Molecular Neurobiology (2019) 56:7408–7419
https://doi.org/10.1007/s12035-019-1616-0

Losartan, an Angiotensin II Type 1 Receptor Antagonist, Alleviates Mechanical Hyperalgesia in a Rat Model of Chemotherapy-Induced Neuropathic Pain by Inhibiting Inflammatory Cytokines in the Dorsal Root Ganglia

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Received: 28 January 2019 / Accepted: 16 April 2019 / Published online: 29 April 2019
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Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) adversely impacts quality of life and a challenge to treat with existing drugs used for neuropathic pain. Losartan, an angiotensin II type 1 receptor (AT1R) antagonist widely used to treat hypertension, has been reported to have analgesic effects in several pain models. In this study, we assessed losartan's analgesic effect on paclitaxel-induced neuropathic pain (PINP) in rats and its mechanism of action in dorsal root ganglion (DRG). Rats received intraperitoneal injections of 2 mg/kg paclitaxel on days 0, 2, 4, and 6 and received single or multiple intraperitoneal injections of losartan potassium dissolved in phosphate-buffered saline at various times. The mechanical thresholds, protein levels of inflammatory cytokines, and cellular location of AT1R and interleukin 1β (IL-1β) in the DRG were assessed with behavioral testing, Western blotting, and immunohistochemistry, respectively. Data were analyzed by two-way repeated-measures analysis of variance for the behavioral test or the Mann-Whitney *U* test for the Western blot analysis and immunohistochemistry. Single and multiple injections of losartan ameliorated PINP, and losartan delayed the development of PINP. Paclitaxel significantly increased, and losartan subsequently decreased, the expression levels of inflammatory cytokines, including IL-1β and tumor necrosis factor α (TNF-α), in the lumbar DRG. AT1R and IL-1β were expressed in both neurons and satellite cells and losartan decreased the intensity of IL-1β in the DRG. Losartan ameliorates PINP by decreasing inflammatory cytokines including IL-1β and TNF-α in the DRG. Our findings provide a new or add-on therapy for CIPN patients.

Keywords Chemotherapy · Paclitaxel · Losartan · Angiotensin II · Analgesic effects · Inflammatory cytokine

Background

Neuropathic pain is a common adverse effect of widely used chemotherapeutic agents, including taxanes, platinum compounds, vinca alkaloids, bortezomib, and thalidomide [1, 2]. The prevalence of chemotherapy-induced peripheral neuropathy (CIPN) in the first month after the end of treatment is about 68% [1]. Owing to its negative impact on daily activities and quality of life, CIPN is increasingly becoming an important problem in cancer patients and survivors [3]. Furthermore, it is dose-limiting adverse effects and leads to the modification or discontinuation of chemotherapy.

Many anticancer drugs such as taxanes, platinum compounds, and vinca alkaloids can cause neuropathic pain that may interfere with cancer patients' treatment and greatly diminish their quality of life. Paclitaxel is one of the drugs to treat breast, cervical, ovarian, and lung cancer [4]. It can induce peripheral sensory neuropathy with a "stocking and glove" pattern characterized by numbness, tingling, and burning pain in both hands and feet [5]. Paclitaxel may affect neuron excitability and survival by disrupting axonal transport, damaging mitochondria, and increasing ion channel activity and neuroinflammation [6]. To date, analgesic drugs such as gabapentin, pregabalin, and tricyclic antidepressants show little or no analgesic effects for cancer patients and survivors [7, 8].

Angiotensin II (Ang II) is the main bioactive component of the renin-angiotensin system that increases blood pressure [9]. Ang II is produced in the several organs including the heart,

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lungs, kidney, bone, reproductive organ, brain, spinal cord, and peripheral nervous system [10]. In addition to its hemodynamic functions, Ang II may affect the central and peripheral sensory system, including nociception [11–13]. Losartan, a selective and competitive nonpeptide Ang II type 1 receptor (AT1R) antagonist, is widely prescribed for hypertensive patients. Losartan may inhibit the vasoconstrictor- and aldosterone-inducing effects of Ang II. Recently, losartan has been reported to have analgesic effects in several models of pain, including inflammatory pain, chronic constriction injury pain, and diabetic neuropathic pain [13–16]. However, the analgesic effect of losartan in CIPN and related mechanism has not been studied.

The purposes of our study were to (1) assess the therapeutic effects of losartan on paclitaxel-induced neuropathic pain (PINP) in rats, (2) assess the preventive effects of losartan on the development of PINP in rats, and (3) identify the mechanisms of action of losartan in the dorsal root ganglia (DRG).

Materials and Methods

Experimental Animals

Male adult Sprague-Dawley rats (200–350 g, Harlan Sprague-Dawley Company, Houston, TX, USA) were used for this experiment. They were housed in plastic cages with soft bedding and freely received food and water under normal light/dark cycle (light cycle 7:00 AM to 7:00 PM). The rats were acclimated to these conditions for 1 week before their use in experiments. All experiment protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. The animals were euthanized with 100% CO_2 and cervical dislocation at the end of the experiments.

Paclitaxel-Induced Neuropathic Pain Model

To induce neuropathic pain in the rats, paclitaxel (GenDEPOT, Katy, TX, USA) was dissolved in a vehicle (4% dimethyl sulfoxide (DMSO) and 4% Tween 80 in sterile saline) and was intraperitoneally injected at a dose of 2 mg/kg on days 0, 2, 4, and 6 (cumulative doses of 8 mg/kg) as previously described [17]. The vehicle group was injected with the same volume of vehicle without paclitaxel.

Behavioral Test for Mechanical Hyperalgesia

We determined rats' degree of mechanical hyperalgesia by assessing the paw withdrawal thresholds of rats in response to the application of a calibrated set of von Frey filaments (0.45–14.45 g) [18]. Briefly, each rat was placed in a transparent cage on a mesh screen. For the behavioral test, we applied von Frey filament to the most sensitive areas including the center of left hind paw or the base of the third or fourth toes. Each rat had either the center of paw or base of toe, respectively for 3–4 s. A filament was applied with up-down method [19]. An abrupt withdrawal, licking, and/or shaking of the foot during stimulation or immediately after stimulation was considered to be a positive response. Fifty percent withdrawal thresholds were calculated using the formula $10^{(X+kd)}/10^4$, where X is the value of the final von Frey filament used in log units, k is the tabular value for the pattern of positive and negative responses, and d (0.22) is the mean difference between stimuli in log units. Personnel performing the behavior tests were blinded to the rats' treatment statuses.

Behavioral Testing for Sedation

To determine whether losartan induced sedation that may affect withdrawal response, we evaluated the rats' sedation status using 5-point (0–4) scales of posture (0 = normal, 4 = flaccid atonia) and righting reflexes (0 = struggles, 4 = no movement) as described previously [20]. Sedation tests were performed immediately after all pain behavioral tests.

