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Over-expression of endothelin-1 in astrocytes, but not endothelial cells, ameliorates inflammatory pain response after formalin injection

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ABSTRACT

Aims: Endothelin-1 (ET-1) has been suggested to be involved in different types of pain due to its neuromodulatory nature. However, its role in inflammatory pain processing, specifically the origin-specific effect, has not yet been clearly defined. Therefore, the aim of this study is to determine the role of cell-type specific ET-1 induction in the modulation of inflammatory pain processing.
Main methods: The current study assesses the effects of ET-1 over-expression specifically targeted to astrocytes (GET-1) or endothelial cells (TET-1) on the expression of pain-like behaviors induced by a model of inflammatory pain, consisting of a formalin injection into the hind paw.
Key findings: The baseline sensitivity thresholds of GET-1 and TET-1 mice to the response elicited by tactile and radiant heat stimulation were similar to those observed in age-matched non-transgenic (NTg) controls. Relative to the NTg controls, GET-1 mice displayed a marked decrease in pain-like behavioral responses during the second phase of formalin-induced pain (i.e., 15–20 min after injection), whereas the responses elicited in TET-1 mice were unaltered. The levels of mRNA encoding adrenomedullin, calcitonin gene-related peptide and calcitonin-like receptor were elevated in the spinal cord of saline-injected GET-1 mice compared to those of NTg mice.
Significance: The current results support a suppressor role for astrocyte-derived ET-1 in inflammatory pain and suggest that the study of GET-1 mice might provide mechanistic insights for improving the treatment of inflammatory pain.

Introduction

Chronic pain is considered as a disease entity, whereas acute pain is simply a symptom of an underlying disease or injury. Inflammatory pain, which contributes significantly to both acute and chronic pain, is triggered by an upset to tissue integrity at cellular level. It is well known that pathological conditions, including burns, arthritis, autoimmune diseases, injuries, operations, infections and vasoconstriction, are closely associated with inflammatory pain (Kidd and Urban, 2001). Recently, unreasonable failure to treat pain is viewed as poor medicine, unethical practice and an abrogation of the fundamental human right (Cheung et al., 2009). Therefore, seeking a new pain-mediator for the development of improved therapeutic approaches is an effective means to improve the current pain management strategies.

Endothelin-1 (ET-1), a 21-amino acid potent vasoconstrictor, is expressed ubiquitously in the central nervous system (CNS), peripheral nervous system (PNS), vascular endothelium and various cell types including endothelial cells and neurons (Yanagisawa et al., 1988; Inoue et al., 1989; Wedgwood et al., 2001). However, its expression is also induced in astrocytes under certain neuropathological conditions such as stroke and Alzheimer's disease (Hama et al., 1997; Zhang et al., 1994). In addition to its role as a vasoconstrictor, ET-1 also serves as a neurotransmitter and/or neuromodulator in the mediation of pain processing. The effects of ET-1 in pain are thought to be differential and depend on the site of originating tissue. Animal studies revealed that exogenous administration of ET-1 induces pain following peripheral subcutaneous administration (Piovezan et al., 2000), but it inhibits pain following a central intrathecal injection [Yamamoto et al., 1994). It is believed that the two subtypes of endothelin receptors, ET_A and ET_B, may contribute differentially to these phenotypic differences (Piovezan et al., 2000; Yamamoto et al., 1994; Khodorova et al., 2003). These studies indicate that

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ET-1-mediated pain processing is tissue specific, or perhaps even pathology specific, although the underlying mechanisms remain elusive.

In the present study, we aimed to investigate the role of cell-type specific ET-1 induction in inflammatory pain processing by making use of transgenic mice, which over-express ET-1 in either astrocytes or endothelial cells. First, we evaluated the impact of ET-1 over-expression in astrocytes (Lo et al., 2005) and in endothelial cells (Leung et al., 2004) on tactile- and radiant heat-associated pain sensations. Then, we compared the pain-like behavioral outcome associated with astrocytic or endothelial cell-specific ET-1 induction under a formalin-induced inflammatory pain condition. Finally, we determined the mRNA expression of common pain signaling molecules in the spinal cord of GET-1 mice to delineate the possible association between astrocytic ET-1 induction and the modulation of inflammatory pain processing.

