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Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone attenuates postincisional pain by regulating macrophage polarization

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ABSTRACT

Acute inflammation triggered by macrophage infiltration to injured tissue promotes wound repair and may induce pain hypersensitivity. Peroxisome proliferator-activated receptor γ (PPAR)γ signaling is known to regulate heterogeneity of macrophages, which are often referred to as classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages have considerable antimicrobial activity and produce a wide variety of proinflammatory cytokines. In contrast, M2 macrophages are involved in anti-inflammatory and homeostatic functions linked to wound healing and tissue repair. Although it has been suggested that PPARγ agonists attenuate pain hypersensitivity, the molecular mechanism of macrophage-mediated effects of PPARγ signaling on pain development has not been explored. In this study, we investigated the link between the phenotype switching of macrophage polarization induced by PPARγ signaling and the development of acute pain hypersensitivity. Local administration of rosiglitazone significantly ameliorated hypersensitivity to heat and mechanical stimuli, and paw swelling. Consistent with the down-regulation of nuclear factor κB (NFκB) phosphorylation by rosiglitazone at the incisional sites, the number of F4/80$^+$iNOS$^+$ M1 macrophages was decreased whereas numbers of F4/80$^+$CD206$^+$ M2 macrophages were increased in rosiglitazone-treated incisional sites 24 h after the procedure. In addition, gene induction of anti-inflammatory M2-macrophage-associated markers such as arginase1, FIZZ1 and interleukin (IL)-10 were significantly increased, whereas M1-macrophage-related molecules such as integrin αX, IL-1β, MIP2α and leptin were decreased at rosiglitazone-treated incisional sites. Moreover, transplantation of rosiglitazone-treated peritoneal macrophages into the incisional sites significantly attenuated hyperalgesia. We speculate that local administration of rosiglitazone significantly alleviated the development of postincisional pain, possibly through regulating macrophage polarity at the inflamed site. PPARγ signaling in macrophages may be a potential therapeutic target for the treatment of acute pain development.

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1. Introduction

Inflammation and nociceptive sensitization are the hallmarks of tissue surrounding surgical incisions. It has become clear that neuroimmune communication in the peripheral nervous system plays an important role in the development of pain hypersensitivity [1]. Notably, macrophages derived from circulating monocytes are predominately activated in the early phase of acute inflammation [2]. Macrophages can acquire distinct functional phenotypes depending on the microenvironment of inflamed sites [3]. Two well-established polarized phenotypes are referred to as proinflammatory (M1) macrophages and anti-inflammatory (M2) macrophages. M1 macrophages produce high levels of toxic intermediates associated with increased microbicidal activity and pronociceptive mediators inducing hyperalgesia such as iNOS and IL-1β, whereas M2 macrophages have homeostatic functions linked to wound healing and tissue remodeling. Balance between these two subsets has a crucial role in regulating inflammation in the peripheral tissues [4].

Peroxisome proliferator-activated receptor (PPAR)γ is a member of the nuclear hormone receptor family and has been implicated in mediating many metabolic, endocrine and cardiovascular disorders [5]. In the absence of PPARγ signaling, macrophages neither appropriately suppress inflammatory cytokine production nor acquire an oxidative metabolic program that is associated with the M2 phenotype. The importance of PPARγ in regulating the M1/M2 phenotypic

Abbreviations: Arg1, arginase-1; HO-1, heme oxygenase-1; IL, interleukin; ItgaX, integrin αX; MIP2α, macrophage inflammatory protein 2α; NF-κB, nuclear factor κB; PPARγ, peroxisome proliferators-activated receptor γ.

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switch has been confirmed by Amine Bouhlel et al. who demonstrated that activation of PPARγ potentiates polarization of circulating monocytes to macrophages of the M2 phenotype [6]. Beyond its peripheral actions, recent studies have indicated that PPARγ agonists regulate inflammation in the central nervous system. PPARγ signaling promotes neuroprotection and neurological improvement following cerebral ischemia and spinal cord injury by altering inflammatory gene induction in macrophages/microglia [7,8]. The link between PPARγ and pain has also been indicated. Intrathecal injection of a PPARγ agonist, rosiglitazone, has a rapid anti-allodynic effect through spinal PPARγ activation [9]. Another PPARγ agonist, pioglitazone, attenuates tactile allodynia when oral administration is started immediately after partial sciatic nerve ligation (PSNL) [10]. We recently have reported that rosiglitazone exerts analgesic effects by regulating macrophage activation in a mouse PSNL model [11]. The previous study provided novel ideas of how neuroinflammatory responses can be controlled by the activation of PPARγ signaling in macrophages during the development of neuropathic pain. However, the effects of PPARγ signaling on the alterations of macrophage polarization during pain development have yet to be elucidated.

