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Resveratrol-decreased hyperalgesia mediated by the $P2X_7$ receptor in gp120-treated rats

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Abstract

Background: Chronic pain is a common symptom in human immunodeficiency virus (HIV)-1 infection/acquired immunodeficiency syndrome patients. The literature shows that the HIV envelope glycoprotein 120 (gp120) can directly cause hyperalgesia by stimulating primary sensory afferent nerves. The $P2X_7$ receptor in the dorsal root ganglia (DRG) is closely related to neuropathic and inflammatory pain. In this study, we aimed to explore the effect of resveratrol (RES) on gp120-induced neuropathic pain that is mediated by the $P2X_7$ receptor in the rat DRG.

Results: Mechanical hyperalgesia in rats treated with gp120 was increased compared with that in the sham group. The $P2X_7$ expression levels in rats treated with gp120 were higher than those in the sham group. Co-localization of the $P2X_7$ receptor and glial fibrillary acidic protein (GFAP, a marker of satellite glial cells [SGCs]) in the DRG SGCs of the gp120 group exhibited more intense staining than that of the sham group. RES decreased the mechanical hyperalgesia and $P2X_7$ expression levels in gp120 treatment rats. Co-localization of the $P2X_7$ receptor and GFAP in the gp120+ RES group was significantly decreased compared to the gp120 group. RES decreased the IL-1β and TNF-α receptor (R) expression levels and ERK1/2 phosphorylation levels as well as increased IL-10 expression in the DRG of gp120-treated rats. Whole cell clamping demonstrated that RES significantly inhibited adenosine triphosphate-activated currents in HEK293 cells that were transfected with the $P2X_7$ plasmid.

Conclusions: RES relieved mechanical hyperalgesia in gp120-treated rats by inhibiting the $P2X_7$ receptor.

Keywords

HIV gp120-associated neuropathic pain, $P2X_7$ receptor, resveratrol, dorsal root ganglia

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Introduction

Chronic pain is a common symptom in human immunodeficiency virus (HIV)-1 infection/acquired immunodeficiency syndrome (AIDS) patients.[1–3] The quality of life of HIV-1/AIDS patients with chronic pain is significantly decreased. To ascertain the pathogenic mechanism of HIV-associated pain, it is pivotal to identify the causative HIV-1 agents. Glycoprotein 120 (gp120) can cause axonal injury of sensory neurons in culture.[4,5] Gp120 is an HIV-1 protein that induces pain behaviors when introduced into animal models.[1,5–8] Pain may arise from the direct effects of HIV on the peripheral nervous system.[4,9] The dorsal root ganglion (DRG) afferent fibers are distributed to both central and peripheral terminals and they transmit noxious stimuli from the periphery to the central nervous system.[10,11] Gp120 appears to bind to the surface of rat

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DRG neurons and may be causative factors in the generation of neuropathic pain in HIV-1-infected patients.[10,12] The HIV-1 gp120 level was significantly higher in ''pain-positive'' HIV-1 patients.[4,5] Therefore, preventing and treating HIV-1 gp120-associated neuropathic pain has become a heavily researched subject.

Resveratrol (RES) is a natural polyphenolic compound found in peanuts, mulberries, grapes, and red wine.[13,14] RES exhibits anti-inflammatory and anti-nociceptive effects.[13–15] Adenosine triphosphate (ATP) is a signaling molecule in neuropathic and inflammatory pain.[16–18] Extracellular ATP can activate the ionotrophic P2X receptors in primary afferent fibers.[16–18] The $P2X_7$ receptor is involved in the induction and maintenance of neuropathic and inflammatory pain.[17,19,20] The interaction between HIV-1 gp120 and macrophages stimulates increased ATP release and P2X receptors activated by ATP are required for HIV entry into macrophages.[21,22] ATP signaling via the $P2X_7$ receptor is related to the regulation of inflammatory responses during acute viral infection.[22] The blocking of purinergic receptors results in a significant reduction in the HIV replication in macrophages.[21] Therefore, the $P2X_7$ receptor may be involved in HIV-associated neuropathic pain. In this study, we investigated the effect of RES on gp120-induced neuropathic pain mediated by the $P2X_7$ receptor in rat DRGs.

Materials and methods

Animals and surgical methods

Adult (200–250 g) male Sprague–Dawley (SD) rats were used in all experiments and housed with an alternating 12-h light/dark cycle. They were provided with food and water ad libitum. The use of the animals was reviewed and approved by the Animal Care and Use Committee of Medical College of Nanchang University. The experiments were conducted under the guidelines of the NIH in the US regarding the care and use of animals for experimental procedures.

