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Spinal cord NMDA receptor-mediated activation of mammalian target of rapamycin is required for the development and maintenance of bone cancer-induced pain hypersensitivities in rats

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Abstract

Mammalian target of rapamycin (mTOR) controls mRNA translation and is critical for neuronal plasticity. However, how it participates in central sensitization underlying chronic pain is unclear. Here, we show that NMDA receptors are required for the functional role of spinal cord mTOR in bone cancer pain induced by injecting prostate cancer cells (PCCs) into the tibia. Intrathecal rapamycin, a specific mTOR inhibitor, dose-dependently attenuated the development and maintenance of PCC-induced mechanical allodynia and thermal hyperalgesia. Rapamycin alone did not affect locomotor activity and acute responses to thermal or mechanical stimuli. Phosphorylation of mTOR and p70S6K (a downstream effector) was increased time-dependently in L₄₋₅ dorsal horn and transiently in L₄₋₅ dorsal root ganglions on the ipsilateral side after PCC injection, although total expression of mTOR or p70S6K was not changed in these regions. The increases in dorsal horn were abolished by intrathecal infusion of DL-AP5, an NMDA receptor antagonist. Moreover, NMDA receptor subunit NR1 colocalized with mTOR and p70S6K in dorsal horn neurons. These findings suggest that PCC-induced dorsal horn activation of the mTOR pathway participates in NMDA receptor-triggered dorsal central sensitization under cancer pain conditions.

Perspective—The present study shows that inhibition of spinal mTOR blocks cancer-related pain without affecting acute pain and locomotor function. Given that mTOR inhibitors are FDA-approved drugs, mTOR in spinal cord may represent a potential new target for preventing and/or treating cancer-related pain.

Keywords

mTOR; NMDA receptors; activation; dorsal horn; cancer pain

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Disclosures

The authors declare no conflict of interests.

Introduction

Cancer, particularly metastatic prostate bone tumor, produces intractable and persistent pain^{5, 22}. The limited success of current treatments for cancer pain is due, at least in part, to our incomplete understanding of the mechanisms that underlie the induction and maintenance of cancer-related pain. Cancer-induced peripheral nerve and tissue damage leads to unique changes in neuronal plasticity in spinal dorsal horn and primary afferent neurons^{6, 27, 29}. These changes are thought to contribute to the generation and maintenance of cancer pain. Understanding the molecular mechanisms that underlie these changes might allow for development of novel therapeutic strategies to treat cancer pain.

Mammalian target of rapamycin (mTOR) is a serine-threonine protein kinase. Its activation, especially in a complex sensitive to rapamycin (mTOR complex 1), promotes the phosphorylation of downstream effectors, such as p70 ribosomal S6 protein kinase (p70S6K), and governs mRNA translation^{7, 13, 14}. Accumulating evidence indicates that mTOR plays an important role in the modulation of long term plasticity, memory processes. Mice with deletions of mTOR downstream effectors exhibit deficits in synaptic plasticity and long-term memory^{2, 4, 7}. Recent studies also reveal that mTOR may participate in transmission and modulation of nociceptive information. mTOR and its downstream effectors are expressed in dorsal root ganglion (DRG) and spinal cord dorsal horn, two major pain-related regions^{12, 17, 34}. Intrathecal (i.th) administration of rapamycin, a specific inhibitor of mTOR, produces antinociception in models of nerve injury and inflammation^{3, 12, 17, 23, 25, 35}. Local perfusion of rapamycin into spinal cord significantly reduces formalin-induced neuronal hyperexcitability in dorsal horn³. These findings indicate that mTOR and its downstream effectors might be activated and have critical roles in the development of spinal central sensitization under persistent pain conditions. However, the mechanisms that underlie these events are unclear.

