receptor coupling in pain

ET_A receptors have been found on a large proportion of the cell bodies of small diameter sensory neurons (DRGs) which are associated with C- and A δ -fibers that carry pain impulses.¹⁹² Moreover, activation of these nociceptive fibers by ET-1 is blocked by BQ-123, an ET_A receptor antagonist.⁸¹ Together, these studies strongly argue for a selective role of peripheral ET_A receptors in the induction of pain through nociceptive fibers.

The receptor pathways involved in pain generation by ET-1 are complex. ET_A receptor activation sometimes elicits a rise in intracellular calcium^{259,272} which is probably mediated by G_{aq}/11, acting through PLC to release Ca²⁺ from intracellular stores, and also through stimulation of guanylyl cyclase (GC). The latter enzyme converts GTP to cGMP, which can

activate protein kinase G, effecting an anti-nociceptive response. 207 (This pathway is complicated, however, since cGMP is also elevated by NO, 60 a substance that has a positive role in mediating inflammatory hyperalgesia. 2 In addition, GTP itself enhances the activation of Na^+ current in small sensory neurons, 11 in a PKC-dependent way, 12 an effect that would be diminished by the nucleotide's conversion to cGMP.) From this complex of information it is not possible to determine the overall change in sensory processing that results from cGMP's elevation by ET-1.

In addition to elevating Ca^{2+} in peripheral sensory neurons, ET-1 stimulates (Ca^{2+} - independent) PKC- ϵ mediated phosphorylation and concomitant functional enhancement of the TRPV1 channels present on nociceptive C-fibers. 188 Potentiation of TRPV1 most likely occurs in concert with a modulatory effect on tetrodotoxin (TTX) -resistant Na^+ channels 273 (see 2.B., below), which is also known to be effected by PKC. 82 The G protein α - or $\beta\gamma$ -subunits mediating these effects are presently unknown, as is the identity of their direct target, although a Phospholipase C enzyme is a probable candidate (see Figure 1).

ET_B receptors appear to be mainly expressed in DRG satellite cells and ensheating Schwann cells 192 where it is thought that they induce the synthesis and release of prostaglandin E_2 , a compound involved in inflammatory pain. 135 ET_B receptors are also found on keratinocytes. Importantly for pain, ET_B receptors are known to mediate the release of β -endorphin from keratinocytes and thereby produce a local analgesic effect. 129 The signaling pathway responsible for this effect remains to be investigated, although G_s , G_{i2} and G_{i3} have all been detected in keratinocytes, the major cell type in the skin's epidermis. 235 In these cells adenylyl cyclase activation is thought to mediate a rise in intracellular calcium 175 along a pathway which might lead to β -endorphin release (see Figure 2).

2.B. In vitro effects on ion channels and transmitter release

Isolated sensory neurons are stimulated by ET-1 to elevate cGMP 60 and to release neuropeptides and glutamate, actions probably resulting from an ET_A -dependent elevation of intracellular $[\text{Ca}^{2+}]$. 60,272

Primary cultures of keratinocytes also are stimulated by ET-1, with separate studies showing ET_B receptor-mediated release of the opioid peptide β -endorphin 129 and of ET-1 itself. 265,266 The latter comprises an autocrine loop, but with a biphasic dynamic response; immediately upon exposure to ET-1 keratinocytes show elevated intracellular Ca^{2+} , which would be expected to cause an acute increase in the release of many substances, including ET-1 itself, but after prolonged (24 h) exposure to ET-1, its basal release by keratinocytes is suppressed, probably by negative feedback on its synthesis. 266 Without this dampening, counteracting mechanism, the initial release of a small amount of ET-1 would further activate ET_B receptors on keratinocytes and continuously stimulate its own release. The β -endorphin released by keratinocytes exerts an analgesic and anti-hyperalgesic action, binding to μ -opiate receptors on the nociceptor nerve endings and opening G protein-sensitive K^+ channels, thereby lowering excitability and the generation of action potentials. 129

ET-1 biphasically modulates N- and L-type Ca^{2+} channels in neurons, causing an initial depression followed by a long lasting facilitation. 169 ET-1 also induces neuronal depolarization and non-selective inward cationic currents, both predominantly calcium-mediated, followed by hyperpolarization, perhaps due to Ca^{2+} -activated K^+ currents. 233 In addition, ET-1 is capable of increasing $[\text{Ca}^{2+}]$ in neurons with an important contribution from store-released Ca^{2+} ions. 272,259

It is noteworthy that TRPV1 and other TRP receptors have been identified in keratinocytes 45,227 which contain neuroactive substances that can be released by stimulation. 102,227 One

scenario for ET-1's peripheral actions might involve its initial release from directly, externally stimulated keratinocytes and the autocrine activation of ET receptors on the same and adjacent keratinocytes, followed by the subsequent release of substances that would activate epidermal nerve endings, e.g., glutamate and CGRP (see below), amplified by the modulation of TRPV1 (and possibly, other TRPs) in both keratinocytes and neurons (Figure 2).

As noted above, the soma and axons of sensory neurons contain functional ET_A receptors, visualized by immunocytochemistry, 192 and causing excitatory actions. ET-1 enhances excitation of isolated sensory nerve soma, particularly of small and medium diameter cells, at least in part by altering the activation of voltage-gated Na⁺ channels. 273 This action is prominent for the slowly inactivating, tetrodotoxin-resistant (TTX-R) Na⁺ channels that occur in nociceptive cells, e.g. Nav1.8 and Nav1.9. The TTX-sensitive channels, which are expressed predominantly in large sensory neurons but are also present in nociceptors, are not altered by ET-1. The effect on TTX-R is a "negative shift" in the membrane potential-dependence of channel opening, to increase the probability of a Na⁺ channel opening at potentials positive to the resting potential and effecting an increase in membrane excitability. Such increased excitability also occurs in vivo when ET-1 is injected around nerve (see 3.F., below).

Very similar effects on TTX-R of sensory neurons follow their exposure to inflammatory mediators such as PGE₂ 66,82 and 5-HT, 39 and further studies of these sensitizing agents indicate that they act through PKC- and PKA-linked pathways. 82 Furthermore, in unpublished results from our laboratory we have observed that ET-1, like NGF 270, suppresses the outward delayed rectifier K⁺ current, I_K, in most sensory neurons, an action that will enhance the pro-excitatory effects of TTX-R's activation shifts. It is likely that ET-1 uses similar or identical pathways to modulate these same channels, and others, e.g. TRPV1 (see above), allowing the confluence of pathways from several types of receptors to effect changes in, e.g., TRPV1, TTX-R and I_K, in order to sensitize nociceptive neurons.

3. EFFECTS OF EXOGENOUS ENDOTHELINS IN PAIN

3.A. ET_A receptor activation triggers acute nociception

There appears to be no tonic, resting contribution of ET-1 to physiological pain. Injection of antagonists to ET_A or ET_B receptors has no effect on the nocifensive responses to noxious thermal or mechanical stimulation. 7,86,163 Exogenous endothelin-1, however, elicits pain when injected into several species of mammals at different peripheral locations: verbally reported pain when injected into the human forearm, 51,91,126 overt nociceptive behavior when injected into the knee joint of dogs or rats, as well as the peritoneal cavity and hindpaw foot pad of mice and rats 55,56,70,81,156,186,195,196 or applied epineurally onto or injected intraneurally into the rat sciatic nerve. 52,68 The peptide also sensitizes the human forearm to mechanical and cold stimulation 70,91 the rat hindpaw to noxious and innocuous mechanical forces, 14,43,49,70,162 and the mouse hindpaw to noxious mechanical stimulation, 7 formalin or capsaicin 184–186 and heat. 156

A single i.p. injection of ET-1, ET-2, or ET-3 into mice reliably elicits an abdominal constriction response within 15 min, similar to the effects of acetylcholine (ACh) or phenyl-*p*-quinone (PpQ).196,197 The percent of mice responding was dose-dependent, with ET-3 significantly less potent than ET-1 and ET-2, and the response was blocked by morphine administered to the CNS, evidence of its nociceptive nature. 195 Administration of the ET_A receptor antagonist BQ-123, i.p., 5 min before ET, blocked the effect of ET-1 and of ET-2, but did not prevent the constrictions induced by ET-3, nor did it inhibit similar behavioral effects produced by ACh or PpQ, establishing the ET_A receptor requirement and the pain-related nature of this response.

Subsequently, ET-1, unlike sarafotoxin S6 (ET_B agonist), caused nociception when injected into the knee-joint of naive rats, 55,56 an effect that was halved by systemic bosentan, a mixed ET_A/ET_B antagonist or local intra-articular injection of the ET_A antagonist BQ-123, but was unaffected by the ET_B antagonist BQ-788. Thus, ET_A receptors also initiate nociception in response to ET-1 injected into the rat knee-joint.