The rats were randomly divided into the following three groups (with six rats in each group): the HIV-gp120 group (gp120 group); HIV-gp120 rats treated with RES group (gp120 + RES group); and sham operation group (sham group). A previously described technique[23] was used for the perineural HIV-gp120 administration. Briefly, under 10% chloral hydrate anesthesia (3 ml/kg, i.p., supplemented as necessary) and aseptic surgical conditions, the left sciatic nerve of the SD rats was exposed in the popliteal fossa without damaging the nerve construction. A 2 × 6 mm strip of oxidized regenerated cellulose was previously soaked in 250 µl of a 0.1% rat serum albumin saline solution containing 200 ng of gp120 (Sigma) or 0.1% rat serum albumin in saline for the sham surgery. A 3–4 mm length of the sciatic nerve that was proximal to the trifurcation was wrapped loosely with the soaked cellulose, did not cause nerve constriction, and was left in situ. The incision was closed with 4/0 sutures. Beginning at 24 h after surgery, the rats in the gp120 + RES group were intraperitoneally treated with RES (30 mg/kg) daily for 14 days.[24] Rats in the sham and gp120 groups were intraperitoneally injected with the same volume of normal saline.

Measurement of the mechanical withdrawal threshold

Determination of the mechanical withdrawal threshold (MWT) was performed at 9:00–12:00 using a BME-404 electronic mechanical stimulator (Institute of Biomedical Engineering, Chinese Academy of Medical Sciences, Tianjin, China). The main technical parameters of this equipment were as follows: end face diameter of the test needle, 0.6 mm; pressure measurement range, 0.1–50 g; and pressure measurement resolution, 0.05 g. An organic glass box (22 × 22 × 12 cm) was placed on the sieve of the metal frame. The rat was placed into the box for 30 min of adaptation. The left hind paws were touched with the test needle until escaping behavior was observed. The pressure value was automatically recorded. The measurement was conducted five times for each rat (interval, $\geqslant$ 5 min), and the mean value was calculated as the MWT for this measurement.[7,25,26]

RNA extraction and Real-time-PCR

The rats in three groups were anesthetized using 10% chloral hydrate (3 ml/kg, i.p.). The L4–6 DRGs were isolated immediately and flushed with ice-cold phosphate-buffered saline (PBS). The total RNA samples were prepared from the L4–6 DRGs of each group using the TRIzol Total RNA Reagent (Beijing Tiangen Biotech Co.). cDNA synthesis was performed with 2 µg of total RNA using a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada). The primers were designed with Primer Express 3.0 software (Applied Biosystems), and the sequences were as follows: $P2X_7$, forward 5'-CT TCGGCGTGCGTTTTG-3', and reverse 5'-AGGA CAGGGTGGATCCAATG-3' as well as β-actin, forward 5'- TAAAGACCTCTATGCCAACACAGT-3', and reverse 5'-CACGATGGAGGGGCCGGACTCA TC-3'. Quantitative PCR was performed using the SYBR® Green MasterMix in an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Inc.: Foster City, CA). The quantification of gene expression was performed using the ΔΔCT calculation with CT as the threshold cycle. The relative levels of target genes, normalized to the sample with the lowest CT, are given

as $2^{-\Delta\Delta CT}$.[27] β-actin was used to be internal control in the three groups. The relative expression levels of mRNA in the three groups were normalized to β-actin.

Western blot analysis

The animals were anesthetized and tissue collection was performed as described above, except that the tissues were snap-frozen in tubes on dry ice during collection.[25] Briefly, on the 14th day after the operation, the animals were anesthetized with 10% chloral hydrate and L4-6 DRGs were isolated immediately and rinsed in ice-cold PBS. The ganglia were homogenized by mechanical disruption in lysis buffer containing the following: 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.02% sodium deoxycholate, 100 μg/mL phenylmethylsulfonyl fluoride, and 1 μg/mL Aprotinin. The cells were incubated on ice for 30 min. The homogenates were then centrifuged at 12,000 r/min for 10 min and the supernatants were collected. The quantity of total proteins in the supernatants was determined using the Lowry method. After dilution with loading buffer (250 mM Tris-Cl, 200 mM Dithiothreitol, 10% SDS, 0.5% Bromophenol Blue, and 50% Glycerol) and heating to 95°C for 5 min, samples containing equal protein levels (20 μg) were separated by 10% SDS–polyacrylamide gel electrophoresis with a Bio-Rad system. The proteins were then transferred onto polyvinylidene difluoride membranes by electrophoretic transfer using the same system. The membrane was blocked with 5% bovine serum albumin (BSA) for anti-p-ERK1/2 and anti-ERK1/2 in 25 mM tris-buffered saline, pH 7.2, plus 0.05% Tween 20 (TBST) for 2 h at room temperature, which was followed by incubation with a rabbit anti-$P2X_7$ (1:800 dilutions, Abcam, USA), rabbit anti-TNFα-receptor (R), rabbit anti-IL-1β and rabbit anti IL-10 (1:500 dilutions, Abcam, USA), rabbit anti-p-ERK1/2 (Thr202/Tyr204) (1:1000, Cell signaling technology, 9101), rabbit anti-ERK1/2 (1:1000, Cell signaling technology, 9102), and mouse monoclonal anti-β-actin antibody (1:800 dilutions, Beijing Zhongshan Biotech Co., China) at 4°C overnight. The membranes were washed three times with TBST and incubated (1 h, room temperature) with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG (1:2000), goat anti-mouse IgG (1:2000), Beijing Zhongshan Biotech Co.) in blocking buffer. After another wash cycle, the labeled proteins were visualized by enhanced chemiluminescence on a high-performance film (Shanghai Pufei Biotech Co.). The chemiluminescent signals were collected on an autoradiography film, and the band intensity was quantified using Image Pro Plus software. The relative band intensity of the target proteins was normalized against the intensity of the respective β-actin internal control.