In this study, we investigated the effects of PPARγ signaling on macrophage polarization in acute pain development and explored the mechanisms involved in mediating analgesic effects.

2. Materials and methods

2.1. Animals

Male C57BL6 mice aged 8–10 weeks were obtained from CLEA Japan (Tokyo, Japan). The Animal Research Committee of Kagoshima University approved all experimental procedures, which were implemented according to the guidelines of the National Institutes of Health and the International Association for the Study of Pain [12]. Mice were housed in groups of four or five per cage with a 12–h light–dark cycle.

2.2. Paw incision model

The mouse hindpaw plantar incision model was performed as described previously [13]. Mice were deeply anesthetized by inhalation of 1.5–2.0% isoflurane (Abbott, Tokyo, Japan) via a nose cone. A 5-mm longitudinal incision was made with a No. 11 blade through the skin and fascia of the plantar foot. The incision was started 2 mm from the proximal edge of the heel and extended toward the toes. The underlying muscle was elevated with a curved forceps leaving the muscle origin and insertion intact. The skin was apposed with a single mattress suture of 8-0 nylon. Rosiglitazone purchased from Cayman Chemical (Ann Arbor, MI), was dissolved in 1:3 solution of DMSO:PBS (pH 7.2) (0.5 mg/ml). Rosiglitazone was injected locally to the incisional sites immediately after the skin was sutured. The suture was removed at the end of postoperative day 2.

2.3. Pain behavior

All behavioral experiments were performed by the same tester in a blinded manner. Withdrawal latencies to heat stimuli were assessed by applying a focused radiant heat source to the unrestrained mouse placed on a heat-tempered glass floor using the Paw Thermal Stimulator (UCSD, San Diego, CA). A thermal stimulus was then applied to the plantar surface of each hind paw. Each mouse was tested at an interval of 2–3 min. The latencies to thermal stimuli were calculated as the mean of three trials. A cut-off time was set at 20.5 s to avoid tissue damage. To evaluate tactile allodynia, calibrated von Frey filaments (0.08–2.0 g) were applied to the plantar surface of the hindpaw from underneath the mesh floor. The 50% paw withdrawal threshold was determined using the updown method [14].

2.4. Measurement of paw edema

Post-incisional edema, reflected by an increase in dorsal-to-ventral paw thickness, was measured by a micro-caliper (Shinwa Measuring Tools; Niigata, Japan). The mean of three measurements at each time-point was calculated.

2.5. Immunohistochemistry

Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused transcardially with saline. Tissue was fixed in 4% paraformaldehyde overnight at 4 °C, and placed in 30% sucrose solution for 24 h at 4 °C. Sections (30 μm thick) were incubated overnight with primary antibody to F4/80 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and inducible NO synthase (iNOS, 1:200; Abcam, Cambridge, UK), or CD206 (1:500; Santa Cruz Biotechnology) at 4 °C overnight and then incubated for 1 h at room temperature with secondary antibody labeled with Alexa Fluor 488 and Alexa Fluor 546 (1:500; Invitrogen, Carlsbad, CA) followed by nuclear staining with DAPI (Vector Laboratories, Burlingame, CA). Fluorescent images were obtained using LSM700 imaging systems (Carl Zeiss, Aalen, Germany). The number of total $F4/80^+$, $F4/80^+iNOS^+$ or $F4/80^+CD206^+$ cells with clearly visible cell bodies in the skin was manually counted using Image J 1.43u 2010 software (National Institutes of Health, Bethesda, MD).

Some sections stained with hematoxylin and eosin were viewed and imaged using an Eclipse TS100 microscope and DS-L2 imaging software (Nikon, Japan).

