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NEW PERSPECTIVES ON THE ENDOTHELIN AXIS IN PAIN

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1. INTRODUCTION

The endogenous peptide endothelin (ET) and its several receptors are now well-known participants in a broad variety of conditions that cause pain. Ample evidence supports a direct role for these molecules in causing overt pain and in sensitizing the nervous system to elevated responses to painful stimuli. Our laboratory has recently published an extensive review on the role of endothelins and their receptors (the “endothelin axis”) on pain that occurs under many conditions including inflammation, nerve injury and cancer [1]. In the present review, we highlight some of the key novel findings over the past two years with regard to the biochemistry and molecular pharmacology of endothelin receptors, the diverse intracellular signaling pathways that might couple ET receptor activation to pain-related processes and the newest and updated findings on the *in vivo* pharmacology of endothelin in various pain states.

2. BIOCHEMISTRY AND MOLECULAR PHARMACOLOGY OF ENODTHELIN RECEPTORS

Endothelin has a complex influence on nociception. ET_A receptor activation appears to always lead to pain or nociceptive sensitization, while the effects attributed to ET_B receptors can be pro- or anti-nociceptive, depending on the experimental conditions. Similarly, ET_B receptor activation has also been shown to have opposing effects in the cardiovascular system, with receptor activation triggering both vasoconstriction and vasodilation [2;3]. These striking differences in ET-1 signaling may be due in part to a complex pharmacology of endothelin receptors, which has been suggested to potentially involve unidentified receptor subtypes, splice variants of known receptors or heterodimers.

2.1. Classification for ET receptors

Endothelin receptors were first categorized into ET_A or ET_B receptors based on their differential affinities for endothelins (ET-1, ET-2 and ET-3) and sarafotoxins (S6b and S6c),

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homologous toxins isolated from snake venom [4;5]. ET-1 and ET-2 bind with similar high affinities to both ET_A and ET_B receptors, while ET-3 shows a much higher affinity for ET_B. Sarafotoxin S6c also has a higher affinity for ET_B compared to ET_A, while S6b is non-selective for these receptors.

This two-receptor model for endothelin has been strongly supported by the cloning of only two mammalian endothelin receptors [6;7]. These receptors were found to be G protein-coupled receptors with ligand selectivity largely conferred by the 2nd through 5th transmembrane domains [8–12]. These receptors also have allosteric sites. Aspirin, aspirin's metabolite sodium salicylate, aldoxime and hydroxamic acid derivatives act as allosteric inhibitors of these receptors [13;14]. In addition, it has been shown that bosentan, a mixed (or dual) antagonist showing high affinity for both ET_A and ET_B receptors, has a separate binding site from ET-1 [15].

Numerous endothelin ligands have been developed and can be broadly grouped into peptide and non-peptide ligands [16]. Some antagonists are selective for either ET_A (e.g. BQ-123) or ET_B receptors (e.g. BQ-788), while others are relatively non-selective between these two subtypes (e.g. PD142893 and SB209670). ET_B receptor-selective agonists have also been described (e.g. IRL-1620 and BQ-3020), although to date, no selective ET_A agonist has been discovered. Therefore, ET_A activity is typically defined by using the non-selective agonists ET-1 or S6b in combination with a selective ET_B receptor antagonist.

The development of selective ligands for ET_A and ET_B has greatly increased our ability to analyze the function of these receptors; however, it has also yielded results that are inconsistent with a model of 2 endothelin receptors acting independently. As described below with reference to possible receptor interactions, these inconsistencies have included synergistic actions of selective endothelin receptor ligands, higher potencies for ET_A antagonists in blocking ET_B-selective compounds compared to the non-selective ligand ET-1 and mismatches in selective antagonist effects.

2.2. Interactions between ET receptors

Synergy between selective ET_A and ET_B ligands has been seen in numerous systems. In primary rat astrocytes for example, saturation binding experiments indicated a single, saturable binding site for [¹²⁵I]-ET-1 [17]. The ET_A selective antagonist BQ-123 and the ET_B selective antagonist BQ-788 both failed to compete for this binding site individually. When combined however, binding of [¹²⁵I]-ET-1 was almost completely blocked. Such apparent interactions raise the question of whether this results from formation of ET_A-ET_B heterodimers (see below) or whether signal processing from one receptor acts through the intracellular network to modify the other receptor. Similar findings have been shown in other tissues. Research in the human bronchus, which expresses both ET_A and ET_B receptors, found that both ET_A-and ET_B-selective antagonists failed to inhibit ET-1 mediated contractions, even when used in combination [18]. Interestingly, however, these contractions were inhibited by the mixed antagonist SB209670. In addition, ET-1, ET-3, S6c, IRL-1620 and BQ-3020 were all potent at initiating contractions in the isolated bronchus, but only IRL-1620 and BQ-3020 contractions were blocked by BQ-788, despite S6c and ET-3 also being ET_B-selective agonists.

Studies have also shown that the presence of ET_A-selective ligands can increase the apparent binding of ET_B-selective ligands. In the rat anterior pituitary gland, the ET_B receptor-selective ligands S6c, ET-3, IRL-1620, and BQ-788 failed to compete with [¹²⁵I]-ET-1 binding except in the presence of BQ-123 [19]. There is also some evidence that the binding of ET_B receptors can reveal binding sites for ET_A receptors. In rat brain, [¹²⁵I]-ET-1 was found to more clearly recognize ET_A receptors when used in conjunction with S6c [20].