Double immunofluorescence

The DRGs isolated from rats in the three groups, six rats in each group, were washed with PBS. The DRGs were dissected immediately and fixed in 4% paraformaldehyde for 24 h at room temperature. Then, they were transferred to 20% sucrose for dehydration at 4°C overnight. The tissues were sectioned at 10 μm using a cryostat and placed onto glass slides that were coated with poly-d-lysine for storage in the refrigerator at −20°C. After washing with PBS three times, the preparations were preincubated with 10% normal goat serum (Jackson ImmunoResearch Inc., West Grove PA, USA) for 40 min in a moist chamber at 37°C. The sections were then incubated with chicken anti-GFAP (1:1000 dilutions; Abcam, USA) and rabbit anti-$P2X_7$ (1:200 dilutions; Abcam, USA), which were diluted in PBS, overnight at 4°C. After three rinses in PBS, the sections were then incubated with fluorescent goat anti-chicken fluorescein isothiocyanate (FITC) and goat anti-rabbit tetramethylrhodamine isothiocyanate (TRITC) secondary antibodies (1:200 dilutions for both secondary antibodies; Jackson ImmunoResearch, PA, USA) in the dark at 37°C for 40 min. The prepared sections were washed three times in PBS before they were mounted in glycerol and cover slipped. After these steps, the sections were examined using fluorescence microscopy. Image-Pro Plus 6.0 image analysis software (Media Cybernetics Inc.) was used to quantify the co-localization of GFAP and $P2X_7$. To specify the immunoreactivity of GFAP and $P2X_7$, normal goat serum and PBS were used as negative controls in place of the primary antibodies.

HEK 293 cell culture and transfection

HEK 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were transiently transfected with the human pcDNA3.0-EGFP-$P2X_7$ plasmid using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The Genbank accession number is NM_019256.1. The hP2X7R plasmid was purchased from Shanghai Generay Biotech Co., Ltd. When HEK293 cells were 70%–80% confluent, cell culture media was replaced with OptiMEM 2 h before transfection. The transfection media were prepared as follows: (a) 4 μg DNA was diluted into a 250 μl final volume of OptiMEM, (b) 10 μl Lipofectimine2000 was diluted into a 250 μl final volume of OptiMEM, and (c) the Lipofectimine-containing solution was mixed with the plasmid-containing solutions, which was incubated at room temperature (RT) for 20 min. Subsequently, 500 μl of cDNA/lipofectamine solution was added to each well. Cells were incubated for 6 h at 37°C in 5% CO_2. After incubation, the cells were

washed in MEM containing 10% FBS and incubated for 24–48 h. The green fluorescent protein (GFP) fluorescence was assessed as a reporter for the efficiency of transfection. Whole-cell patch clamp recordings were performed one to two days after transfection.

Electrophysiological recordings

The electrophysiological recording was performed using a patch/whole cell clamp amplifier (Axopatch 200B).[28] The micropipette was filled with internal solution (in mM) containing KCl 140, $MgCl_2$ 2, HEPES 10, EGTA 11, and ATP 5. The osmolarity was adjusted to 340 mOsmol/kg with sucrose and pH was adjusted to 7.4 with KOH. The external solution (in mM) contained NaCl 150, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES 10, and D-glucose 10. Its osmolarity was adjusted to 340 mOsm with sucrose and pH was adjusted to 7.4 with NaOH. The resistance of recording electrodes was in the range of 1–4 MΩ; 3 MΩ was the best. A small patch of membrane underneath the pipette tip was aspirated to form a seal (1–10 GΩ). Then, a more negative pressure was applied to rupture it and establish a whole-cell mode. The holding potential was set at –60 mV. The drugs were dissolved in an external solution and delivered by gravity flow from an array of tubules (500 μm O.D. and 200 μm I.D.) connected to a series of independent reservoirs. The distance from the tubule mouth to the examined cell was approximately 100 μm. Rapid solution exchange was achieved by horizontally shifting the tubules with a micromanipulator.

Statistical analysis

The data were analyzed using SPSS 20 software. The numerical values were reported as the mean ± SEM. Statistical significance was determined by one-way analysis of variance followed by the Fisher's post hoc test for multiple comparisons. A p-value < 0.05 was considered statistically significant.