2.6. Quantitative PCR

Total RNA of hindpaw within 2 mm of the incision site was extracted using Sepazol reagent (Nacalai Tesque, Kyoto, Japan). The synthesis of first-strand cDNA was performed using High Capacity RNA-to-cDNA (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR analysis was performed using SYBR Green normalized to G3PDH according to the manufacturer's instructions (Thermal Cycler Dice TP800; Takara, Japan). The sequences of primers used for quantitative PCR were as follows; macrophage inflammatory protein (MIP)2α: forward: 5′-TCCAGAGCTTGAGTGTGACG-3′, reverse: 5′-CAGTTAGCCTTGCCTTTGTTCAG-3′; leptin: forward: 5′-GATCCCACGTGCCACAGTCT-3′, reverse: 5′-AGCCATAGTGCAAGGTTCTCTGA-3′ arginase (Arg)1: forward: 5′-CTCCAAGCCAAAGTCCTTAGAG-3′, reverse: 5′-AGGAGCTGTCATTAGGGACATC-3′ interleukin (IL)-1β: forward: 5′-GCTTCAGGCAGGCAGTATC-3′, reverse: 5′-AGGATGGGCTCTTCTTCAAAG-3′ integrin αX(ItgaX): forward: 5′-CTGGATAGCCTTTCTTCTGCTG-3′, reverse: 5′-GCACACTGTGTCCGAACTC-3′, IL-10: forward: 5′-GCTCTTACTGACTGGCATGAG-3′, reverse: 5′-CGCAGCTCTAGGAGCATGTG-3′ HO-1: forward: 5′-TGGTGCAAGATACTGCCCCTGC-3′, reverse: 5′-TGGGGGACAGCAGTCGTGGT-3′; FIZZ1: forward: 5′-TCCCAGTGAATACTGATGAGA-3′, reverse: 5′-CCACTCTGGATCTCCCAAGA-3′ G3PDH: forward: 5′-TGAAGCAGGCATCTGAGGG-3′, reverse: 5′-CGAAGGTGGAAGAGTGGGAG-3′.

2.7. Western blotting

Samples taken from the hindpaws of mice were homogenized in RIPA lysis buffer containing phosphatase inhibitor (Nacalai Tesque). Protein samples (20 μg) were separated on sodium dodecyl

sulfate polyacrylamide gel and transferred to PVDF membrane. The blots were blocked with 2% milk and incubated overnight at 4 °C with antibody to phospho-nuclear factor (NF)-κB or NF-κB (1:1000; Cell Signaling Technology, Denvers, MA). The blots were further incubated with horseradish peroxidase-conjugated secondary antibody (1:2000, Cell Signaling Technology), developed with the Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL), and exposed to Hyperfilm (Amersham Bioscience, Arlington Heights, IL). Specific bands were evaluated by the apparent molecular size. The intensity of the selected bands was captured and analyzed by Image J 1.43u 2010 software (National Institutes of Health). The optical density (OD) of phospho-NF-κB for each band was normalized to that of NF-κB.

2.8. Transplantation of peritoneal macrophages into the incisional sites

Mice were intraperitoneally injected with 3 ml of 4% thioglycollate (Sigma, St Louis, MO). After 3 days, peritoneal macrophages were collected by peritoneal lavage with 8 mL of cold PBS. Cells were incubated overnight in 24-well tissue culture plates. Nonadherent cells were removed with PBS by repeated washing. Then, cells were incubated with 50 μM rosiglitazone for 72 h and fluorescently labeled with PKH26 (Sigma) according to manufacturer's instructions. Peritoneal macrophages (1×10^5 cells in 10 μm PBS) were locally injected to the incisional sites immediately after the procedure.

2.9. Statistical analysis

Values are presented as mean ± SEM. Differences among groups were analyzed using one-way or two-way ANOVA with Bonferroni test, or Mann–Whitney *U* test (Graphpad Prism 5.0, La Jolla, CA). $P < 0.05$ was considered to be significant.

3. Results

Local administration of rosiglitazone immediately after the procedure ameliorates hyperalgesia.

To evaluate the effects of rosiglitazone on the development of acute hyperalgesia, rosiglitazone was injected locally into the incisional sites immediately after the procedure. Hyperalgesia to thermal stimuli was significantly attenuated in mice that received 10 μg rosiglitazone at 48 h after the procedure (vehicle, 4.52 ± 0.56; 3 μg rosiglitazone, 7.84 ± 1.46; 10 μg rosiglitazone, 11.29 ± 2.26 s), although withdrawal latency declined to the vehicle level at 72 h (Fig. 1A). Contrary to transient effects and late onset of thermal stimuli, rosiglitazone significantly inhibited the decrease in withdrawal threshold to mechanical stimuli from 3 to 72 h with a peak at 48 h after the injection, consistent with the time point of maximum effect on thermal stimuli (vehicle, 0.18 ± 0.14; 3 μg rosiglitazone, 0.30 ± 0.15; 10 μg rosiglitazone, 1.33 ± 0.23 g at 48 h) (Fig. 1B).