When applied epineurally onto the exposed rat sciatic nerve, ET-1 produced unilateral hindpaw flinching, an effect blocked by preemptive i.p. morphine, and completely prevented, or reversed, by an epineural ET_A antagonist, with no effect from an ET_B antagonist. 52

Activation of distal nerve endings by subcutaneous (s.c.) injection of ET-1 into the rat plantar hindpaw induced both flinching behavior, in awake rats, and impulse firing in nociceptor afferents recorded from anesthetized rats. 81 The flinching developed within minutes after injection, and was strongly inhibited by BQ-123, similar to that from neuronal application (see above). Recordings from single, physiologically characterized sensory fibers showed that almost all nociceptive C-fibers A δ -fibers were excited by ET-1 injected into their receptive fields. In contrast, the large majority of "light touch" A β -fibers (9 of 12) did not respond to ET-1. BQ-123, when co-injected with ET-1, blocked ET-1-induced activation in all C- and A δ -fibers tested. Therefore, nociceptive fibers are selectively excited through ET_A with the same doses of s.c. ET-1 that cause pain behavior when injected into the rat hindpaw.

Recent results confirm that exogenous ET-1 is capable of evoking acute pain in humans. Spontaneous pain in response to ET-1 was found to develop rapidly after intradermal injection of ET-1 at high concentrations (10^{-7} and 10^{-6} M) into the volar aspect of the forearm of healthy males, where it decreased gradually, respectively ending at 30 and 60 min after ET-1 administration. 91

3.B. ET_A receptor mediates hyperalgesia

The nature of the response to exogenous ET-1 (acute nociception vs. sensitization/hyperalgesia) seems to depend on the concentration of the peptide. Endothelin-1 injected into the footpad of rats 14,49,70 and mice 7,156 at 30 nM-10 μ M induced hyperalgesia and sensitization at the site of injection, rather than the overt pain behavior produced by higher doses, 100–500 μ M. 81

Different test species may also produce differences in the detected pain response. An enhanced responsiveness to mechanical stimulation, evoked by exogenous ET-1 in rodents' plantar footpad, appeared as a slowly developing and prolonged reduction in the force withdrawal threshold of the injected paw. In rats such dose-dependent allodynia reached its maximum in tens of minutes 14 or a few hours, 162 and then lasted for 3–8 hours. This hyper-responsiveness, unlike overt nociception, was only partially attenuated by ET_A receptor antagonists. In mice, ET-1 injected s.c. into the paw also produced tactile allodynia, but this was completely prevented by the local ET_A antagonist BQ-123. 7 Thermal hyperalgesia induced by s.c. injection of ET-1 in mice (0.8–8 μ M, i.pl.), manifested as a unilateral decrease in paw withdrawal latency in response to heat stimuli, was inhibited solely and completely by an ET_A receptor antagonist. 156

The behavioral sensitivity produced by the ET_A receptor was reflected in BQ-123's prevention of the amplification of Fos immunoreactivity in the nociceptive-processing outermost laminae of the spinal cord, in species pre-treated with ET-1 and then subjected to thermal stimulation. To the extent that such immediate early gene expression is indicative of cellular activation that may precede longer duration neural plasticity, the blockade of peripheral ET_A receptors may provide a pre-emptive treatment to minimize central sensitization and long-lasting pain.

3.C. ET-1 potentiates effects of other algogens

Exogenous ET-1 enhances the nociceptive actions of other algogens, administered into the knee-joint of dogs or rats 55,56,70 or into plantar skin in mice. 185,186 When injected intra-articularly (i.a.), ET-1 potentiated the effects of PGE₂ injected i.a. 1.5 or 3 h later. Incapacitation in dogs, caused by elevated joint pain, was produced by PGE₂ given, after ET-1, at the usually ineffective dose of 1 µg/joint. 70 The role of specific ET receptors in this process has not been determined.

In studies of the rat knee-joint (see 3.a., above), low doses of ET-1 (15 or 30 pmol/joint), that did not cause incapacitation per se, potentiated the effect of subsequently given carrageenan on hindpaw lifting. Systemic (i.v.) treatment with the non-selective ET receptor antagonist bosentan, given 15 min before joint stimulation, did not affect paw elevation in naïve animals in response to carrageenan or to lipo-polysaccharide (LPS), but reduced by ~50% the ET-1 - enhanced response to LPS 55. No such inhibitory action was seen with BQ-788, showing that potentiation of inflammation-induced incapacitance in joints by ET-1 involves only ET_A receptors.

Endothelin-1 induces sensitization to the nociception caused by formalin and capsaicin injections in mice.184–186 Intraplantar formalin induced a “classic” nocifensive response evaluated by the amount of time spent licking the injected paw, both in an early, phase 1, period of activity (0–10 min after injection) and in the phase 2 period (from 15 min to 60–90 min). Both phases of nociception were significantly potentiated by simultaneous injection of formalin with either ET-1 or ET-3, but the first phase was unaffected by the ET_B agonist sarafotoxin S6c. Pre-treatment with the mixed ET_A/ET_B receptor antagonist bosentan attenuated the potentiation by ET-1 of both phases. From these data viewed together, the contribution of the ET_A receptor to the first phase of the formalin effect appears to be more relevant than that of the ET_B receptor. 184

With regard to capsaicin's actions, local injection of ET-1 30 min prior to ipsilateral injection of capsaicin (0.1 µg/paw) increased the amount of time spent licking the injected paw, with a maximum ~2.5-fold increase. 185 Importantly, the selective ET_B receptor agonists S6c and IRL-1620 did not change the capsaicin response. ET_A receptor blockade fully prevented the potentiation, whereas blockade of ET_B receptors was ineffective.

Altogether, exogenous ET-1 enhanced the algesic inflammatory actions of PGE₂ and LPS in experimental articular pain in dogs and rats, and potentiated capsaicin-, and likely the first phase of formalin-induced pain in plantar skin in mice, all via the ET_A receptor.

3.D. ET_B receptor mediates both pro-nociceptive and anti-nociceptive effects of ET-1

Pro-nociceptive effects via ET_B receptor—Certain pro-nociceptive effects of exogenous ET-1 are also mediated by ET_B receptors (Figure 3). Injection of ET_B receptor agonists i.p. into mice rapidly elicited abdominal constrictions which, unlike those from ET-1, were unaffected by an ET_A receptor antagonist, implying a role for ET_B receptors. 196

The ET_B receptor, along with ET_A, also partially mediates mechanical hyperalgesia from exogenous ET-1. In mice, BQ-788 completely prevented the decrease in paw withdrawal threshold from ET-1 7. In rats, however, BQ-788 only partially reduced mechanical allodynia (S-D rats, von Frey hairs), as did BQ-123, suggesting involvement of both receptor subtypes. 14 Mechanical hyperalgesia induced by a lower ET-1 concentration (1.5 µM, in Wistar rats, tested with von Frey dynamic aesthesiometer), was equally partially inhibited by either of the antagonists, BQ-788 or BQ-123. 162 In contrast, the mechanical hyper-nociception from lower concentrations of ET-1, 30–300 nM, in rats is purely ET_B receptor-dependent, since it was inhibited only by BQ-788 and not by BQ-123. 49

Again it appears that different concentrations of exogenous ET-1 may activate different receptor subtypes to produce different behavioral effects. Such differences in response could arise from coupling between receptor sub-types such that activation of one influences the coupling of another (see 1.B., above). Alternatively, the difference could reflect different ET-1 distributions that are differentially affected by ET-1 diffusing in the skin; after s.c. injection those cells or axon regions in the more superficial skin, e.g. the epidermis, are reached and affected only by the higher concentrations of ET-1, but the deeper structures are affected by both high and low concentrations. This latter explanation is consistent with the very high concentrations of exogenous ET-1, and its receptor antagonists, compared to their K_D s determined in vitro, that are required for induction of acute nociception or its inhibition 68, 81, 128 and with the very slow responses that occur when much lower concentrations are used, e.g., 3 h to peak mechanical allodynia from 1.5 μ M ET-1162 vs. 20 min from 10 μ M ET-1.14

Selective activation of the ET_B receptor potentiated the second phase of formalin-induced nociception in mice, to about the same extent as was seen with ET-1 per se, indicating that this potentiation, in contrast to that of the first phase, is largely mediated by ET_B .184 Capsaicin-induced hypernociception, however, remained unaffected by ET_B receptor activation, 186 showing the selective actions of ET_B modulation between different chemical algogens (see 3.C., above). In contrast, the ET_B receptor does not appear to mediate heat allodynia; the thermal nociceptive response was equivalent between wild-type and homozygous ET_B knockout mice. 86 Consistent with this, blockade of ET_B receptors by BQ-788 was ineffective against ET-1-induced thermal hyperalgesia (hot plate test) in wild-type mice. 156

Anti-nociceptive effects involving ET_B receptors—In contrast to the above-listed pronociceptive effects, ET_B receptor activation has also been shown to provide an anti-hyperalgesic/anti-nociceptive action. 128,129,185,186,187 These apparently opposite effects may arise because of different ET-1 doses and/or different local conditions such as inflammation (see 4.A, below). For example, hyperalgesia from capsaicin-injected into the mouse hindpaw was enhanced by only a narrow range of ET-1 concentrations. 185 Ten pmol/paw of ET-1 gave the maximum enhancement, above which this potentiation decreased and was absent at 30 pmol/paw (given intraplantar 30 min beforehand). However, pretreatment with ET-1 plus a local ET_B receptor antagonist BQ-788 (10 nmol/paw) unmasked a significant hyperalgesic response at that otherwise ineffective, higher dose of ET-1.186 This finding suggested that at high exogenous ET-1 doses an anti-hyperalgesic action of ET-1 was occurring via ET_B receptors, a notion that was supported by results showing that co-injection of selective agonists of ET_B receptors could abolish the ET_A -mediated potentiation. Thus, simultaneous activation of ET_B and ET_A receptors by exogenous ET-1 limited the hyperalgesic effect of the peptide. 186 It is likely that this ET_B mediated inhibition represents the analgesic mechanism resulting from release of β -endorphin from adventitial tissues, such as skin, and its anti-hyperalgesic actions through μ -opiate receptors (see 2.B., above).128,129