Evaluation of the Therapeutic Effect of Losartan

For the experiments assessing the therapeutic effect of losartan, rats received single or multiple intraperitoneal injections of the drug. Single injection was done to investigate whether losartan has analgesic effect on PINP and to decide effective dose of losartan for multiple injections. Multiple injections were done to evaluate the effect of repeated application of losartan to prolong it efficacy. For the single intraperitoneal injection of losartan, 32 rats were randomly divided into 4 groups (3 losartan groups and 1 vehicle group; 8 rats per group) on day 21 after the first paclitaxel injection (Fig. 1a). The rats in losartan group received 20, 50, or 100 mg/kg losartan potassium dissolved in phosphate-buffered saline (PBS), and the rats in the vehicle group received PBS (4 ml/kg) only. The rats' degree of mechanical hyperalgesia was assessed before injection and 1, 1.5, 2, 3, 4, and 5 h after injection.

For the multiple intraperitoneal injections of losartan, 12 rats were divided into two groups (1 losartan group and 1 vehicle group, 6 rats per group) on day 21 after the first paclitaxel injection (Fig. 1b). The losartan group received 50 mg/kg losartan twice daily (at 9 AM and 7 PM) for 5 days, and the vehicle group received 4 ml/kg PBS twice daily for 5 days. The rats' degree of mechanical hyperalgesia was assessed before the first injection on day 21 and at 8 AM on days 22–28.

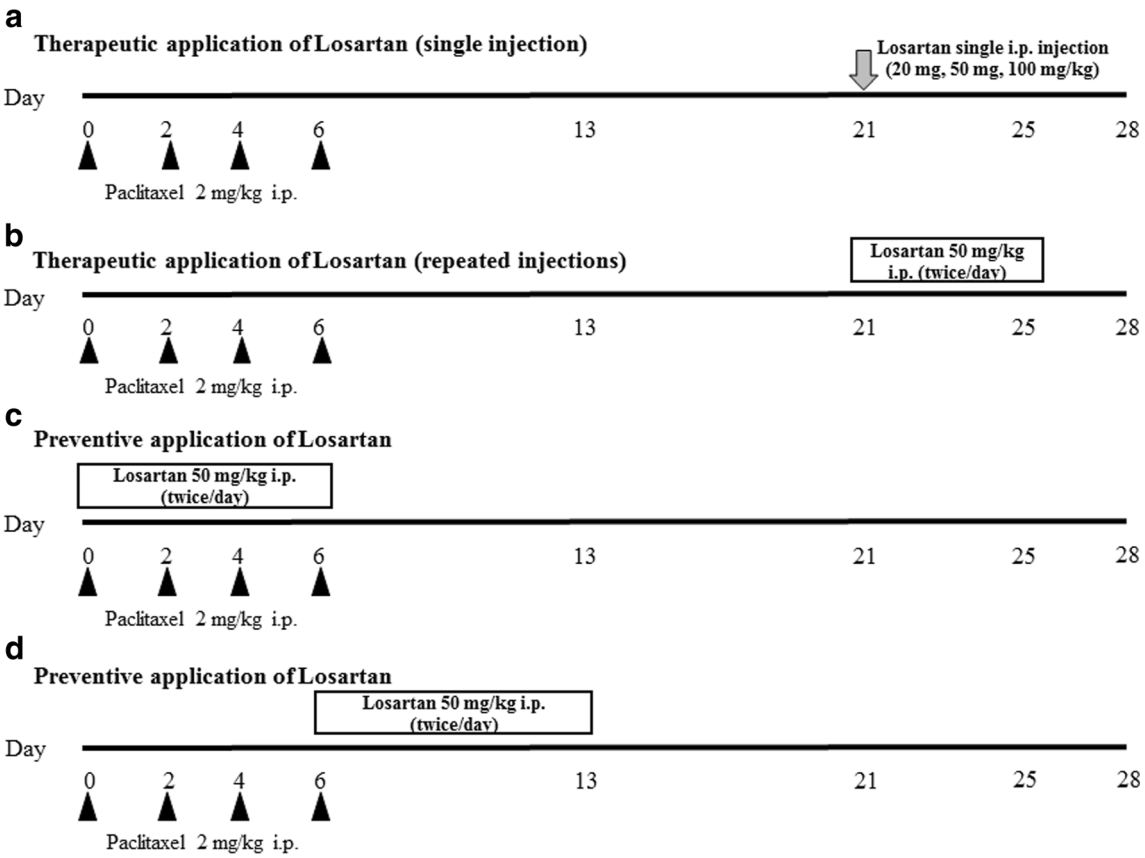

Fig. 1 Schematic overview of the experimental design. **a** Schematic for assessing the therapeutic effects of a single intraperitoneal injection of losartan on day 21 after the first paclitaxel injection. **b** Schematic for assessing the therapeutic effects of repeated twice-daily intraperitoneal injections of losartan on days 21–26 after the first paclitaxel injection. **c** Schematic for assessing the preventive effects of repeated twice-daily intraperitoneal injections of losartan on days 0–6 after the first paclitaxel injection (schedule I). **d** Schematic for assessing the preventive effects of repeated twice-daily intraperitoneal injections of losartan on days 6–14 after the first paclitaxel injection (schedule II). Triangles represent single intraperitoneal injections of paclitaxel; arrows represent single intraperitoneal injections of losartan; and squares represent repeated, twice-daily intraperitoneal injections of losartan

Evaluation of Preventive Effect of Losartan

For the experiments assessing the preventive effect of losartan, rats received multiple intraperitoneal injections of losartan on days 0–6 (schedule I; Fig. 1c) or on days 6–13 (schedule II; Fig. 1d). Our previous reports indicated that the mechanical threshold maintains more than 20 g on days 0–6 (schedule I) and then that gradually decreased to 1 g do days 6–13 (schedule II). Two dosing schedules were used to investigate whether the administration of losartan before or during decreasing mechanical threshold by paclitaxel made any differences.

For the schedule I, 12 rats were divided into two groups (1 losartan group and 1 vehicle group, 6 rats per group). The losartan group received intraperitoneal injections of losartan (50 mg/kg) twice daily (11 AM and 7 PM) on day 0–6 and paclitaxel (2 mg/kg/ml vehicle) once (9 AM) on days 0, 2, 4, and 6. The peritoneal injection of PAC and losartan had the interval of at least 2 h due to block of direct interaction in the peritoneal cavity. In addition, the pain behavioral testing was measured at 8 AM. The vehicle group received intraperitoneal injections of PBS (4 ml/kg) twice daily on 0–6. The rats' degree of mechanical hyperalgesia was measured before the injection of paclitaxel on day 0 and every 2 days thereafter.

For schedule II, 14 rats were divided into two groups (1 losartan group and 1 vehicle group; 7 rats per group). The losartan group received intraperitoneal injections of losartan (50 mg/kg) twice daily on days 6–14. The vehicle group received intraperitoneal injections of PBS (4 ml/kg) twice daily on days 6–14. The rats' degree of mechanical hyperalgesia was measured before the injection of paclitaxel on day 0 and every 2 days thereafter.

Western Blot Analysis

We performed Western blot analysis to evaluate the levels of proteins associated with the mechanism of losartan's anti-CIPN action. We first divided rats into three groups (1 vehicle group, 1 paclitaxel group, and 1 paclitaxel + losartan group; 3 rats per group).