Plantar incision rapidly induces paw swelling and infiltration of immune cells such as macrophages and neutrophils in the acute phase of the inflammatory process [15]. Consistent with a previous report that rosiglitazone significantly reduced paw edema induced by carrageenan [16], intraplantar injection of 10 μg rosiglitazone significantly reduced paw swelling compared with vehicle injection at 6 h after the procedure (vehicle, 3.47 ± 0.24; 3 μg rosiglitazone, 3.28 ± 0.17; 10 μg rosiglitazone, 2.74 ± 0.10 mm) (Fig. 1C).

Consistent with decreased paw swelling in rosiglitazone-treated incisional sites, infiltration of acute phase immune cells at 24 h after incision was markedly attenuated (SFig. 1A). Monocytes are immune cells that are commonly recruited to the sites of injury and mature within hours to increase the proportion of macrophages in the inflamed area [17]. It has been shown previously that

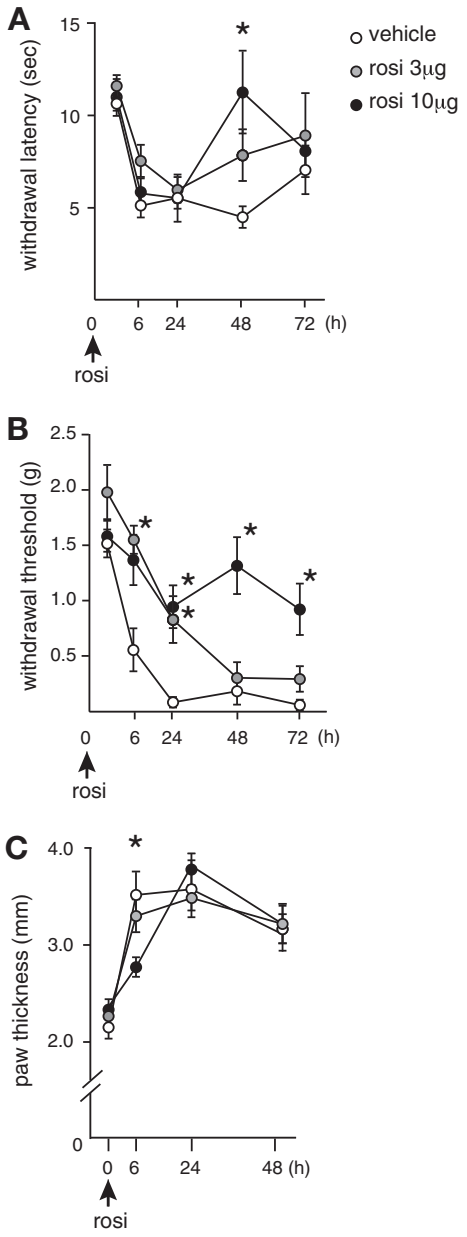

Fig. 1. Local administration of rosiglitazone alleviated pain hypersensitivity and inflammation. Rosiglitazone was injected immediately after the surgical procedure. Withdrawal latency to thermal stimuli (A) and withdrawal threshold to mechanical stimuli (B) were examined. (C) Paw swelling was suppressed by local administration of 10 μg rosiglitazone at 6 h after the procedure. $^*P < 0.05$. Each bar represents the mean ± SEM ($n = 8$ for each).

rosiglitazone regulates macrophage properties through NF-κB activation at the inflamed sites [18]. Indeed, phosphorylation of NF-κB was decreased at 6 h after the procedure in mice with local injection of rosiglitazone (vehicle, 0.88 ± 0.30; 10 μg rosiglitazone, 0.31 ± 0.26 OD at 6 h) (SFig. 1B). In addition, basal NF-κB phosphorylation at 6 h was higher than that at 24 h at vehicle-injected incisional sites, suggesting that the activation of NF-κB in macrophages at the initial phase might have an essential role in regulating macrophage polarity during the inflammatory process and acute pain development.