Importantly for nociception, a recent report shows that ET_A and ET_B receptors are co-expressed in the trigeminal ganglion neurons that mediate pain in the facial region [5]. It will be interesting to determine if there are interactions between these two receptors in such sensory neurons and what consequences this has for pain sensation.

2.3 Dimerization of ET receptors

Evidence for the ability of ET_A and ET_B receptors to form homo- and heterodimers has come from studies using stably transfected HEK and CHO cells. In these studies, interactions between ET_A and ET_B receptors in co-transfected cells were indicated by co-immunoprecipitation [22;23]. These studies have also shown differences between endothelin receptor coupling and internalization in cells co-transfected with both ET_A and ET_B receptors [22]. When ET_A receptors were expressed alone in HEK cells, ET-1 caused a transient increase in intracellular calcium that was not dependent on extracellular calcium. When ET_B receptors were expressed alone in HEK cells, ET-1 also caused a transient rise in intracellular calcium, but this was blocked by removal of extracellular calcium. Each of these responses was inhibited by the appropriate selective antagonist. This indicates that both ET_A and ET_B receptors can mediate a rise in intracellular calcium, but do so by coupling to different second messenger pathways. However, when ET_A and ET_B receptors were co-expressed in HEK cells, ET-1 caused a sustained intracellular calcium increase that was not reduced by removal of extracellular calcium [24]. This sustained response was not prevented by either BQ-123 or BQ-788 alone, but was prevented by the combined administration of these antagonists.

These studies using heterologous expression systems provide intriguing evidence that ET_A and ET_B receptors can dimerize, resulting in changes in second messenger coupling and internalization kinetics. In addition, a study analyzing ET_B-expressing Girardi heart cells transfected with ET_A receptors has provided evidence that ET_A and ET_B receptor heterodimers may account for some of the unusual binding results that have been previously reported [25]. Binding experiments in these cells showed that the presence of BQ-123 increased the affinity of the ET_B ligands BQ-3020 and BQ-788. The synergistic binding results were paralleled by the coupling of these receptors to second messenger pathways. BQ-788 alone failed to inhibit ET_B receptor-mediated intracellular calcium increases, but BQ-788 in combination with BQ-123 did inhibit this response. Therefore, it appears that heterodimers of ET_A and ET_B may explain the synergistic effects of endothelin receptor ligands seen in some systems.

3. CELLULAR PHYSIOLOGY AND INTRACELLULAR PATHWAYS MEDIATING PAIN BY ENDOTHELIN

Various pathological conditions are known to elevate ET-1 levels and to alter expression of ET receptors in the affected tissues. These changes, in turn, are known to activate or sensitize axons on nociceptor terminals [26–30]. However, the molecular and cellular mechanisms underlying ET-1-mediated pain processes are very complex and are slowly being unraveled. Peripheral ET-1 acts through receptors on nociceptor membranes which subsequently modify voltage-gated ion channels and transduction channels to increase membrane excitability and amplify signal generation.

3.1 ET-1 elevates intracellular calcium and mediates the release of algogenic peptides from primary sensory neurons

Pioneering studies on primary sensory neurons of the somatosensory system uncovered endothelin binding sites [31] as well as production of endothelins [31;32] at both dorsal root ganglia (DRG) and spinal cord levels. ET-1 was localized to a subset of peptidergic neurons

expressing substance P (SP) and calcitonin gene-related peptide (CGRP) [32] and it was suggested that ET-1 evokes the release of SP from neuronal terminals through a mechanism involving dihydropyridine-sensitive calcium channels [31]. Subsequently, it was shown that ET-1 does not promote spontaneous SP or CGRP release by isolated rat DRGs, but rather potentiates the release of SP and CGRP evoked by capsaicin [33]. ET-1 also enhanced the capsaicin-stimulated production of cyclic GMP [33]. These findings suggest that ET-1 acts on ET receptors in TRPV1 expressing peptidergic sensory neurons. The role of TRPV1 in ET-1's sensitization of nociceptors and its specific modulation of ET-1 expression levels in these cells has also been reported. (34; 35; and see below "ET-1 modulation of TRP receptor activity on nociceptors").

ET-1 is able to trigger calcium release from intracellular stores in isolated DRG neurons through ET_A receptor activation [36]. Although ET_A has been reported to couple to G_s in other tissues, the absence of intracellular cAMP production following stimulation of dissociated DRG with ET-1 [33] argues in favor of a mechanism involving coupling to G_q, activation of PLC and subsequent production of DAG and IP₃.

3.2. ET-1 acts through ET_A receptors on nociceptors to trigger pain-like behavior

Exogenous ET-1 elicits pain when injected subcutaneously at different peripheral locations in several species of mammals [1]. In adult rats, subcutaneous injection of high doses of ET-1 (100 to 500 μ M, 1–5 nmol) in the plantar region of the hindpaw elicits pain-like flinching behavior and induces selective firing of nociceptors [37]. This behavior is strongly inhibited by BQ-123, a selective ET_A-receptor antagonist. Similarly, epineural application or intraneural injection of ET-1 into the rat sciatic nerve causes pain-like behavior which also is inhibited by the administration of BQ-123 [38;39].