For the vehicle group, vehicle (4% DMSO and 4% Tween 80 in saline, 1 ml/kg) was intraperitoneally injected on days 0, 2, 4, and 6. On day 14, the rats were anesthetized with 4% isoflurane and perfused with cold saline, and the L1-6 DRGs were removed frozen immediately in liquid nitrogen, and then stored at − 80 °C until use.

For the paclitaxel group, paclitaxel (2 mg/kg) was intraperitoneally injected on days 0, 2, 4, and 6, and PBS (4 ml/kg) was intraperitoneally injected twice daily (9 AM and 7 PM) on day 14 and at 9:00 AM–10:30 AM on day 15. Two hours (11:00 AM–12:30 PM) after the last injection of PBS on day 15, the L1-6 DRGs were removed and stored as described above.

For the paclitaxel + losartan group, paclitaxel (2 mg/kg) was intraperitoneally injected on days 0, 2, 4, and 6, and losartan (50 mg/kg) was intraperitoneally injected twice daily (9 AM and 7 PM) on day 14 and at 9:00 AM–10:30 AM on day 15. Two hours after the last injection of losartan on day 15, the L1-6 DRGs were removed and stored as described above.

For the Western blot analysis, the L1-L6 DRGs (about 15 mg) were collected from the rat because we needed enough protein amount. They were homogenized in RIPA cell lysis buffer with a protease inhibitor on ice by homogenizer and centrifuged at 17,000*g* at 4 °C for 10 min. The supernatants were loaded onto 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies against phosphorylated nuclear factor kappa B (p-NFκB; 1:1000; 65KDa; rabbit monoclonal antibody, no. 3033; Cell Signaling Technology, Danvers, MA), interleukin 1β (IL-1β; 1:1000; 17/31KDa; rabbit polyclonal antibody, sc-7884; Santa Cruz Biotechnology, Dallas, TX, USA), tumor necrosis factor alpha (TNF-α; 1:1000; 26KDa; rabbit polyclonal antibody, ab9635; Abcam, San Francisco, CA, USA), monocyte chemoattractant protein 1 (MCP-1; 1:500; 12KDa; rabbit polyclonal antibody, sc-28879; Santa Cruz Biotechnology), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH; 1:5000; 37KDa; rabbit polyclonal antibody, sc25778; Santa Cruz Biotechnology) overnight at 4 °C. The blots then were incubated with anti-rabbit IgG-HRP (1:5000; anti-rabbit IgG conjugated with HRP, W3902, GenDEPOT) for 1 h at room temperature. The blots were applied with a chemiluminescence detection method. The bands were scanned with Spot Advanced and Adobe Photoshop 8.0 (Adobe Inc., San Diego, CA, USA). Band densities were analyzed with image J software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemical Analysis

We performed immunohistochemical analysis to assess the localization of AT1R and IL-1β in the L5 DRG as described previously [21]. The L5 DRG is one of major parts of sensory nerve of sciatic nerve. Rats were divided into three groups (1 vehicle group, 1 paclitaxel group, and 1 paclitaxel + losartan group; 3 rats per group) and treated as described above in the "Western Blot Analysis" section.

Briefly, the L5 DRGs were removed, frozen, cryosectioned, and mounted on microscope slides. The sections were incubated with the primary antibodies including anti-Angiotensin II Type 1 Receptor antibody (rabbit polyclonal antibody, ab18801, 1:100; Abcam), the neuronal marker anti-NeuN (mouse monoclonal antibody, PA303, 1:50; GenDEPOT), anti-IL-1β (rabbit polyclonal antibody, sc-7884, 1:50; Santa Cruz Biotechnology), and the satellite cell marker anti-glial fibrillary acidic protein (GFAP, mouse monoclonal antibody, sc-51908, 1:100; Santa Cruz Biotechnology). The sections were incubated with combinations of the following primary antibodies followed by secondary antibodies conjugated with either Alexa Fluor 568 (SA101–015, 1:100, GenDEPOT, red) or Alexa Fluor 488 (SA802, 1:100, GenDEPOT, green). In addition, ProLong Diamond antifade mountant (Thermo Fisher Scientic, Waltham, MA, USA) was applied to the sections with the nuclear and chromosome marker 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) for 1 day at room temperature. The sections were coverslipped and stored at − 20 °C until imaging. The stained tissue sections were viewed under a Vectra 2 microscope from PerkinElmer. For analysis of AT1R and IL-1β co-localization with NeuN, GFAP, or DAPI, DRG sections from 3 rats were double-stained. For IL-1β quantification, 3 DRG sections were selected per rat and 4 fields of view per section were selected by an experimenter under blind condition and IL-1β intensity was analyzed using the inform Software from PerkinElmer. The experiment using a microscope was performed in the Flow Cytometry and Cellular Imaging Facility, which is supported in part by the National Institutes of Health through MD Anderson's Cancer Center Support Grant CA016672.

Statistical Analysis

All rats were randomly assigned to different experimental groups. Data were expressed as means with standard errors of the means for the behavioral tests and as means with standard deviations for the Western blot analysis and immunohistochemistry. Data analyses were performed using the GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). We performed a two-way repeated-measures analysis of variance with one repeated factor (time) followed by the Tukey's post hoc test for the behavioral test or the Mann-Whitney *U* test for the Western blotting analysis and immunohistochemistry. For the behavioral tests, the sample sizes per group were 6, 7, or 8. These sample sizes of more than 6 supported sufficient power using an alpha of 0.05 and two

tails. However, the sample sizes in Western blot is only 3, which does not support sufficient power and normal distribution for Mann-Whitney *U* test. However, many articles have used small sample size (for example, 3) because of experimental limitations [22, 23]. Therefore, we have used 3 rats per group for Western blotting. *P* values less than 0.05 were considered statistically significant. In all experiments, a sample size of $n \geq 6$ of each group for the behavioral tests was enough to find the effects of losartan.

Results

Losartan Does Not Have Sedative Effects

All rats receiving losartan, PBS, and paclitaxel had scores of 0 on measures of posture and righting reflex, indicating that losartan did not produce any sedative effects. These data suggest that losartan-induced increases of the mechanical threshold were due to the drug's analgesic effect.

Losartan Increases the Mechanical Threshold in Rats with PINP

Single injection of 100 mg/kg losartan significantly increased the mechanical threshold from 0.8 to 2.6 g, 3.9 g, 3.8 g, 2.7 g, and 1.8 g at 1, 1.5, 2, 3, and 4 h after injection, respectively, in a time-dependent manner (Fig. 2a; $P < 0.05$ by 2-way repeated ANOVA followed by Tukey post hoc test). The peak effect of losartan on PINP occurred 1.5 and 2 h after injection.

Repeated injections of 50 mg/kg losartan significantly increased the mechanical threshold from 0.7 g on day 21 to 3.5 g, 3.6 g, 4.8 g, 6.2 g, and 5.5 g on days 22, 23, 24, 25, and 26, respectively; the mechanical threshold returned to the level (1.1 g) before losartan treatment on day 27 (Fig. 2b; $P < 0.05$ by 2-way repeated ANOVA followed by Tukey post hoc test). These data indicate that single and repeated injections of losartan may produce an analgesic effect without sedation.

