Research Article

MOLECULAR PAIN

Molecular Pain
Volume 15: 1–11

DOI: 10.1177/1744806919842473
journals.sagepub.com/home/mpx
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ET_AR and protein kinase A pathway mediate ET-1 sensitization of TRPA1 channel: A molecular mechanism of ET-1-induced mechanical hyperalgesia

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Abstract

Endothelin-1 (ET-1) is a potent endogenous vasoconstrictor that has been widely known as a pain mediator involved in various pain states. Evidence indicates that ET-1 sensitizes transient receptor potential cation channel, subfamily A, member 1 (TRPA1) *in vivo*. But the molecular mechanisms still remain unknown. We aim to explore whether ET-1 sensitizes TRPA1 in primary sensory neurons and the molecular mechanisms. Ca^{2+} imaging, immunostaining, electrophysiology, animal behavioral assay combined with pharmacological experiments were performed. ET-1 sensitized TRPA1-mediated Ca^{2+} responses in human embryonic kidney (HEK)293 cells as well as in cultured native mouse dorsal root ganglion (DRG) neurons. ET-1 also sensitized TRPA1 channel currents. ET-1 sensitized TRPA1 activated by endogenous agonist H_2O_2. ET_A receptor (ET_AR) colocalized with TRPA1 in DRG neurons. ET-1-induced TRPA1 sensitization *in vivo* was mediated via ET_AR and protein kinase A (PKA) pathway in HEK293 cells and DRG neurons. Pharmacological blocking of ET_AR, PKA, and TRPA1 significantly attenuated ET-1-induced mechanical hyperalgesia in mice. Our results suggest that TRPA1 acts as a molecular target for ET-1, and sensitization of TRPA1 through ET_AR–PKA pathway contributes to ET-1-induced mechanical hyperalgesia. Pharmacological targeting of TRPA1 and ET_AR-PKA pathway may provide effective strategies to alleviate pain conditions associated with ET-1.

Keywords

TRPA1, endothelin, pain, protein kinase A, sensitization

Date Received: 9 February 2019; revised: 10 March 2019; accepted: 11 March 2019

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide that has been implicated in the pathogenesis of tissue inflammation and pain.[1] Injection of ET-1 induced overt pain-like behavior and thermal and mechanical allodynia in animals.[2–4] In humans, ET-1 injection produced severe pain and prolonged, touch-evoked allodynia.[5] ET-1 exerts its effects mainly via acting on ET_A and ET_B receptors (ET_AR and ET_BR), both of which are G protein-coupled receptors.[1] ET_ARs are abundantly expressed in primary sensory neurons, whereas ET_BR are found exclusively in satellite glial cells.[6,7] Extensive studies have been carried out to elucidate the mechanisms underlying ET-1-induced pain responses. ET-1 can induce hyperpolarizing shifts in voltage-dependent

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activation of TTX-R Na^+ channels.[8] ET-1 also potentiates TRPV1 channel via ET_AR-mediated protein kinase C (PKC) signaling in both expression system and sensory neurons.[6]

Transient receptor potential cation channel, subfamily A, member 1 (TRPA1) is a nonselective cation ion channel mainly distributed in sensory neurons where it functions as a molecular detector to sense a variety of noxious stimuli. TRPA1 can be activated by a wide variety of endogenous and exogenous substances that elicit pain and irritation.[9] Activation of TRPA1 depolarizes nociceptors and contributes to the perception of noxious stimuli.[9] In addition, TRPA1 channel activity can be sensitized by inflammatory mediators, including bradykinin, trypsin, and nerve growth factor (NGF).[10–12] TRPA1 can be sensitized via protein kinase A (PKA) and phospholipase C (PLC) pathways.[10,13–15] ET-1 can potentiate cinnamaldehyde (a TRPA1 agonist)-induced nociception.[16] ET-1-induced mechanical allodynia is inhibited by specific antagonists against TRPA1 and ET_AR *in vivo*, suggesting a possible interaction between ET_AR and TRPA1 in mediating pain responses.[17] However, little is known about whether ET-1 sensitizes TRPA1 in primary sensory neurons and the detailed mechanisms.

Here, we investigated whether ET-1 sensitizes TRPA1 channel expressed in heterologous expression system and in cultured mouse primary sensory neurons. We further studied the molecular mechanisms underlying ET-1's effect on TRPA1. Lastly, we examined the contribution of ET_AR–PKA pathway and TRPA1 in ET-1-induced nocifensive response. Our results suggest that ET-1 sensitizes TRPA1 via ET_AR and PKA signaling pathway both *in vitro* and *in vivo*, and this pathway contributes to ET-1-induced mechanical hyperalgesia.

Material and Methods

Animals

Male C57BL/6 mice (from Laboratory of Animal Research Center, Zhejiang Chinese Medical University, Hangzhou, China and Charles River Laboratories, Wilmington, MA, USA), six to eight weeks old, were used in this study. $Trpa1^{-/-}$ mice were a gift from David Julius (University of California, San Francisco, CA, USA). The mice were housed 5 per cage on a 12 h light/dark cycle with controlled temperature. Food and water were provided ad libitum. This study was carried out in accordance with the guidelines of National Institutes of Health guide for the care and use of laboratory animals and approved by the Animal Ethics Committee of Zhejiang Chinese Medical University.

Chemicals

Dimethyl sulfoxide (DMSO), HQ, ionomycin, capsaicin, and mustard oil (MO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). HC-030031, ET-1, H89, BQ-123, BQ-788, edelfosine, forskolin, and bisindolylmaleimide (BIM) were purchased from Tocris (Minneapolis, MN, USA).

Cell culture

Human embryonic kidney (HEK)293 cells (ATCC, CRL-1573) were cultured in Dulbecco's modified Eagle's medium (Lonza, Belgium) supplemented with 10% fetal bovine serum (Lonza, Belgium), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. Human TRPA1 (hTRPA1) is a gift from Professor David Julius (University of California, San Francisco), and ET_AR is purchased from OriGene (Rockville, MD).