Materials and methods

Animals

In this study, homozygous GET-1 (Lo et al., 2005) and TET-1 (Leung et al., 2004) mice were used. The original transgenic mouse lines have been previously described (Lo et al., 2005; Leung et al., 2004). In brief, GET-1 and TET-1 heterozygous mice were generated by microinjection of constructs containing mouse ET-1 cDNA containing SV40 polyA sequence in which the transgene expression was driven by astrocyte-specific glial fibrillary acidic protein (GFAP) or endothelial cell-specific receptor tyrosine kinase-1 (Tie-1) promoter, respectively. Heterozygous GET-1 or TET-1 mice were backcrossed for five generations with wild-type C57BL/6N mice. Genotyping procedures were performed according to the initial publications using Southern blot and PCR analyses. All animals were bred and maintained under strictly controlled environmental conditions at a temperature of 20 °C and a light/dark cycle of 12-hr day/night with the light cycle beginning at 8:00 AM. The experimental mice were fed with a sterilized diet and water ad libitum until at the age of 8–12 wks, when they were used for experiments. Prior to the behavioral assessments, mice were habituated to the experimental setting for at least 2 hrs, and all observations were conducted during the lights-on part of the phase. The experimental design and protocols used in the study were previously approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong (CULATR).

Baseline measurement of pain sensations

The baseline sensitivity level of pain sensation of the 8–12-week-old naïve GET-1 ($n=25$) and TET-1 ($n=25$) mice and their NTg controls ($n=25$) (body weight 25–30 g) were assessed by measuring the nociceptive thresholds to tactile and radiant heat stimuli by von Frey filament (Barr et al., 2011) and Hargreaves' test (Held et al., 1998), respectively. Briefly, the tactile threshold was determined by applying a punctate stimulus to the skin of the plantar surface of the hind paw using a calibrated von Frey filament (IITC Life Science, Inc., USA) in which 50% of mice showed hind paw withdrawal. The mean value of the applied force (g) in three consecutive measurements within a 5-min interval was taken as a threshold to the tactile stimulus. For radiant heat threshold assessment, a focal light beam was applied to the plantar region of the hind paw using an IITC plantar analgesia meter for thermal paw (IITC Life Science, Inc., USA). The mean paw withdrawal latency obtained from three consecutive measurements within a 5-min interval was recorded and taken as a parameter of threshold to radiant heat of the animals.

Formalin-induced pain-like behavioral measurement

Acute inflammatory responses were induced in the animals by intraplantar injection of 20 μl of 1% formalin in saline to the right (ipsilateral) paw. Saline-injected animals served as no-pain controls. The total amount of time each animal spent manifesting pain-related nociceptive behaviors (including licking, lifting and/or shaking of the injected paw) was monitored and recorded continuously in 5-min periods for up to 30 min after injection. Foot-lifting associated with exploratory behavior, locomotion, body repositioning, and grooming were excluded by the experimenters in accordance with the behavior observed on the left (contralateral) paw. To avoid experimental bias, two experimenters who were blind to genotype and treatment scored the behaviors separately, and the data were averaged.

Sample collection, RNA extraction and cDNA preparation

Following the formalin-induced pain-like behavioral assessment, spinal cords from the animals were dissected out following quick cervical dislocation after anesthetized with sodium pentobarbital. Spinal segments L1–4 were isolated, separated into the ipsilateral and contralateral sides, snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Spinal cord segments were homogenized in 10 volumes (w/v) of ice-cold TRI Reagent (Invitrogen, by Life Technologies, USA). Total mRNA was extracted using the phenol:chloroform extraction method according to the manufacturer's instructions. The quality and quantity of the extracted RNA were determined by measuring the optical density at 260 and 280 nm using a spectrophotometer (GeneQuant, Pharmacia). cDNA preparation was achieved by reverse transcription using 1 μg of the extracted total RNA with High Capacity RNA-to-cDNA™ master mix kit (Applied Biosystems, by Life Technologies, USA) according to the manufacturer's protocol. Prepared cDNA samples were diluted and stored at −20 °C until further analyses.