3. E. Peripheral vasoconstriction in ET-1's actions

Endothelins were first and foremost recognized as potent vasoconstrictive agents. Vascular constriction was documented in almost every organ and tissue, including peripheral nerve 274 and skin, 28,47 yet the potential physiological importance of this action has been largely overlooked in recent work. ET-1 application to rat peripheral nerve had been shown to cause conduction block in vivo but not in isolated nerve in vitro; 274 the conduction block corresponded to physiological deficits and to a reduction in blood flow in the circulation supplying the nerve, suggesting that local ischemia from ET-1-induced vasoconstriction had secondary consequences for neural physiology, and thus for behavior. The importance of this effect may differ among different tissues, where the type of blood vessels, their neural control and their importance for neuronal activity may differ. 199,200 At present it seems prudent to

not dismiss a role for ET-1-induced vasoconstriction, potentially leading to local ischemia, in the overall actions of ET-1 on intact organisms.

3. F. In vivo neurophysiological actions of ET-1

Peripheral nerve in vivo can be activated by ET-1, applied at the trunk of peripheral axons 68 or at cutaneous endings. 81 Action potentials appeared in the absence of acute electrical or physiological stimulation in functionally identified nociceptors, within a minute or so after injection of subcutaneous ET-1 (e.g. 200 μ M). Such neurophysiological actions, which occur almost exclusively in almost all cutaneous C- and A-delta nociceptors, are totally prevented by ET_A antagonists, 81 and also strongly suppressed by *agonists* of ET_B receptors, 129 consistent with the ET_B-mediated release of β -endorphin from keratinocytes (see 2.B., 3.D., above). There is virtually no stimulation of low threshold mechano-receptive A β -fibers by ET-1.

The peripheral nerve fiber types activated by ET-1 differ depending on the site of application. Pain behavior from high concentrations of ET-1 applied directly to rat sciatic nerve could be prevented by the non-selective conduction block caused by the proximal application of lidocaine to the sciatic nerve, which also blocked pain responses to toe pinch. However, application of TTX at the same proximal site, at doses that also blocked the response to toe pinch, had no effect on ET-1-induced pain. 107 Earlier studies of impulses in functionally identified sciatic fibers had shown that such doses of TTX failed to block only a small population of axons, the slowest conducting C-fibers, which were almost unanimously unresponsive to noxious cutaneous stimulation. 80 These are probably identical to the ones stimulated by activation of ET_A receptors in peripheral nerve. In contrast, subcutaneous injection of high concentrations of ET-1 in the paw led to pain behavior that was partially reduced by TTX at the proximal sciatic nerve (and totally abolished by lidocaine). This difference in TTX susceptibility dependent on the locus of administered ET-1 requires that different fiber types are activated at these different locations, and therefore the mechanism of impulse initiation and the cellular distribution of impulse-generating ET_A receptors must also differ. Perhaps ET_A receptors at cutaneous nerve terminals in the paw are located on more types of fibers, or even other cell types, than those in the nerve axons of the trunk.

In vivo behavioral studies have identified a number of receptors, signaling enzymes and pathways involved in ET-1-induced hypernociception. For example, mechano-sensitization appears to engage local, cutaneous NMDA and CGRP1 receptors, and may result from an ET-1-enhanced release of glutamate and/or CGRP, e.g., from peripheral nerve endings, replicating in nerve terminals the ET-1-enhanced release of glutamate and CGRP seen in isolated sensory nerves (see 2.B., above). Consistent with this, tactile allodynia after s.c. plantar injection of ET-1 (10 μ M) in rats was virtually abolished by local injection of the NMDA receptor inhibitors (+)MK-801 or D-AP5 (5 mM).¹³⁰ In contrast, hindpaw flinching caused by high concentrations of ET-1 was not affected by either of these antagonists, although it was suppressed by blockade of the CGRP1 receptor. It thus appears that overt pain behavior, in response to high concentrations of exogenous ET-1 and mediated by ET_A receptors, is coupled through the neuropeptide-dependent reactions, while mechano-sensitivity, from low [ET-1], and mediated by both ET_A and ET_B receptors, is modulated by both NMDA and CGRP1 receptors. Perhaps activation of the ET_B receptor leads to the release of glutamate and causes tactile allodynia, whereas overt pain behavior requires both glutamate and CGRP, which are released by ET_A receptor activation.

Taken together, these findings suggest that in skin ET-1 increases the level of free neurotransmitter/neuropeptides to sensitize nociceptive afferents, subserving tactile allodynia. Because sensory neurons also express receptors for CGRP and glutamate, this ET-1-enhanced

autocrine release would increase the subsequent neuronal responses, causing a gain in a positive feedback cycle for sensory activation in the periphery.

3. G. Intracellular pathways mediating endothelins' peripheral actions

The intracellular pathways that couple ET receptor activation to changes in pain behavior are challenging to identify. This is, at least in part, due to the complexity of the integrated peripheral responses, which involve multiple cell types, including keratinocytes in skin, endothelial cells that modify blood flow and thereby indirectly alter local physiology, 274 the autonomic fibers that modify the skin micro-environment and can modulate sensory afferent sensitivity, 115, 211 and the variety of nociceptive and tactile nerve fibers themselves, generating impulses whose integration in the spinal and supra-spinal parts of the CNS results in the eventual behavior. By examining only the behavioral outcome it is therefore impossible to know which of these, often interactive, processes is the target of an inhibitor of an intracellular pathway, so the following must be interpreted cautiously.

Several downstream signaling pathways appear to be targets of ET receptor activation in vivo, leading to pro-nociceptive responses. As indicated by behavioral studies, these include cyclic AMP (cAMP), various isoforms of Protein Kinase C and mitogen activated protein kinases (MAP kinases). 49,162 Hypernociception could be induced by ET-1, as well as by ET-2, or ET-3, and can be inhibited by antagonists of ET_B and ET_A receptors. 14,162 When rats were pre-treated with a phosphodiesterase inhibitor, which will elevate resting and stimulated levels of intracellular cAMP, mechanical hypernociception induced by ET-1 was more than doubled. On the other hand, hypernociception was not affected by the PKA inhibitor H89, implying that cAMP must be stimulating some other target, e.g., Epac. 42,76 In contrast, ET-1-induced hyperalgesia was lessened by i.pl. pre-treatment with inhibitors of PKC, staurosporine or calphostin C. Allowing for the possibility of non-selective actions of these inhibitors at the doses required to have an effect in skin, these data nevertheless suggest that endothelins, acting through ET_B or ET_B receptors, induce mechanical hypernociception in the rat hindpaw that depends on the activity of PKCs and can be enhanced, although not directly driven, by elevated cAMP.

MAP kinases appear to contribute to hyperalgesia from ET-1 162. Inhibitors of p38 MAPKinase, extracellular signal-regulated kinases (ERK_{1/2}) and c-Jun N-terminal kinase (JNK), as well as those of phospholipase C (PLC) and PKC, locally injected 15 min before ET-1, caused long-lasting reductions of the mechanical hyperalgesia. A smaller inhibition occurred at 4 h after ET-1 injection from an inhibitor of Phospholipase A2, PACOCF3. Thus, mechanical hyperalgesia triggered by ET-1 in the rat hind paw appears to include contributions from several signaling pathways. 162. Whether all of the MAP kinases are activated in the same cell types, or in different neurons, or even in keratinocytes, etc., and whether they act in parallel or sequentially, seems to be an important unanswered question related to the complex, interacting pathways that contribute to nociceptive sensitivity.

Evidence for the involvement of bioactive lipids produced by the cyclo-oxygenase (COX) enzymes in ET-1 nociceptive signaling is contradictory. Endothelin-1 potentiates the pain and sensitization from pro-inflammatory mediators, e.g., PGE₂, 70 or agents inducing inflammation, such as carrageenan, 55 or formalin (the second phase) 184 (see 3.C., above). Despite the known potentiation by bioactive lipids in the inflammatory processes that were also enhanced by ET-1, broadly acting cyclo-oxygenase inhibitors such as indomethacin and ibuprofen did not block the ET-1-induced nociceptive writhing in mice. Even traditional non-steroidal anti-inflammatory drugs were 30-fold (aspirin) to > 100-fold (ibuprofen, naproxen, tolmetin) less potent in antagonizing ET-1 responses than in antagonizing abdominal constriction from Ach 197. On the other hand, a PGE₂ signaling cascade, effected downstream by endogenous ET-1, mediates IL-15-, IL-18-, and IL-33-dependent inflammatory hyper-

nociception in mice 247–249 (see 4.A., below). Perhaps the only clear conclusion from this collection of results is that there is no single, linear sequential pathway that connects the reactions that drive ET-1's modulation of hyperalgesia to the reactions that involve COX-derived products, e.g. prostaglandins and thromboxanes.