Adult mouse dorsal root ganglia (DRGs) were dissociated using 0.28 Wünsch units/ml Liberase Blendzyme 1 (Roche Diagnostics, Mannheim, Germany) as described previously.[18] Neurons were cultured in Neurobasal-A medium (Invitrogen, Grand Island, NY) with B-27 supplement, 0.5 mM glutamine, and 50 ng/ml NGF (Calbiochem, La Jolla, CA) on an 8-well chambered coverglass coated with poly-D-lysine (Sigma, St. Louis, MO) and mouse laminin (Invitrogen, Carlsbad, CA, USA).

Immunofluorescence and confocal imaging

Mice were euthanized by CO_2. Bilateral L3-5 DRGs were collected and immersed immediately in 4% paraformaldehyde overnight at 4°C. Then, DRGs were transferred to 15% and 30% sucrose for dehydration. Mouse DRGs were then frozen in frozen tissue matrix (OCT) and cut by cryostat in 8-μm sections. For immunostaining, the sections were first blocked with 1% BSA plus 10% donkey serum for 2 h at room temperature. The sections were then incubated overnight at 4°C with primary antibody against TRPA1 (1:200, Alomone Labs, Jerusalem, Israel) and ET_AR (1:200, Abcam, Carlsbad, CA, USA). After washout, corresponding secondary antibodies (1:1000, Abcam, Carlsbad, CA, USA) were used for staining. Fluorescence signals were detected by Nikon A1R laser scanning confocal microscope (Nikon, Japan) and analyzed by ImageJ software. For quantification of immunofluorescent staining, two images were randomly selected per mouse tissue, and three mice were included in the present study.

Ca^{2+} imaging

For Ca^{2+} imaging of HEK293 cells, cells were used within 48 h after transfection. For DRG neurons, neurons were used 24 h after dissociation. Cells were loaded with Fura 2-AM (10 μM, Invitrogen) for 45 min in a loading buffer containing (mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES (pH 7.4, adjusted with NaOH). Cells were subsequently washed three times and imaged in the loading buffer. Ratiometric Ca^{2+} imaging was performed on an Olympus IX51 microscope with a Polychrome V monochromator (Till Photonics, Hillsboro, Oregon, USA) and a PCO Cooke Sensicam QE CCD camera and Imaging Workbench 6 imaging software. Fura-2 emission images were obtained with exposures of 0.5 ms at 340 nm and 0.3 ms at 380 nm excitation wavelengths. Ratiometric images were generated using ImageJ software. A cell or neuron was considered responsive if the peak Ca^{2+} response is above 20% of the baseline.

Patch-clamp recordings

Recordings were carried out with borosilicate glass pipettes with initial series resistance of 2 to 4 MΩ after loading the pipette solution. Currents were filtered at 2.3 kHz and digitized at 100μs intervals using an EPC-10 amplifier and PatchMaster acquisition software (HEKA, Germany). Perforated whole-cell hTRPA1 currents in HEK293 cells were recorded by patch-clamp recordings with a pipette solution containing (in mM) 140 CsAsp, 2 $MgCl_2$, 10 HEPES, and 10 Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (pH 7.4, adjusted with CsOH), with ~30 pM of amphotericin B added. The perfusion solution contained (in mM) 140 NaCl, 4 KCl, 2 EGTA, 2 $MgCl_2$, 10 HEPES, and 8 glucose (pH 7.4, adjusted with NaOH).

Animal behavioral assay

Mechanical hyperalgesia was examined by von Frey hair test as described before.[19,20] Briefly, mice were habituated for 30 min to the wire mesh surface before testing. Paw withdrawal thresholds (PWTs) were determined using a series of von Frey filaments (0.008–4.00 g) pressed against the plantar surface of the hind paw in ascending order beginning with the finest fiber following standard procedures. The minimum force (g) that caused the mouse to withdraw its hind paw away from the filament was considered as the withdrawal threshold. For each paw, a von Frey hair was applied 5 times at 10-s intervals. The threshold was determined when paw withdrawal was observed in more than three of five applications. ET-1 (20 ng/paw, dissolved in phosphate-buffered saline) was injected into the hind paw of mice using 1-ml syringe and 30-gauge needle in a volume of 20 μl. HC-030031 (10 μg/paw), H89 (5 μg/paw), and BQ-123 (1 μg/paw) was coinjected with ET-1, and the behavioral test was carried out thereafter at 0, 0.5, and 2.5 h after the injection. All behavioral tests were performed by an experimenter blinded to experimental conditions.

Statistics

Student's *t*-test was used for comparison of data between two groups. One-way or two-way analysis of variance followed by Tukey post hoc test was used for comparison of ≥ 3 groups. Comparison is considered significantly different if the p value is less than 0.05. Data in bar graphs are expressed as means ± SE.

Results

ET-1 sensitizes hTRPA1 channel expressed in heterologous expression system

We transiently expressed hTRPA1 and ET_AR together in HEK293 cells. To examine whether ET-1 sensitizes TRPA1, we measured its effects on Ca^{2+} responses to TRPA1 agonist MO in HEK293 cells. We tested the effects of 100 nM ET-1 in our *in vitro* experiments, which is a commonly used concentration in other studies and falls within the *in vivo* ET-1 concentration range.[6,7,21] Pretreatment of HEK293 cells for 2 min with ET-1 (100 nM) significantly increased the magnitude of Ca^{2+} responses to MO (5 μM) compared to pretreatment with vehicle (0.1% DMSO), indicating sensitization effect of ET-1 (Figure 1(a) and (b)). ET-1 per se induced robust Ca^{2+} responses in HEK293 cells expressing both ET_AR and TRPA1, which gradually returned to baseline level after 2 min (Figure 1(b)). Ionomycin (Iono) was applied at the end of Ca^{2+} imaging to identify all live cells (Figure 1(a) and (b)). We also tested HEK293 cells which are expressed with hTRPA1+empty vector pcDNA3.1 but with no ET_AR. We found that ET-1 did not induce Ca^{2+} responses or potentiate MO's response in cells expressing hTRPA1+pcDNA3.1 compared with cells expressing hTRPA1+ET_AR (Figure 1(c) and (d)).