Determination of mRNA expression of pain signaling molecules by real-time PCR analysis

Diluted cDNA samples (4 μl) were served as template for gene expression analyses using Real-time Polymerase Chain Reaction (RT-PCR) with the SYBR green chemistry. The specific primers for substance P (SP), enkephalin (ENK1), adrenomedullin (AM), calcitonin gene-related peptide (αCGRP), calcitonin-like receptor (CLR) and 18S rRNA were employed as published elsewhere (Fernandez et al., 2010). Briefly, the PCR reactions were carried out in a total volume of 20 μl containing 10 μl Fast SYBR green master mix (Applied Biosystems, by Life Technologies, USA), 1 μl of the corresponding 10 μM primer pair (Invitrogen, by Life Technologies, USA), and 4 μl of diluted cDNA samples, which was brought up to 20 μl with DEPC-treated water (Applied Biosystems, by Life Technologies, USA). The reactions were performed using the StepOnePlus™ system and the data generated were analyzed using the software provided (Applied Biosystems, by Life Technologies, USA).

Statistical analysis

All statistical analyses were performed using the GraphPad Prism software (Version 5.02, GraphPad Software, Inc.). The data are presented as the mean ± standard error of the mean. The results were analyzed using one- or two-way ANOVA followed by Tukey post-hoc test. For all statistical analysis, $p<0.05$ was considered statistically significant.

Results

Effects of cell type-specific ET-1 expression on pain sensation

To evaluate the effect of astrocyte- and endothelial cell-specific over-expression of ET-1 on pain sensation, nociceptive thresholds to tactile and radiant heat stimuli were first determined in GET-1 and TET-1 mice. Upon tactile stimulus, GET-1, TET-1 and their corresponding NTg control groups exhibited similar threshold level of mechanical sensitivity in the right paw (mean forced applied: GET-1, 6.40 ± 0.16 g vs. NTg, 5.97 ± 0.21 g; TET-1, 6.11 ± 0.17 g vs. NTg, 5.68 ± 0.41 g; $p > 0.05$; $n = 25$) (Fig. 1A) and in the left paw (mean forced applied: GET-1, 6.43 ± 0.74 g vs. NTg, 5.55 ± 0.20 g; TET-1, 6.41 ± 0.51 g vs. NTg, 5.51 ± 0.18 g; $p > 0.05$; $n = 25$). Similarly, upon radiant heat stimulation, all groups displayed a similar threshold level of thermal sensitivity in the right paw (latency for response: GET-1, 4.01 ± 0.30 s vs. NTg, 3.71 ± 0.27 s; TET-1, 4.10 ± 0.35 s vs. NTg, 3.94 ± 0.23 s; $p > 0.05$; $n = 25$) (Fig. 1B) and in the left paw (latency for response: GET-1, 3.99 ± 0.29 s vs. NTg, 3.98 ± 0.38 s; TET-1, 3.87 ± 0.27 s vs. NTg, 3.49 ± 0.36 s; $p > 0.05$; $n = 25$).

Differential effects of cell type-specific ET-1 expression on formalin-induced inflammatory pain

Apart from physiological pain sensations, the susceptibility to inflammatory pain was also assessed in GET-1 and TET-1 mice with formalin test. As observed in NTg mice, after 1% formalin injection, two distinct phases of nociceptive response could be identified during the 0–5 min (1st phase) and 15–20 min (2nd phase) intervals. In GET-1 mice, compared to the NTg mice, no significant change could be observed in the 1st phase (mean accumulated time: 0–5 min, NTg, 152.2 ± 14.1 s; GET-1, 139.4 ± 10.2 s; $p > 0.05$; $n = 15$), but the total duration of nociceptive response was significantly reduced in the 2nd phase (mean accumulated time: 15–20 min, NTg, 60.5 ± 10.3 s; GET-1, 12.5 ± 4.1 s; $p < 0.0001$; $n = 15$) (Fig. 2A). Nociceptive responses of TET-1 mice to formalin were unchanged relative to those of NTg mice during the 1st phase (mean accumulated time: 0–5 min, NTg, 141.6 ± 8.2 s; TET-1, 140.6 ± 10.2 s; $p > 0.05$; $n = 15$) and also the 2nd phase (mean accumulated time: 15–20 min, NTg: 68.3 ± 11.5 s; TET-1: 90.9 ± 8.8 s; $p > 0.05$; $n = 15$), despite the trend towards an increase in the latter (Fig. 2B).

