Involvement of pro-inflammation signal pathway in inhibitory effects of rapamycin on oxaliplatin-induced neuropathic pain

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Abstract

Background: Oxaliplatin is a third-generation chemotherapeutic agent that is commonly used to treat metastatic digestive tumors; however, one of the main limiting complications of oxaliplatin is painful peripheral neuropathy. The purpose of this study was to examine the underlying mechanisms by which mammalian target of rapamycin (mTOR) and its signal are responsible for oxaliplatin-evoked neuropathic pain.

Methods: Neuropathic pain was induced by intraperitoneal injection of oxaliplatin in rats. ELISA and Western blot analysis were used to examine the levels of pro-inflammatory cytokines (including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α) and the expression of mTOR signal pathway.

Results: Oxaliplatin increased mechanical and cold sensitivity as compared with control animals ($P < 0.05$ vs. control rats). Oxaliplatin also amplified the expression of p-mTOR and mTOR-mediated phosphorylation of p70 ribosomal S6 protein kinase 1 and 4E-binding protein 1 in the lumbar dorsal root ganglion. Blocking mTOR using rapamycin attenuated peripheral painful neuropathy observed in oxaliplatin rats ($P < 0.05$ vs. vehicle control). This inhibitory effect was accompanied with decreases of IL-1β, IL-6, and TNF-α. In addition, inhibition of phosphatidylinositide 3-kinase (p-PI3K) attenuated the expression of p-mTOR and the levels of pro-inflammatory cytokines in oxaliplatin rats, and this further attenuated mechanical and cold hypersensitivity.

Conclusions: The data revealed specific signaling pathways leading to oxaliplatin-induced peripheral neuropathic pain, including the activation of PI3K-mTOR and pro-inflammatory cytokine signal. Inhibition of these pathways alleviates neuropathic pain. Targeting one or more of these molecular mediators may present new opportunities for treatment and management of neuropathic pain observed during chemotherapeutic application of oxaliplatin.

Keywords

Oxaliplatin, neuropathic pain, mTOR, rapamycin, pro-inflammation

Introduction

One of the most common and distressing symptoms suffered by patients with progression of cancer is pain.[1] Cancer pain mainly arises from a tumor compressing or infiltrating tissue, from nerve and other changes caused by a hormone imbalance or immune response, and/or from treatments and diagnostic procedures.[1,2] It should be noted that chemotherapy and radiotherapy may produce painful conditions that persist long after treatment has ended.[1,3,4] As a result, how to effectively manage cancer pain related to these therapies becomes

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an important issue for treatment and management of cancer patients in clinics.

Oxaliplatin (OXL) is an organoplatinum compound, and as a third-generation chemotherapeutic agent, it is commonly used to treat the cancer.[5] Especially, it has a significant activity against advanced and/or metastatic digestive tumors, but one of the main limiting complications of OXL is painful neuropathy.[6] The signs of neuropathy start with paresthesia, followed by hyperesthesia.[2] Also, a heightened cold sensitivity is observed in cancer patients with OXL treatment.[6] Overall, treatment options for these abnormal sensations have been restricted partly due to a poor understanding of the underlying mechanisms responsible for neuropathic pain induced by chemotherapeutic OXL.

Mammalian target of rapamycin (mTOR) is a serine threonine protein kinase. There are two distinct mTOR forms of protein complexes, mTOR complex 1 (mTORC1) and mTORC2. In general, mTORC1 is composed of raptor, mammalian lethal with SEC13 protein 8 (mLST8) and mTOR, and is known to gate translation of most proteins by phosphorylation of specific downstream effectors including p70 ribosomal S6 protein kinase (p70 S6Ks) and 4E-binding proteins (4E-BPs).[7]

Activation of mTOR, in particular, mTORC1 that is more sensitive to rapamycin, leads to the promotion of the phosphorylation of downstream effectors such as p70 ribosomal S6 protein kinase 1 (p70 S6K1) and this further governs mRNA translation.[7] The mTORC1 is well known for its critical roles in the regulation of protein synthesis and growth, and evidence demonstrates that mTOR plays a key role in the modulation of long-term neuronal plasticity.[8,9] Particularly, mTOR, S6K1, and 4E-BP1 are expressed in the sensory nerve and contribute to transmission and modulation of pain,[9–11] that is, intrathecal injection of rapamycin, an inhibitor of mTOR, produces antinociception in animal models of inflammation.[11–13] Local perfusion of rapamycin into the spinal cord attenuates formalin-induced neuronal hyperexcitability in the dorsal horn.[14] Note that rapamycin can attenuate pain response, and this is accompanied with downregulated mTOR, S6K1, and 4E-BP1.[15] These findings indicate that mTOR and its downstream signals are activated under persistent pain conditions and contribute to the development of pain sensitization.