Given that NMDA receptors play a critical role in spinal central sensitization^{18, 33} and that blocking NMDA receptors produces a significant analgesic effect on bone cancer pain^{20, 21, 26, 32}, we propose that spinal cord NMDA receptor-mediated activation of mTOR and its downstream effectors is required for development and maintenance of bone cancer-induced pain hypersensitivities. We used a rat model of bone cancer pain produced by prostate cancer cell (PCC) injection of the tibia to determine first whether pre- and post-treatment with i.th. rapamycin affects the development and maintenance of PCC-induced pain hypersensitivity. We then examined whether PCC-induced peripheral noxious input changes the activity of mTOR and p70S6K in spinal cord dorsal horn and DRG. Finally, we addressed whether spinal cord NMDA receptors were involved in such changes in dorsal horn.

Methods

Animals

Male Copenhagen rats weighing 200-225 g (Harlan, Frederick, MD) were housed on a standard 12-h light/dark cycle, with water and food pellets available *ad libitum*. To minimize intra- and inter-individual variability of behavioral outcome measures, animals were trained for 1-2 days before behavioral testing was performed. Animal experiments were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University School of Medicine and were consistent with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experimenters were blind to drug treatment condition during the behavioral testing.

Drugs

The AT-3.1 PCC line was obtained from American Type Culture Collection (ATCC, Manassas, VA). Rapamycin and ascomycin were purchased from LC laboratories (Woburn, MA) and dissolved in 50% DMSO. DL-2-amino-5-phosphonovaleric acid (DL-AP5) was purchased from Tocris Bioscience (Ellisville, MO) and dissolved in saline. All drug dosages used were based on data from previous studies^{3, 12, 17, 34, 35} and our pilot work.

Cancer cell preparation

The PCCs were grown in RPMI 1640 medium (Sigma, St. Louis, MO) that contained L-glutamine and was supplemented with 250 nM dexamethasone and 10% fetal bovine serum. Cells were maintained in T-75 plastic flasks (Corning Glass) and cultured in a water-saturated incubator in an atmosphere of 5% CO₂:95% air. For passage, cells were detached by rinsing gently with calcium- and magnesium-free Hanks' balanced salt solution (HBSS) and a trypsin solution containing 0.05% trypsin and 0.02% EDTA. Before being injected into the rats, the detached cells were first collected by centrifuging 10 mL of medium for 3 min at 1,200 rpm. The resulting pellet was washed twice with 10 mL of calcium- and magnesium-free HBSS and centrifuged again for 3 min at 1,200 rpm. The final pellet was resuspended in 1 mL of HBSS. The cells were counted by using a hemocytometer and diluted to a final concentration of 4.5×10^5 cells/15 μ L HBSS for injection.

Intrathecal catheter implantation, drug injection, and drug infusion

Rats were fully anesthetized with 2% isoflurane, a 1-cm midline incision was made from the back, and the muscles were retracted to expose the L₄₋₅ vertebrae. Sterile polyethylene tubing (PE-10 catheter) was inserted into the subarachnoid space and advanced 3.6 cm rostrally at the level of spinal cord lumbar enlargement segments. The catheter was secured to the paraspinal muscle of the back and then tunneled subcutaneously to exit in the dorsal neck region, where it was secured to the superficial musculature and skin. The rats were allowed to recover for 5-7 days; rats that showed neurologic deficits or the presence of fresh blood in the cerebral spinal fluid postoperatively were excluded from the study. The position of the PE-10 catheter was confirmed after behavioral testing.

For drug injection, the drugs were administered intrathecally in a 10- μ L volume followed by 12 μ L of saline to flush the catheter.

For drug infusion, PE-10 catheter was connected to a syringe pump (Kent Scientific, Torrington, CT) and saline or DL-AP5 (2 μ g/ μ L) were continuously infused for the experimental period at a rate of 1 μ L/h.

PCC injection

Five to seven days after catheter implantation, rats were anesthetized with 2% isoflurane. A 1-cm rostrocaudal incision was made in the skin over the upper medial half of the tibia on the right leg. The tibia was carefully exposed with minimal damage to the muscle. The bone was pierced with a 23-gauge needle 5 mm below the knee joint medial to the tibial tuberosity. A 50- μ L Hamilton syringe was used to inject 15 μ L of PCCs (4.5×10^5 cells) or vehicle (HBSS) into the bone cavity. After injection, the syringe was kept in place for 2 min and was then slowly pulled out. The bone hole was filled with bone wax (Ethicon, Somerville, NJ), and the skin was sutured with 4-0 silk threads.