Results

Effects of RES on hyperalgesia in gp120-treated rats

Mechanical hyperalgesia was tested with a mechanical stimulator. There was no difference in the MWT between the gp120 and sham groups before the operation ($p > 0.05$). At 4 to 14 days after the operation, the MWT in the gp120 group was lower than the sham group ($p < 0.05$) and there was a significant difference from days 7 to 14 ($p < 0.01$). The MWT in the gp120 + RES group was higher than the gp120 group from days 4 to 14 ($p < 0.05$), and there was a significant difference from days 12 to 14 ($p < 0.01$) (Figure 1).

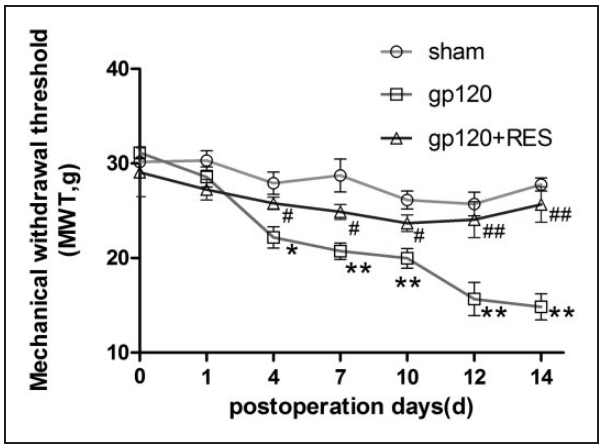

Figure 1. Effects of RES on the mechanical withdrawal threshold (MWT) in gp120-treated rats. Each group consisted of six rats ($n = 6$ per group). The data represent the mean ± SEM. The significant differences are noted as "*" for $p < 0.05$ and "**" for $p < 0.01$ compared to the sham group; significant differences are denoted as "#" for $p < 0.05$ and "##"for $p < 0.01$ compared to the gp120-treated group.

Effects of RES on the expression of the P2X$_7$ mRNA and protein in the DRG of the gp120-treated rats

The expression of the $P2X_7$ mRNA in the DRG was measured by RT-PCR. The relative levels of the $P2X_7$ mRNA in the gp120 group were significantly increased compared to the sham group ($p < 0.01$). The expression levels of the $P2X_7$ mRNA in the gp120 + RES group were significantly decreased compared to the gp120 group ($p < 0.01$) (Figure 2(a)).

The expression levels of the $P2X_7$ protein in the DRG were analyzed by Western blot analysis. Using image analysis, the $P2X_7$ protein expression (normalized to each β-actin in the internal control) in the gp120 group was significantly enhanced compared to the sham group ($p < 0.01$). The relative levels of the $P2X_7$ protein expression in the gp120 + RES group were lower than the gp120 group ($p < 0.01$) (Figure 2(b)).

Effects of RES on the co-localization of P2X$_7$ and GFAP by double immunofluorescence in the DRGs of the gp120-treated rats

The co-localization of the $P2X_7$ receptor and GFAP (a marker of SGCs) was measured by double immunofluorescence. The upregulated expression of GFAP was a typical characteristic of active SGCs. The immunofluorescence results showed that the $P2X_7$ receptor and GFAP were co-localized in the DRG SGCs. The co-localization of the $P2X_7$ receptor and GFAP in the gp120 group exhibited more intense staining than the sham group. The co-localization of the $P2X_7$ receptor