Accordingly, we hypothesized that OXL amplifies mTOR signal, which subsequently increases pro-inflammatory cytokines (PICs), namely, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in the dorsal root ganglion (DRG) and thereby resulting in mechanical and cold hypersensitivity. Blocking of mTOR attenuates the amplified levels of PICs and alleviates OXL-evoked neuropathic pain. We further hypothesized that OXL amplifies the expression of phosphatidylinositide 3-kinase (p-PI3K), an upstream signal of mTOR.[7] Blocking PI3K attenuates mTOR and PIC signals thereby leading to a reduction in neuropathic pain.

Materials and methods

Animal

All animal protocols were in accordance with the guidelines of the International Association for the Study of Pain and approved by the Institutional Animal Care and Use Committee of Jilin University. Adult male Sprague–Dawley rats (200–250 g) were housed in individual cages with free access to food and water and were kept in a temperature-controlled room (25°C) on a 12/12-h light/dark cycle.

A model of neuropathic pain and administration of drugs

OXL (Tocris Bioscience) was dissolved in a 5% glucose solution at a final concentration of 2 mg/ml. Acute neurotoxicity was induced in rats by a single intraperitoneal (i.p.) injection of OXL (6 mg/kg), as described previously.[16,17] Control rats received the same volume of i.p. injection of glucose vehicle. Mechanical and cold hypersensitivity were fully developed by OXL three days after injection and experiments were performed.

Prior to each of experiments, 0.5 ml of dimethyl sulfoxide as vehicle control, antagonists to mTOR (rapamycin, 5 mg/kg; Selleck Chem), and inhibitor of PI3K (LY294002, 5 mg/kg; Sigma-Aldrich) were i.p. given. The volume of injected rapamycin and LY294002 was 0.5 ml. In a subset of experiments, in order to examine the effects of blocking mTOR and PI3K on PICs vehicle (n = 12), rapamycin (5 mg/kg, n = 15) and LY294002 (5 mg/kg, n = 15) were i.p. given, respectively. Then, rats were euthanized by sodium pentobarbital (120 mg/kg, i.p.), and DRG tissues (L4–L6) were removed for ELISA measurements and Western blotting analysis.

Behavioral test

To quantify the mechanical sensitivity of the hindpaw, rats were placed in individual plastic boxes and allowed to acclimate for > 30 min. Mechanical paw withdrawal threshold (PWT) of rat hindpaw in response to the stimulation of von Frey filaments was determined. A series of calibrated von Frey filaments (ranging from 0.5 to 18.0 g) were applied perpendicularly to the plantar surface of the hindpaw with a sufficient force to bend the filaments for 60 s or until paw withdrew. In the presence of a response, the filament of next lower force was applied. In the absence of a response, the filament of

next greater force was applied. To avoid injury during tests, the cutoff strength of the von Frey filament was 18 g. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the "up-down" method.[18] Each trial was repeated two times at approximately 2-min intervals. The mean value was used as the force produced a withdrawal response.

To examine cold sensitivity, Thermal Place Preference System (Coulburn Instruments) was used to perform the thermal place preference test in order to assess a cold avoidance behavior. Two connecting metal plates were surrounded by a plastic enclosure. The first plate was kept at neutral temperature (25°C), and the second plate was kept at cold temperature (12°C). The test was performed in darkness and each session lasted 3 min. During the session, the rats were left free to explore both plates. The time spent on the cold plate during the entire session was recorded using an infrared camera connected to a computer to determine cold avoidance behavior. To better control behavior test, the rats were repeatedly placed on the apparatus with both plates held at room temperature (25°C) during 3 min two days before the beginning of the experiment. Note that rats spent an equal amount of time on each plate under these conditions, suggesting that animals showed no place preference. Also, to avoid learning or any place preference unrelated to cold, the temperature of the plates were inverted between two consecutive sessions. Two trials were performed for each of dosages, and data were averaged. It is noted that all behavioral tests were performed in a blind fashion.