Losartan Delays the Development of PINP

We used two injection schedules to assess the preventive effects of losartan. The schedule I losartan injections did not affect the development of pain behaviors (Fig. 3a). In contrast, the schedule II injections significantly impeded the development of pain behaviors (Fig. 3b; $P < 0.05$ by 2-way repeated ANOVA followed by Tukey post hoc test). However, after the schedule II injections were discontinued, the rats' mechanical threshold returned to the fully developed pain levels (0.9–1.1 g) measured after the paclitaxel injection. These data indicate that losartan may impede the development of PINP but has no long-term preventive effect.

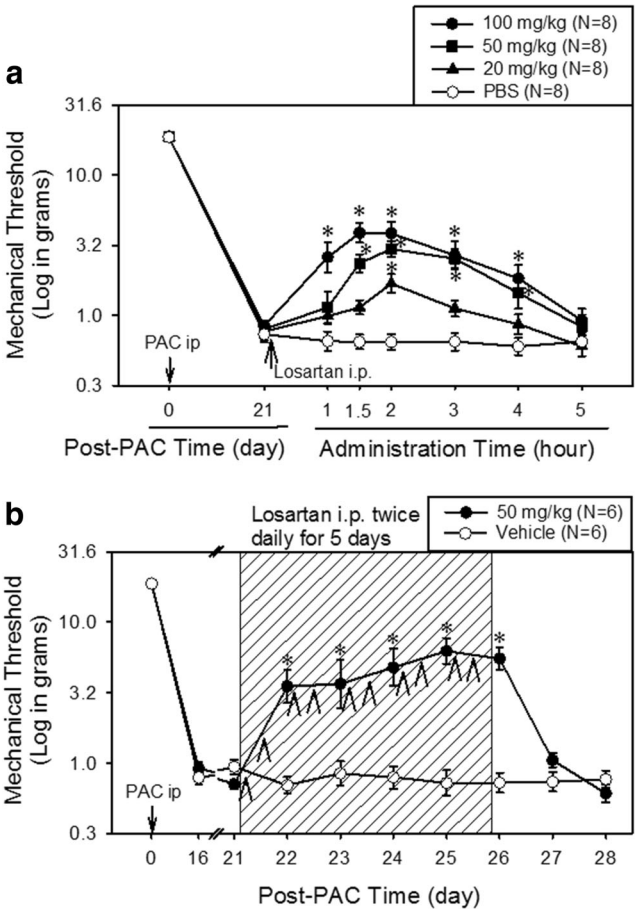

Fig. 2 Therapeutic effects of single intraperitoneal injections and repeated intraperitoneal injections of losartan on PINP in rats. **a** On day 21 after paclitaxel injection, rats received single intraperitoneal injections of PBS or 20, 50, or 100 mg/kg losartan (arrow). The mechanical thresholds of the rats that received 100 mg/kg losartan remained significantly higher than those of the PBS-injected rats for 4 h. **b** Starting on day 21 after the first paclitaxel injection, rats received twice-daily intraperitoneal injections of PBS or 50 mg/kg losartan (arrowheads) for 7 days (hatched box). On days 22–26, the mechanical thresholds of the rats that received repeated intraperitoneal injections of losartan were significantly higher than those of the PBS-injected rats. Data are means with standard errors; asterisks indicate significant differences ($P < 0.05$) compared with the PBS group as determined by a two-way repeated-measures analysis of variance with one repeated factor (time) followed by the Tukey's post hoc test

Paclitaxel Increases, and Losartan Subsequently Decreases, Levels of P-NFκB, TNF-α, IL-1β, and MCP-1 in DRGs

Western blot analysis revealed that the levels of p-NFκB, IL-1β, TNF-α, and MCP-1 in the lumbar DRGs of rats in the paclitaxel group were 1.5, 1.7, 2, and 2.6 times as high, respectively, as those in the lumbar DRGs of rats in the vehicle group (VEH vs PAC; 1.0 ± 0.03 vs 1.5 ± 0.08 for p-NFκB; 1.0 ± 0.02 vs 1.7 ± 0.08 for IL-1β; 1.0 ± 0.02 vs 2.0 ± 0.05 for TNF-α; 1.0 ± 0.05 vs 2.6 ± 0.09 for MCP-1; $P < 0.05$ by Mann-Whitney *U* test). Subsequent administration of losartan

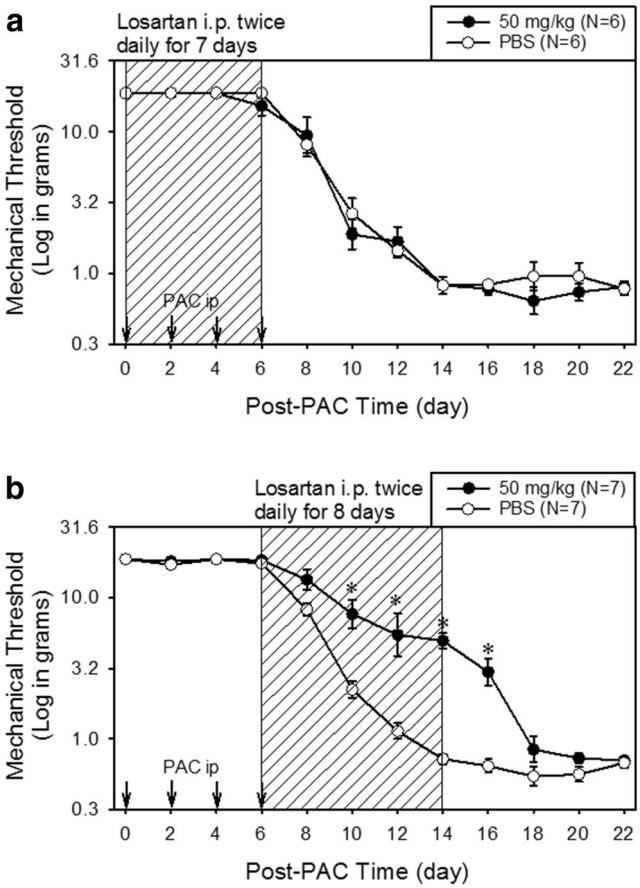

Fig. 3 Losartan delays PINP development (**a**, **b**). In addition to receiving injections of paclitaxel (PAC) on days 0, 2, 4, and 6 (arrows), rats received twice-daily injections of PBS or 50 mg/kg losartan on days 0–6 (A) or days 6–14 (**b**; hatched boxes). Whereas the repeated injections of losartan on days 0–6 had no effect on PINP development, the repeated injections of losartan on days 6–14 delayed the development of PINP but had no lasting preventive effect. Data are means with standard errors; asterisks indicate significant differences ($P < 0.05$) compared with the PBS group as determined by a two-way repeated-measures analysis of variance with one repeated factor (time) followed by the Tukey's post hoc test

decreased these levels (1.03 ± 0.02 for p-NFκB; 1.11 ± 0.02 for IL-1β; 0.71 ± 0.01 for TNF-α; 1.17 ± 0.03 for MCP-1) in paclitaxel-treated rats to those in vehicle-injected rats (Fig. 4a–d). These data indicate that losartan may decreases the protein levels of inflammatory cytokines in the DRGs.