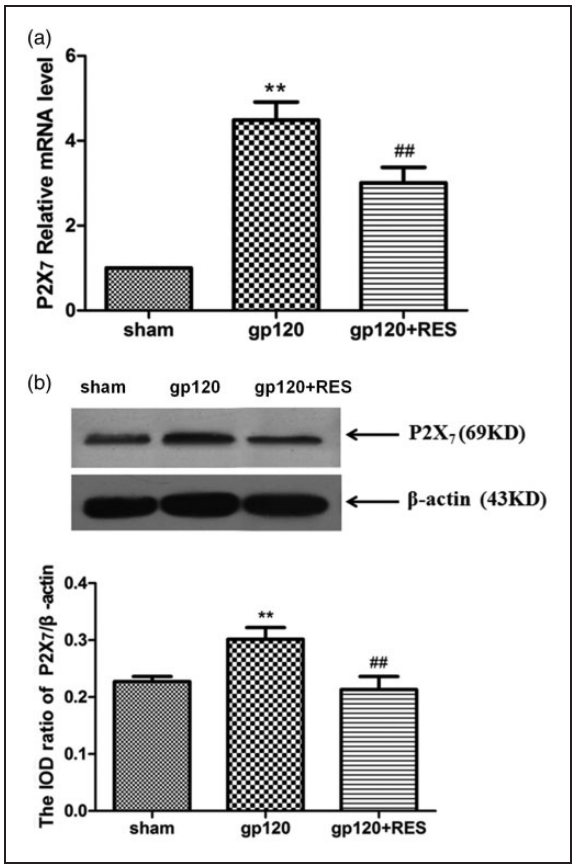

Figure 2. Effects of RES on the expression of $P2X_7$ mRNA and protein in L4–6 DRGs from each group. (a) The $P2X_7$ mRNA expression level in the DRG of the gp120 group was increased compared to the sham group ($p < 0.01$). The $P2X_7$ mRNA level in the DRG of the gp120 + RES group was decreased compared to the gp120 group ($p < 0.01$). The experiment was performed in triplicate and repeated three times. β-actin was an internal control to normalize the mRNA expression level of the three groups to different amounts of tissue. And the expression levels of each group were normalized to the sham group. The data represent the mean ± SEM. **$p < 0.01$, compared to the sham group and ##$p < 0.01$, compared to the gp120-treated group. (b) The expression of $P2X_7$ protein in the DRG of the gp120-treated group was increased compared to the sham group ($p < 0.01$). The $P2X_7$ protein expression in the DRG of the gp120 + RES group was decreased compared to the gp120 group ($p < 0.01$). The bar histograms show the ratio of the $P2X_7$ protein level to the β-actin level in each group (Note: The data are shown as the mean ± SEM. **$p < 0.01$, compared to the sham group and ##$p < 0.01$, compared to the gp120 group).

and GFAP in the gp120 + RES group was significantly decreased compared to the gp120 group (Figure 3).

Effects of RES on the expression of TNFα-R, IL-1β, and IL-10 proteins in the DRG of gp120-treated rats

The expression levels of TNFα-R, IL-1β, and IL-10 proteins in the DRG were analyzed by Western blot analysis. Using image analysis, the values for the TNFα-R and IL-1β protein expression levels (normalized to each β-actin internal control) in the gp120 group were significantly augmented compared to the sham group ($p < 0.01$). The relative values of the TNFα-R and IL-1β protein expression levels in the gp120 + RES group were lower than the gp120 group ($p < 0.01$) (Figure 4).

The IL-10 protein expression levels in the gp120 group were decreased compared to the sham group ($p < 0.01$). The IL-10 protein expression levels in the gp120 + RES group were increased compared to the gp120 group ($p < 0.01$) (Figure 4).

Effects of RES on the ERK1/2 and p-ERK1/2 expression levels in the DRG of gp120-treated rats

The phosphorylation and activation of ERK1/2 are involved in inflammatory pain. The ERK1/2 and p-ERK1/2 expression levels in the DRG were analyzed by Western blot analysis. The integrated optical density (IOD) ratio of p-ERK1/2 to ERK1/2 was higher in the gp120 group than in the sham group ($p < 0.01$, $n = 6$ for each group).The results indicated that the role of extracellular signal-regulated protein kinase (ERK) phosphorylation in the DRG is related to the $P2X_7$ receptor-mediated hyperalgesia in the gp120-treated rats.

In addition, we examined whether the administration of RES was able to affect the phosphorylation of ERK in the gp120 group DRG. The IOD ratio of p-ERK1/2 to ERK1/2 in the gp120 + RES group was significantly lower than the gp120 group ($p < 0.01$, $n = 6$ for each group) (Figure 5). These results suggest that the RES effects on the $P2X_7$ receptor-mediated hyperalgesia may help decrease the ERK1/2 phosphorylation and activation in the DRG of the gp120-treated rats.

Depressive effects of RES on ATP-induced current in HEK293 cells expressing the hP2X7 receptor

The ATP-activated currents in HEK293 cells transfected with pEGFP-h$P2X_7$ plasmid were recorded by whole cell patch clamping. The ATP-activated currents in HEK293 cells can be inhibited by RES (Figure 6(a)). The concentration dependence of ATP on the peak amplitude of current responses by the $P2X_7$ receptor in the absence (closed symbols) and presence of RES (100 μM) (open symbols) ($p < 0.05$, $n = 8$–10) was showed in Figure 6(b).

Discussion

Elucidation of how HIV-1 infection causes chronic pain is essential for developing effective therapy. HIV-1 gp120, as a potential pathogenically relevant factor, is involved in neuropathic pain.[1,5,8] Our data showed that the MWT in the peripheral gp120-treated rats was