Electrophysiological recordings of sensory fibers from sciatic nerves of rats further demonstrated that most nociceptive (C- and A δ) fibers, but not the usually non-nociceptive (A β) fibers, respond in a dose-dependent way to ET-1 (200–400 μ M, 1–20 nmol), and that co-injections of BQ-123 blocked these effects [37]. This is consistent with autoradiographic and immunohistochemistry experiments on DRG neurons of rats, rabbits and monkeys demonstrating that ET_A receptors are present in a subset of small peptidergic (CGRP⁺) and non-peptidergic neurons and their axons, as well as in a small subset of medium size neurons [30;40;41]. ET_B receptors, which are expressed by satellite cells and non-myelinating ensheathing Schwann cells, but not neurons, do not appear to contribute to this phenomenon.

3.3. Ionic mechanisms of pain generation in nociceptors

ET-1 enhances excitation of isolated sensory nerve soma of small and medium diameter neurons, in part by selectively altering activation of tetrodotoxin-resistant (TTX-R) Na⁺ channels that are selectively expressed on many nociceptors (i.e., Na_v 1.8 and Na_v 1.9), [42], and also by suppressing the delayed outward rectifier current, I_K [43].

In the DRG-derived cell line ND7/102, ET-1 induces a transient rise of intracellular calcium, emanating from intracellular stores, which is blocked by BQ-123 but not by the ET_B-selective antagonist BQ-788 [44]. In freshly dissociated mouse DRG neurons, 10 nM ET-1 induced a very small intracellular calcium increase from intracellular stores which was blocked by BQ-123 but not by BQ-788. Conversely, in satellite cells the large intracellular calcium increase induced by 10 nM ET-1 was blocked by BQ-788 but not BQ-123. These data support the immunohistological findings and further suggest that ET_A and ET_B receptors both couple to a calcium pathway in their respective cell types [36]. The exact nature of the signal transduction components (e.g. G-proteins) involved in the release of

calcium from intracellular stores in sensory neurons remains unknown and warrants further investigation. It is tempting to speculate that G_q and the phospholipase C enzyme, both shown to act downstream of ET_A in other systems [44], are involved here. However, such a narrow conclusion may be premature, since both PKC- and PKA-linked pathways are affected by other pro-nociceptive mediators modifying Na^+ currents in DRG, including 5-HT, adenosine, epinephrine and PGE_2 [45–48].

Recently, it was reported that the tissue specific ablation of ET_A receptors in nociceptors expressing the sodium channel $Na_v 1.8$ completely suppressed tactile allodynia as well as late phase thermal hyperalgesia resulting from ET-1 injection into the plantar region without affecting “physiological” nociception (i.e. normal pain) [49]. It seems that ET_A receptors on sensory neurons are responsible for the ET-1 mediated late stage thermal hyperalgesia, while acute thermal hyperalgesia is mediated by non-nociceptive sensory neurons, or non-neuronal cells. While $Na_v 1.8$ has been used to specifically target nociceptors, it is important to note that keratinocytes have also been shown to express this channel, along with both ET_A and ET_B receptors [50–53]. Thus, it is possible that some of the deficits in late phase thermal hyperalgesia were due to the lack of keratinocyte ET_A receptor signaling. In addition, it is possible that ET_B receptors on keratinocytes mediate the remaining acute thermal hyperalgesia.

In summary, the ET_A receptor, PKC_ϵ , TRPV1 and most probably PLC, define the molecular components underlying ET-1 mediated thermal hyperalgesia and, possibly, tactile allodynia in nociceptors. Direct modulation of this neuronal signaling by keratinocyte responses to ET-1 has yet to be determined.

3.4. ET-1 modulation of TRP receptor activity on nociceptors

The nomenclature of behavioral studies of pain defines “hyperalgesia” as an umbrella term for all conditions of increased pain sensitivity. “Allodynia” is a special case of hyperalgesia where normally innocuous stimuli are perceived as painful, or cause “pain-like” behavior [54]. Thermal and mechanical hyperalgesia as well as tactile allodynia are well documented pain states induced by subcutaneous injection of a low concentration of ET-1 (30nM, 3pmol - 200uM, 2nmol) [55–59]. A recent study conducted in rats demonstrated that the TRPV1 blocker capsazepine efficiently reduced the thermal hyperalgesia induced by ET-1 (10nM, 1pmol - 600nM, 30pmol) [59] suggesting that TRPV1 receptors contribute to this phenomenon. Furthermore, this effect was attenuated by antagonists of both ET_A and ET_B receptors, suggesting that both receptors participate in this phenomenon. TRPV1 is a non-specific cation channel, part of the transient receptor potential ion channel superfamily predominantly found on unmyelinated C-nociceptors [60]. TRPV1 is activated/opened by several noxious agents including, H^+ , endocannabinoids, noxious heat, and capsaicin, a compound naturally found in hot peppers. About half of the DRG neuron population expressing TRPV1 is immunopositive for ET_A receptors. Like ET-1, other pro-inflammatory compounds such as bradykinin and ATP also mediate their effects, at least in part, by inducing or potentiating TRPV1 activity in nociceptors [61–63].