AT1R Is Colocalized in Both Neurons and Satellite Cells in DRGs

Immunohistochemical analysis revealed that AT1R was expressed in the L5 DRG in vehicle- and paclitaxel-injected rats (Fig. 5a–h). In addition, AT1R was co-expressed in both NeuN-positive neurons (green) and DAPI-positive cells (blue) in the DRG (Fig. 5a–h). DAPI is probe for nuclei, not satellite cell. We found the DAPI-positive cells around neuron in the center (Fig. 5d and h, inset). It is reported that satellite cells are located around neuron [24, 25], which suggests that DAPI-positive cells may be satellite cells. Therefore, DAPI-positive cells in the DRG may be satellite cell because (1) DAPI-positive cells were located around the neuron and (2) satellite cells are also located around the neuron in the DRG. These data indicate that both neurons and satellite cells in the DRG may express AT1R. In detail, AT1R may be expressed in the membrane of neuronal cells in the DRG in vehicle-injected rats (Fig. 5a) and may be expressed in both membrane and cytosol of neuronal cells in the DRG in paclitaxel-injected rats (Fig. 5e and h). It likely was receptor internalization that is common in physiological process (Fig. 5e and h).

Paclitaxel Increases, and Losartan Subsequently Decreases, the Intensity of IL-1β in DRGs

IL-1β was expressed in the L5 DRG in both vehicle- and paclitaxel-treated rats (Fig. 6 a and e). The intensity of IL-1β staining in the paclitaxel-treated rats was significantly higher than that in the vehicle-treated rats (Fig. 6a and e; VEH vs PAC; 100 ± 1 vs 128 ± 9; $P < 0.05$ by Mann-Whitney U test). Furthermore, losartan significantly decreased the intensity of IL-1β staining in the DRG cells (Fig. 6e and i; PAC vs PAC+LOS; 128,128 ± 9 vs 98 ± 7; $P < 0.05$ by Mann-Whitney U test). In Fig. 6, we have stained slide with GFAP and DAPI, no NeuN. It was reported that neuron may be surrounded by satellite cell [24, 25]. Therefore, DAPI-positive cells in the DRG may be satellite cell. These results demonstrate that AT1R and IL-1β may be colocalized in both neurons and satellite cells in DRG cells and losartan decreased the intensity of IL-1β in the DRG cells. IL-1β might be expressed in neurons and satellite cells in the L5 DRG (Fig. 6d, h, and l). These results demonstrate that AT1R and IL-1β might be colocalized in both neurons and satellite cells in DRG cells and losartan decreased the intensity of IL-1β in the DRG cells.

Discussion

Our results indicate that the systemic administration of losartan has an analgesic effect on paclitaxel-induced neuropathic pain without sedation by inhibiting p-NFκB, IL-1β, TNF-α, and MCP-1 in DRGs. Losartan delayed the development of PINP. In addition, AT1R was localized in both neuronal cells and satellite cells in the DRG. Taken together, these results suggest that losartan may have potential for the analgesic effects on CIPN without sedation.

The renin-angiotensin system is a hormone system that has a key role in regulating blood pressure and electrolyte and fluid balance [26]. Intrathecal administration of Ang II induces pain behavior through AT1R in the spinal cord, demonstrating Ang II's role in the spinal transmission of nociceptive information [12]. Patil et al. demonstrated the formation of

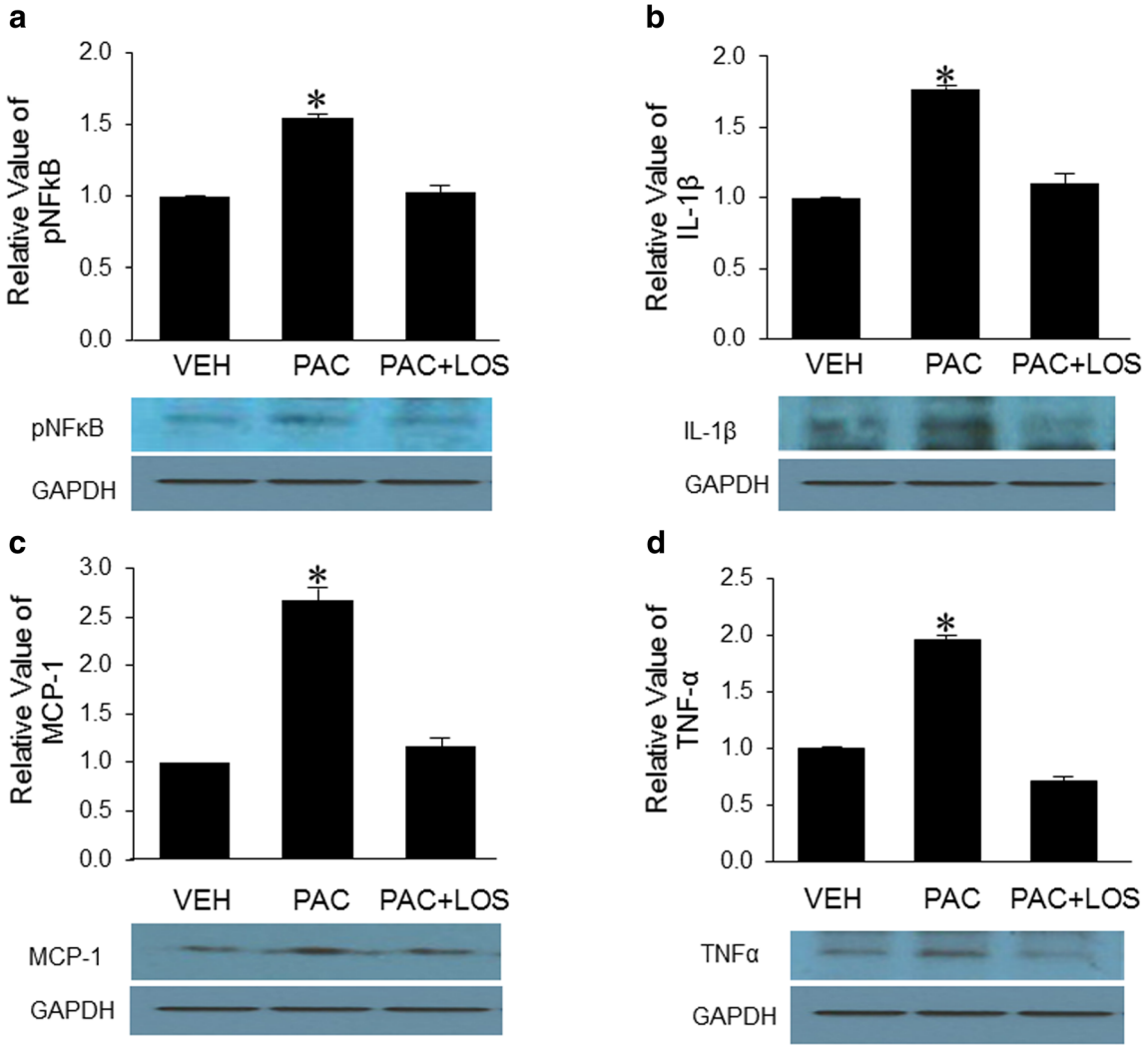

Fig. 4 Paclitaxel increases and losartan subsequently decreases the levels of p-NFκB, IL-1β, MCP-1, and TNF-α in rat DRGs (**a**–**d**). Western blotting revealed that compared with vehicle (VEH), paclitaxel (PAC) increased the levels of p-NFκB (65KDa, **a**), IL-1β (17/31KDa, **b**), MCP-1 (12KDa, **c**), and TNF-α (26KDa, **d**) in rat DRGs; and losartan (LOS) subsequently decreased these protein levels. Data are the means with standard deviations for three rats; asterisks indicate significant differences ($P < 0.05$) compared with the vehicle group as determined by the Mann-Whitney *U* test