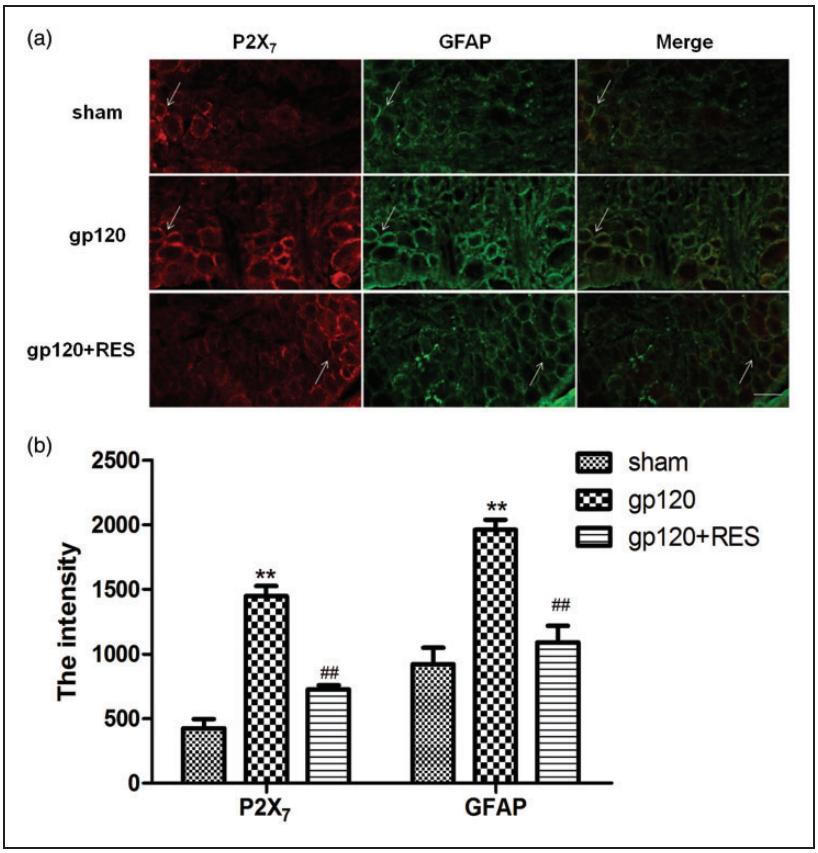

Figure 3. Double immunostaining for GFAP and the $P2X_7$ receptor in the DRG. (a) GFAP and $P2X_7$ immunoreactivity levels were analyzed in DRG sections. Double labeling for GFAP and $P2X_7$ in a single section of the DRG. The green signal represents GFAP staining with a FITC-conjugated secondary antibody, and the red signal indicates $P2X_7$ staining with a TRITC-conjugated secondary antibody. The scale bars denote 100 μm. (b) Quantification of $P2X_7$ (red) and GFAP (green). The GFAP and $P2X_7$ intensity of the immunoreactivity in the gp120 group were increased compared to the sham group ($p < 0.01$, $n = 6$). The GFAP and $P2X_7$ intensity in the gp120 + RES group were decreased compared to the gp120 group ($p < 0.01$, $n = 6$). Data presented as means ± SEM. **$p < 0.01$, compared to sham group, and ##$p < 0.01$, compared to gp120 group.

decreased compared to the sham rats, which was consistent with previous reports.[8,23,29] The DRG can transmit pain signals from the periphery to the central nervous system.[10,11] The $P2X_7$ receptor in the DRG is related to inflammatory and neuropathic pain.[17,19,20,30] Our study demonstrated that the $P2X_7$ mRNA and protein levels in the HIV-1 gp120-treated rats were significantly enhanced compared with those in the sham rats. The increased $P2X_7$ receptor in the DRG may be involved in HIV-associated neuropathic pain. After treatment with RES, the $P2X_7$ receptor mRNA and protein levels were decreased. Meanwhile, the MWT in the gp120 + RES group was higher than that in the gp120 group. Our results indicated that RES might decrease the upregulated expression of the $P2X_7$ receptor and inhibit the transmission of nociceptive signaling. This effect may eventually alleviate the HIV-associated pain behavior in the gp120 group rats.

The SGCs enwrap the neuronal soma in the DRG.[31] Double immunohistochemical staining showed that co-localization of the $P2X_7$ receptor and GFAP in the DRG in the gp120-treatment rats was increased. GFAP is a marker of SGCs.[32] Our data indicated that the expression of the $P2X_7$ receptor in the DRG SGCs was increased. The GFAP upregulation in the DRG SGCs of gp120 treatment rats indicates the activation of SGCs.[32,33] The activation of SGCs can release cytokines and thus augment neuronal excitation.[31,32] The results showed that the TNFα-R and IL-1β protein levels in the gp120 group were significantly increased compared to the sham group, and the IL-10 protein levels (anti-inflammatory factor) in the gp120 group were decreased compared to the sham group. RES inhibited the increased expression levels of $P2X_7$ and GFAP in the DRG SGCs. After the gp120 rats were treated with RES, the relative TNFα-R and IL-1β protein levels were lower than the gp120 rats and the IL-10 protein levels were increased compared to the gp120 rats. RES may inhibit the upregulated $P2X_7$ and GFAP levels in the DRG and decrease the activation of SGCs. RES may