Activation of ET_A receptors enhances a capsaicin-induced increase in intracellular calcium in dissociated mouse DRG neurons [35]. This effect is mediated by ET_A activation of PKC_ϵ which in turn phosphorylates TRPV1 at residue S800 to potentiate its activity [59;58]. PKC_ϵ is a group II PKC “novel” isoform which requires only DAG and not calcium for its activation [64]. ET-1 also can raise intracellular calcium in neurons independent of TRPV1 [36], but how such elevation of calcium affects the TRPV1 response is not known. In addition, the cascade of events leading to the production of DAG and subsequent translocation of PKC_ϵ from the cytosol to the cell membrane following ET-1 application to TRPV1-positive nociceptors remains unexplored.

Interestingly, heterologous expression of ET_B receptors with TRPV1 in HEK293 cells showed that ET_B was also able to elicit potentiation of TRPV1 responses, although less effectively than ET_A [34]. This effect is not recapitulated in isolated DRG neurons because ET_B is not normally expressed in these cells. However, ET_A and ET_B do appear to co-localized in a subset of small diameter, non-myelinated TrpV1 expressing fibers in the trigeminal ganglia, [21;65] where they both seem to participate in the development of thermal hyperalgesia. Whether the concerted activity of ET_A and ET_B modulates TRPV1 in these neurons or if they act independently remains unknown.

A recent study found ET-1-induced (300nM, 3pmol - 4μM, 400pmol) licking, flinching and biting behavior is attenuated in TRPV1-deficient mice, where the relatively non-selective PKC inhibitor bisindolylmaleimide I is inactive, thus implicating a PKC-TRPV1-independent pathway [66]. PKA, calmodulin kinase II and phosphatidyl-4,5-bisphosphonate are enzymes that have all been reported to modulate TRPV1 activity [67–69] and therefore represent potential candidates for ET-1 effects on TrpV1. However, given that no increase in intracellular cAMP levels was observed following stimulation of dissociated DRG with ET-1 [33], PKA seems unlikely to be involved.

In mice, TRPV1 is involved in both pain-like behavior [66] and thermal hyperalgesia induced by ET-1 [58]. However, ET-1-induced thermal hyperalgesia is PKC-mediated [58], whereas nociception is PKC-independent [66]. In rats, by comparison, TRPV1 does not mediate acute nociception [57;70], but is involved in mechanical allodynia [57] and thermal hyperalgesia [70]. A potentiating effect of ET-1 (2nM, 1pmol - 20nM, 10pmol) on capsaicin-induced overt nociception is ET_A receptor mediated, whereas both ET_A and ET_B receptors contribute to ET-1's potentiation of capsaicin-induced thermal hyperalgesia [70].

Transient receptor potential ankyrin subfamily member 1 (TRPA1) is a non-selective cation channel known to be activated by noxious cold [71]. TRPA1 is expressed by unmyelinated peptidergic nociceptors that also express TRPV1 [71;72]. Recent findings implicate its involvement in acute and chronic cold nociception in mice and rats [73–76].

TRPA1 is involved in ET-1-induced spontaneous pain-like behavior in wild-type mice. Pain-like behavior induced by a low concentration (10 μM, 100pmol) of ET-1 was significantly reduced when it was co-injected with highly selective TRPA1 antagonists [77]. The pain-like behavior induced by 10 μM ET-1 was reduced by local inhibitors of phospholipase C, but not protein kinase C. ET-1, at a concentration (300 nM, 3pmol) that did not induce significant pain on its own, enhanced nociception from the TRPA1 agonist, cinnamaldehyde. These results suggested that TRPA1 is involved in ET-1-induced spontaneous pain-like behavior in mice.

3.5. Recent findings on pain in humans from exogenous ET-1

Previously, pain had been reported in humans after intradermal and intravenous injection of ET-1 [78;79]. Now, a quite recent study reported that intracutaneous ET-1 (10pM, 10fmol - 1μM, 10pmol) injected into humans caused stinging and burning pain in 9/34 subjects and a mixture of the above accompanied by itch sensation in 14/34 subjects. Itch alone occurred in 11/34 subjects [80]. Micro-neurography showed that only mechanosensitive C-nociceptors (65%), but none of the mechano-insensitive fibers were activated by ET-1, unlike the effect of capsaicin, where mechano-insensitive fibers were predominantly activated. No activation occurred at (or below) 10⁻¹⁰ M, whilst 93% of fibers were activated by 10⁻⁶ M ET-1. 47% of these showed very long-lasting, vigorous responses, and some had burst-like firing for up to 15 min. At 10⁻⁶ M, ET-1 sensitized 31% of previously insensitive fibers to mechanical stimulation, and 60% of such fibers to heat. Mechano-insensitive fibers were also sensitized to heat (5/13 by 1 μM ET-1).

Intracutaneous injection of the H1 blocker Tavegil before 1 μ M ET-1, both prevented the axon reflex flare response and decreased pain and itch from ET-1 [80]. Hence, axon reflex erythema appears to be mediated by histamine, released possibly from ET receptor-expressing mast cells, following injection of ET-1.

3.6. ET-1's action on peri-neuronal tissue and nociception

In addition to its direct action on nociceptors as described above, ET-1 can trigger a variety of indirect effects via ET receptors expressed on other cell types which are capable of releasing signaling molecules that can in turn modulate nociception. Here we will focus on the action of ET-1 on satellite and glial cells wrapping around DRG neurons as well as on keratinocytes surrounding nerve terminals in the skin.