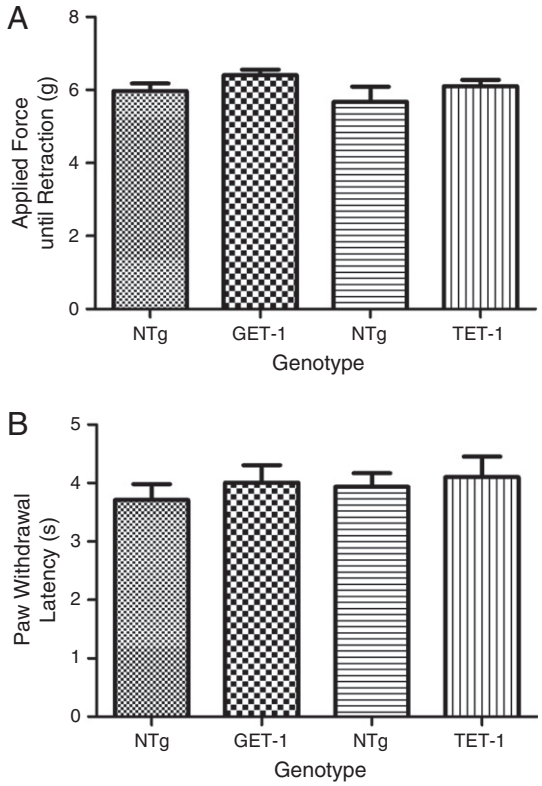

Fig. 1. Over-expression of ET-1 in astrocytes or endothelial cells does not affect the nociceptive pain response upon tactile or radiant heat stimuli. The nociceptive thresholds in response to tactile and radiant heat stimuli were determined in GET-1, TET-1 and NTg mice using von Frey filament (A) and Hargreaves' (B) tests. The data are presented as the mean ± standard error of the mean ($n = 25$).

Astrocytic ET-1 over-expression leads to an alteration in the mRNA expression of pain signaling molecules in the spinal cord

Because the formalin-induced hyperalgesic effect was significantly altered in the 2nd phase in the GET-1 mice, we investigated the possible involvement of some of the key pain signaling molecules in mediating the pain suppression effect associated with astrocytic ET-1 over-expression. In this study, gene expression levels of pain mediators (SP, AM and αCGRP), modulator (ENK1) and receptor (CLR) were compared between GET-1 and NTg mice. In saline-injected GET-1 and NTg mice, the mRNA expression levels of AM (NTg, 0.86 ± 0.1 vs. GET-1, 1.76 ± 0.09, $p < 0.05$; $n = 4–6$), αCGRP (NTg, 0.73 ± 0.08 vs GET-1, 1.54 ± 0.09, $p < 0.05$; $n = 4–6$) and CLR (NTg, 1.04 ± 0.13 vs. GET-1, 2.84 ± 0.10, $p < 0.05$; $n = 4–6$) were significantly increased in the spinal L1–4 region of the GET-1 mice, whereas no obvious difference was observed in SP (NTg, 1.26 ± 0.06 vs. GET-1, 1.29 ± 0.05, $p > 0.05$; $n = 4–6$) or ENK1 (NTg, 1.18 ± 0.05 vs GET-1, 1.34 ± 0.04, $p > 0.05$; $n = 4–6$). In addition, in the GET-1 spinal cord after formalin injection, significant decreases in the levels of mRNA for AM (Con, 1.78 ± 0.17 vs. Ipsi, 0.97 ± 0.09, $p < 0.05$; $n = 4–6$) and ENK1 (Con, 1.27 ± 0.11 vs. Ipsi, 0.77 ± 0.20, $p < 0.05$; $n = 4–6$) were detected in the ipsilateral side, when compared to those measured on the contralateral side. More importantly, by comparing the ipsilateral side of GET-1 and NTg mice after formalin-injection, the αCGRP (NTg, 1.02 ± 0.06 vs. GET-1, 2.03 ± 0.26, $p < 0.05$; $n = 4–6$), CLR (NTg, 1.24 ± 0.13 vs. GET-1, 3.26 ± 0.11, $p < 0.05$; $n = 4–6$) and SP (NTg, 1.24 ± 0.07 vs. GET-1, 1.68 ± 0.14, $p < 0.05$; $n = 4–6$) mRNA expression levels were dramatically induced in the GET-1 mice (Fig. 3).