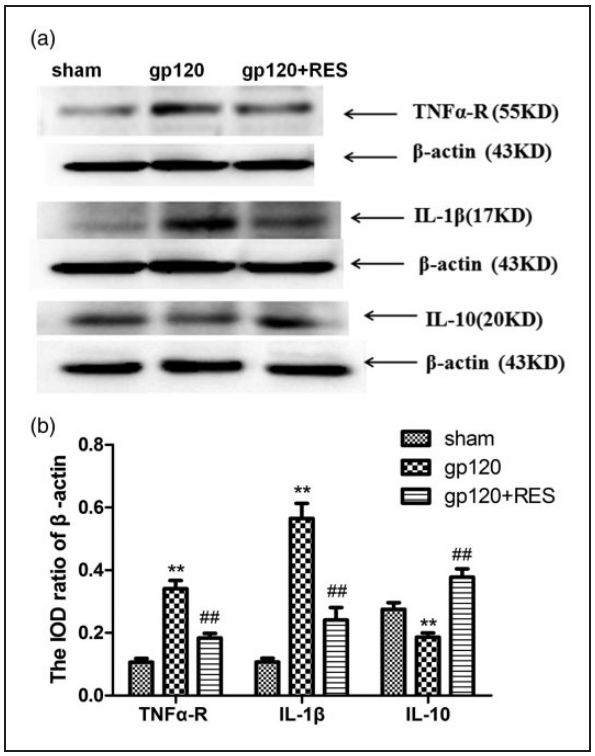

Figure 4. Effects of RES on the TNFα-R, IL-1β, and IL-10 protein expression levels in L4–6 DRGs. (a) The TNFα-R and IL-1β protein expression levels in the DRG of the gp120 group were increased compared to the sham group ($p < 0.01$). The TNFα-R and IL-1β protein expression levels in the gp120 + RES group were decreased compared to the gp120 group ($p < 0.01$). The expression of IL-10 protein in the DRG of the gp120 group was decreased compared to the sham group ($p < 0.01$). The IL-10 protein expression in the DRG of the gp120 + RES group was increased compared to the gp120 group ($p < 0.01$). (b) The bar histograms show the ratio of TNFα-R, IL-1β, and IL-10 protein levels to the β-actin level in each group. (Note: The data are shown as the mean ± SEM. **$p < 0.01$, compared to the sham group and ##$p < 0.01$, compared to the gp120 group.)

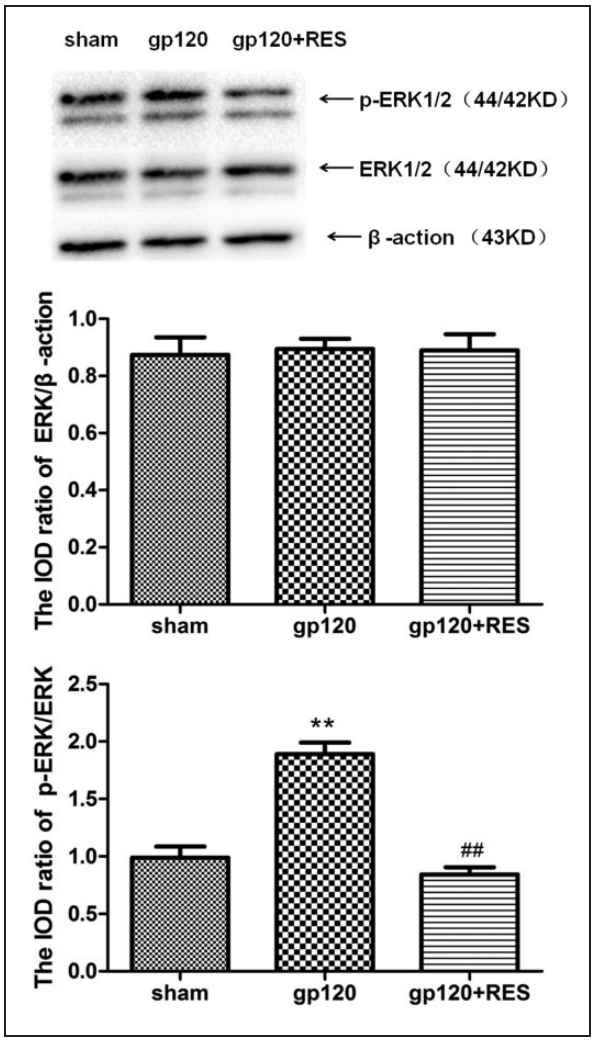

Figure 5. Effects of RES on the ERK1/2 and p-ERK1/2 expression levels in L4–6 DRGs. The ERK1/2 and p-ERK1/2 expression levels in the DRG were analyzed using Western blot analysis. The IOD ratio of ERK1/2 to β-actin was not significantly different between the two groups ($p > 0.05$). The IOD ratio of p-ERK1/2 to ERK1/2 in the gp120 group was higher than the sham group ($n = 6$). The IOD ratio of p-ERK1/2 to ERK1/2 in the gp120 + RES-treated rats was significantly lower than that of the gp120 group ($p < 0.01$, $n = 6$ for each group). The data represent the mean ± SEM, $n = 6$. **$p < 0.01$ compared to the sham group and ##$p < 0.01$ compared to the gp120 group.

then reduce the release of cytokines. Inflammatory factors can increase the activation of the $P2X_7$ receptor, aggravating the neuropathic damage.[34,35] Anti-inflammatory effects could decrease the nociceptive signal of the aggravated DRG neuronal excitation. RES has anti-inflammatory effects.[13–15] Our results suggested that RES relieved the HIV-associated pain behavior in the gp120 rats by influencing the $P2X_7$ receptor in the DRG SGCs.