Studies in rat, rabbit and monkey peripheral nervous systems revealed that ET_A receptors are expressed on primary afferent axons and on the cell bodies of small-diameter dorsal root ganglion neurons, whereas ET_B receptors are distributed on the ensheathing non-myelinating Schwann cells of the sciatic nerve and the DRG satellite cells [40]. Recently, ET_A and ET_B receptors were found to be co-expressed in neurons of all three divisions of rat trigeminal ganglion (TG) [21]. In mouse, ~21% of small and medium diameter and 5% of large diameter DRG neurons were shown to express ET_A receptors and essentially every non-myelinating Schwann cell expressed ET_B receptors [30]. Despite the known expression of ET receptors in neuronal cell bodies and axons, the presence of ET receptors on nerve endings in the skin or other target tissues has been more difficult to determine.

3.6.1. ET-1 acts through ET_B receptors on keratinocytes to induce antinociception and local analgesia—Recent evidence indicates that an endogenous ET_B mediated analgesic mechanism is present in the epidermis [50]. Our laboratory has previously shown that pain caused by the subcutaneous injection of ET-1 (40 μ M, 400 pmol) is mediated by ET_A receptor activation and that this pain is countered by an anti-nociceptive action of ET_B receptors. This analgesic effect of ET_B receptor activation was prevented by naloxone, indicating the involvement of endogenous opioids. Cultured keratinocytes were found to release β -endorphin upon ET_B receptor activation, implicating these cells as the source of the endogenous opioids.

4. THE PRIMARY ROLE OF THE ENDOTHELIN AXIS IN THREE TYPES OF PATHOLOGIC PAIN

Exciting new findings have occurred over the past 2–3 years about the involvement of endothelins and ET receptors in the biology of pain related to sickle-cell disease, complex regional pain syndrome and several types of cancer. In this last section of this review we summarize these findings.

4.1. Variations of pain during sickle cell disease

Pain during a “crisis” in sickle-cell anemia has been successfully treated by ET-R antagonists. One hallmark of these episodic crises, which occur more frequently in males than females and appear early in childhood, is increasing pain intensity with repeated episodes. This phenomenon may be one example of the general observation that pain experienced by neonates early in life can have long-term effects on nociception and opioid analgesia. The mechanistic basis for this sensitization was explored in rats by injecting repeated doses of ET-1 (330 μ M, 3.3 nmol) at post-natal days 7 and 11, time points chosen on the assumption that postnatal day 7 (P7) is approximately equivalent to the nervous system development of a full-term human infant, and postnatal day 11 (P11) is approximately equivalent to a human toddler [81].

In male rats, flinching from ET-1 increased at the repeat exposure (P11 after P7), and was correlated with a significant decrease in ET_B receptor expression in plantar skin at P11. However, in young females decreased flinching occurred at the second injection at P11, and was correlated with a significant increase in ET_B receptor expression on that day. A subsequent paper on neonatal ET-1 responses revealed that total ET_A receptor expression was unaffected by previous ET-1 injection (330μM, 3.3nmol) [82].

Apparently, at this stage of development, alteration of responses to repeated high doses of ET-1 are shaped by sex-specific changes in peripheral ET_B receptors, which mediate anti-nociceptive actions [50]. Why these changes in ET_B receptor expression are sex-dependent, how soon they occur, how long they persist, and whether they also occur in adult rats are all intriguing questions that remain to be answered. In adult male rats only a de-sensitization to a second injection of ET-1 (200μM, 2nmol), occurs in the injected paw, and this resolves by 72 hours and appears to involve the ET_A receptor [83].

4.2. Endothelin-1 in complex regional pain syndrome

Complex regional pain syndrome (CRPS) is a painful disorder that primarily occurs as a complication after limb trauma or surgery, and is more clearly diagnosed when a nerve lesion is present (CRPS Type 2). Symptoms like those of CRPS may also appear without definitive evidence of nerve injury (CRPS Type 1). CRPS is characterized by spontaneous and stimulus-evoked pain, motor dysfunction, changes in tissue blood flow and skin surface temperature, edema and trophic changes in the affected limb; the severity of symptoms is usually disproportional to the initial event [84]. The pathogenesis of this syndrome is unclear, but growing evidence indicates the involvement of an exaggerated inflammatory process, mediated by cytokines and/or neurogenic inflammation by neuropeptides; this autoimmune etiology of CRPS is a new pathophysiological concept [85;86].

Several studies have sought correlations between levels of ET-1 and the occurrence of CRPS as evidence for ET-1's involvement in the pathogenesis of this disease. ET-1 levels in plasma from severely affected CRPS patients are the same as those from patients with non-CRPS chronic painful conditions, or healthy volunteers [87]. ET-1 levels in cerebrospinal fluid levels of chronic CRPS patients are also the same as those in controls [88]. However, a marked increase of ET-1 was found in the fluid collected from artificial blisters induced on the dorsal skin of the involved hand of patients in the intermediate phase of CRPS Type 1, compared with those in fluid obtained from the contralateral, non-symptomatic forearm. This elevated ET-1 correlated with a significant increase of the levels of IL-6 and TNF-α and reduced NO levels in this blister fluid [89]. These findings suggest that CRPS-related elevations of ET-1 occur in the skin (and perhaps elsewhere in the periphery) but are not present in blood or in the central nervous system. The pro-inflammatory cytokines that are important in inflammatory and intermediate phases of CRPS will promote ET-1 production [90] and ET-1 itself stimulates production of TNF- α and IL-6 in skin-derived mast cells [91]. This implies that a positive feedback cycle exists among these components that can result in large and long-lasting changes in the expression and release of pain-sensitizing substances.