ELISA measurements

The levels of PICs were examined using an ELISA assay kit (Promega Co., Madison, WI) according to the provided description and modification. Briefly, polystyrene 96-well microtitel immunoplates were coated with affinity-purified rabbit anti-IL-1β, anti-IL-6, and anti-TNF-α antibodies. Parallel wells were coated with purified rabbit IgG for the evaluation of nonspecific signal. After overnight incubation, plates were washed. Then, the diluted samples and these PIC standard solutions were distributed in each plate. The plates were washed and incubated with anti-IL-1β, anti-IL-6, and anti-TNF-α galactosidase. Then, the plates were washed and incubated with substrate solution. After incubation, the optical density was measured using an ELISA reader (wavelength of 575 nm).

Western blot analysis

Briefly, DRG tissues (L4–L6) were removed, and total protein of DRG tissues was then extracted by homogenizing sample in ice-cold immunoprecipitation assay buffer. The lysates were centrifuged, and the supernatants were collected. After being denatured, the supernatant samples containing 20 μg of protein were loaded onto gels and electrically transferred to a polyvinylidene fluoride membrane. The membrane was blocked and incubated overnight with respective primary rabbit antibodies, including anti-p-mTOR/p-S6K1/p-4E-BP1 antibodies (1:200), anti-mTOR/S6K1/4E-BP1 antibodies (1:200–1:500), and anti-p-PI3K p85 antibodies (1:250). Next, the membranes were washed and incubated with an alkaline phosphatase-conjugated antirabbit secondary antibody (1:1000). All these primary antibodies were purchased from the Abcam Co. and/or Cayman Chemical Co., and goat antirabbit secondary antibody was purchased from Santa Cruz Biotech. The immunoreactive proteins were detected by enhanced chemiluminescence. The bands recognized by the primary antibody were visualized by exposure of the membrane onto an X-ray film. The membrane was stripped and incubated with anti-β-actin antibody to show equal loading of the protein. Then, the film was scanned, and the optical density of all protein bands was first analyzed using the Scion Image software (National Institutes of Health), and values for densities of immunoreactive bands/β-actin band densities from the same lane were determined. Each of the values was then normalized to a control sample.

Statistical analysis

All data were analyzed using a two-way repeated measures analysis of variance for different time courses between two experimental groups. Values were presented as means ± standard deviation of mean. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL).

Results

Mechanical and cold sensitivity

OXL injection significantly decreased PWT as compared with vehicle injection. PWT was 8.7 ± 0.5 g in control rats (n = 24) and 4.1 ± 0.4 g in OXL rats (n = 28, $P < 0.05$ vs. control rats).