4. ROLE OF ENDOGENOUS ET-1 IN PAIN FROM INJURY AND DISEASE

Elevated tissue or plasma concentrations of ET-1 occur in a variety of pathological states, e.g., in metastasized prostate and breast cancer cells 167, following cutaneous injury 1 or ischemic injury related to acute respiratory distress syndrome, sepsis, and disseminated intravascular coagulation 57,112,252. In many of these pathologies ET-1 contributes to pain.

4.A. Inflammatory pain

Inflammation releases substances that excite or sensitize primary afferent nerve fibers and cause pain/hyperalgesia, 131,212 and ET-1 is significantly over-secreted in inflammatory conditions. 194 Carrageenan injected into peripheral tissues rapidly increased local and plasma ET-1 levels 17 and chronic constriction of the rat sciatic nerve, with a substantial contribution from local inflammation, 151 caused thermal and mechanical hyperalgesia and elevated both ET-1 and ET_A at the injury site. 132 Inflammation of the rat hind paw led to elevated TRPV1 expression in sciatic nerve, 190 providing an increase in one of the critical receptor molecules with which elevated ET-1 interacts to promote hyperalgesia (see 2.B., above).

Endogenously released ET-1 mediates nociception in inflammatory models *in vivo*. The nociceptive response (abdominal constriction) to i.p. injection of pro-inflammatory phenyl-*p*-quinone (PpQ), or ET-1 in mice was desensitized by ET-1 pre-injection. 197 Both ET_A and ET_B receptor subtypes contributed positively to pain from intraperitoneal acute inflammation in mice. 196

Abdominal constrictions from i.p. phenylbenzoquinone (PBQ) had a reduced intensity in heterozygous (+/–) ET_B knockout mice that was 80% less than in wild type controls, and such constrictions were totally absent in the homozygous (–/–) knockouts. 86 Abdominal constrictions from PBQ recorded over 5–15 min after i.p. injection were strongly inhibited in wild-type mice by a systemically delivered ET_B receptor-selective antagonist, but unaffected by a systemic ET_A receptor antagonist. 86

In a different inflammatory model, wild-type (+/+) ET_B-receptor mice had a marked inflammatory response to topical arachidonic acid (AA), but the (+/–), and (–/–) mice had significantly reduced fluid phase responses, measured by edema and by secreted metalloproteinase activity (37 and 65% inhibition, respectively) 86. The cellular phase of the inflammatory response, marked by neutrophil infiltration, was correspondingly reduced in the (+/–) and (–/–) mice (by 51 and 65%, respectively). Topical administration of an ET_B receptor antagonist on (+/+) mice inhibited AA-induced swelling (39%), whereas an ET_A receptor antagonist was ineffective. Collectively, these results implicate endogenous ET-1 acting through the ET_B receptor in mediation of both inflammatory pain and cutaneous inflammatory cellular responses in mice.

Endothelin-1 levels in synovial fluid were elevated in rheumatoid arthritis (RA), osteoarthritis and gout, and plasma levels of ET-1 in active RA exceeded the values in non-active RA, whereas ET-1-like immunoreactivity in synovial fluid was found at levels several-fold higher than those in plasma 159. In a rat model of experimental arthritis, endogenously-released ET-1 mediated pain in the inflamed knee-joint via both ET_A and ET_B receptors, with the higher contribution from the ET_B receptor 56. Thus, when ET-1 was injected into a “primed” rat joint (wherein 300 µg of carrageenan had been injected 72 h beforehand, to initiate inflammation

and sensitize the joint), the pain and incapacitation re-stimulated by ET-1 was equi-sensitive to inhibition of ET_A or ET_B receptors (each causing approximately = 80% inhibition). When LPS (lipo-polysaccharide) was used to induced hyperalgesia in the primed joint, the pain and incapacitation responses were inhibited ~50% (maximally) by either the non-selective ET receptor antagonist bosentan or by ET_B receptor-selective BQ-788, but were unaffected by BQ-123. Thus, unlike the predominantly ET_A mediated nociception induced by *exogenous* ET-1 in the naïve joint 55,56, both ET_A and ET_B receptors contributed to the stimulation of nociception by ET-1 in the carrageenan-primed (pre-inflamed) joint, ET_B receptors account for the LPS-induced nociception in the primed joint, dependent to a large extent on release of *endogenous* ET-1. The pro-nociceptive role of ET_B receptors was confirmed by the fact that its highly selective agonist, sarafotoxin S6c, when injected 72 h after priming with carrageenan, increased the pain-indicating incapacitation.

Surprisingly, the same agonist for the ET_B receptor produced an *anti-nociceptive* effect when it was given 24 h before either the initial injection of carrageenan into the naïve joint, or re-stimulation of the primed joint with any of the following: carrageenan, ET-1, or S6c 50. Apparently ET_B activation exerts a prophylactic action, inhibiting the development of inflammatory (carrageenan-induced) pain, and ET_B receptor-operated mechanisms limit the priming effect of carrageenan to nociception evoked by subsequent inflammatory insult. These findings dramatically illustrate the dual roles of ET_B receptors, pro- and anti-nociceptive on the same inflammatory conditions, depending on the order in which the processes occur. There may be an inflammation-induced shift in the expression or the coupling of ET_B receptors that explains this duality of action.

ET-1 is derived from various cells in skin: keratinocytes, 244 vascular endothelial cells, 262 immune cells 61,217 and mast cells. 62 Sensory afferents themselves 72,77,234 and satellite cells of DRG 124 contain ET-1. Thus, both cells of the skin and those that innervate it may release ET-1 in normal and pathological conditions.

Local inflammation in skin clearly involves ET-1 and its receptors. Thermal hyperalgesia in rats during acute inflammation following s.c. complete Freund's adjuvant (CFA, 20 µg/paw) was partially (~50%) attenuated by an ET_A receptor antagonist 276. A similar degree of inhibition occurred with an ET_B receptor antagonist. In contrast, ET_B receptor activation with the highly selective agonist IRL-1620, given before and with CFA, almost totally reversed maximum thermal hyperalgesia; the latter actions of IRL-1620 were reversed by co-administration of BQ-788, naloxone or antiserum against β-endorphin confirming an opiate-dependence of ET_B receptor-linked peripheral analgesia in the inflamed skin (see 3.D., above). These experiments thus revealed pro-and anti-nociceptive roles of ET_B receptors at the acute inflammation site.

Cutaneous ET_A receptors participate in thermal hyperalgesia, in both acute (induced by carrageenan, 3.5 h before testing) and chronic (induced by CFA, 1 week before testing) inflammation in mice 7. When mice were injected s.c. (intraplantar, i.pl.) with carrageenan or CFA the paw withdrawal latency (PWL; unilateral hot plate test) significantly decreased, with the decrease during acute inflammation being half as much as at chronic inflammation. Pre-treatment with BQ-123 (i.pl.), before inflammation, completely blocked the thermal hyperalgesia measured 90 min after this ET_A antagonist was administered, in both inflammatory conditions, whereas identically given BQ-788 was without effect. Unlike this exclusively ET_A-dependent thermal hyperalgesia, mechanical allodynia (Randall and Selitto paw pressure test) induced by the same inflammation stimulus involved both ET_A and ET_B receptors.

In summary, the ET_A receptor always appears to promote inflammatory pain/hyperalgesia, but ET_B receptor activation is capable of providing opposing actions, likely depending on the inflammatory state and other factors, the species, procedure, even the type of skin, glabrous or hairy, etc. These mechanisms, dynamically regulating the roles of the ET_B receptor in different pathological conditions, remain an intriguing area for future investigation.

4. B. Injury associated pain

Damage to the structure of tissues most often leads to inflammation. Although at first it may appear that the same cellular and molecular responses are involved in all injury- and inflammation-related pain states, recent evidence indicates that the neurobiology of different pain states differ from each other in important ways. 30,146,147 Nevertheless, there are some events common to all injury. Overproduction of ET-1 is a characteristic of injured skin, 110, 121,194 and sites of tissue injury also have elevated inflammatory cytokines, and other substances that are released from broken resident cells, e.g. ATP, or imported by inflammatory cells, such as neutrophils, that have bold enzymatic, e.g., oxidative, activity. After UV damage the ET-1 level in skin peaked in 1–2 days 1 and elevated ET-1 was also found after local administration of cytokines TNF- α or IL-1 α . 1,110,229 Local, transient inflammatory response at a wound resulted in a robust activation of primary afferent nociceptors in the skin 229 that far outlasts the incision-induced injury discharge, and may spread well beyond the region of cutaneous injury, probably through central sensitization in the spinal cord.

The complex reactions to incision are likely to involve responses to local injury and inflammation, including those that trigger wound healing, recruit immune cells, release cytokines and other cellular activators, and also trigger changes in the expression of genes in the sensory neurons (DRG) and activate second order sensory neurons and various types of glia in the spinal cord. 117,198,240,254 These complex factors render the overall response after incisions different from that from inflammation alone.