intra-neuronal Ang II and co-localization of Ang II with substance P and calcitonin gene-related peptide in the DRG, demonstrating Ang II's interaction with pain perception pathways [27]. In the present study, AT1R was expressed in neuronal cells and satellite cells in the DRG. Another study showed that AT1R is mostly located in neuronal cells in the DRG and that a small subpopulation of these cells overexpress AT1R after chronic constriction injury [28]. Taken together, these findings suggest that Ang II may be associated with pain perception through AT1R in the DRG.

In the present study, the 50 and 100 mg/kg of losartan significantly increased the mechanical thresholds for only 4 h by single injection (Fig. 2). So the prolonged analgesic effects of losartan was induced by intraperitoneal injections at a dose of 50 mg/kg twice daily for 5 days. This repeated doses may produce analgesic effects without sedation. Recently, Kalynovska et al. reported that oral administration of losartan produced analgesic effects on thermal hypersensitivity in spinal nerve ligation model, but it failed to prevent the development of mechanical allodynia [29]. The results showed similar effects in the present study. In Figs. 2 and 3, we used log scale in grams value for the mechanical threshold as *y*-axis because the log scale value decreases in proportion to the increase in pain intensity in human [30]. To date, many drugs including acetyl-L-carnitine, tricyclic antidepressants, cannabinoids, anticonvulsants, α-lipoic acid, and venlafaxine did not recommend for CINP [31]. Only duloxetine is approved for CINP by Food and Drug Administration. Its recommended dose is 30–60 mg once a day for neuropathic patients. Intraperitoneal injection of 30 and 60 mg/kg of duloxetine produced analgesic effects in the oxaliplatin-induced neuropathic pain in mice [32]. Therefore, losartan may be one of options for CINP.

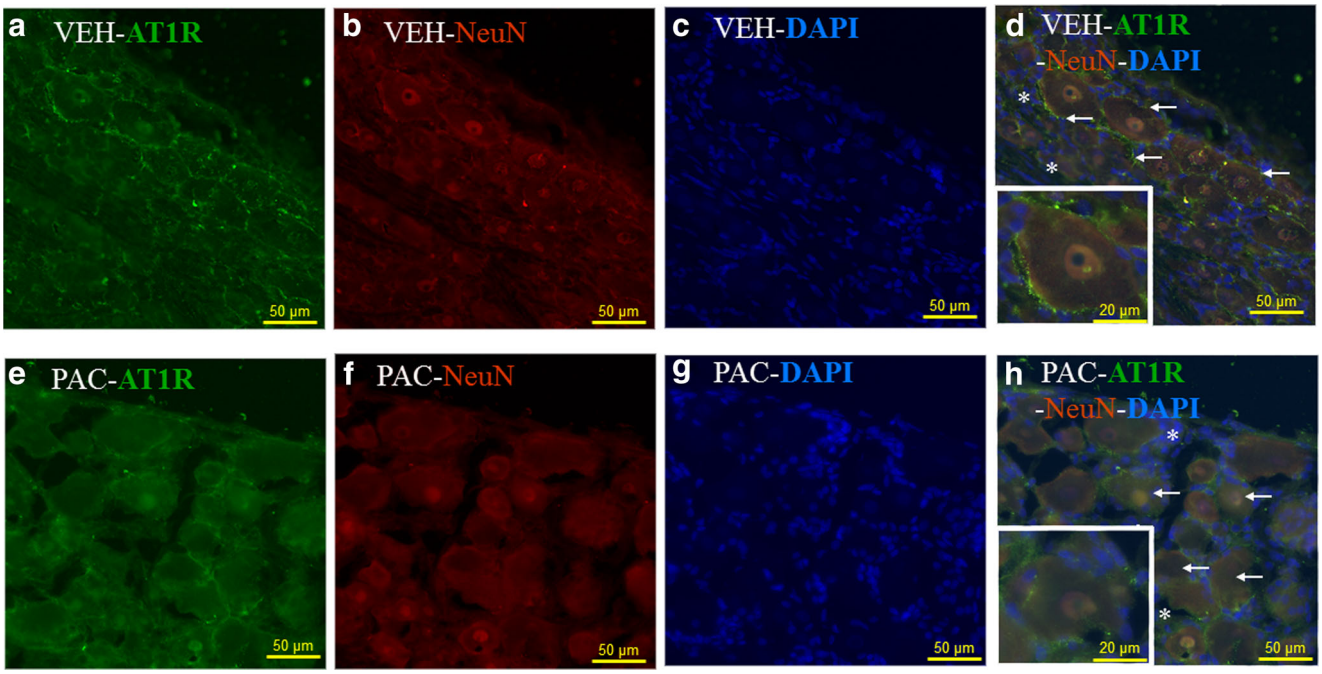

Fig. 5 Co-localization of AT1R, NeuN, and DAPI in DRGs (low magnification). **a** AT1R (green, Alexa Fluor 488) in the L5 DRG of a rat injected with vehicle (VEH; 4% DMSO and 4% Tween 80 in saline) only. **b** NeuN (red, Alexa Fluor 594) in the L5 DRG of a vehicle-injected rat. **c** DAPI (blue) in the L5 DRG of a vehicle-injected rat. **d** AT1R (green, Alexa Fluor 488), NeuN (red, Alexa Fluor 594), and DAPI (blue) in the L5 DRG of a vehicle-injected rat (in inset, scale bar = 20 μm). **e** AT1R (green, Alexa Fluor 488) in the L5 DRG of a rat injected with paclitaxel (PAC; 2 mg/kg). **f** NeuN (red, Alexa Fluor 594) in the L5 DRG of a paclitaxel-injected rat. **g** DAPI (blue) in the L5 DRG of a paclitaxel-injected rat. **h** AT1R (green, Alexa Fluor 488), NeuN (red, Alexa Fluor 594), and DAPI (blue) in the L5 DRG of a paclitaxel-injected rat. Stars and arrows indicate satellite cells and neurons, respectively (in inset, scale bar = 20 μm). Scale bars, 50 μm

For high blood pressure, infusion of losartan at a dose of 20 mg/kg/day showed antihypertensive effects in the spontaneously hypertensive rats [33], which means the 50 mg/kg of losartan for analgesic effect may be higher than its doses for hypertension. Furthermore, we considered toxicity of losartan. For acute toxicity of losartan (Material Safety Data Sheet by Pfizer), the LD_{50} of lactose monohydrate form is 29,700 mg/kg (oral in rat) and minimum lethal dose of potassium form is 1000 mg/kg (oral in mouse) and 2000 mg/kg (oral in rat). Therefore, losartan has high safety margin. In the preliminary experiment, we injected losartan at a dose of 100 mg/kg in normal rats. This injection did not change the mechanical threshold, which means losartan did not induce pain in normal condition (data not shown).