Figure 1(a) (left panel) shows that PWT was increased after i.p. injection of rapamycin in control rats and OXL rats. As rapamycin was given, the effects were observed 30 min after its administration, peaked at 60 min and lasted for >2 h in control rats and OXL rats. In addition, the increase in PWT evoked by rapamycin was smaller in OXL rats (n = 16) than that in control rats (n = 12), that is, PWT was increased by 58% in OXL rats
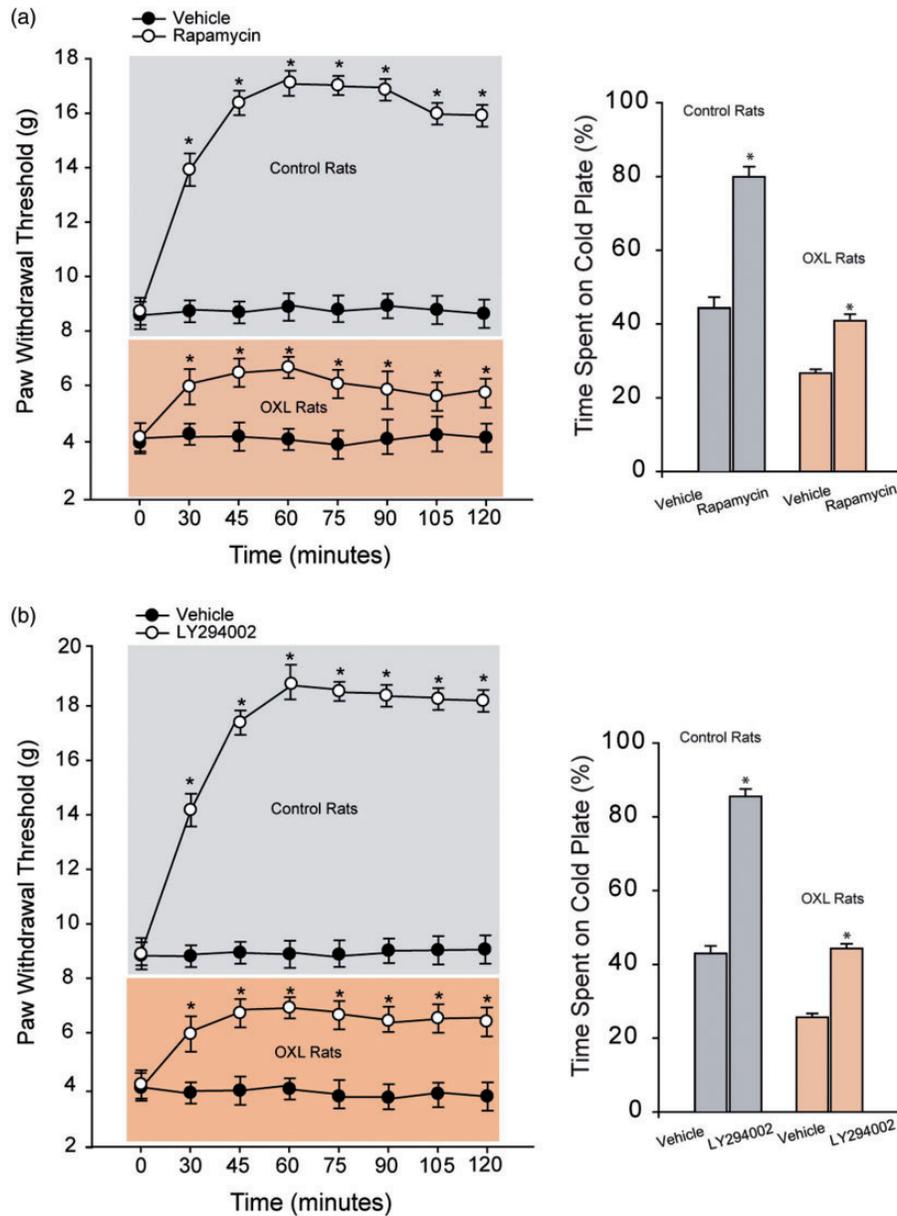
Figure 1. (a) Left panel: effects of blocking mTOR by the administration of rapamycin on paw withdrawal threshold (PWT) in control rats and OXL rats. As compared with vehicle injection, rapamycin increased PWT in control rats and OXL rats. Note that the increases in PWT evoked by rapamycin were smaller in OXL rats than that in control rats. *P < 0.05 versus vehicle control. n = 12 in control and n = 16 in rapamycin. Right panel: effects of blocking mTOR in sensory nerves on cold sensitivity expressed as time spent on the cold plate (%) in control rats and OXL rats. Rapamycin elevated the percentage of time spent on the cold plates in control rats and OXL rats, as compared with vehicle injection. *P < 0.05 versus vehicle. n = 12 in each of control and rapamycin groups. (b) Left panel: effects of blocking PI3K by the administration of LY294002 on PWT in control rats and OXL rats. LY294002 increased PWT in control rats and OXL rats as compared with vehicle injection, but the amplitude of PWT increases evoked by LY294002 was smaller in OXL rats than that in control rats. *P < 0.05 versus vehicle control. n = 12 in each group. Right panel: effects of blocking PI3K in sensory nerves on cold sensitivity expressed as time spent on the cold plate (%) in control rats and OXL rats. LY294002 elevated percentage of time spent on the cold plates in control rats and OXL rats. *P < 0.05 versus vehicle control. n = 8 in control group and n = 12 in group of LY294002 injection. OXL: oxaliplatin.

($P < 0.05$ vs. control rats) and 96% in control rats 60 min after injection of rapamycin.