We proceeded to examine the mechanisms underlying ET-1-induced sensitization of TRPA1 in HEK293 cells. We used percent of ionomycin response, in which cell responses to MO were normalized to ionomycin, to compare Ca^{2+} responses among different groups. ET_AR couples to PLC and PKA pathway, respectively.[22,23] Since TRPA1 can be sensitized by PLC and PKA, we therefore examined the contributions of these two pathways to ET-1-induced sensitization of TRPA1 in HEK293 cells. ET_AR-specific antagonist BQ-123 (10 μM), but not ET_BR-specific antagonist BQ-788 (5 μM), at effective

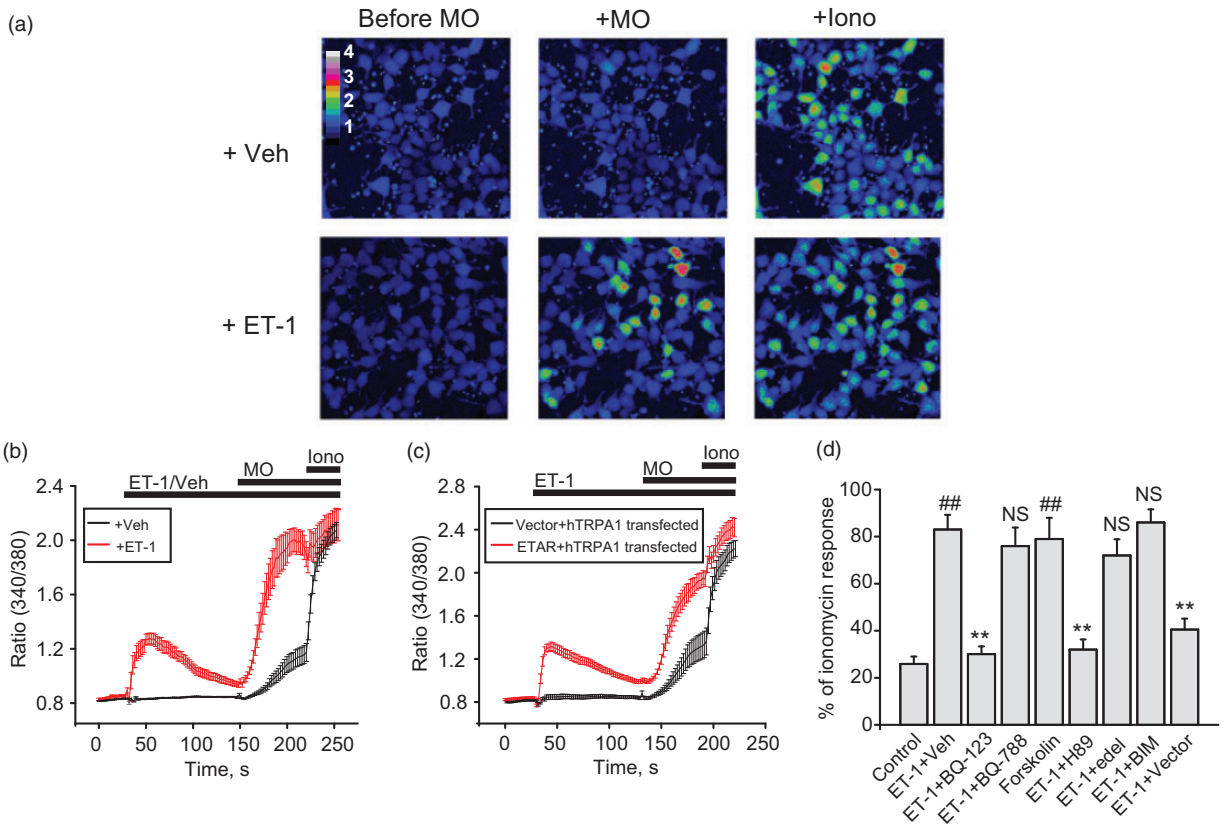

Figure 1. ET-1 sensitizes TRPA1 channel exogenously expressed in HEK293 cells. (a) Pseudo color images from Fura-2 ratiometric imaging showing Ca^{2+} responses in HEK293 in response to TRPA1 agonist MO (5 μM) with or without pretreatment of ET-1 (100 nM). Ionomycin (1 μM) was applied at the end of the recording to determine all active cells. HEK293 cells were cotransfected with hTRPA1 and ET_AR. (b) Averaged Ca^{2+} responses from experiments shown in panel (a). Red and black lines depict conditions with or without ET-1 pretreatment, respectively. $n > 30$ cells/group. (c) Averaged Ca^{2+} responses of HEK293 cells expressing hTRPA1 with ET_AR or with empty vector (pcDNA3.1). $n > 30$ cells/group. (d) Pharmacological analysis of ET-1-induced TRPA1 sensitization in HEK293 cells. Cells were preincubated with ET_AR antagonist BQ-123 (10 μM), ET_BR antagonist BQ-788 (5 μM), PKA activator forskolin (15 μM), PKA inhibitor H89 (10 μM), PLC inhibitor edelfosine (10 μM), PKC inhibitor BIM (100 nM), or corresponding vehicle (0.1% DMSO) for 5 min, and then these reagents were coapplied with ET-1 (100 nM) during the imaging test. Ca^{2+} responses were normalized to ionomycin applied at the end of the tests for comparison (% response of ionomycin). $n = 3$–5 tests/group. Each test contains up to 30 cells. $^{\#\#}p < 0.01$ versus control group. $^{**}p < 0.01$ versus ET-1+Veh group ($p > 0.05$).
ET-1: endothelin-1; ET_AR: ET_A receptor; hTRPA1: human TRPA1; MO: mustard oil; NS: no significance; Iono: ionomycin; BIM: bisindolylmaleimide.