As noted above, CRPS is characterized by enhanced neurogenic inflammation, mediated by neuropeptides. Krämer and colleagues [92] hypothesized that patients with a lower capacity to degrade neuropeptides are at a greater risk to develop CRPS after nerve injury, and suggested a role in CRPS for neutral endopeptidase (NEP) that degrades several neuropeptides, including ET-1 and SP. This group demonstrated the development of a CRPS-like syndrome, increased pain behavior and signs of neurogenic inflammation after soft tissue trauma, with and without nerve injury in NEP knockout mice. This knockout also led to an increased sensitivity to hot, cold and mechanical stimuli compared to wild type

mice following chronic constriction injury (CCI) of the sciatic nerve [93]. After CCI, increased levels of ET-1 and SP, but not CGRP, were found in traumatized sciatic nerves in NEP KO mice, suggesting a role for ET-1 (and/or SP) in the pathogenesis of CRPS with nerve injury.

In a rodent model of CRPS Type 1 [94], prolonged (3 hrs) ischemia/reperfusion (I/R) of the rat hind paw produced chronic post-ischemic pain (CPIP), as well as mechanical and cold allodynia beginning 2 days after I/R and lasting for several weeks. CPIP mice also exhibited increased sensitivity to an intraplantar injection of ET-1 (3nM, 0.3pmol - 20uM, 200pmol) [95]. Neither mechanical, nor cold allodynia were reduced by local ET_A or ET_B receptor antagonists. Conversely, the ET_B receptor antagonist BQ-788 significantly enhanced cold allodynia in CPIP, but not sham mice, although the ET_B receptor agonist IRL-1620 was without effect. In contrast, IRL-1620 reduced mechanical allodynia in CPIP mice, and this anti-nociceptive effect appeared naloxone-insensitive, unlike the ET_B mediated analgesic mechanism in the epidermis [50].

Spontaneous nociceptive behavior (SNB) induced by ETs at EC₅₀ concentrations (7 μM ET-1 and 20 μM ET-2) was inhibited by local pre-treatment (20 min before) with an ET_A receptor antagonist (BQ-123) and enhanced by an ET_B receptor antagonist (BQ-788), in both CPIP and sham mice. The ET_B receptor agonist IRL-1620 was anti-nociceptive for SNB in CPIP, but not sham mice. Therefore, it appears that the respective analgesic and analgesic roles for ET_A and ET_B receptors remain in the CPIP condition and that the ET_B mediated analgesic mechanism may actually be enhanced. This is consistent with the finding that the distribution of ET_A and ET_B receptors was unchanged in plantar skin of CPIP mice, although both receptors appeared over-expressed (by Western blotting) in plantar muscles.

Overall, the probability of ET-1 involvement in CRPS-relevant pain is high and deserves further investigation.

4.3 Recent Findings on the Role of the Endothelin Axis in Cancer Pain

The role of endothelins and the ET axis in cancer states has been intensively studied over the last decade. To date, the etiology of tumor-induced pain is unclear. ET-1 was initially identified in animal models to be a key player responsible for pain states accompanying tumor growth. These experimental models, utilizing direct injection of different types of cancer cells into the bone or soft tissues, presented a spectrum of pain conditions that allowed for the evaluation of the ET level in and around tumor tissue, and identification of the role for ET receptors in cancer pain.

4.3.1. Increased endothelin levels accompany severe pain following implantation of tumor cells into bone and soft tissue—Ongoing (spontaneous) pain and tactile allodynia were exhibited by tumor-bearing mice with fibrosarcoma cells implanted into the rodent's calcaneus bone [96]. Ongoing pain and touch-evoked hyperesthesia developed in mice with osteolytic (2472) sarcoma cell inoculation into the medullary space of the femur [30]. Tactile allodynia in fibrosarcoma-bearing mice occurred prior to the onset of bone destruction and grew with progressing bone destruction, while cold hyperalgesia developed at a later time [96]. Mechanical hyperalgesia of a significantly lower level was exhibited by mice with subcutaneous fibrosarcoma implants not involving bone [96]. Tactile allodynia also developed following inoculation into mouse plantar soft tissue of squamous cell carcinoma (SCC, HSC-3 cell line), melanoma (WM 164 cells) [97;98], or highly invasive and metastatic murine melanoma (B16-BL6 cells) [99]. In all experimental models, the pain level (defined by the degree of tactile allodynia) correlated with tumor growth (volume).