Likewise, OXL injection also significantly diminished the percentage of time spent on the cold plate as compared with vehicle injection. The percentage of time spent was $43 \pm 4\%$ in control rats (n = 20) and $26 \pm 1\%$ in OXL rats (n = 24, $P < 0.05$ vs. control rats). Figure 1(a) (right panel) further shows that rapamycin significantly attenuated cold sensitivity in control rats and OXL rats (n = 12 in each group). Injection of rapamycin attenuated cold sensitivity to a greater degree in control rats (percentage of time increased by 87%, n = 12) than in OXL rats (percentage of time increased by 53%, n = 12, $P < 0.05$).

We also examined the role played by PI3K signal in mediating mechanical and cold sensitivity. Figure 1(b) (left panel) shows that LY294002 attenuated mechanical sensitivity in OXL rats and control rats as compared with vehicle injection. The inhibitory effects were observed 30 min after its administration, peaked at 60 min, and lasted for >2 h. The effects were smaller in OXL rats than in control rats ($P < 0.05$ vs. vehicle control, n = 12 in each group). In addition, Figure 1(b) (right panel) shows that inhibition of PI3K in the sensory nerves using LY294002 attenuated cold sensitivity to a greater degree in control rats (n = 8) than in OXL rats (n = 12, $P < 0.05$, LY294002 vs. vehicle control for both control rats and OXL rats).

Levels of PICs

In additional experiments, we examined the effects of OXL on the levels of PICs including IL-1β, IL-6, and TNF-α in DRG. Figure 2 shows that IL-1β, IL-6, and TNF-α were significantly increased in OXL rats with vehicle (n = 12) as compared with control rats (n = 10). Furthermore, blocking individual mTOR and PI3K signaling pathways by the administration of rapamycin and LY294002 (n = 15 in each group) significantly attenuated amplifications of PICs evoked by OXL. It is noted that LY294002 had a greater inhibitory effect on IL-1β, IL-6, and TNF-α than rapamycin did.

Expression of mTOR and PI3K signal pathways

Figure 3(a) shows the protein expression of p-mTOR, p-S6K1, and p-4E-BP1 as well as mTOR, S6K1, and 4E-BP1 in control rats and OXL rats. OXL significantly increased the protein levels of p-mTOR and mTOR-mediated p-S6K1 and p-4E-BP1 in the DRG tissues as compared with control rats (n = 8–10). Note that total protein levels of mTOR, S6K1, and 4E-BP1 were not significantly increased in OXL rats. Furthermore, Figure 3(b) demonstrates that the ratio of p-mTOR, p-S6K1, and p-4E-BP1 levels versus total protein of mTOR, S6K1, and 4E-BP1 levels was significantly increased in OXL rats.

In addition, Figure 3(c) shows that the expression of PI3K was upregulated in OXL rats as compared with control rats (n = 6 in each group). We further examined the effects of blocking PI3K on the expression of p-mTOR and p-S6K1. Figure 3(d) demonstrates that the protein expression of p-mTOR and p-S6K1 was significantly increased in OXL rats (n = 6–10) as compared with saline control rats (n = 6–10). When LY294002 was given in OXL rat, the amplified expression of p-mTOR and p-S6K1 evoked by OXL were significantly attenuated (n = 6–10 in each group). Nonetheless, total protein levels of mTOR and S6K1 were not significantly altered by LY294002 in OXL rats.
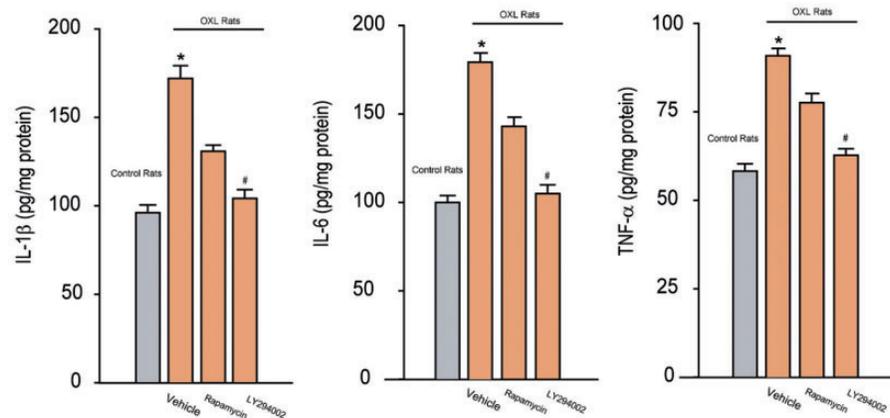