The specific molecules responsible for sensitizing nociceptors after incision have been investigated in studies using different experimental animal models. A rat model of post-incisional pain 58 has been used to examine the contribution of endogenous ET-1 and ET receptors subtypes to injury-induced pain. 163 Mechanical hyperesthesia from an incision through the hairy skin of the rat developed next to the wound (primary responses) and at a distance (secondary responses, involving spinal circuits). Control incisions showed both primary tactile allodynia and hyperalgesia, and a weaker secondary hyperesthesia, peaking 3–4 h after surgery and lasting at least 72 h. Primary allodynia, but not hyperalgesia, was suppressed by an ET_A receptor antagonist given locally just before surgery. 163 The antagonist had no effect on mechano-responsiveness of intact skin. This suppression of post-incisional primary allodynia by local ET_A antagonist disappeared in 24 h. Secondary responses to incision, however, both allodynia and hyperalgesia, were inhibited even more strongly by these local ET_A antagonists, and this inhibition remained effective for at least 24 h. These results are evidence that while the endogenous basal level of ET-1 does not modulate mechano-sensitivity of intact skin, ET-1 released from hairy skin by incision activates nociceptors to cause primary allodynia and to sensitize spinal circuits through central sensitization.

Post-incisional hyperalgesia, paralleled by local tissue acidosis, 228,256 appeared more resistant than allodynia to ET_A receptor antagonism and is likely less dependent on endogenous ET-1. The important clinical consequence is the indication that local blockade of ET_A receptors in the immediate peri-operative period may be able to prevent the later development of central sensitization that supports prolonged and persistent post-operative pain.

4.C. ET-1 in acute pain of sickle cell disease

In patients with sickle cell disease, hemoglobin-S (Hgb-S)-containing erythrocytes have a reduced deformability that can greatly increase the viscosity of blood and even result in mechanical blockage of the microcirculation, creating ischemic tissue and leading to the occurrence of painful crises, an episode of severe pain lasting several days.

ET-1 levels are elevated in the plasma of sickle cell patients (see Ref. 89). Levels of circulating ET-1, along with other inflammatory mediators, have been measured in asymptomatic sickle cell disease, and also, during and after a pain crisis. 83 Highly elevated levels of ET-1 were found in symptomatic sickle cell patients during acute pain crises (130.9 ± 23.1 pg/ml) compared with healthy African-American controls (0.535 ± 0.508 pg/ml). These levels decreased, but never to normal values, 1 to 3 weeks after patient discharge from the hospital (post-crisis, 23.69 ± 9.52 pg/ml). Interestingly, the plasma level of PGE₂ was also elevated during a crisis, ~2.6-fold, but it remained elevated afterwards, ~2-fold, compared to healthy controls. The other inflammatory mediators that were measured (TNF- α , IL- β , IL-6, IL-8, and IL-10) were neither elevated in asymptomatic sickle cell disease nor during an acute vaso-occlusive crisis. ET-1 appears to contribute to both the prolonged vasospasm and to inflammation in acute painful sickle cell crisis, and ET-1 was suggested to play a key role in the cycle of ischemia and inflammation that initiates and sustains pain at crisis. The elevated level of PGE₂ was linked to the down-regulatory effects of PGE₂ on immune cell function, which could contribute to the increased susceptibility to infection observed in patients with sickle cell disease. 83

The age-sensitivity in rats of acute nociception and mechanical hyperalgesia in response to exogenous ET-1 suggests that there may be greater pain to stimuli that release endogenous endothelins in younger *versus* older (and possibly male *versus* female) mammals 153. Such differences may be relevant to painful vaso-occlusive episodes that occur most dramatically in children with sickle cell disease. 13,19,113 Importantly, exposure to ET-1 in infancy may result in sex-dependent modulation of ET-1-induced pain later in life (sensitization in males and desensitization in females), correlating with changes in ET_B receptor expression. 154 Further developments should help to define a beneficial approach for treatment of repetitive painful vaso-occlusive episodes.

4. D. Endothelin-1 role in immune-mediated hypernociception

One of the most important roles of endothelins, in general, is their influence on the immune system and contribution to the inflammatory diseases that involve immune components. Among complex disorders with a strong immunological component are asthma, rheumatoid arthritis 137, type 1 (autoimmune) diabetes 251, autoimmune pancreatitis 123,142, autoimmune prostatitis 161,255, autoimmune fatigue syndrome 238, retroperitoneal fibrosis with associated autoimmune diseases 32. Erythema nodosum, a painful disorder of the subcutaneous fat, is the most common type of panniculitis 214, and psoriatic arthritis, a chronic, autoimmune, seronegative inflammatory arthritis characterized by varying degrees of axial and peripheral arthritis 145. All of these are associated with pain.

Elevated ET-1 is pathogenic in asthma, and may cause some of the pain associated with this disease.191 A similar argument can be made for the pain-associated immune disease - Henoch-Schönlein purpura (HSP) - one of the common types of vasculitis disorders seen in childhood. It is characterized by abdominal pain and arthritis, along with a rash and renal complications. Cytokines have been implicated in its pathogenesis, and ET-1 levels were found significantly higher in HSP patients during the acute phase compared with the control group and the HSP patients in the remission phase 164.

An immune-mediated Type I hypersensitivity reaction can be induced by i.pl. ovalbumin (OVA, 0.3–1 µg) challenge in mice sensitized to this antigen 14 days beforehand, and utilized to examine the involvement of ET-1. Sensitized mice exhibited greater total licking time in response to intraplantar OVA injection than controls. Nocifensive behavior induced by this OVA challenge was inhibited by systemic morphine or local depletion of mast cells, or by systemic pre-treatment with a number of ET_A and ET_B receptor selective antagonists or agonists. ET_B receptor activation induced nocifensive responses only in OVA-sensitized mice. Compound 48/80 (a synthetic polyamine, inducing degranulation of mast cells) potentiated the nociception induced by i.pl. capsaicin (0.1 µg/paw), and this potentiation was inhibited by local BQ-123, showing a pro-nociceptive role for ET_A receptors, whereas blockade of ET_B receptors by BQ-788 was ineffective. Therefore, non-immune activation of mast cells could also trigger an ET-dependent change in nocifensive responsiveness. The authors concluded that mast cell- and ET-dependent nociception, that was evoked by immune-mediated Type I hypersensitivity reactions, is mediated locally by both types of ET receptors in mice. In agreement with this pro-nociceptive role of both ET receptors are the responses to systemic treatment with dual ET_A/ET_B or selective ET_A receptor antagonists, which inhibited the nocifensive response to the antigen.

A cascade of cytokines constitutes a link between inflammatory stimuli and release of the final mediators that directly sensitize nociceptors. Rheumatoid arthritis (RA) is characterized by a T helper 1 (Th1) inflammatory immune response in joints. 15,189 In the pathogenesis of RA, for which a major symptom is mechanical allodynia, cytokines IL-12, IL-15, and IL-18 play a key role by means of elevated IFN-γ production. 15,31,69,120,141,189,205,230 This signaling cascade, effected downstream by endogenous ET-1, is intimately involved in cytokine-dependent inflammatory hypernociception in mice.247–249

To elucidate the pathophysiology of RA, experimental models were established in which an antigen challenge (intraplantar injection of OVA, on day 21(D21)) was mounted in previously immunized mice (subcutaneously injected OVA, on D 0, D 7 and D14). Ovalbumin induced a Th1 inflammatory response with early neutrophil influx followed by mononuclear cell infiltration and similar features of joint inflammation to those in RA patients 38,243. Of relevance to pain, the ovalbumin challenge evoked IFN-γ and ET-1 production, both of which were inhibited in immunized IL-18^{-/-} mice.

Signaling pathways revealed in the OVA-induced model for RA in mice, wherein IL-15 or IL-18 triggers the sequential productions of IFN-γ → ET-1 → PGE₂, 247,248 or IL-33 triggers the cascade TNFα → IL-1β → IFN-γ → ET-1 → PGE₂, 249 suggest that ET receptors as well as the cytokines could be targets for the treatment of antigen-induced inflammatory pain.