Discussion

In the past decade, ample evidence was obtained in support of a pathological role of ET-1 in pain processing associated with various types of inflammatory and non-inflammatory painful conditions, including arthritis, cancer and neuropathies (Barr et al., 2011; Imhof et al., 2011; Hans et al., 2009; Werner et al., 2010; Khodorova et al., 2009a). It has been suggested that ET-1 produced peripherally can participate in the promotion of both acute and chronic pain by acting on ET_A receptor, though these effects are limited by ET_B receptor activation (Khodorova et al., 2009a). In contrast, in the CNS, ET-1 induces anti-nociception via ET_A receptor coupled to L-type calcium channel-dependent Ca^{2+} influx (Held et al., 1998). However, the mechanisms underlying these opposite effects on nociception have not yet been clearly identified.

The current views on the participation of ET-1 in pain modulation are largely based on experimental data involving the influence of pharmacological blockade of ET_A and/or ET_B receptors with selective or dual receptor antagonists in animal models of neuropathic or inflammatory nociception, or against the effects of administration of exogenous ET-1 to different sites (Piovezan et al., 2000; Yamamoto et al., 1994; Khodorova et al., 2003; Nikolov et al., 1992). Such experimental approaches may fail to reveal nuances in the contribution of ET-1 synthesized by specific cell types or tissues to alteration in

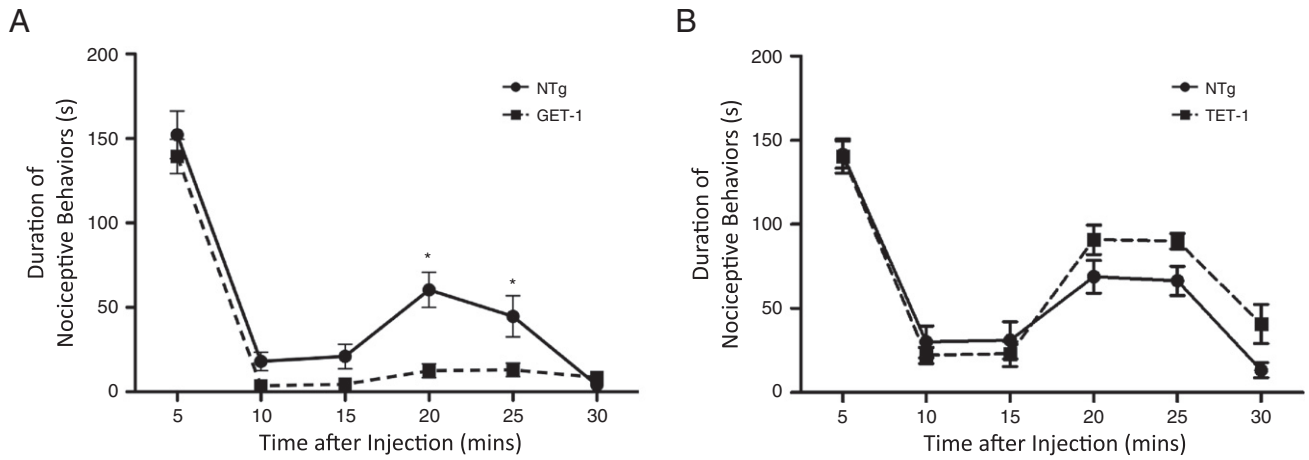

Fig. 2. Comparison of the nociceptive responses of GET-1, TET-1 and NTg mice to formalin injection into the hind paw. The values shown represent the amount of time GET-1 (A), TET-1 (B) or NTg control mice presented nociceptive paw lifting and/or licking behaviors, displayed in 5-min periods for up to 30 min after injection of 1% formalin. * denotes statistically significant difference ($p<0.05$) compared with the corresponding NTg control groups by two-way ANOVA followed by Tukey post-hoc test. The data are presented as the mean ± standard error of the mean ($n=15$).