Repeated injections of losartan increased the mechanical threshold to 6.2 g on day 25 (Fig. 2b). In addition, repeated injections of losartan during days 6–14 delayed the development of PINP but had no lasting preventive effects (Fig. 3b). Also, these injections reduced inflammatory cytokines in the lumbar DRGs to their level of vehicle group (Fig. 4). In summary, losartan may have partial analgesic effects but completely block inflammatory cytokines in the DRG. Therefore, inflammatory cytokines may be one of modulators for the induction and maintenance of CINP. Furthermore, other modulators may be reactive oxygen species, sodium channel, and calcium channel.

Inflammation may contribute to the induction and maintenance of persistent and neuropathic pain [34] and is involved in the release of inflammatory cytokines, chemokines, lipid mediators, and vasoactive peptides [35]. They may sensitize peripheral nerves and be released by glial cells (satellite cell, microglia, Schwann cell, astrocyte, microglia) and infiltrating cells (macrophage, polymorphonuclear cell) [35]. Phosphodiesterase 4 inhibitor including rolipram and pentoxifylline ameliorates pain behavior in chemotherapy-induced neuropathic pain in animals by decreasing inflammatory cytokines in the DRG [21, 23, 36], but did not prevent the development of PINP. Phosphodiesterase 4 is located in only immune cells and nervous cells and releases inflammatory cytokines [37]. Therefore, inflammatory cytokines may be involved in part of analgesic effects in the maintenance of PINP and slightly involved in the development of PINP.

In the present study, we found that paclitaxel increased, and losartan subsequently decreased, the level of p-NFκB in the DRGs. This finding is in agreement with those of previous studies with mouse models of collagen-induced arthritis, in which NFκB inhibition decreased the production of pro-inflammatory cytokines such as TNF-α and IL-1β, thereby reducing inflammation and joint destruction [38]. Recently, it was shown that paclitaxel increased p-NFκB, which in turn increased TNF-α and IL-1β in DRGs [23]. NFκB activity in neurons on the spinal cord is increased by sciatic nerve