Figure 2. Levels of PICs in control rat and OXL rats. OXL increased IL-1β, IL-6, and TNF-α as compared with control rats (n = 10). Blocking individual mTOR and PI3K signaling pathways by the administration of rapamycin and LY294002 (n = 15 in each group) significantly attenuated amplifications of PICs evoked by OXL as compared with vehicle injection (n = 12). It is noted that LY294002 had a greater inhibitory effect on IL-1β, IL-6, and TNF-α than rapamycin did. *$P < 0.05$ versus control rats and OXL rats with rapamycin or LY294002. #$P < 0.05$ indicated rapamycin in OXL rats versus LY294002 in OXL rats. OXL: oxaliplatin; IL: interleukin; TNF: tumor necrosis factor.
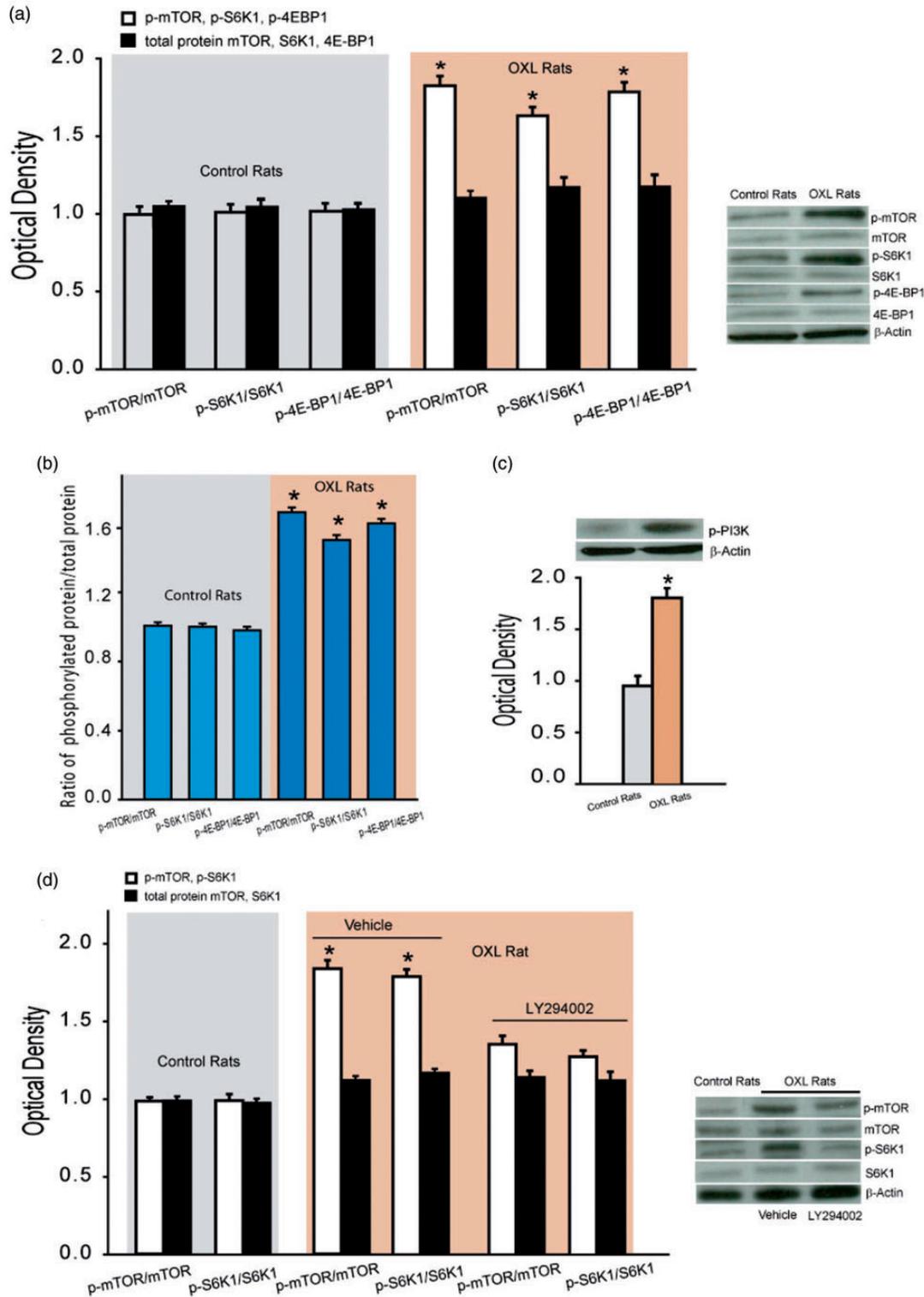
Figure 3. (a) Expression of mTOR pathways in control rats and OXL rats. Left panel and right panel are averaged data and typical bands, showing that p-mTOR, p-S6K1, and p-4E-BP1 in the DRG were upregulated in OXL rats. *P <0.05 versus control rats (n = 8–10 in each group). There were insignificant differences in total protein expression of mTOR, S6K1, and 4E-BP1 in control rats and OXL rats (P > 0.05. n = 8–10 in each group). (b) The ratio of p-mTOR, p-S6K1, and p-4E-BP1 levels versus total protein of mTOR, S6K1, and 4E-BP1 levels was significantly increased in OXL rats. *P < 0.05 versus control rats (n = 8–10 in each group). (c)\Top panel: typical bands and bottom panel: averaged data showing the expression of upregulated p-PI3K in OXL rats as compared with control rats. *P < 0.05, control versus OXL (n = 6 in each group). (d) Effects of blocking PI3K on mTOR expression. Averaged data and typical bands: p-mTOR and p-S6K1 was amplified in DRG of OXL rats with vehicle treatment as compared with control rats. Blocking PI3K signal pathway by injection of LY294002 attenuated increases in p-mTOR and p-S6K1 in OXL rats. *P < 0.05 versus control animals and OXL animals injected with LY294002 (n = 6–10 in each group). Note that total protein of mTOR and S6K1 levels was not significantly increased in control rats and OXL rats. OXL: oxaliplatin; mTOR: mammalian target of rapamycin; S6K1: ribosomal S6 protein kinase 1; 4E-BP1: 4E-binding protein 1.