concentration, significantly reduced ET-1-induced sensitization of Ca^{2+} responses to MO in HEK293 cells.[24–27] H89 (10 μM), a PKA antagonist, significantly reduced the sensitization, whereas BIM (100 nM), a PKC antagonist, had no effect (Figure 1(c)). In line with this observation, pretreating HEK293 cells with forskolin (15 μM), a PKA agonist, sensitizes the Ca^{2+} responses to MO in HEK293 cells, mimicking the effect of ET-1. On the contrary, the PLC-specific antagonist edelfosine, at effective concentration (10 μM), had no effect on ET-1-induced TRPA1 sensitization.[28]

We further studied the effects of ET-1 on TRPA1/ET_AR-expressing HEK293 cells via whole-cell patch-clamp recording. TRPA1 channel current was recorded under Ca^{2+}-free extracellular solution to avoid channel inactivation as in our previous study.[29] It was observed that 5 μM of MO induced small TRPA1 currents showing typical outward rectification property (Figure 2(a) and (b)). After 2 min pretreatment with 100 nM ET-1, reapplication of MO at the same dose (5 μM) elicited much larger current compared with control group (Figure 2(a) to (c)). The above results indicated that ET-1 sensitized TRPA1 via ET_AR-mediated PKA signaling pathway in HEK293 cells.

ET-1 sensitizes TRPA1 channel in mouse primary sensory neurons

It is reported that ET_ARs are mainly expressed in DRG neurons.[6,7] We therefore performed double

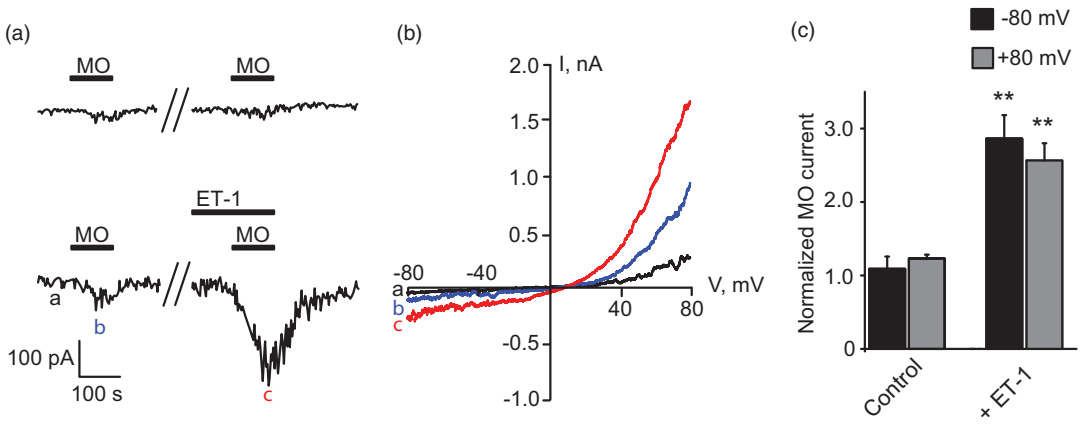

Figure 2. ET-1 sensitizes TRPA1 channel currents in HEK293 cells expressing TRPA1 and ET_AR. (a) Representative inward current traces recorded at –80 mV in whole-cell configuration by patch clamp. MO (5 μM) and ET-1 (100 nM) were applied as indicated. (b) Current–voltage (I–V) curve recorded from HEK293 cell shown in the lower panel of (a). The letters a, b, c denote corresponding time point recorded in lower panel of (a). (c) Summarized data showing MO-activated TRPA1 currents were sensitized after ET-1 pretreatment. Currents were normalized to values first induced by MO application in the absence of ET-1. Inward and outward TRPA1 currents were recorded at –80 and +80 mV, respectively. $n = 6$ cells/group. $^{**}p < 0.01$ versus control group.
ET-1: endothelin-1; MO: mustard oil.

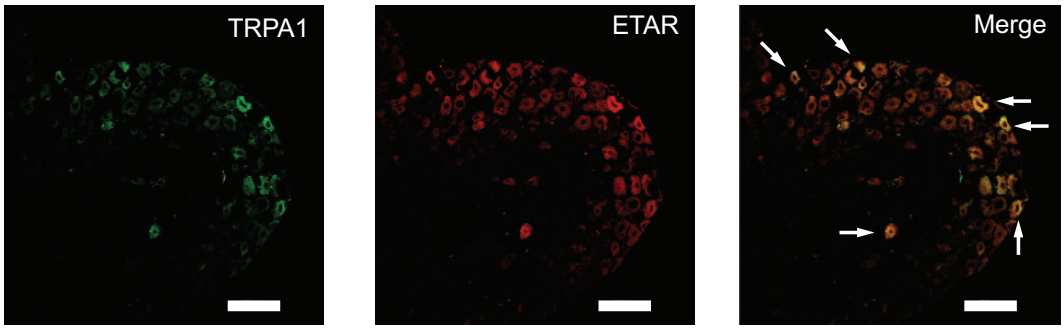

Figure 3. TRPA1 showed coexpression with ET_AR in mouse DRG neurons. Representative immunofluorescence images showing the expression of TRPA1 (in green, left panel), ET_AR (in red, middle panel), and their coexpression (in yellow, right panel) in mouse DRG neurons. Scale bar indicates 100 μm.
TRPA1: transient receptor potential cation channel, subfamily A, member 1; ET_AR: ET_A receptor.

immunostaining experiments using specific antibody against ET_AR in conjunction with antibody against TRPA1. We found that a large population (over 80%) of TRPA1 positive neurons was labeled for ET_AR (Figure 3). Out of 78 ET_AR-positive neurons, 69 were stained positive for TRPA1 (6 sections obtained from 3 mice). This high percentage of coexpression suggests a possible functional interaction between TRPA1 and ET_AR in mouse DRG neurons.