P2X receptor-mediated pain transmission is related to ERK signaling.[36] ERK pathway activation participates in the sensitized primary afferents in pain transmission.[37,38] ERK1/2 phosphorylation generates the activation form of ERK1/2. Our data revealed that the IOD ratio of p-ERK1/2 to ERK1/2 was higher in the gp120 group than the sham group. The role of ERK phosphorylation in the DRG may be involved in the $P2X_7$ receptor-mediated hyperalgesia in the gp120-treated rats. After the administration of RES, the IOD ratio of p-ERK1/2 to ERK1/2 in the gp120 + RES group was significantly decreased compared with that in the gp120 group. RES may decrease the phosphorylation of ERK1/2 in the DRG of the gp120-treated rats, relieving the $P2X_7$ receptor-mediated hyperalgesia. To identify whether RES can specially act on the $P2X_7$ receptor, HEK293 cells that were transfected with the $P2X_7$ plasmid were evaluated. RES significantly inhibited the

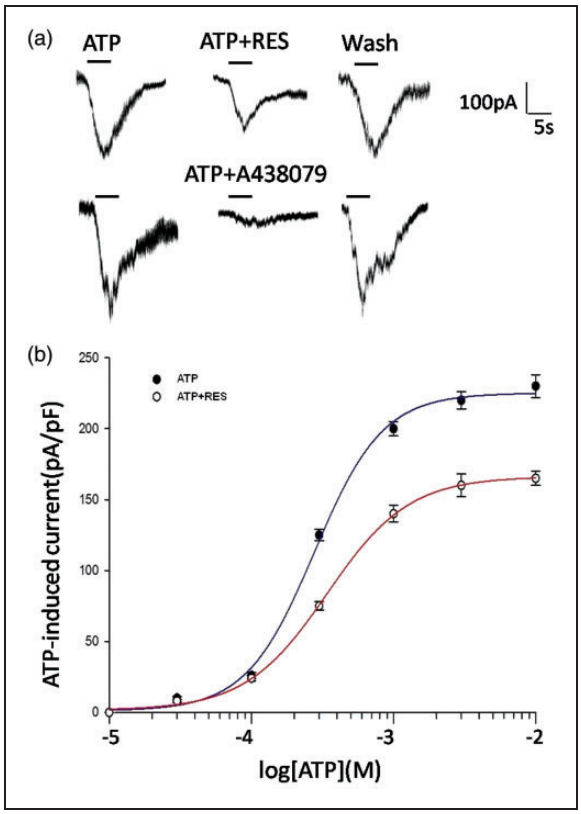

Figure 6. Depressive effects of the RES on the ATP-activated current in HEK293 cells transfected with pEGFP-hP2X$_7$ plasmid. (a) Current traces showed that RES inhibited ATP-activated currents in HEK293 cells expressing the hP2X$_7$ receptor and the current can be inhibited by specific P2X$_7$ receptor antagonist A438079. All ATP-activated currents were obtained by application of 10 mM ATP. (b) Concentration dependence of ATP on the peak amplitude of current responses by the P2X$_7$ receptor in the absence (closed symbols) and presence of RES (100 μM) (open symbols). Data points are the mean ± SEM values from 8 to 10 cells per dose. The EC_{50} values in the absence and presence of RES were approximately 270 ± 20 μM and 340 ± 10 μM, respectively.

ATP-activated currents in the HEK293 cells that were transfected with P2X$_7$ plasmid. These data confirmed that RES relieved the HIV-associated pain behavior in the gp120 rats by acting on the P2X$_7$ receptor. The electrophysiological data supported the phenomenon, RES treatment relieved the HIV-associated pain behavior relating to downregulation of the P2X$_7$ receptor expression. In conclusion, the gp120 protein treatment enhanced the expression the P2X$_7$ receptor in DRG SGCs. The upregulated P2X$_7$ and GFAP levels in the DRG indicated the activation of SGCs. The activation of SGCs increased the release of inflammatory cytokines (IL-1β and TNF-α) and decreased the release of anti-inflammatory cytokine (IL-10). IL-1β and TNF-α could increase the sensitization of neurons in the DRG, resulting in gp120-induced neuropathic pain behavior. RES dampened the release of inflammatory cytokines, enhanced the release of an anti-inflammatory cytokine, and reduced the upregulation of the P2X$_7$ receptor. Inhibition of the P2X$_7$ receptor in DRG SGCs may decrease the sensitization of neurons in the DRG of the gp120-treated rats. Therefore, RES relieved the mechanical hyperalgesia in the gp120-treated rats relating to inhibition of the P2X$_7$ receptor in DRG SGCs.

Author contributions

Bing Wu performed experiments and wrote the manuscript. Bing Wu, Yucheng Ma, Zhihua Yi, Shuangmei Liu, Shenqiang Rao, Lifang Zou, Shouyu Wang, Yun Xue, Tianyu Jia, Shanhong Zhao, Lin Li, Huilong Yuan, and Liran Shi performed the experiments. Bing Wu and Shuangmei Liu performed the electrophysiological experiments. Shandong Liang designed the study, supervised the work, wrote, and revised the manuscript. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declare that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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