Discussion

Data of the current study showed that OXL increased mechanical and cold sensitivity, and this was accompanied with the upregulation of PI3K-mTOR and mTOR-mediated signals as well as IL-1β, IL-6, and TNF-α. As PI3K or mTOR signal was inhibited, mechanical and cold hypersensitivity were attenuated in OXL rats, and the levels of PICs were decreased.

Prior studies have shown that a single injection of OXL produces neuropathic pain in rats including mechanical hyperalgesia and cold hypersensitivity after initiation of the chemotherapy regimen in rats.[16,17] The signs of mechanical hyperalgesia and cold hypersensitivity were ablated several weeks after discontinuation of OXL.[16,17] Thus, in this study, we employed this well-established rat model to examine the mechanisms leading to neuropathic pain induced by OXL. Using the same intervention, in our current study, we observed that PWT significantly declined to evoke mechanical withdrawal and less time (%) spent on the cold plate three days after OXL injection. This is consistent with the previous findings.[16,17,19] Results of the current study also demonstrated that the expression of p-mTOR, mTOR-p-S6K1, p-4E-BP1, and p-PI3K pathway was upregulated in DRG of OXL rats as compared with control rats. Blocking mTOR by systemic administration of rapamycin attenuated mechanical hyperalgesia and largely restored shortened time spent on the cold plate evoked by OXL. Blocking PI3K signal attenuated activities of mTOR and also decreased mechanical and cold hypersensitivity. Notably, increases in IL-1β, IL-6, and TNF-α in sensory nerves evoked by OXL were inhibited after blocking mTOR or PI3K signal pathways.