We began to examine the effects of ET-1 on primary sensory neurons. Cultured mouse dorsal root ganglion (DRG) neurons were loaded with Fura-2 for Ca^{2+} imaging. Application of ET-1 (100 nM) did not induce strong Ca^{2+} signals in mouse DRG neurons as in HEK293 cells (Figure 4(a) and (b)). The summarized percent of responding neurons to ET-1 application is not significantly different from vehicle-treated group (3. 3 $\pm 1.5\%$ vs. $2.0 \pm 0.9\%$, $p > 0.05$). We set to examine whether ET-1 was capable of sensitizing TRPA1 channel in DRG neurons. We used low concentrations of MO (5 μM) and capsaicin (10 nM) to activate TRPA1 and TRPV1 subsequently. In control group (vehicle-treated), 5 μM MO barely induced any Ca^{2+} signal, whereas subsequent application of 10 nM capsaicin induced only small Ca^{2+} signal in DRG neurons (Figure 4(a) and (b)). When DRG neurons were pretreated with ET-1 (100 nM), however, larger Ca^{2+} responses were recorded with low concentration of MO (5 μM) (Figure 4(a) and (b)). Subsequent application of 10 nM capsaicin also induced higher Ca^{2+} responses (Figure 4(a) and (b)).

MO-induced Ca^{2+} signal sensitized by ET-1 was mediated via TRPA1, since it was completely eliminated