4. Rosiglitazone alters macrophage polarity at the inflamed site

iNOS, an M1 marker, is a down-stream molecule of NF-κB signaling and its induction is negatively regulated by PPARγ agonists

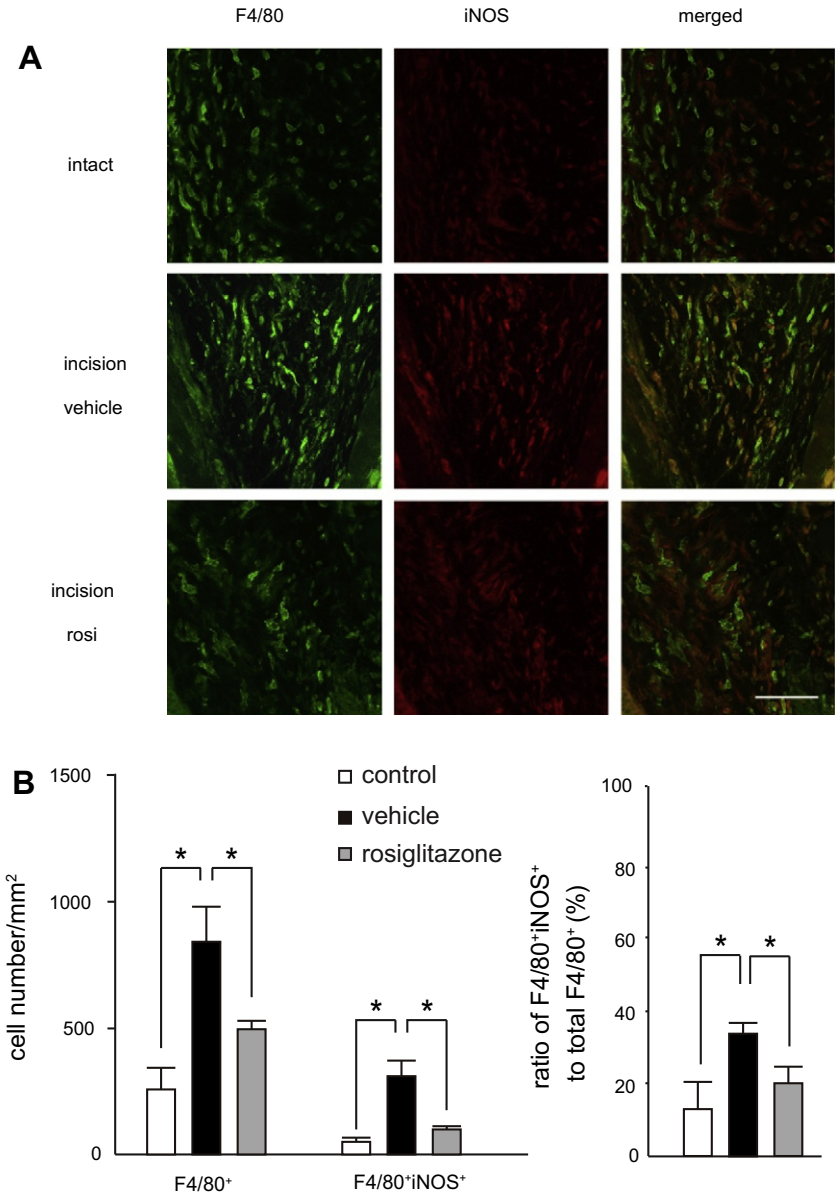

Fig. 2. F4/80$^+$iNOS$^+$ M1 macrophages were decreased in rosiglitazone-treated incisional sites. (A) Macrophages at the incisional sites were immunostained 24 h after the procedure with antibody to F4/80 and iNOS. Green, F4/80; red, iNOS. Scale bar, 50 μm. (B) The numbers of F4/80$^+$ total macrophages and F4/80$^+$iNOS$^+$ M1 macrophages were counted at intact, vehicle- and rosiglitazone-treated incisional sites. $^*P < 0.05$. Each column represents the mean ± SEM ($n = 5$ for each). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[19]. To evaluate the macrophage polarity at the incisional sites, local macrophages were immuno-stained with iNOS and pan-macrophage marker, F4/80 (Fig. 2A). The total number of F4/80$^+$ macrophages was significantly reduced at rosiglitazone-treated sites 24 h after the procedure (intact, 253 ± 88; vehicle, 852 ± 134; 10 μg rosiglitazone, 488 ± 37 cells/mm^2) (Fig. 3B). In addition, F4/80$^+$iNOS$^+$ M1 macrophages were also decreased by rosiglitazone treatment (intact, 44 ± 24; vehicle, 294 ± 69; 10 μg rosiglitazone, 94 ± 21 cells/mm^2). The ratio of F4/80$^+$iNOS$^+$ M1 to total F4/80$^+$ macrophages was 33.3 ± 3.7% and 12.7 ± 1.9% in vehicle- and rosiglitazone-treated sites, respectively, suggesting that rosiglitazone decreased the ratio of M1 macrophages to intact levels (13.1 ± 7.2%). In contrast, F4/80$^+$CD206$^+$ M2 macrophages were significantly increased by rosiglitazone (intact, 137 ± 29; vehicle, 127 ± 8; 10 μg rosiglitazone, 292 ± 40 cells/mm^2) (SFig. 2A and B). The ratio of M2 macrophages was markedly increased in rosiglitazone-treated sites (intact, 27.8 ± 6.0%; vehicle, 12.7 ± 1.9%; rosiglitazone, 41.0 ± 4.6%)