nociception. The current study has used a transgenic approach to address the importance of cell-specific ET-1 synthesis on nociception induced by formalin, namely mice over-expressing ET-1 specifically in astrocytes (GET-1) (Lo et al., 2005) or in endothelial cells (TET-1) (Leung et al., 2004), as evidenced by immunohistochemistry and in-situ hybridization, which do not present any gross structural or morphological abnormalities in the vasculature or the brain. The behavioral responses of GET-1 and TET-1 mice to stimulation of their hind paws with either tactile or radiant heat was unchanged relative to that of their NTg counterparts, suggesting that specific ET-1 over-expression in astrocytes or in endothelial cells does not normally interfere with pain transmission. Moreover, no differences were observed regarding the behavioral responses of TET-1 and NTg mice to formalin injection into their hind paws, a model of inflammatory pain. It thus appears that over-expression of ET-1 in endothelial cells does not alter responsiveness of TET-1 mice to formalin. In sharp contrast, even if GET-1 mice responded normally during the first (nociceptive) phase (over first 5 min after injection) of the formalin test, they manifested a clear reduction in nociceptive behavior during the second (inflammatory) phase of the test (15 to 30 min after injection). These results suggest that astrocyte-derived ET-1 most likely has an anti-hyperalgesic (rather than truly anti-nociceptive) role in the pathogenesis of inflammatory pain.

It is interesting that ET-1 derived from astrocytes exerts such an evident anti-nociceptive action, whereas that from endothelial cells does not, as both types of cell are so abundant in the spinal cord. In