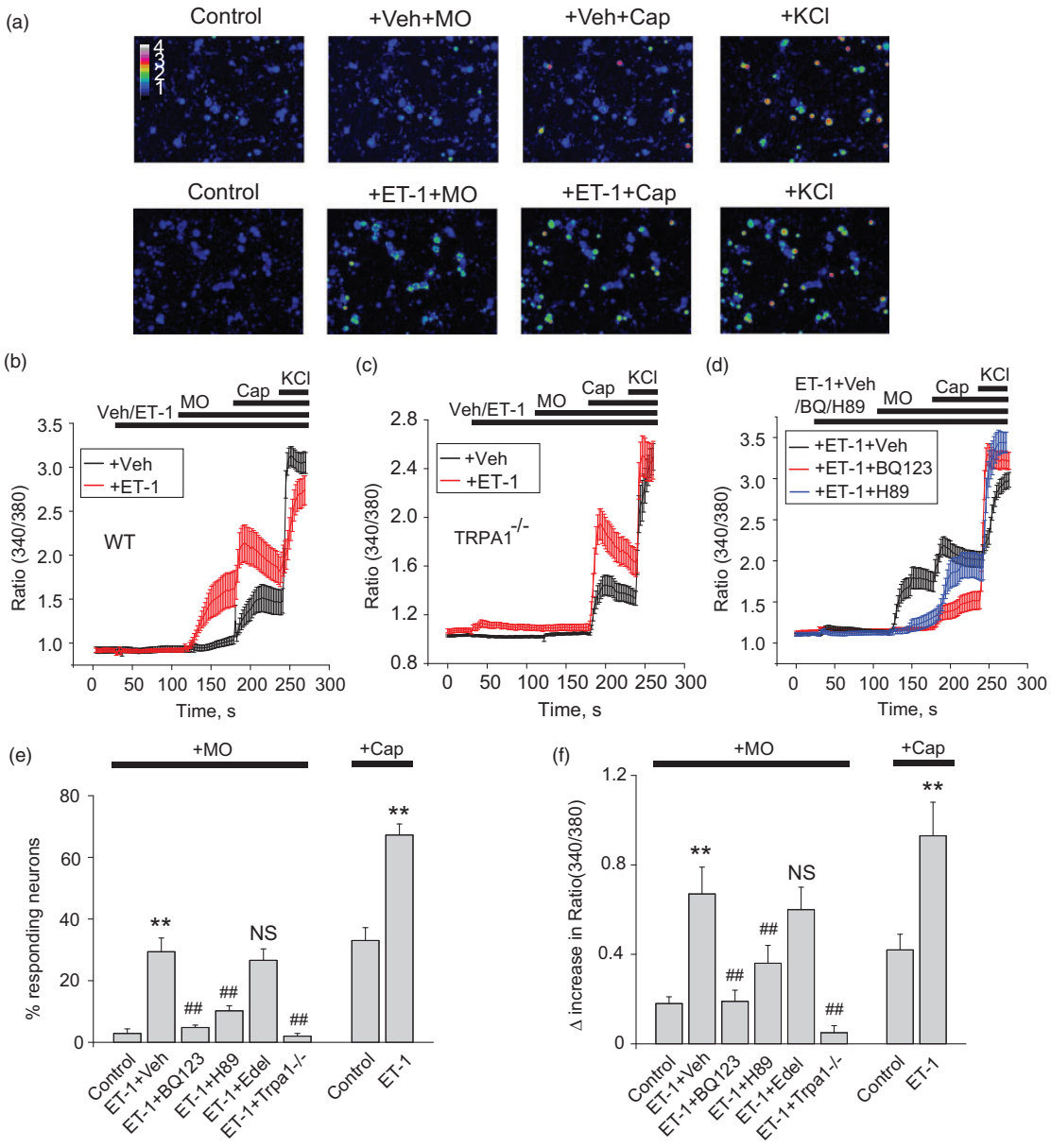

Figure 4. ET-1 sensitizes TRPA1 in cultured mouse DRG neurons. (a) Pseudo color images from Fura-2 ratiometric imaging showing Ca^{2+} responses in mouse DRG neurons in response to MO (5 μM) with or without pretreatment of ET-1 (100 nM). Capsaicin (10 nM) was applied after MO application for comparison. KCl (40 mM) was applied at the end of recording to determine all live DRG neurons. (b) Averaged Ca^{2+} responses from experiments shown in panel (a). Red and black lines show conditions with or without ET-1 pre-treatment, respectively. $n > 20$ cells/group. (c) Averaged Ca^{2+} responses of DRG neurons obtained from Trpa1$^{-/-}$ mice. $n > 20$ cells/group. (d) Pharmacological studies of ET-1-induced TRPA1 sensitization in mouse DRG neurons. Cells were preincubated with ET_AR antagonist BQ-123 (10 μM), PKA antagonist H89 (10 μM), PLC antagonist edelfosine (10 μM), or corresponding vehicle (0.1% DMSO) for 5 min and then coapplied with ET-1. $n > 20$ cells/group. (e) Percentages of mouse DRG neurons responding to MO or Cap in control condition (no ET-1 added) and conditions of ET-1 with vehicle, BQ-123, H89, edelfosine, and TRPA1$^{-/-}$. $n = 5$–6 tests/group, each group contains 150–200 neurons from 3 mice. (f) Summarized Δ increase in ratio of 340/380 of MO or Cap-induced Ca^{2+} responses in mouse DRG neurons as recorded in (e). $^{**}p < 0.01$ versus control group, $^{\#\#}p < 0.01$ versus ET-1+Veh group.
TRPA1: transient receptor potential cation channel, subfamily A, member 1; ET-1: endothelin-1; MO: mustard oil.

in neurons derived from Trpa1 knockout (Trpa1$^{-/-}$) mouse (Figure 4(c), (e), and (f)). In contrast, the sensitizing effect of ET-1 on capsaicin-induced Ca^{2+} signal remained unaltered in Trpa1$^{-/-}$ neurons (Figure 4(c), (e), and (f)). ET_AR antagonist BQ-123 (10 μM) and PKA antagonist H89 (10 μM) largely eliminated ET-1-induced sensitization on TRPA1 (Figure 4(d), (e), and (f)). PLC antagonist edelfosine (10 μM) had no effect on ET-1-

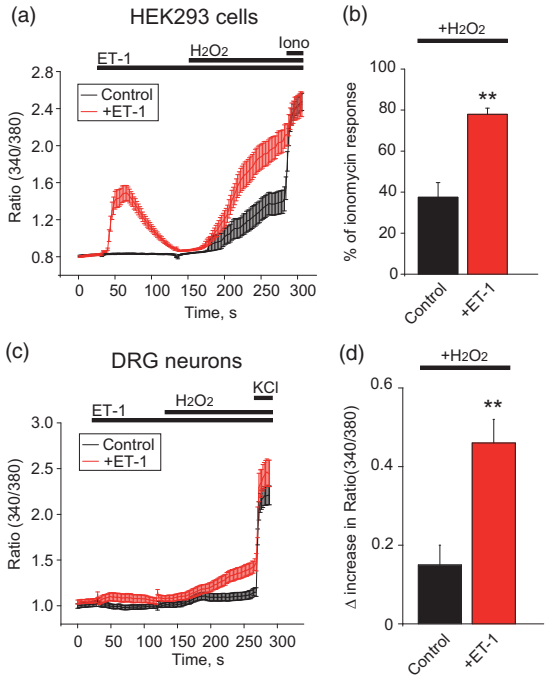

Figure 5. ET-1 sensitizes TRPA1 channel activated by endogenous agonist H_2O_2 in both HEK293 cells and mouse DRG neurons. (a) Averaged Ca^{2+} responses from HEK293 cells in control condition or treated with ET-1 (100 nM). HEK293 cells were expressed with TRPA1 and ET_AR. H_2O_2 (1 mM) was applied as indicated to induce TRPA1 activation in HEK293 cells. $n > 30$ cells/group. (b) Summarized amplitude of H_2O_2-induced Ca^{2+} responses in control condition or treated with ET-1. Ca^{2+} responses were normalized to ionomycin (1 μM) applied at the end of the tests (% response of ionomycin). $n = 3$ tests/group. Each test contains up to 30 cells. (c) Averaged Ca^{2+} responses from mouse DRG neurons in control condition or treated with ET-1 (100 nM). H_2O_2 (1 mM) was applied as indicated to induce TRPA1 activation in DRG neurons. $n > 20$ cells/group. (d) Summarized Δ increase in ratio of 340/380 of H_2O_2-induced Ca^{2+} responses in DRG neurons as recorded in (c). $n = 4$ tests/group, each group contains 120–160 neurons from 3 mice. $^{**}p < 0.01$ versus control group.
HEK293: human embryonic kidney 293 cells; ET-1: endothelin-1; DRG: dorsal root ganglion; Iono: ionomycin.

induced sensitization of TRPA (Figure 4(e) and (f)). Therefore, the above results demonstrated that ET-1 sensitizes TRPA1 in DRG neurons via ET_AR and PKA pathway.

TRPA1 can be activated by a variety of endogenous agonists that activate TRPA1 to produce pain signals. We examined whether ET-1 sensitizes TRPA1 activated by hydrogen peroxide (H_2O_2), a well-established endogenous agonist for TRPA1.[30] In HEK293 cells expressing ET_AR and TRPA1, ET-1 (100 nM) treatment significant increased the magnitude of Ca^{2+} responses to H_2O_2 (1 mM) compared with vehicle-treated group (control) (Figure 5(a) and (b)). ET-1 (100 nM) treatment also significantly increased magnitude of Ca^{2+} responses to H_2O_2 in mouse DRG neurons (Figure 5(c) and (d)). These results suggest that ET-1 sensitizes TRPA1 channel activated by endogenous agonist H_2O_2.

TRPA1 mediates ET-1-induced mechanical hyperalgesia in vivo

We examined the contribution of TRPA1 in ET-1-induced nocifensive behavior *in vivo*. Mice injected with ET-1 into hind paw (20 ng/paw) showed obvious signs of mechanical hyperalgesia compared with mice receiving vehicle injection only, measured 0.5 and 2.5 h after injection (Figure 6). HC-030031 (10 μg/paw), the TRPA1 antagonist, significantly reduced the mechanical hyperalgesia induced by ET-1. PKA antagonist H89 (5 μg/paw) and ET_AR antagonist BQ-123 (1 μg/paw) both significantly attenuated the mechanical hyperalgesia induced by ET-1 (Figure 6). Injection of these antagonists alone in the same dosage as above did not produce any effects on PWTs compared with vehicle (control) group (Table 1). In all, these results demonstrated that ET-1 elicited mechanical hyperalgesia through TRPA1 via ET_AR and PKA pathway.