The PI3K/Akt pathway is an intracellular signaling pathway in regulating the cell cycle. This important mechanism is directly related to cellular quiescence, proliferation, and longevity. PI3K can phosphorylate and activate Akt in the plasma membrane.[20] The Akt leads to several downstream effects, which alters transcription of p70 ribosomal S6K1 or 4E-BP1 and activating Cyclic adenosine monophosphate (cAMP) response element-binding protein and inhibition of p27, and so on.[7,8,20,21] Our present study demonstrated that blocking PI3K attenuated p-mTOR and p-S6K1 expression and that systemic infusion of PI3K inhibitor attenuated hyperalgesia and restored shortened time spent on the cold plate observed in OXL rats. This suggests that PI3K is necessary to play a regulatory role in mediating the effects of mTOR on OXL-evoked mechanical pain and cold hypersensitivity responses.

Moreover, mTOR inhibitors have been used as an immunosuppressive agent, and the prior study indicates that mTOR plays an important role in the immune system.[22] Rapamycin, the prototype of selective mTOR inhibitor, enhances the anti-inflammatory activities of regulatory T cells and decreases the production of PICs and chemokines by macrophages in addition to other immune cells.[23] Recent studies have suggested the role of mTOR in regulating a number of inflammatory and autoimmune diseases.[24,25] Data of our current study showed that IL-1β, IL-6, and TNF-α in sensory nerve of OXL rats were amplified. Particularly, blocking mTOR or PI3K signal pathways using rapamycin and LY294002 decreased the amplified levels of these PICs whereas they also attenuated mechanical and cold hypersensitivity. Interestingly, we observed that LY294002 had a greater effect on IL-1β, IL-6, and TNF-α than rapamycin did, indicating the role played by PI3K as a upstream pathway in regulating hypersensitive pain and cold responses observed in OXL rats via mTOR signals. This suggests that mTOR is unlikely only downstream pathway of PI3K. Nevertheless, there is lacking of evidence specifically showing the role played by mTOR and PI3K in PICs in a neuropathic pain model induced by OXL. Results of the present report suggest that IL-1β, IL-6, and TNF-α regulated by mTOR and PI3K in sensory nerves contribute to mechanical pain and cold hypersensitivity in OXL-induced neuropathy.

It is well known that stimulation of nociceptive receptors in the sensory nerves leads to the releases of substance P and calcitonin gene-related peptide (CGRP) from the nerve terminal of DRG into the superficial dorsal horn.[26,27] Numerous receptors present on the presynaptic site of the nerve terminals and contribute to the releases of substance P and CGRP in regulating inflammatory and neuropathic pain.[28] Recent studies have demonstrated that OXL increases the levels of substance P and CGRP in the dorsal horn, and inhibition of receptors such as transient receptor potential vanilloid 1 (TRPV1) stimulating the releases of substance P and CGRP can also alleviate mechanical and cold hypersensitivity induced by systemic administration of OXL in rats.[29] PI3K-mTOR pathway can affect TRPV1 receptors and thereby decrease the levels of substance P and CGRP.[13,30] Thus, it is assumed that blocking PI3K-mTOR may decrease the levels of those two neuropeptides in involvement of OXL-induced neuropathic pain.

In conclusion, in OXL rats, p-mTOR and mTOR-mediated p-S6K1 and p-4E-BP1 are upregulated in sensory nerves, which result in mechanical pain and cold hypersensitivity. Systemic administration of rapamycin has a significant analgesic effect in a rat model of neuropathic pain induced by OXL. Moreover, blocking PI3K blunts the amplified expression of p-mTOR pathways and thereby alleviates OXL-evoked neuropathic pain. Nonetheless, our data for the first time reveal specific signaling pathways leading to mechanical pain and cold hypersensitivity evoked by OXL, including the

activation of PI3K and mTOR pathways, and stimulation of PICs including IL-1β, IL-6, and TNF-α. Results of our study provide a base for the mechanisms responsible for OXL-induced neuropathic pain. Targeting one or more of these signaling molecular mediators involved in the activation of PI3K-mTOR at the levels of sensory inputs may present new opportunities for treatment and management of peripheral painful neuropathy often observed in cancer patients during chemotherapeutic application of OXL.

Author Contributions

Duan Z, Su Z, and Pang X contributed to experimental performance and data analysis; Duan Z and Su Z were also participated in experimental designs and drafting the manuscript. Wang H and Pang X designed and oversaw the experiments and reviewed the paper. Duan Z and Su Z had equal contributions to this work.

Declaration of Conflicting Interests

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

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