4.E. ET-1 in neuropathic pain of peripheral origin

Neuropathic pain, a common disorder that originates from pathology of the peripheral nervous system, is presented by patients with various diseases, including diabetes, infection (herpes zoster), “channelopathies” (inherited diseases caused by mutations in genes coding for ion channel subunits or the proteins that regulate them, and which include a wide range of neurologic diseases, including periodic paralysis, congenital myasthenic syndromes, malignant hypothermia, etc.16,37), small-fiber neuropathy (a peripheral nerve disease in older people, who develop burning pain in their feet 71), as well as autoimmune diseases. In addition, neuropathic pain frequently develops after nerve compression or nerve trauma. Neuropathic pain reflects both peripheral and central nervous system sensitization, and abnormal impulse activity may arise from the intact afferent neurons that share the receptive field or the common nerve trunk with injured axons. 37

Only a few studies of intact animals link ET-1 to neuropathic pain. One is the investigation of tactile allodynia in the streptozotocin-induced diabetic rat model of neuropathic pain. 116 Tactile allodynia, the perception of pain in response to normally non-painful stimulation, is a common complication of diabetic neuropathy. Diabetic neuropathy is associated with decreased neurovascular blood flow and concomitant endoneurial hypoxia resulting in impaired nerve conduction 35,36,245. Early degeneration of small nerve fibers in diabetes leads to symptoms ranging from hyperalgesia to loss of pain and temperature sensation (for review see Ref. 251). The abnormal activation of ET_A receptors in peripheral nerves has been implicated in diabetes-induced reductions in peripheral neurovascularization and concomitant endoneurial hypoxia 258,275. Increased nociception in streptozotocin-induced diabetic rats is not attributable to streptozotocin neurotoxicity 34, but derives, indirectly, from hyperglycemia 108. Rats injected with streptozotocin, after 8–12 weeks developed stabilized, high blood glucose levels, evidence of their diabetetic condition, along with tactile allodynia 116. The lower withdrawal thresholds that characterized this tactile allodynia were about doubled by morphine (8 mg/kg, i.p.), indicating the nociceptive nature of the response. A novel ET_A receptor antagonist, ABT-627 (Atrasentan®), approved for clinical use, was administered systemically, either acutely (i.p.) or chronically (p.o., given in the drinking water for 7 days) to these rats. When injected intraperitoneally, ABT-627 reversed the allodynia by 40–50%, but only for 0.5–2-h after injection. Chronic ABT-627 treatment (10 mg/kg/day, p.o.), similarly to its acute effect, resulted in a ~40% increase in tactile allodynia thresholds. In contrast, an ET_B receptor-selective antagonist injected i.p. did not significantly alter tactile allodynia. Combined i.p. administration of this ET_A antagonist and the ET_B receptor antagonist produced an acute relief of tactile allodynia that was significantly less than that from the ET_A receptor inhibitor ABT-627 alone. Selective blockade of ET_A receptors, resulting in an attenuation of tactile allodynia in the streptozotocin-treated rats, is evidence of endogenous ET-1 mediating diabetic neuropathy via ET_A receptors. In our interpretation, the reduced effectiveness of ET_A receptor blockade by coincident ET_B receptor blockade clearly indicates that anti-nociceptive actions derive from endogenous ET-1 through ET_B receptors (see 3.D., above).

The just-stated hypothesis is confirmed by another recent study. Reduced expression of ET_B receptors (by ~30–35%) was revealed in sciatic nerves (with no changes in DRGs) from rats with experimental chronic diabetes, and these diabetic animals also exhibited both an increase in the withdrawal responses to “high threshold” stimuli (mechanical hyperalgesia) and to light touch (tactile allodynia) 18. Mechanical hyperalgesia and tactile allodynia in ET_B receptor-deficient transgenic rats appeared similar to those observed in diabetic rats. These results suggest that decreased expression of ET_B receptors in peripheral nerve may contribute to the development of neuropathic pain in chronic diabetes, and that ET_B receptors contribute to anti-nociception in that situation. The mechanistic basis for these behavioral changes is not apparent, however, since ET_B receptors have been localized on the Schwann cells but not the axons of peripheral nerve 192, and do not have an obvious function in impulse conduction at this location.

ET_A receptors mediate neuropathic pain in the rat sciatic nerve chronic constriction injury model (CCI). Endothelin-1 and ET_A receptor are locally up-regulated at the site of chronic constriction (nerve ligation) in rats with neuropathic pain behavior 5 days after surgical manipulation, and systemic delivery of the ET_A receptor antagonist ABT-627 acutely reduced thermal hyperalgesia and completely reversed mechanical hyperalgesia in those animals.132

Contrasting the role of ET_A receptors in driving diabetic and nerve-injury induced neuropathy, ET_B receptors appear to trigger trigeminal neuropathy 43. Sustained bilateral mechanical allodynia in a rat model of trigeminal neuropathic pain results from unilateral constriction of the infraorbital nerve (ION). Mechanical allodynia on the face 12–15 days after nerve constriction, that was abolished for up to 90 min by subcutaneous morphine, was unchanged

by systemic bosentan (10 mg/kg, iv) or ABT-627 (10 mg/kg, i.v.). In contrast, systemic delivery of the ET_B receptor antagonist A-192621 resulted in a ~60% reversal of allodynia, evidence of ET_B receptors positive contribution. However, co-injection of A-192621 with ABT-627 (co-inhibition of ET_A and ET_B receptors, mimicking the actions of bosentan) also did not modify ION injury-induced mechanical allodynia. Control injection of ET-1 into the upper lip of naive rats caused ipsilateral mechanical allodynia, although the effected receptor type was not identified. These data show that endogenous ET-1 contributes to orofacial mechanical allodynia via ET_B receptors, but in order to engage the anti-allodynic effect of ET_B receptor blockade, functional ET_A receptors are required. Whether these receptors are present on the same cells, e.g., neurons, where converging intracellular signaling pathways allow cross-modulation, or are present on different cell types, which are coupled by diffusible substances released by ET receptor activation, is an interesting question.

Furthermore, orofacial cold hyperalgesia also occurs after chronic constriction of the infraorbital nerve.⁴⁴ This symptom, which peaks at 4–6 days after surgery, appeared to lack an inflammatory component (was unaffected by indomethacin and celecoxib), but was ameliorated by systemically administered antagonists of ET_A and of ET_B receptors. These inhibitions, reaching 65–90%, lasted for 1–2.5 hours. Bosentan (the dual ET_A/ET_B receptor antagonist) abolished this injury-induced cold hyperalgesia, for up to 6 h. Thus, the cold allodynia after ION injury involves ET receptors in a different way than that for mechanical hypersensitivity from the same injury, suggesting that a single mechanism is not common to all types of hypernociception, and that peripheral sensory neurons of different modality can be modulated very differently by endogenous endothelins.

4.F. Endothelin-1 in Raynaud's phenomenon

Raynaud's phenomenon (RP), a painful condition that occurs in response to cold or stress, is characterized by episodic ischemia of digits of the hands or feet. Primary RP, without tissue loss, may precede by years the onset of visceral and/or cutaneous sclerosis. Secondary Raynaud's phenomenon, following tissue loss or damage, eventually develops in 95% of patients with systemic sclerosis (SSc) and other collagen or vascular diseases and reflects the microvascular damage of distal extremities, complicated by functional disability and digital ulcerations.^{46,193}

Endothelin-1 appears to be crucial in the pathogenesis of RP and in the development of skin alterations in SSc.²⁵⁷ Bosentan, a non-selective ET receptor antagonist, prevented digital ulcers in SSc,¹³⁴ and when used in a small number of patients with severe RP (associated with pre-scleroderma and systemic sclerosis, independent of digital ulcers), it significantly decreased pain. This treatment also lessened Raynaud's disease activity, judging from the number and severity of vasospastic attacks and reflected by improved thermoregulation in all patients after a 16-week treatment.²¹⁵ These findings may point to a therapeutic use of ET receptor antagonists in severe RP, and perhaps in delaying the onset and suppressing the symptoms of early stages of this disease.

4. G. The Endothelin Axis in cancer pain

Endothelin-1 is involved in the growth and progression of many types of tumors. These include prostatic, ovarian, renal, pulmonary, colorectal, cervical, breast, bladder, endometrial carcinomas, bone metastases, melanoma and others, where ET and ET-receptors, collectively are referred as the "ET axis". The activation of ET receptors contributes to cell proliferation, escape from apoptosis, neo-vascularization, invasion and metastatic spread (see reviews 10, 40,166,223). Endothelin-1 activates a network of intracellular events resulting in tumorigenesis. The ET_A receptor mediates ET-1's effects in prostate, ovarian, breast, renal, bladder, cervical cancer and bone malignancies, 8,40,88,100,182,202,223 while both ET_A and

ET_B receptor subtypes are presumably responsible for ET-1's actions in colon cancer and Kaposi's sarcoma. 180,201 Endothelins also modulate the trafficking, differentiation and activation of tumor-infiltrating immune cells. 10,85

Over 70% of patients with advanced breast or prostate carcinoma present with metastatic disease, and bone is a common site for metastasizing prostate and breast cancer 79. Endothelin-1, via ET_A receptors, stimulates mitogenesis in osteoblasts leading to new bone formation 87,88,263. Tumor-produced ET-1 enhances osteoblast proliferation, decreases osteoclastic bone resorption and osteoclast motility, the events resulting in osteoblastic metastases 263, while osteoblasts and endothelial cells in the bone of the host are secreting certain factors (e.g., IL-1, TNF- α and transforming growth factor- β) which in turn stimulate more ET-1 production by the metastatic cancer cells 40. Thus, the ET axis serves a critical role in a positive feedback loop that can accelerate the growth of tumors in bone.

Bone cancer is often associated with severe pain. Contemporary experimental models of bone cancer pain utilize a direct injection of cancer cells into calcaneus, humerus, or the medulla of the femur. 33,103,155,181,206,216,253,271 The first of two studies of ET-1's contribution to pain from bone tumors used a murine fibrosarcoma model. 33,253 Fibrosarcoma-bearing mice presented signs of ongoing, resting (spontaneous) pain along with hyperalgesic responses to mechanical and cold stimuli. Tactile allodynia, tested with a very weak von Frey hair, was evident at a time before any bone destruction and then advanced, in parallel with progressing bone destruction. Cold hyperalgesia appeared at a later time. Systemic morphine inhibited both mechanical and cold hyperalgesia.