Discussion

In the present study, we found that ET-1 sensitizes TRPA1 via ET_AR and PKA-mediated signaling pathway both *in vitro* and *in vivo*. Our findings are based upon the following observations: First, ET-1 sensitizes TRPA1 channel in HEK293 cells via ET_AR and PKA-mediated pathway. Second, ET-1 does not produce robust Ca^{2+} signals in DRG neurons but sensitizes MO-activated TRPA1 channel activity. Third, ET-1 sensitizes TRPA1 channel activated by endogenous agonist H_2O_2. Last, blocking TRPA1, ET_AR, and PKA all significantly alleviated ET-1-induced mechanical hyperalgesia.

ET_ARs are widely expressed in small and medium-to-large diameter neurons and, in particular, in TRPV1-expressing small sensory neurons, whereas ET_BRs are mainly found in satellite glial cells but not in sensory neurons.[6,7] TRPA1 are distributed in sensory neurons that also express TRPV1.[31] Previous studies revealed ET_AR coexpressed largely with TRPV1 in mouse DRG neurons.[6] This suggests that TRPA1 is likely to coexpress with ET_AR in sensory neurons. Our immunostaining results demonstrated that a large population (over 80%) of TRPA1-positive DRG neurons also express ET_AR. The high percentage of coexpression suggests a possible functional interaction between TRPA1 and ET_AR in DRG neurons.

ET_AR couples through $G_{\alpha q/11}$ to PLCβ and the release of inositol trisphosphate (IP_3) to induce intracellular Ca^{2+} release.[23,32] In HEK293 cells which are

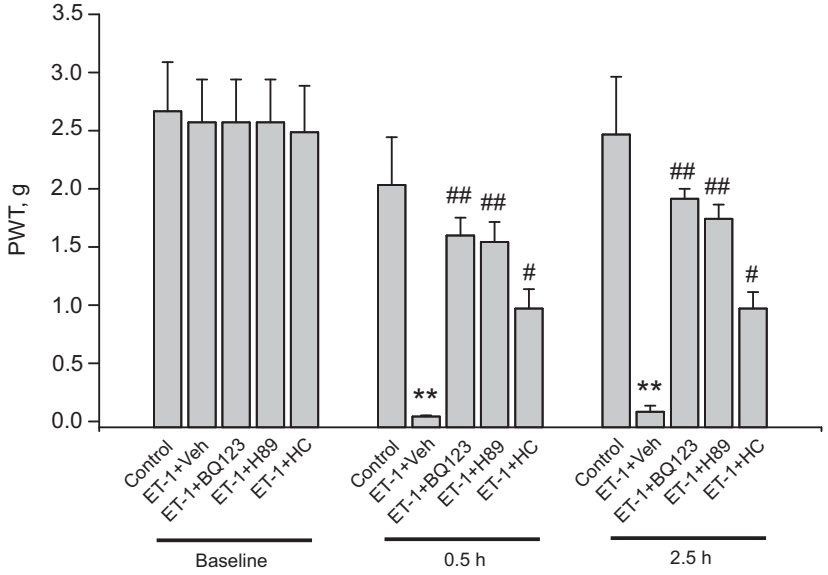

Figure 6. Pharmacological blocking of ET_AR, PKA, and TRPA1 attenuates ET-1-induced mechanical hyperalgesia in mice. PWT of mice was measured by von Frey hair test. BQ-123 (1 μg/paw), H89 (5 μg/paw), or HC-030031 (10 μg/paw) was coinjected with ET-1 (20 ng/paw) into the hind paw of mice. Control group received vehicle (1% DMSO in PBS) injection only. PWT was measured before injection (baseline) and 0.5, 2.5 h after the injection. $n = 7$ mice/group. $^{**}p < 0.01$ versus control group, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ versus ET-1+Veh group.
ET-1: endothelin-1; PWT: paw withdraw threshold.

Table 1. Effect of intraplantar injection of BQ-123, H89, or HC-030031 on the PWTs of mice.

	PWT (g)		
Group	0 h	0.5 h	2.5 h
Control	2.7 ± 0.4	2.0 ± 0.4	2.5 ± 0.5
+BQ-123	2.3 ± 0.3 (NS)	2.1 ± 0.4 (NS)	2.1 ± 0.4 (NS)
+H89	2.7 ± 0.4 (NS)	2.0 ± 0.4 (NS)	2.2 ± 0.4 (NS)
+HC-030031	2. 7± 0.4 (NS)	2.2 ± 0.4 (NS)	2.6 ± 0.5 (NS)

PWT: paw withdrawal threshold; NS: no significance.
BQ-123 (1 μg/paw), H89 (5 μg/paw), or HC-030031 (10 μg/paw) and corresponding vehicle (1% dimethyl sulfoxide in phosphate-buffered saline, control) were administered intraplantarly into the hind paws of mice. PWTs were measured before and 0.5 and 2.5 h after drug/vehicle treatment.

overexpressed with ET_AR, we found that ET-1 application elicited large Ca^{2+} responses, which gradually subsided in the continued presence of ET-1. This suggests that ET-1 binds with ET_AR and initiates Ca^{2+} signals which is likely mediated via PLCβ-IP_3 pathway in HEK293 cells. In contrast, ET-1 did not elicit obvious Ca^{2+} signals in native DRG neurons in our study. It is reported that ET-1 only induced quite small intracellular Ca^{2+} transients in mouse DRG neurons, but its effect on satellite nonneuronal cells is much larger.[33] Recently, one study reported that only a small proportion (approximate 3%) of DRG neurons respond to ET-1 application in Ca^{2+} imaging.[7] This responding rate to ET-1 is similar with our findings. But unfortunately, no comparisons between ET-1-responding and vehicle-responding rate were included in that study. In the present study and our previous publication, we found that even vehicle application produces small and random Ca^{2+} transients in DRG neurons during Ca^{2+} imaging.[18] Therefore, it still remains to be investigated whether ET-1 can truly induce reliable and robust Ca^{2+} signals in mouse DRG neurons.