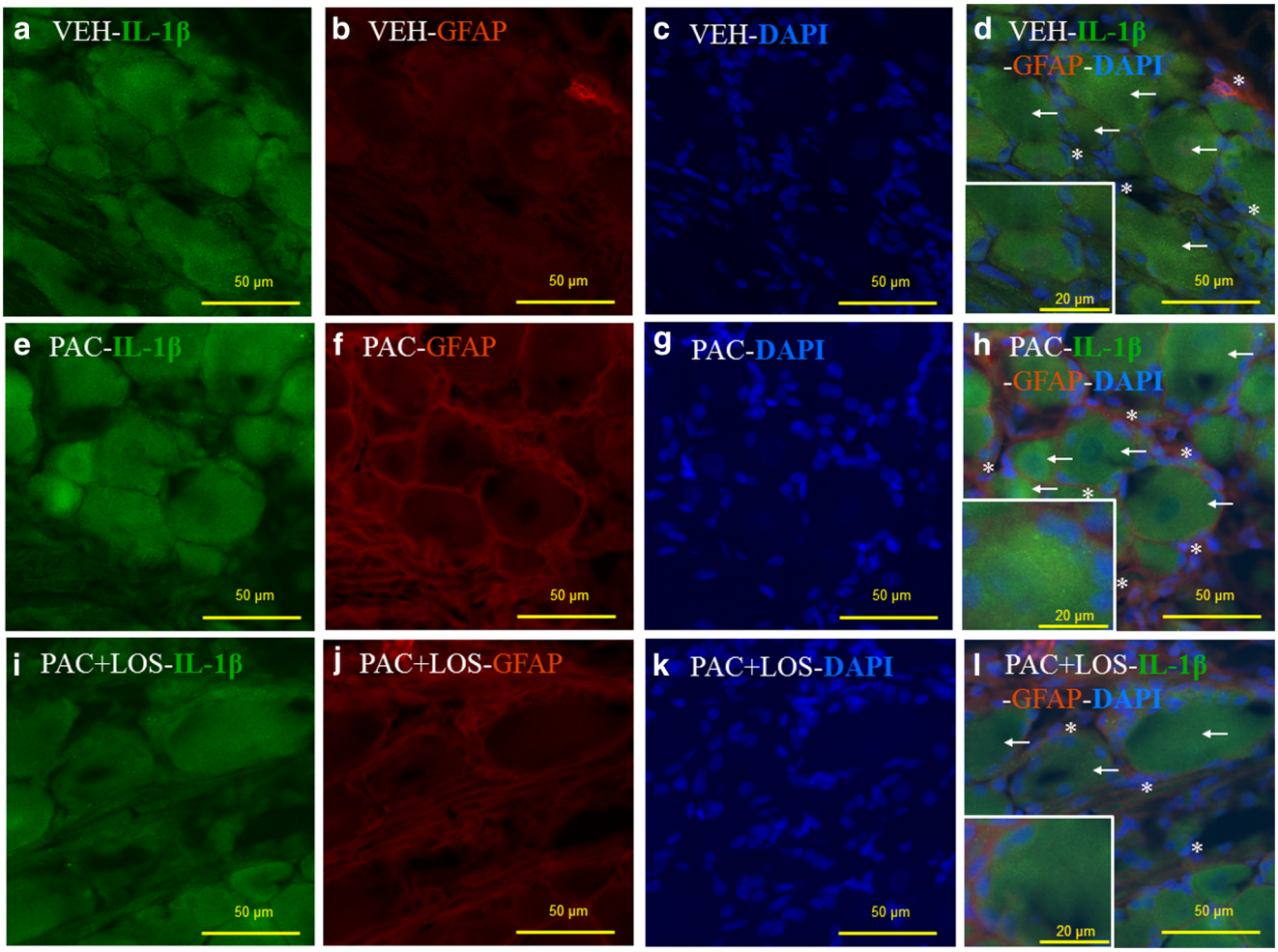

Fig. 6 Co-localization and intensity of IL-1β, GFAP, and DAPI in DRGs (low magnification). **a** IL-1β (green, Alexa Fluor 488) in the L5 DRG of a rat injected with vehicle (VEH; 4% DMSO and 4% Tween 80 in saline) only. **b** GFAP (red, Alexa Fluor 594) in the L5 DRG of a vehicle-injected rat. **c** DAPI (blue) in the L5 DRG of a vehicle-injected rat. **d** IL-1β (green, Alexa Fluor 488), NeuN (red, Alexa Fluor 594), and DAPI (blue) in the L5 DRG of a vehicle-injected rat (in inset, scale bar = 20 μm). **e** IL-1β (green, Alexa Fluor 488) in the L5 DRG of a rat injected with paclitaxel (PAC; 2 mg/kg). **f** GFAP (red, Alexa Fluor 594) in the L5 DRG of a paclitaxel-injected rat. **g** DAPI (blue) in the L5 DRG of a paclitaxel-injected rat. **h** IL-1β (green, Alexa Fluor 488), GFAP (red, Alexa Fluor 594), and DAPI (blue) in the L5 DRG of a paclitaxel-injected rat (in inset, scale bar = 20 μm). **i** IL-1β (green, Alexa Fluor 488) in the L5 DRG of a rat injected with paclitaxel + losartan (PAC+LOS). **j** GFAP (red, Alexa Fluor 594) in the L5 DRG of a rat injected with paclitaxel + losartan. **k** DAPI (blue) in the L5 DRG of a rat injected with paclitaxel + losartan. **l** IL-1β (green, Alexa Fluor 488), GFAP (red, Alexa Fluor 594), and DAPI (blue) in the L5 DRG of a rat injected with paclitaxel + losartan. Stars and arrows indicate IL-1β in the satellite cells and neurons, respectively (in inset, scale bar = 20 μm). Scale bars, 50 μm

transection [39]. In addition, NFκB in neurons is activated by TNF-α and IL-1β [40, 41]. Therefore, NFκB activation produces inflammatory cytokines including TNF-α and IL-1β, which may then themselves activate NFκB.

We also found that the systemic administration of paclitaxel increased the level of MCP-1 in the DRG. MCP-1 is produced by many cells, including endothelial cells, fibroblasts, astrocytes, monocytes, and microglial cells [42]. It also is either produced constitutively or induced by oxidative stress or inflammatory cytokines [42]. MCP-1 regulates the migration and infiltration of monocytes, T lymphocytes, and natural killer cells and is involved in many disease processes, including multiple sclerosis, rheumatoid arthritis, and diabetes [42]. Losartan's inhibition of MCP-1 may block the chemotaxis of inflammatory cells and the infiltration of macrophages in the DRG, thereby diminishing CIPN.

In addition, we found that losartan decreased the protein levels of inflammatory cytokines associated with Ang II. Ang II mediates oxidative stress by activating free radicals that generate enzymes such as nicotinamide adenine dinucleotide phosphate oxidase and inducible nitric oxide synthase [43]. This oxidative stress may produce inflammatory cytokines that contribute to neuropathic pain [22]. In addition, Ang II stimulates inflammatory processes through the activation of NFκB and the production of inflammatory cytokines [43]. Furthermore, Ang II is a pro-inflammatory mediator, mainly through AT1R [44]. Thus, by blocking Ang II, losartan's inhibition of

AT1R may decrease inflammatory cytokines and diminish CIPN.

In the present study, paclitaxel treatment increased IL-1β expression in the DRG cells and losartan reduced the paclitaxel-induced increase in IL-1β intensity (Fig. 6). In Fig. 6, we were focused on IL-1β because its overproduction may be a common cause of neuropathic pain and a key mediator in the regulation of calcium channels in pain pathway [45, 46].

Paclitaxel may produce inflammatory cytokines including IL-1β in the DRG [22, 23]. Also, Ang II may produce inflammatory cytokines including IL-1β through the activation satellite cells [13]. Losartan decreased paclitaxel-induced IL-1β intensity, which means AT1R may be involved in the induction of inflammation including increased inflammatory cytokines in the DRG cells through increases of intracellular calcium.

Losartan has been used for years to treat hypertension and other diseases. For example, losartan may prevent cardiovascular morbidity and mortality [47] and also delay the progression of renal disease in patients with diabetic nephropathy [48]. In present study, losartan produce analgesic effect on CIPN. Therefore, losartan or other AT1R antagonists may be a good treatment option or add-on therapy for CIPN in cancer patients with hypertension or medical comorbidities.

This study has clear limitations. First, the present study was not performed to measure the blood pressure of the rats. Usual adult dose of losartan for hypertension is 50 to 100 mg a day. But, 10 to 50 mg/kg/day losartan is usually employed in experimental studies. In this study, the rats received twice higher dose of losartan than conventional treatment. However, previous study has shown that the pain perception is dissociated from blood pressure regulation in spontaneously hypertensive rats [49, 50]. Irvine et al. reported that losartan showed on effects on tail flick latencies in SHR [36]. In addition, Sitse and de Jong reported that the antihypertensive drugs including hydralazine and captopril did not affect nociceptive responses in DOCA-salt hypertensive in Wistar rats and Wistar-Kyoto rats [23]. This dissociation of pain perception from blood pressure may be influenced from noxious stimuli including heat or pain network of central nerve tissues. Second, the present study was performed with a small number of rats; a higher number of rats is needed for greater statistical power. Third, the mechanisms of CIPN vary greatly depending on several factors, including drug type; most of our study's results are limited to paclitaxel. Although the present observations cannot be applied to humans without careful clinical trial, our results suggest that losartan may be a good alternative for patients suffering CIPN that is difficult to treat.

In the present study, we found analgesic effects of losartan in chemotherapy-induced neuropathic pain in rats. For the next study, we will focus on how activation of AT1R modulates downstream pathways including (1) ERK pathway, (2) cAMP pathway, (3) NOX pathway, and (4) catecholamine pathway [51, 52].

In conclusion, the systemic administration of losartan ameliorated PINP; inhibited p-NFκB, TNF-α, IL-1β, and MCP-1 in the DRG; and delayed the development of mechanical allodynia. In addition, AT1R was expressed in both neuronal cells and satellite cells in the DRG. Taken together, our results suggest that the therapeutic modulation of Ang II with AT1R antagonists represents a promising new add-on therapy for cancer patients or survivors with CIPN.

Acknowledgements This work was supported by grants to S.A. from the Peggy and Avinash Ahuja Foundation and the Helen Buchanan and Stanley Joseph Seeger Endowment at The University of Texas MD Anderson Cancer Center. The authors thank Joe Munch (Department of Scientific Publications, The University of Texas MD Anderson Cancer Center) for the editorial assistance.

Author's Contributions EK: conception, design, data acquisition, analysis, interpretation, and writing of the manuscript. S-HH: design, data acquisition, analysis, and interpretation and preparation of the manuscript. H-KK: conception and interpretation, and preparation of the manuscript. SA: conception, design, interpretation, and writing of the manuscript. HKK: conception, design, data acquisition, analysis, interpretation, and writing of the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

The animals used in the experiment were approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Texas MD Anderson Cancer Center. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflict of Interest The authors declare that they have no competing interests.

Abbreviations CINP, chemotherapy-induced peripheral neuropathy; AT1R, angiotensin II type 1 receptor; PINP, paclitaxel-induced neuropathic pain; DRG, dorsal root ganglion; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; MCP-1, monocyte chemoattractant protein 1; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; DAPI, 4′,6-diamidino-2-phenylindole; p-NFκB, phosphorylated nuclear factor kappa B; NFκB, nuclear factor kappa B

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