Levels of ET-1 measured in the whole tumor of a cancerous paw increased ca. 4.8 times compared to the contralateral paw and up to 2.5 times in perfusates collected from the tumor sites of fibrosarcoma-bearing mice [96]. Immunohistochemistry revealed high levels of ET-1 in cultured 2472 sarcoma cells, while ET-1 plasma levels increased about 5 times in sarcoma-bearing mice compared to sham controls (1.56 ± 0.48 vs. 0.319 ± 0.36 pg/ml) [30]. ET tissue content increased with the tumor mass over time after inoculation of metastatic murine melanoma into the plantar mouse hind paw [99]. A direct correlation was demonstrated between tactile allodynia and the ET-1 level produced by the melanoma tumors in the skin. Tactile allodynia was more pronounced in mice bearing squamous cell carcinoma (HSC-3 cell line) that was characterized by significantly greater peripheral concentration of ET-1 within the cancer microenvironment and of intra-tumor expression of both ET-1 mRNA and ET-1 protein, even though the tumor size was smaller than that from melanoma (WM 164 cell line) tumors [98]. Thus, ET-1 concentration appeared a more important factor than tumor volume in producing cancer pain in this model.

When ET-1 (133nM, 4pmol - 40μM, 1.2 nmol) was injected into the tumor-affected paw it enhanced acute nociception, both in mice with sarcoma inoculated into bone (calcaneous) [96] and in mice with subcutaneous inoculation of androgen-independent human prostate cancer cells (PPC-1 into the plantar hind paw) [100].

4.3.2. Role for ET_A and ET_B receptors in cancer pain—Administration of the selective antagonists of ET receptors in murine models defined a role for ET_A receptors in sarcoma- and carcinoma- related nociception. Injection of the ET_A receptor antagonist BQ-123 into the tumor site partially attenuated mechanical hyperalgesia in mice with fibrosarcoma calcaneous inoculation [96]. The ET_A receptor antagonist ABT-627 suppressed both ongoing and movement-evoked pain in osteolytic sarcoma-bearing mice after acute systemic delivery [30]. Its chronic administration (p.o. in drinking water, over post-inoculation days (PID) 6–14) was similarly effective even at later stages of tumor development. BQ-123 injected at the site of greatest tumor development (on PID 32) into the plantar hind paw, was highly effective against tactile allodynia [98]. However, the ET_A receptor antagonism of BQ-123 was recently found to be more complicated than the direct inhibition of pain induction via these receptors (see below).

ET_B receptors appear to mediate anti-nociceptive actions in both sarcoma and carcinoma models. The ET_B receptor antagonist A-192621 (given i.p.) enhanced both spontaneous and palpation-induced guarding, although without altering the flinching behavior in osteolytic sarcoma-bearing mice [30]. Intra-tumor injection of the ET_B receptor agonist BQ-3020 in mice inoculated with squamous cell carcinoma (SCC) attenuated tactile allodynia by approximately 50% for up to 3 hours post injection, while μ-opioid receptor antagonist injection transiently reversed ET_B receptor agonist-induced antinociception [101]. In addition, oral SCC cells in culture were shown to significantly increase β-endorphin production upon ET_B receptor agonist treatment [101]. These results taken together with the data on elevated levels of ET-1 produced by SCC cells, allowed the authors to suggest that the endogenous activation of ET_B receptors could release β-endorphin from malignant keratinocytes to act on peripheral opioid receptors in the cancer microenvironment, the anti-nociceptive mechanism resembling the one described previously for normal skin [50]. This kind of endogenous autocrine ET-1 regulation is plausible, although possibly compromised by significantly decreased levels of ET_B receptor mRNA in SCC cells compared to normal oral keratinocytes [101]. Down- regulation of ET_B receptor expression, one of the key components of the ET-1 clearance pathway, is characteristic for different types of cancer, including prostate carcinoma and colorectal cancer [102–104].

Interestingly, the ET_B receptor-mediated opioid mechanism in SC carcinoma was found to be modulated by ET_A receptor antagonism with BQ-123. Intra-tumor injections of μ -opioid or δ -opioid receptor antagonists transiently reversed antinociception previously induced by local BQ-123 in cancer-bearing mice [105]. Treatment of SCC cells in culture with BQ-123 increased secretion of the opioid receptor agonists β -endorphin and leu-enkephalin peptides [105]. Whether this ET_A receptor antagonistic action on an opioid system is common for all ET_A receptor antagonists or for other types of tumors remains to be determined, however these data are further evidence of interactions between two types of ET receptors (see above sections on ET-R interactions and dimerization).

These studies indicate that the endogenous ET_B mediated analgesic mechanism remains in cancerous keratinocytes (SCC) and that ET_A and ET_B receptors may have opposing roles on β -endorphin production and release from these cells. These studies also provide more evidence that keratinocytes can play significant underlying roles in peripheral effects of ET-1 on nociception.

In contrast to their anti-nociceptive actions in sarcoma and carcinoma, ET_B receptors were pro-nociceptive in melanoma cancer models [99]. Mechanical allodynia evoked in naïve mice by intraplantar injection of tumor extract (paw-grown melanoma, B16-BL6 cells) was reduced by local inhibitors of ET_B receptors, as well as by antagonists of ET_A receptors.