Next, we investigated the macrophage polarization by quantifying the expression of inflammatory-related mediators and phenotype-specific markers of macrophages by real-time PCR analysis (Fig. 3). Consistent with the data for immunohistochemical analysis, local administration of 10 μg rosiglitazone significantly reduced neutrophil chemoattractant MIP2α and proinflammatory mediators such as IL-1β, which directly contribute to inflammatory pain hypersensitivity, and are secreted mainly by neutrophils immediately after tissue incision [20]. In addition, the gene induction of M1 marker ItgaX and leptin, which promotes the differentiation of monocytes to M1 macrophages, was significantly decreased by rosiglitazone treatment. In contrast, the expression of M2 cell marker Arg1 and Fizz1, and anti-inflammatory cytokine IL-10 were significantly increased at 24 h after the procedure at the rosiglitazone-treated sites. HO-1, involved in macrophage polarization to an M2 phenotype [21], was markedly increased in macrophages at rosiglitazone-treated sites 24 h after the incision.

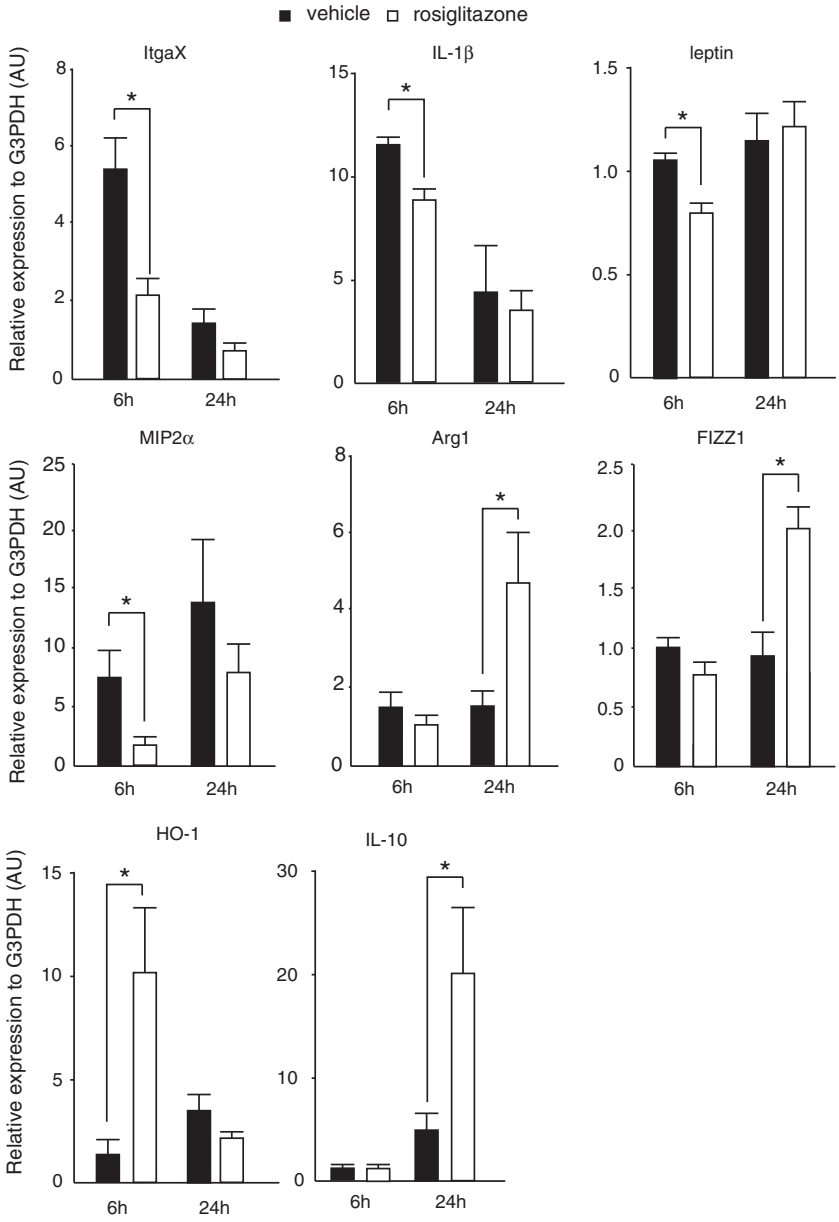

Fig. 3. Rosiglitazone altered the gene induction of inflammation-related molecules underlying macrophage polarity and pain development. The expression changes of inflammatory mediators and phenotype-specific markers for M1/M2 macrophages were evaluated by quantitative PCR. Each experiment was performed in triplicate. $^{*}P < 0.05$. Each column represents the mean ± SEM ($n = 5$ for each).

5. Rosiglitazone ameliorates hyperalgesia by a macrophage-mediated mechanism

To determine whether the effects of rosiglitazone on macrophage polarity are sufficient to ameliorate incisional pain, rosiglitazone-treated peritoneal macrophages prelabeled with PKH26 were transplanted to the incisional sites. Transplanted peritoneal macrophages remained localized at the