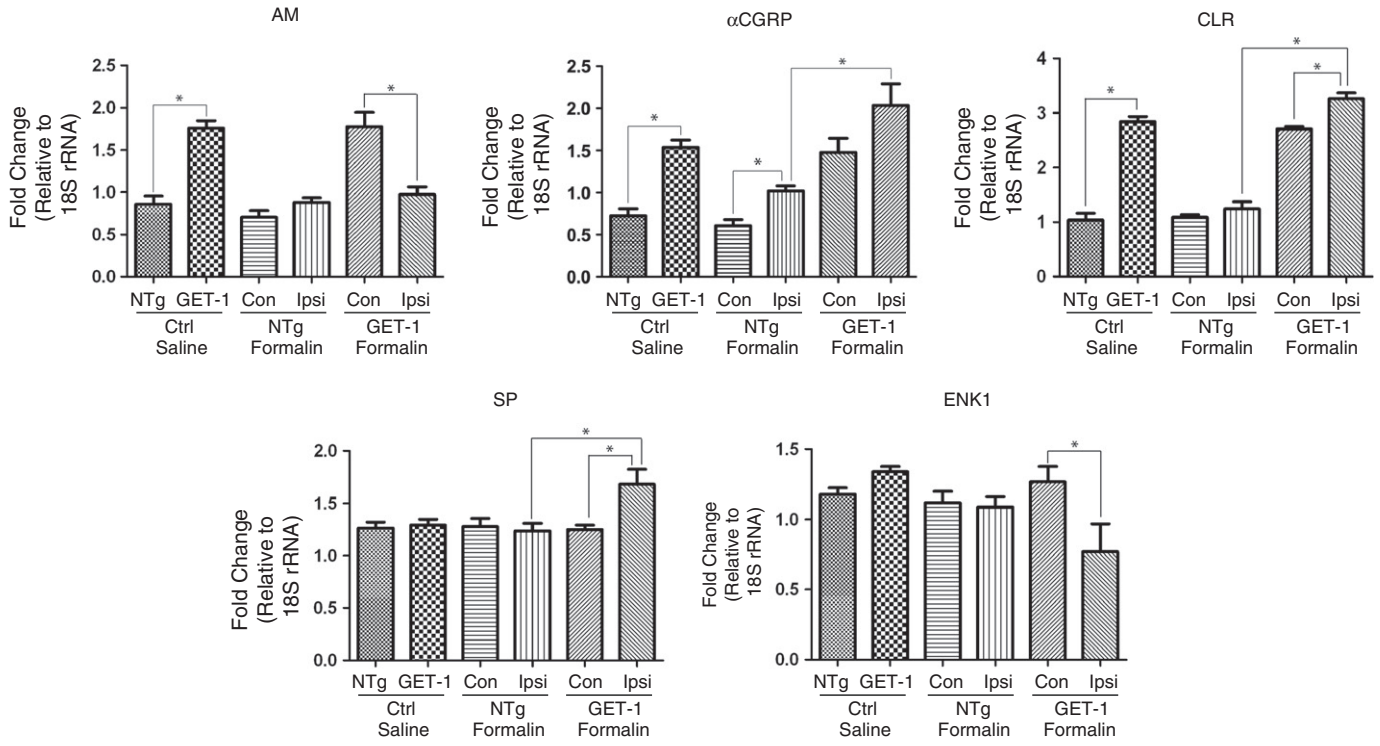

Fig. 3. Increased gene expression of AM, αCGRP and CLR in GET-1 mice. Real-time PCR analyses showing the mRNA expression levels of AM, αCGRP, CLR, SP and ENK1 in contralateral (Con) or ipsilateral (Ipsi) GET-1 and NTg spinal cord at 30 min after saline- (Ctrl) or formalin-injection. * denotes statistically significant difference ($p<0.05$) compared with the NTg mice by one-way ANOVA followed by Tukey post-hoc test. The data are presented as the mean ± standard error of the mean ($n=4$–6).

fact, a nearly 30-fold of ET-1 mRNA elevation was detected in the spinal cord of the naïve GET-1 mice when compared to that of the NTg mice (unpublished data), which is similar to our previous investigation in brain (Lo et al., 2005). However, it is possible that while the actions of astrocyte-derived ET-1 were restricted to the spinal cord by the blood-spinal cord barrier, this barrier most likely prevented ET-1 derived from endothelial cells from entering into the spinal cord. It should be interesting to examine in the future what changes GET-1 and TET-1 mice display in hind paw edema induced by formalin, and also how this algogen affects ET-1 levels in the spinal cord and periphery.

Astrocytic ET-1-dependent neuro-modulation may be one of the possible mechanisms mediating the anti-hyperalgesic effect. Thus, the relative expression level of pain signaling molecules was determined in saline- and formalin-injected animals. In the spinal L1–4 region of saline-injected GET-1 mice, the AM, αCGRP and CLR mRNA expression levels were significantly induced compared with NTg mice, whereas only negligible changes in the expression levels of SP and ENK1 were found. In addition, the levels of αCGRP and CLR were further up-regulated after formalin injection. Interestingly, the expression levels of mRNA for AM and ENK1 in the ipsilateral side of the GET-1 spinal cord were drastically decreased after formalin challenge. Although it is generally believed that ENK exerts an inhibitory influence on transmission of nociceptive information at the synaptic level between the primary afferent fibers and the second order spinal nociceptive neurons, our results implicate that it is worthwhile to investigate the interaction between astrocytic ET-1 and other neurotransmission systems including ENK1 and AM in pain perception. Taken together, our gene expression analysis suggests that astrocytic ET-1 exerts its anti-hyperalgesic effect via AM- and/or αCGRP/CLR-mediated pathways, although pharmacological blockade experiments should be conducted to validate this hypothesis. Indeed, the correlation between ET-1 and αCGRP has been illustrated recently in study showing that the release of αCGRP is involved in mediating the late-phase response (>30 min) of ET-1-dependent tactile allodynia (Khodorova et al., 2009b). However, it is also possible that AM and αCGRP induction is a consequence of the vasoconstrictive effect of astrocytic ET-1 over-expression in the spinal cord, as these proteins are potent vasodilators. Hence, local blood flow in the spinal cord of GET-1 and TET-1 mice should be examined in detail to clarify the contribution of cell type-specific ET-1 over-expression in regulating local vascular tone of the spinal cord.

Conclusion

Taken together, astrocytic ET-1 over-expression leads to less severe formalin-induced nociceptive response to pain although it does not affect the mechanical and thermal sensitivity to pain. However, endothelial ET-1 over-expression does not affect the nociceptive response to either mechanical, heat or formalin stimuli. In addition, AM and/or αCGRP-mediated pathways may be involved in the ET-1-mediated pain modulation. Hence, our present study not only provides a new insight into cell type-specific ET-1 induction in inflammatory pain modulation but also highlights the potential of using GET-1 and TET-1 mice in mechanistic studies aimed at developing new targets for the management of inflammatory pain.

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Conflicts of interest/disclosures

The authors declare that there is no conflict of interest/disclosures in this study.

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