ET\textsubscript{A}R and protein kinase A pathway mediate ET-1 sensitization of TRPA1 channel: A molecular mechanism of ET-1-induced mechanical hyperalgesia

Xiaoli Zheng\textsuperscript{1,*}, Yan Tai\textsuperscript{2,*}, Dongwei He\textsuperscript{3,*}, Boyu Liu\textsuperscript{1,*}, Chuan Wang\textsuperscript{4}, Xiaomei Shao\textsuperscript{1}, Sven-Eric Jordt\textsuperscript{5}, and Boyi Liu\textsuperscript{1}

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) \textit{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\textsuperscript{2+} imaging, immunostaining, electrophysiology, animal behavioral assay combined with pharmacological experiments were performed. ET-1 sensitized TRPA1-mediated Ca\textsuperscript{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 H2O2. ET\textsubscript{A} receptor (ET\textsubscript{A}R) colocalized with TRPA1 in DRG neurons. ET-1-induced TRPA1 sensitization \textit{in vivo} was mediated via ET\textsubscript{A}R and protein kinase A (PKA) pathway in HEK293 cells and DRG neurons. Pharmacological blocking of ET\textsubscript{A}R, 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\textsubscript{A}R–PKA pathway contributes to ET-1-induced mechanical hyperalgesia. Pharmacological targeting of TRPA1 and ET\textsubscript{A}R-PKA pathway may provide effective strategies to alleviate pain conditions associated with ET-1.

Keywords
TRPA1, endothelin, pain, protein kinase A, sensitization

Introduction
Endothelin-1 (ET-1) is a potent vasoconstrictor peptide that has been implicated in the pathogenesis of tissue inflammation and pain.\textsuperscript{1} Injection of ET-1 induced overt pain-like behavior and thermal and mechanical allodynia in animals.\textsuperscript{2–4} In humans, ET-1 injection produced severe pain and prolonged, touch-evoked allodynia.\textsuperscript{5} ET-1 exerts its effects mainly via acting on ET\textsubscript{A} and ET\textsubscript{B} receptors (ET\textsubscript{A}R and ET\textsubscript{B}R), both of which are G protein-coupled receptors.\textsuperscript{1} ET\textsubscript{A}Rs are abundantly expressed in primary sensory neurons, whereas ET\textsubscript{B}R are found exclusively in satellite glial cells.\textsuperscript{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
activation of TTX-R Na\textsuperscript{+} channels.\textsuperscript{8} ET-1 also potenti-
ates TRPV1 channel via ET\textsubscript{A}R-mediated protein kinase
C (PKC) signaling in both expression system and senso-
ry neurons.\textsuperscript{6}

Transient receptor potential cation channel, subfam-
ily 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 vari-
ety of endogenous and exogenous substances that elicit
pain and irritation.\textsuperscript{9} Activation of TRPA1 depolarizes
nociceptors and contributes to the perception of noxious
stimuli.\textsuperscript{9} In addition, TRPA1 channel activity can be
sensitized by inflammatory mediators, including brady-
kinin, trypsin, and nerve growth factor (NGF).\textsuperscript{10–12}

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\textsubscript{A}R–PKA pathway and TRPA1 in ET-1-induced
nocifensive response. Our results suggest that ET-1 sen-
sitizes TRPA1 via ET\textsubscript{A}R 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\textsuperscript{1/−} 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 labo-
ratory 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 bisin-
dolylmaleimide (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-glu-
tamine, 100 units/ml penicillin, and 100 \( \mu \)g/ml strepto-
mycin. Cells were transfected by Lipofectamine 2000
(Invitrogen, Carlsbad, CA, USA) according to manufac-
turer’s instruction. Human TRPA1 (hTRPA1) is a gift
from Professor David Julius (University of California,
San Francisco), and ET\textsubscript{A}R is purchased from OriGene
(Rockville, MD).

Adult mouse dorsal root ganglia (DRGs) were disso-
ciated using 0.28 Wänisch units/ml Liberase Blendzyme 1
(Roche Diagnostics, Mannheim, Germany) as described
previously.\textsuperscript{18} Neurons were cultured in Neurobasal-A
medium (Invitrogen, Grand Island, NY) with B-27 sup-
plement, 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\textsubscript{2}. Bilateral L3-5 DRGs
were collected and immersed immediately in 4% para-
formaldehyde 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-\( \mu \)m sections. For immu-
nostaining, 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\textsubscript{A}R (1:200, Abcam,
Carlsbad, CA, USA). After washout, corresponding sec-
ondary antibodies (1:1000, Abcam, Carlsbad, CA, USA)
were used for staining. Fluorescence signals were
detected by Nikon A1R laser scanning confocal micros-
cope (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 \(\text{Ca}^{2+}\) 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 \(\mu\)M, 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 \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) 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 M\(\Omega\) after loading the pipette solution. Currents were filtered at 2.3 kHz and digitized at 100\(\mu\)s 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 \(\mu\)l. HC-030031 (10 \(\mu\)g/paw), H89 (5 \(\mu\)g/paw), and BQ-123 (1 \(\mu\)g/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 \(\geq\) 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\(_A\)R 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 \textit{in vitro} experiments, which is a commonly used concentration in other studies and falls within the \textit{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 \(\mu\)M) 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\(_A\)R 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\(_A\)R. 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\(_A\)R (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 \(\text{Ca}^{2+}\) responses among different groups. ET\(_A\)R couples to PLC and PKA pathways, 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\(_A\)R-specific antagonist BQ-123 (10 \(\mu\)M), but not ET\(_B\)R-specific antagonist BQ-788 (5 \(\mu\)M), at effective
concentration, significantly reduced ET-1-induced sensitization of Ca$^{2+}$ responses to MO in HEK293 cells. 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/ETAR-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 ETAR-mediated PKA signaling pathway in HEK293 cells.