In order to influence nociception, ET receptors need to be expressed by primary afferent nociceptors themselves, or by non-neuronal cells in close enough proximity to primary afferent nociceptors to allow for interactions. ET receptors are found in a variety of non-neuronal cell types in peripheral tissues, including immune cells, keratinocytes and endothelial cells, activation of which can lead to the release of neuroactive mediators capable of modulating nociception. Stosser and colleagues [49] used mice with a conditional deletion of ET_A receptors in peripheral nociceptors (Nav1.8- ET_A^{-/-} mice) to obtain strong evidence for a significant role of ET_A receptors expressed on peripheral nociceptors in tumor associated hyperalgesia. In this study, lung carcinoma cells were injected subcutaneously in the hind paws of the mutant mice and of their control littermates (ET_A^{fl/fl} mice). Despite similar tumor growth, both mechanical and thermal hyperalgesia following tumor induction was significantly reduced in the Nav1.8- ET_A^{-/-} mice. In controls, sequential injections of BQ-123 subcutaneously into the tumor-laden paw decreased thermal hyperalgesia, and the termination of this treatment resulted in a recurring high level of hyperalgesia. Importantly, BQ-123 was without any effect in Nav1.8- ET_A^{-/-} mice, indicating the lack of a role for peripheral ET_A receptors located on non-neuronal cells in this pain. However, this conclusion does not account for the finding that keratinocytes also express Nav1.8 and therefore may also have had reduced ET_A receptor expression in this model. These data provide strong evidence that peripheral ET_A receptors located on nociceptors and/or keratinocytes are responsible for most of the painful sensitivity of skin surrounding such tumors.

4.3.3. Nociceptor activation by ET-1 in cancer pain—Sensitization of primary afferent nociceptors innervating tissue near the tumor likely contributes to chronic pain and hyperalgesia. Electrophysiological properties of C- nociceptors undergo substantial changes evoked by tumor growth and progression. Initial recordings from primary afferent fibers in control and hyperalgesic mice revealed the development of spontaneous activity in about one-third of cutaneous C-fibers adjacent to a tumor implanted around the calcaneous bone [106]. A more recent report found about 80% of C nociceptors had ongoing activity in fibrosarcoma-bearing mice that demonstrated mechanical hyperalgesia [107]. A subpopulation (14%) of these C nociceptors exhibited bursting patterns of ongoing activity, similar to the bursting patterns induced by injection of ET-1 (100 μ M, 1nmol) into the

receptive field of C-nociceptors in non-tumor-bearing mice [107] or of C-nociceptors in naive rats [37]. Given that increased spontaneous firing in intact C-nociceptors is related to both neuropathic and inflammatory spontaneous pain [108] and that a bursting impulse pattern is known to sensitize second-order spinal neurons [109], these changes may contribute to persistent pain associated with bone cancers and cancers that metastasize to bone.

The spontaneous activity of C-nociceptors in tumor-bearing mice was reduced, although not fully abolished, by injection of BQ-123 into their receptive field [107]. In these tumor-bearing mice, the heat-response threshold of heat-sensitive C-nociceptors was lower by ~ 5°C compared to controls and each heat stimulus (35–51°C) evoked a greater number of impulses than in controls. This level of sensitization in fibrosarcoma-bearing mice was similar to that of C-nociceptors in control mice after injection of ET-1 into the receptive field, and injection of ET-1 did not further increase the tumor-elevated responsiveness. Injection of BQ-123 into the receptive field of C-nociceptors in tumor-bearing mice decreased the mean number of impulses evoked by heat stimuli to the baseline values of non-tumor-bearing mice. Therefore, inhibition of ET_A receptors appeared to both partially suppress the spontaneous baseline activity of sensitized C-nociceptors and return the responses to supra-threshold heat stimuli back to the control levels. These data provide the electrophysiological evidence for an endogenous ET-1 contribution to tumor-evoked changes in C-nociceptors and the resulting pain under such conditions.

By comparison, no spontaneous activity was observed in myelinated A δ -nociceptors in tumor-bearing mice [106]. Also, no significant differences were found for heat threshold of A δ -fibers between control mice and fibrosarcoma-bearing mice [106]. The latter observations emphasize the contribution to pain of C nociceptors that are apparently more susceptible to tumor-derived algogens, including ET-1, secreted in high concentrations by cancer cells. The susceptibility to sensitization by ET-1 might depend on the degree of excitatory ET_A receptors on primary afferents along with that of TRPV1 receptors, implicated in noxious heat transduction [110–111].

5. OVERALL CONCLUSION

A role for the endothelin axis in a range of different pathological pain conditions is now well established. However, the mechanisms by which endothelin and its receptors contribute to these conditions remain to be fully described. Although ET_A receptors appear to be uniformly pro-hyperalgesic, the effect of ET_B receptor activation can be either pro- or anti-hyperalgesic. Both receptor types have now been identified on sensory neurons (ET_A in DRG, ET_A and ET_B in TG), but are also expressed by a number of ancillary cells, such as glia and keratinocytes. An ET_B mediated analgesic mechanism has already been identified in keratinocytes and similar interactions may exist between neurons and other ET-R expressing cells. The second messenger pathways coupled to ET-R activation vary widely among different cell types and are only beginning to be elucidated. The interactions between ET receptors themselves (i.e. dimers) and those between ET-R activated pathways and other pathways known to mediate hyperalgesia, or normal, nociceptive pain, will be interesting to discern in the near future, as will be the dynamic regulation of the ET axis under conditions that elevate pain, such as nerve injury and disease.

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