Increased levels of ET-1 or ET-2 (but not ET-3) were found in perfusates collected from the tumor sites of these hyperalgesic mice. Injection of the ET_A receptor antagonist BQ-123 into the tumor site partially attenuated mechanical hyperalgesia, although systemic (i.p.) delivery was ineffective. An antagonist of the ET_B receptor, BQ-788, was much less effective at reducing hyperalgesia. These findings suggest that endogenous ET-1 makes a partial contribution to fibrosarcoma-induced pain, primarily through tumor-associated ET_A receptors.

In addition, hyperalgesia was present but significantly less after subcutaneous tumor implantation that did not involve bone. Mice with calcaneus inoculation of melanoma cells (G3.26, a B6 subclone) developed neither ongoing pain, nor hyperalgesia nor bone destruction, nor did ET-1 levels increase in the collected perfusates. 253 From this it appears that engagement of the ET axis depends both on the type of cancer and on the anatomical location of tumors.

When ET-1 was injected into a tumor-bearing paw it induced enhanced acute nociception, both in mice with sarcoma inoculated into bone (calcaneus) 253 and in mice with subcutaneous inoculation of androgen-independent human prostate cancer cells (PPC-1 into the plantar hind paw. 269 This suggests that primary afferents can be sensitized or activated by ET-1 released from tumors. Systemic ET_A receptor antagonists YM598 and ABT-627 inhibited this ET-1-induced potentiation of nociception, 268 probably by acting at receptors around the tumor.

In the other study of ET-1 in bone cancer pain, osteolytic (2472) sarcoma cells were injected into the medullary space of the mouse femur, and the injection site was sealed to prevent leakage of cells outside the bone. 181 These sarcoma-bearing mice exhibited ongoing pain and touch-evoked hyperesthesia. Immunohistochemistry revealed high levels of ET-1 in cultured 2472 sarcoma cells, but the levels of ET_A and ET_B receptors appeared low or undetectable. On the other hand, ~21% of small and medium diameter and 5% of large diameter DRG neurons expressed ET_A receptors and essentially every non-myelinating Schwann cell expressed ET_B receptors. Acute systemic delivery of the ET_A receptor antagonist ABT-627 strongly suppressed the indices of ongoing and movement-evoked pain. In contrast, the ET_B receptor

antagonist A-192621 (i.p.) enhanced both spontaneous and palpation-induced guarding, although without altering the flinching behavior. Chronic administration of ABT-627 (p.o. in drinking water, over PIDs 6–14) inhibited both ongoing and movement-evoked pain behavior in bone cancer. Such chronic delivery of this ET_A receptor antagonist also partially reduced *c-Fos* expression in dorsal horn laminae I–II, where it is a marker of sensitization of primary afferent terminals, 104,170 and in deeper loci, laminae III–VI, where dynorphin expression was also reduced. Neither tumor growth nor bone destruction were influenced by chronic ABT-627.

These studies identify a triggering role for ET_A receptors in sarcoma cancer pain. The chronic, systemic administration of ABT-627 was shown to be effective against sarcoma cancer pain, even at later stages of tumor development, unlike the earlier results with systemic BQ-123. 253 Whether this difference in effectiveness is due to receptor affinity or to pharmacokinetic factors of distribution or biotransformation is not obvious. The anti-nociceptive actions of ET_B receptors (likely present on Schwann cells), present in this model of sarcoma cancer pain, could be explained by the ET-1 clearance function of ET_B receptors, or by some anti-nociceptive mechanism resembling the one found for skin. 129 The latter mechanism might have more support since ET-1 clearance mechanisms are decreased in cancer cells with ET_A receptor-mediated growth and progression, e.g., prostate cancer cell lines. 167,179,246

Murine models of other types of cancer pain have been developed, with some similarities for ET involvement. 213 Recently, either of two skin tumors that differed significantly in production of ET-1, squamous cell carcinoma (SCC, HSC-3 cell line) or melanoma (WM 164 cell line), were inoculated into the plantar hind paw of female mice, and indices of pain, tumor volume and ET-1 content were compared. 183 Tactile allodynia correlated with tumor volume, with a stronger correlation in the melanoma group. However, despite significantly greater tumor volumes in the melanoma group, tactile allodynia was more pronounced in the SCC group. The expression of both ET-1 mRNA and ET-1 protein were higher in the SCC tumor compared to the melanoma tumor. BQ-123 injected into the mid-plantar paw, at the site of greatest tumor development on PID 32, was effective against tactile allodynia in the SCC model only. The results indicate that the intensity of cancer pain more likely depends on a tumor's ET-1 content rather than its size, and that ET_A receptors mediate pain in squamous cell carcinoma, but not in melanoma cancer in skin. Whether this distinction remains when these tumors metastasize to other regions, such as bone, remains to be tested.

Inoculation of B16-BL6 cells, a highly invasive and metastatic murine melanoma, into the plantar region of the mouse hindpaw resulted in a progressing mechanical tactile allodynia from PID 11 until PID 16–20, 74 which is opposite to the effects described previously for implantation of the melanoma G3.26, B6 cell line. 253 Intraplantar BQ-123 inhibited tumor-induced allodynia (PID15–18), while BQ-788 was without effect. An intraplantar injection of ET-1 elicited acute nocifensive licking that was greater in the melanoma-bearing hind paw than in controls, and was inhibited by the ET_A antagonist BQ-123. In naive mice, an intraplantar injection of tumor extract, prepared from the paw-grown tumor, evoked mechanical allodynia which was reduced, although only partially, by local inhibitors of both ET_A and ET_B receptors. The levels of mRNA coding for the ET_A, but not the ET_B, receptor were increased in the ipsilateral DRGs (where both neuronal and glial cells were present). Immunochemical staining showed that the cultured melanoma cells expressed an ET, probably ET-1, and that ET content increased with the tumor mass over the time after inoculation. These results suggest that endogenous ET-1 contributes to melanoma-induced hyperalgesia, and that in melanoma ET_B receptors are contributing to pain, along with ET_A receptors.

The findings in this study are in agreement with the role of ET_B receptors that has been proposed for melanoma. ET_B receptors are over-expressed in melanoma cells and correlated with tumor

progression. ET_B receptors probably mediate all of ET-1's tumor promoting effects. Thus, BQ-788 affects apoptosis most effectively in metastatic melanoma cells lines 138, and an orally delivered, non-peptide ET_B antagonist, A-192621, inhibited melanoma growth *in vivo* in nude mice 9. Bosentan, a dual ET_A/ET_B receptor antagonist, stabilized the cancer in patients with stage IV metastatic melanoma. 127 We suggest that the ET_B receptor, playing a leading role in the tumorigenesis of melanoma, effects pain-related signaling, as well. The latter is worth further investigation.

Given the expanding evidence for a role of the ET-1 axis in initiation and progression of malignancies, the use of ET receptor inhibitors is an obvious approach to cancer treatment. Promising results have been obtained with ET_A receptor antagonists in pre-clinical models, i.e. inhibiting tumor cell proliferation, invasiveness, or angiogenesis, as well as attenuating osteoblastic and pain-related responses to tumor. Among the ET_A antagonists that have been tested, ABT-627 (Atrasentan) is an orally bioavailable, highly receptor-specific and rapidly absorbed antagonist. 40 In a Phase III clinical trial that included 809 men with metastatic hormone-refractory prostate cancer (HRPC), Atrasentan suppressed the increase of prostate-specific antigen and the progression of bone turnover and of pain. However, enthusiasm for further developments of Atrasentan treatment was dampened by the fact that significant improvement in survival or time to cancer progression was not demonstrated. 41,139 Although disease stabilization or reversal is the most desired goal of such therapy, improvement in the quality of life for patients with advanced disease is an important achievement. That various ET receptor antagonists, alone or in combination, have been able to achieve substantial relief of cancer-associated pain in many pre-clinical tests, shows that they deserve further studies and clinical evaluation. Also, a combination of ET receptor blockade with inhibition of other "pro-algesic" receptors, 79 such as TRPV1, could present an effective multi-target approach in pain treatment.

5. PAIN MODULATION BY ENDOTHELINS IN THE CNS

In contrast to its generally pro-nociceptive actions in the periphery, ET-1 inhibits nociception when administered centrally. The administration of ET-1 into the lateral ventricle (i.c.v.) and into the periaqueductal gray area (PAG) produced a significant increase in hot plate and tail flick latencies. 48,168 Spinal (i.t.) administration of ET-1 also produced a dose-dependent antinociceptive effect in both the tail flick test in mice 122 and in the formalin test in rats. 260 The latter suppression of acute nociception by i.t. ET-1, appearing as a dose-dependent decrease in phases 1 and 2 of formalin-induced flinching behavior, was antagonized by an ET_A selective antagonist. In contrast, ET_B activation by i.t. agonists enhanced phase 2 flinching, indicating that ET receptor subtypes might have opposing roles in spinal nociceptive processing. 260

The pathways and cellular events underlying central ET-1-induced antinociception are not clearly identified yet. ET-1 injected i.c.v. increased hot plate latencies in mice, like the effect of morphine, but this antinociception was naloxone-insensitive and was antagonized by the calcium entry blocker cinnarizine, given pre-emptively i.p. 168. These results, along with the antinociception produced by the L-type calcium channel activator BAY K 8644, 27 led to a suggestion that the CNS actions of ET-1 might be due to mechanisms involving an increased influx of extracellular Ca²⁺ into cells. In cultured rat cerebellar granule neurons, however, ET-1 inhibited the L-type Ca²⁺ current, via ET_A. 98 This inhibition of Ca²⁺ current was neither due to a change in cell membrane potential nor because of Ca²⁺-dependent inactivation of Ca²⁺ channels, but resulted from a decrease in the frequency of L-type Ca²⁺-channel opening. 98 It is noteworthy that ET-1's effects on Ca²⁺ channels in central neurons are opposite to those on peripheral neurons (see 2. A., B., above); whether this difference is related to the opposite effects on pain caused by ET-1 in these different locations is an interesting consideration.