ET_AR also couples to PKA signaling pathway.[22,34] ET-1 induces intracellular cAMP level increase in HEK293 cells expressing ET_AR but not ET_BR, suggesting ET_AR couples with PKA signaling in HEK293 cells.[6] TRPA1 can be sensitized by inflammatory mediators, such as bradykinin and tryptase, via PKA and PLC pathways.[10,13,15,35] Further study identified the amino acid residues involved in PKA-mediated phosphorylation and sensitization of TRPA1.[14] We examined the contribution of these two pathways to ET-1-induced sensitization of TRPA1. Pharmacological blockage of ET_AR by specific antagonist BQ-123 abolished ET-1-induced sensitization of TRPA1 Ca^{2+} signals in both HEK293 cell lines and DRG neurons. Furthermore, PKA antagonist H89 largely abolished ET-1-induced sensitization of TRPA1 Ca^{2+} signals in both HEK293 cell lines and DRG neurons. The PKA-specific agonist, forskolin, mimics the effect of ET-1 in sensitizing TRPA1 mediated Ca^{2+} signals. However, pretreating HEK293 cells and DRG neurons with PLC antagonist edelfosine did not affect ET-1-induced sensitization of

TRPA1. These results demonstrate that ET-1-induced sensitization of TRPA1 *in vitro* requires ET_AR-mediated PKA signaling pathway.

ET-1 potentiates TRPV1 channel, which underlies ET-1-induced nocifensive behavior.[36] However, ET-1-induced nociceptive response is not completely inhibited in $Trpv1^{-/-}$ mice or by TRPV1 antagonist, suggesting other mechanisms are involved as well.[3,37] In addition to TRPV1, TRPA1 is involved in ET-1-induced mechanical hypersensitivity.[17] We found that ET-1 induced mechanical hyperalgesia is significantly reduced by TRPA1-specific antagonist HC-030031, as well as by PKA and ET_AR antagonists. These findings suggest that TRPA1 is a molecular target of ET-1 in mediating nociceptive responses.

Oxidative stress occurs during many pathophysiological conditions including inflammation and tissue injury, which produces a variety of highly reactive oxygen species (ROS) including H_2O_2, lipid peroxidation products, like 4-hydroxy-2-nonenal and Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphatidylcholine (OxPAPC).[38] These ROS products act as endogenous TRPA1 agonists and are involved in many inflammatory and neuropathic pain conditions.[20,39] ET-1 is generated during tissue inflammation and damage and involved in pathogenesis of pain.[1] The concentration of ET-1 (100 nM) we tested falls well within ET-1's endogenous concentration range reported.[21] Thus, our findings suggest that ET-1-induced TRPA1 sensitization is likely to occur in pathological conditions.

In addition to causing pain, ET-1 is also known to cause pruritus.[7] This property is shared with many other stimulators of peripheral sensory neurons. Pain and itch sensations are activated by excitation of separate populations of peripheral sensory neurons, the nociceptors, and the pruriceptors, respectively.[40] Recent studies demonstrated that nociceptors and pruriceptors engage spinal circuits that, depending on stimulus strength, duration, and lateral spread of inputs, control whether itch or pain is transduced, or whether itch is suppressed (by scratching, for example).[40,41] It is possible that high local concentrations of ET-1 may favor pain since widespread nociceptor activation is known to suppress itch sensations, while lower local concentrations favor itch sensation. The role of TRPA1 in ET-1-induced pruritus remains controversial. While TRPA1 inhibitors were found to increase ET-1-induced scratching responses in mice immediately after injection, a study in $Trpa1^{-/-}$ mice observed that ET-1 induced scratching was attenuated.[7,42] Additional studies, potentially using more selective inhibitors and longer observation time, may be necessary to clarify the role of TRPA1 in ET-1-induced pruritus.

It has been reported that ET-1 can potentiate TRPA1 agonist cinnamaldehyde-induced nociception *in vivo*.[16] Further studies demonstrated that ET-1-induced mechanical allodynia is inhibited by specific antagonists against TRPA1 or ET_AR *in vivo*, suggesting a possible interaction between ET_AR and TRPA1 in mediating pain responses.[17] However, little is known about whether ET-1 acts on TRPA1 in primary sensory neurons and the detailed molecular mechanisms. Our findings showed for the first time that ET_AR couples with TRPA1 in primary sensory neurons, which provide another molecular mechanism for explaining ET-1-induced pain response. Our results demonstrate that TRPA1 acts as a novel molecular target for ET-1 and sensitization of TRPA1 through ET_AR–PKA pathway contributes to ET-1-induced mechanical hyperalgesia. Targeting of TRPA1 and ET_AR-PKA pathway may offer effective strategies to alleviate pain conditions related with ET-1.

Author Contributions

SEJ and BL conceived and designed the project. XZ, YT, DH, BL, CW and XS carried out the experiments and collected and analyzed the data. BL and SEJ prepared the manuscript.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Sven-Eric Jordt serves on the Scientific Advisory Board of Hydra Biosciences LLC (Cambridge, MA), a biopharmaceutical company developing TRP ion channel inhibitors for the treatment of pain and inflammation. Other authors state no conflict of interest.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This project is supported by funding from Zhejiang Provincial Natural Science Funds for Distinguished Young Scholars (LR17H270001), the National Natural Science Foundation of China (81873365, 81603676, 81770407, 81574056), Qianjiang Talent Program (QJD1702020), research fund from Zhejiang Chinese Medical University (2018ZY37, 2018ZY19), Scientific Research Foundation for the Returned Overseas Chinese Scholars of Hebei Province, Administration of Traditional Chinese Medicine of Hebei Province (2018139), and from the National Institute of Arthritis and Musculoskeletal and Skin Disease (R21AR070554 to S.E.J.).

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