incisional sites 72 h after the procedure (Fig. 4A). Transplantation of rosiglitazone-treated peritoneal macrophages significantly reduced hyperalgesia to mechanical and thermal stimuli 24 h after the procedure, whereas vehicle treatment resulted in the progression of hyperalgesia (Fig. 4B), indicating that the effects of rosiglitazone on local macrophages might partially ameliorate pain hypersensitivity after the incision.

6. Discussion

We demonstrated that local administration of PPARγ agonist rosiglitazone attenuated postincisional pain and altered polarization of infiltrating macrophages at the incisional sites. In addition, transplantation of macrophages pretreated with rosiglitazone significantly reduced incision-induced hyperalgesia. These data indicate that rosiglitazone might exert its analgesia effects by regulationg macrophage polarity at the incisional sites.

We previously demonstrated that early treatment with rosiglitazone attenuated tactile allodynia in mice with PSNL and reduced gene induction of proinflammatory mediators such as iNOS around the PSNL sites [11]. Although the previous data suggested that rosiglitazone ameliorated pain hypersensitivity by regulating macrophage activation, the molecular mechanism of how rosiglitazone,

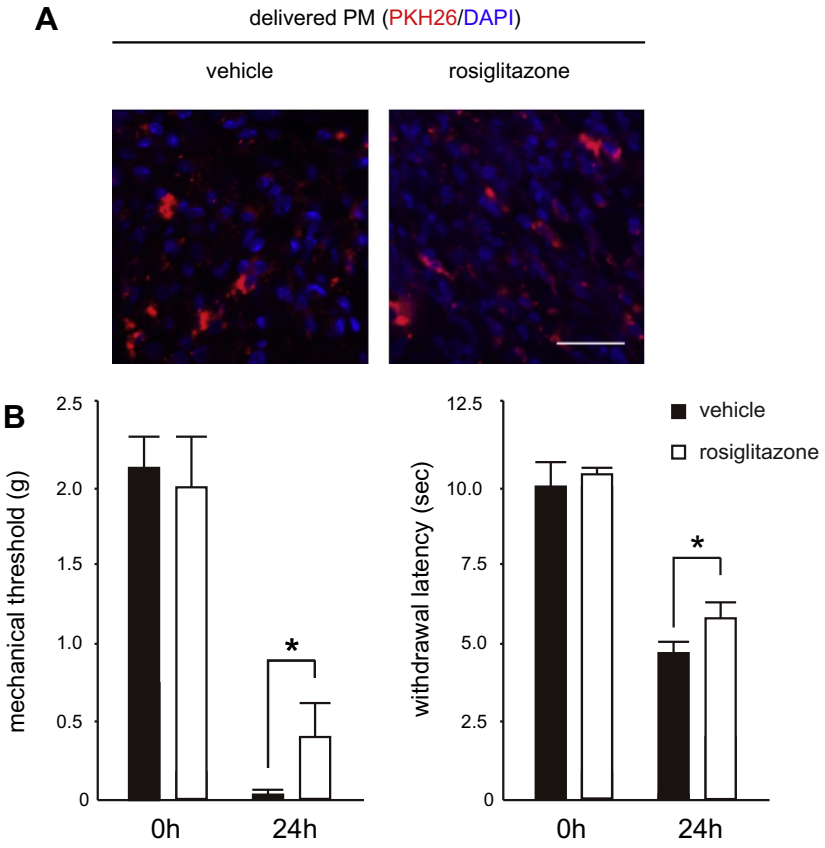

Fig. 4. Local transplantation of rosiglitazone-treated peritoneal macrophages to the incisional sites produced analgesic effects. (A) Peritoneal macrophages pretreated with rosiglitazone *ex vivo* were transplanted. Engraftment of peritoneal macrophages prelabeled with PKH26 was assessed 72 h after the procedure. Red, PKH26; blue, DAPI. Scale bar, 50 μm. (B) Hyperalgesia to both thermal and mechanical stimuli was attenuated by transplantation of rosiglitazone-treated peritoneal macrophages. $^{*}P < 0.05$. Each column represents the mean ± SEM (n = 6–7 for each).