NMDA receptors are likely implicated in certain of the central antinociceptive effects of ET-1. Stimulation of the peri-aqueductal gray region with ET-1 resulted in enhanced hot plate latencies, an antinociception that was prevented by the NMDA receptor antagonist 2-APV, but not by an antagonist of the AMPA-kainate type of glutamate receptor, injected into the same area prior to ET-1. 48 On the other hand, NMDA-mediated nociception can be inhibited via activation of protease-activated receptor-1 (PAR-1) sites by intrathecal thrombin, using a pathway involving ET-1 and its ET_A receptor subtype. Thrombin generates a tethered ligand for activating PAR-1, whose activation potentiates NMDA receptor activity in hippocampal neurons. 78 Intrathecally injected thrombin, or selective PAR-1 agonists, however, inhibit nocifensive writhing behavior induced in mice by either i.t. NMDA or by inter-abdominal acetic acid (which behavior is NMDA-mediated), although thrombin has no such effect on hot plate or tail flick latencies, which are independent of NMDA. 67 These anti-nociceptive actions were prevented by inhibition of ET_A, but not by ET_B receptors, indicating an essential role for ET-1 in central antinociception, opposite of the circumstance in the periphery. The source of the pain-inhibiting endogenous ET-1 is not known, although endothelin is produced by both neuronal and non-neuronal cells, including glial, 144 and thrombin has been shown to release ET-1 from cultured astrocytes. 63 Endothelial cells of cerebral microvessels produce and release ET-1 through the adventitial side of the cell to the extracellular space of the CNS. 267 Such non-neuronal ET-1 in the CNS may reach ET receptors on neurons in a paracrine manner or by way of CSF, and affect transmission in pain pathways.

ET-1 of neuronal origin may also participate in pain-inhibiting actions in the CNS. It has been shown that neuron-specific ET-1-knockout mice are hyper-responsive to radiant heat and mechanical stimulation, and to hyperalgesia from intraplantar carrageenan-evoked inflammation or after spinal nerve ligation. The same mice have less stress-induced analgesia in a swim-stress model, compared to wild-type mice. 95 These changes suggest that a basal release of ET-1 from neurons likely affects baseline pain thresholds, and that additional ET-1 released during persistent pain states acts to further suppress pain, and to contribute to stress-induced analgesia. In fact, neuronal ET-1 in normal mice was increased in the hypothalamus after induction of peripheral inflammation with intraplantar carrageenan. 95 Interestingly, [ET-1] measured in other parts of the CNS, midbrain, medulla and spinal cord, all involved in endogenous pain inhibitory systems, was unchanged.

Interactions of ET-1 with opioid receptors indicate another central pathway for endogenous modulation of pain. Antinociceptive, naloxone-sensitive effects of intrathecally administered ET-1 on the tail flick latencies in mice were inhibited by intrathecal verapamil, an L-type Ca²⁺ channel blocker, and by the δ -opioid receptor-selective antagonist naltrindole, but not by antagonists of μ - or κ -opioid receptors 122. ET-1 may thus mediate a Ca²⁺ entry-dependent release of endogenous opiates acting on δ -opioid receptors. Importantly, although ET-1's peripheral actions can be complicated by its potent vasoconstrictive actions (see above), ET-1 does not affect spinal blood flow, since the highest dose of ET-1 used for intrathecal microinjections was ~30-fold lower than the dose just able to increase spinal cord microvascular resistance. 90

In contrast to the antinociception from ET-1 itself, centrally administered (i.c.v.) antagonists of ET_A potentiated morphine analgesia on tail-flick latencies in rats and mice. 20,21,150 Stimulation of ET_B in the CNS did not affect the actions of morphine. 22 While chronic morphine treatment results in functional uncoupling of μ -opioid receptors from their G-proteins, antagonists of ET_A receptors enhanced the coupling of G-proteins to opioid receptors (as shown *in vitro* in neuroblastoma cells), an effect that has been proposed as a mechanism for potentiation and restoration of morphine anti-nociception in morphine-tolerant animals. 23 It is tempting to speculate that hyper-nociceptive input elevates the release of endogenous

ET-1 in the CNS which in turn prevents opiate-induced desensitization of specific opiate receptors, thereby promoting the inherent anti-nociception.

CONCLUSIONS

Endothelin-1 and its receptors are implicated in many forms of long-lasting, elevated pain, from inflammation, injury and diseases that affect peripheral tissues, while normal physiological pain is unaffected. Many different ion channels and transmitter pathways appear to be altered by this “endothelin axis”, suggesting that diverging signaling pathways, such as MAPKs, account for these broad effects. Early therapeutic results, e.g. in cancer pain, encourage the further development of drugs targeted at endothelin receptors for the prevention or relief of elevated pain.

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Figure 1.

Schematic of the actions of ET-1 on a sensory nerve ending and on local keratinocytes in the epidermis. ET-1 is secreted by many different resident and invading cells and binds to receptors on both nerve and skin cells. Activation of the GPCR ET_A receptors on nerve cells results in acute changes in ion channels that enhance excitability: delayed rectifier (D-R) type K⁺ channels are inhibited, TTX-R Na⁺ channel activation is enhanced, as are TRPV1 channels. Calcium is released rapidly from intracellular stores and in turn activates PKC (and, possibly, PKA) as part of the coupling between ET_A activation and its downstream targets. Keratinocytes express ET_B receptors and, in addition to releasing ET-1, keratinocytes also release β-endorphin. That opioid, in turn, binds to μ-opiate receptors (MOR) on nerve endings, which are coupled through G-proteins to increase the outward currents through the G protein-coupled inward rectifier K⁺ channels (GIRK2). The hyperpolarization of the nerve's membrane by this action lowers excitability by moving the resting potential away from threshold and by lowering the membrane resistance. This same GIRK may also be a target of NK1 receptors, activated by Substance P, but in this case the K⁺ channels close, the membrane depolarizes and the nerve becomes more excitable. (Dashed lines show speculative pathways; Circled + or – depict stimulatory or inhibitory effects.) After ref. 135.

Figure 2.

Schematic of the signaling pathways initiated by endothelin-1 in keratinocytes. Endogenous production and subsequent release of ET-1 is proposed to activate resident ET_A receptors which mediate cell proliferation as well as the release of noxious chemical stimuli such as glutamate and CGRP. ET_B receptors on keratinocytes have a dual function: i. Acute stimulation of ET-1 release, followed by slow negative regulation of ET-1 synthesis and secretion, as part of an autoregulatory loop, ii. Acute stimulation of β-endorphin release, as part of the local analgesic action of endothelin-1. (Dashed lines show speculative pathways; Circled + or – depict stimulatory or inhibitory effects.)

Figure 3.

A diagrammatic display of the documented ways in which ET-1 causes pain and hyperalgesia. The upper section lists the manner of pain caused by exogenous ET-1, and the lower section lists the various disease states wherein ETs and their ET receptors are thought to contribute to pain and hyperalgesia. Question marks indicate the conditions where the role of ETs is not established. Numbers in parentheses refer to citations in References.

PERIPHERAL EFFECTS of ENDOTHELIN

ETs-induced anti-nociception↓
ET-Bexogenous local ET-1 (tested by local ET-B agonists/antagonists)

1. **anti-hyperalgesic action** of ETs along with pro-hyperalgesic action in **capsaicin-induced** nociception (i.pl., mice; 185, 186)
2. **anti-nociception via β -endorphin release from keratinocytes** (for ET-1-induced overt flinching in rats; 128,129)

selective ET-B activation (by local ET-B agonist)

1. **inhibition of carrageenan-induced nociception and priming** in the naive joint (ET-B operated mechanism limits priming effect of carrageenan to nociception evoked by subsequent inflammatory insults) (rats, 50)
2. **anti-hyperalgesic effect in acute inflammation** for **thermal hyperalgesia** (in rats, CFA; 276)

endogenous ET (tested by systemic ET-B antagonists)

1. **anti-hyperalgesia in a rat model of diabetic neuropathic pain** (116)
2. **anti-nociception in ongoing and movement-evoked pain** in murine **ostelytic sarcoma model of bone cancer** (181)
3. **anti-nociception in antigen-induced pain response** in immunized mice (187)

Figure 4.

A diagram of the cases where ETs and the ET_B receptors cause analgesia or anti-hyperalgesia. The three sections list: upper-effects of exogenous ET-1; middle-ET_B's selective activation by local agonists; lower – evidence for anti-hyperalgesic actions from endogenously released ET. Numbers in parentheses refer to citations listed in References.