**ET-1 sensitizes TRPA1 channel in mouse primary sensory neurons**

It is reported that ETARs are mainly expressed in DRG neurons.6,7 We therefore performed double...
immunostaining experiments using specific antibody against ETAR in conjunction with antibody against TRPA1. We found that a large population (over 80\%) of TRPA1 positive neurons was labeled for ETAR (Figure 3). Out of 78 ETAR-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 ETAR 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 ± 1.5\% vs. 2.0 ± 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 \(\mu\)M) and capsaicin (10 nM) to activate TRPA1 and TRPV1 subsequently. In control group (vehicle-treated), 5 \(\mu\)M 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 \(\mu\)M) (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 mM) 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 pretreatment, 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 ETAR antagonist BQ-123 (10 nM), PKA antagonist H89 (10 nM), PLC antagonist edelfosine (10 nM), 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$\lambda$R antagonist BQ-123 (10 nM) and PKA antagonist H89 (10 nM) largely eliminated ET-1-induced sensitization on TRPA1 (Figure 4(d), (e), and (f)). PLC antagonist edelfosine (10 nM) had no effect on ET-1-
ET-1 sensitizes TRPA1 channel activated by endogenous agonist H$_2$O$_2$ in both HEK293 cells and mouse DRG neurons (Figure 5(a) and (b)). Summarized amplitude of H$_2$O$_2$-induced Ca$^{2+}$ responses from HEK293 cells in control condition or treated with ET-1 (100 nM). HEK293 cells were expressed with TRPA1 and ETAR. H$_2$O$_2$ (1 mM) was applied as indicated to induce TRPA1 activation in HEK293 cells. n > 30 cells/group. (b) Summarized amplitude of H$_2$O$_2$-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$_2$O$_2$ (1 mM) was applied as indicated to induce TRPA1 activation in DRG neurons. n > 20 cells/group. (d) Summarized A increase in ratio of 340/380 of H$_2$O$_2$-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.

Discussion

In the present study, we found that ET-1 sensitizes TRPA1 via ETAR 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 ETAR 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$_2$O$_2$. Last, blocking TRPA1, ETAR, and PKA all significantly alleviated ET-1-induced mechanical hyperalgesia.

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

ETAR couples through G$_{q/11}$ to PLCβ and the release of inositol trisphosphate (IP$_3$) to induce intracellular Ca$^{2+}$ release. In HEK293 cells which are
overexpressed with ETAR, 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 ETAR and initiates Ca\(^{2+}\) signals which is likely mediated via PLCb-IP3 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.

ETAR also couples to PKA signaling pathway.\(^{22,34}\) ET-1 induces intracellular cAMP level increase in HEK293 cells expressing ETAR but not ETBR, suggesting ETAR 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 ETAR 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 Ca\(^{2+}\) signals.

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

| Group             | PWT (g)   | 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.
TRPA1. These results demonstrate that ET-1-induced sensitization of TRPA1 in vitro requires ET\textsubscript{AR}-mediated PKA signaling pathway.

ET-1 potentiates TRPV1 channel, which underlies ET-1-induced nocifensive behavior.\textsuperscript{36} However, ET-1-induced nociceptive response is not completely inhibited in Trpv1\textsuperscript{-/-} mice or by TRPV1 antagonist, suggesting other mechanisms are involved as well.\textsuperscript{3,37} In addition to TRPV1, TRPA1 is involved in ET-1-induced mechanical hypersensitivity.\textsuperscript{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\textsubscript{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\textsubscript{2}O\textsubscript{2}, lipid peroxidation products, like 4-hydroxy-2-nonenal and Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphatidylcholine (OxPAPC).\textsuperscript{38} These ROS products act as endogenous TRPA1 agonists and are involved in many inflammatory and neuropathic pain conditions.\textsuperscript{20,39} ET-1 is generated during tissue inflammation and damage and involved in pathogenesis of pain.\textsuperscript{1} The concentration of ET-1 (100 nM) we tested falls well within ET-1’s endogenous concentration range reported.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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).\textsuperscript{40,41} It is possible that high local concentrations of ET-1 may favor pain since widespread nociceptor activation is known to suppress itch sensation, 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\textsuperscript{-/-} mice observed that ET-1 induced scratching was attenuated.\textsuperscript{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.\textsuperscript{16} Further studies demonstrated that ET-1-induced mechanical allodynia is inhibited by specific antagonists against TRPA1 or ET\textsubscript{AR} in vivo, suggesting a possible interaction between ET\textsubscript{AR} and TRPA1 in mediating pain responses.\textsuperscript{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\textsubscript{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\textsubscript{AR}-PKA pathway contributes to ET-1-induced mechanical hyperalgesia. Targeting of TRPA1 and ET\textsubscript{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.

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ORCID iD
Boy Liu https://orcid.org/0000-0001-9870-4548

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