during the early phase, maintained long-lasting analgesic effects have not been clarified.

Although the role of macrophage polarization in pain development is not clear, however, Komori et al. recently reported that M1 macrophages and neutrophils predominantly infiltrate to the PSNL site [22]. It has been established that PPARγ regulates monocyte phenotypic differentiation to M1 by repressing target genes of the transcription factor, NF-κB [4]. In the present study, we found that NF-κB activation was decreased 6 h after the procedure at the rosiglitazone-treated incisional sites (SFig. 1B), followed by changes in polarity of localized macrophages (Fig. 2 and SFig. 2), and the expression of M1/M2 macrophage-specific markers and inflammation-related molecules (Fig. 3). The ratio of M1 ($F4/80^{+}$ $iNOS^{+}$) to total macrophages at rosiglitazone-treated sites was decreased comparable to intact level (Fig. 2B) whereas M2 ($F4/80^{+}CD206^{+}$) to total macrophages was increased 2.3-fold by rosiglitazone treatment compared with vehicle injection (SFig. 2B). These data suggest that rosiglitazone not only decreases total macrophages at the incisional sites, but also regulates the polarity of local macrophages. Indeed, mice lacking chemotactic cytokine receptor 2, a marker for M1 macrophages, exhibited impaired inflammatory pain development and decreased macrophage infiltration to inflamed sites [2], suggesting that increased infiltration of M1 macrophages might exacerbate local inflammation and the development of hyperalgesia. It has been reported that administration of leptin in the early but not late phase of neuropathic pain development significantly reverses the failure of tactile allodynia in leptin-deficient *ob/ob* mice, through leptin receptor signaling in macrophages [23]. In our study, rosiglitazone significantly reduced gene induction of leptin at 6 h (Fig. 3). Since leptin deficiency is associated with the accumulation of proinflammatory M1 monocyte/macrophage lineage [24], the previous study supports the idea that macrophage polarity may be important for the development of tactile allodynia.

On the other hand, recent studies have demonstrated that activation of PPARγ signaling has a protective role for tissue remodeling and wound repair by reducing oxidative stress and inflammation [25]. Indeed, local administration of rosiglitazone increased the expression of M2-specific markers such as Arg1, FIZZ1, and anti-inflammatory cytokine IL-10 (Fig. 3). It has been reported that $CD206^{+}$ M2 macrophages that express HO-1 have antioxidant and antiinflammatory properties [26,27]. In addition, HO-1 itself promotes macrophage polarization toward an M2 phenotype [21] and has antinociceptive effects against inflammatory pain induced by formalin injection in mouse hindpaws [28,29]. In our study, HO-1 was markedly increased at 6 h after the procedure (Fig. 3), suggesting that rosiglitazone might regulate the macrophage phenotype towards M2 and reduced hyperalgesia through antinociceptive molecules such as HO-1.

To identify the target cells through which rosiglitazone exerts its analgesic effects, we transplanted peritoneal macrophages pretreated with rosiglitazone into the incisional sites. Transplantation of rosiglitazone-treated peritoneal macrophages was sufficient to ameliorates pain hypersensitivity (Fig. 4). We previously demonstrated that the expression of M1 markers such as iNOS and CCR2 was significantly suppressed in rosiglitazone-treated peritoneal macrophages [11], suggesting that rosiglitazone reduced hyperalgesia by suppressing polarization of local macrophages

towards an M1 phenotype, and probably promoting differentiation to M2.

In summary, we demonstrated that administration of rosiglitazone markedly alleviated pain hypersensitivity by regulating macrophage polarity at incisional sites. PPARγ signaling can ameliorate inflammatory responses by switching macrophage polarity to be tissue protective, which may result in reduced pain hypersensitivity. We propose that PPARγ signaling in macrophages might be a potential therapeutic target for the treatment of acute pain development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.039.

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