Radiology and histochemical staining

To confirm whether cancer developed in the tibia, we anesthetized the rats and radiographed their legs 12 days after behavioral testing. The tibias were then collected, demineralized in

EDTA (10%) for 2-3 weeks, and embedded in paraffin. The sections were cut at a thickness of 5 mm on a microtome and stained with Harris' hematoxylin and eosin to verify cancer cell infiltration and bone destruction.

Behavioral testing

To measure paw withdrawal latency to noxious heat stimuli, we placed each animal in a plastic chamber on a glass plate located above a light box. Radiant heat from a Model 336 Analgesia Meter (IITC, Inc. /Life Science Instruments, Woodland Hills, CA) was applied by aiming a beam of light through a hole in the light box through the glass plate to the middle of the plantar surface of each hind paw. When the animal lifted its foot, the light beam was turned off. The length of time between the start of the light beam and the foot lift was defined as the paw withdrawal latency. Each trial was repeated five times at 5-min intervals for each paw. A cutoff time of 20 s was used to avoid paw tissue damage. Thermal testing was performed 1 day before PCC inoculation (baseline) and on days 3, 5, 7, 9, and 12 after PCC inoculation.

For measurements of paw withdrawal threshold to mechanical stimuli, each animal was placed in a plastic chamber on an elevated mesh screen. Von Frey filaments in log increments of force (3.61, 3.84, 4.08, 4.31, 4.56, 4.74, 4.93, 5.18 g) were applied to the plantar surface of the left and right hind paws. The 4.31-g stimulus was applied first. If a positive response occurred, the next smaller von Frey hair was used; if a negative response was observed, the next higher von Frey hair was used. The test was ended when (1) a negative response was obtained with the 5.18-g hair, (2) four stimuli were applied after the first positive response, or (3) nine stimuli were applied to one hind paw. The pattern of positive and negative paw withdrawal responses to the von Frey filament stimulation was converted to a 50% threshold value using the formula provided by Dixon¹⁰. Mechanical testing was performed 1 day before PCC inoculation (baseline) and on days 3, 5, 7, 9, and 12 after PCC inoculation.

To assess locomotor function, we tested rats for the following three reflexes^{30, 31, 37}. (1) Placing reflex: The rat was held with the hind limbs slightly lower than the forelimbs, and the dorsal surfaces of the hind paws were brought into contact with the edge of a table. The experimenter recorded whether the hind paws were placed on the table surface reflexively; (2) Grasping reflex: The rat was placed on a wire grid and the experimenter recorded whether the hind paws grasped the wire on contact; (3) Righting reflex: The rat was placed on its back on a flat surface and the experimenter noted whether it immediately assumed the normal upright position. Scores for placing, grasping, and righting reflexes were based on counts of each normal reflex exhibited in five trials.

Western blot analysis

The ipsilateral and contralateral L₄₋₅ dorsal horns and L₄₋₅ DRGs were collected after behavioral testing or at different time points after PCC injection. The tissues were homogenized in homogenization buffer [10 mM Tris-HCl (pH 7.4), 5 mM NaF, 1 mM sodium orthovanadate, 320 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 M leupeptin, and 2 μM pepstatin A]. After centrifugation at 1,000 × g for 20 min at 4°C, the supernatant was collected and the pellet (nuclei and debris fraction) discarded. The samples were heated for 5 min at 95°C and then loaded onto 4% stacking/10% separating SDS-polyacrylamide gels. The proteins were electrophoretically transferred onto nitrocellulose membrane. The blotting membranes were blocked with 3% nonfat dry milk for 1 h and incubated overnight at 4°C with rabbit anti-phospho-mTOR (1:500; Cell Signaling Technology, Inc, Danvers, MA), rabbit anti-mTOR (1:500; Cell Signaling Technology, Inc), rabbit anti-phospho-p70S6K (1:500; Cell Signaling

Technology, Inc), rabbit anti-p70S6K (1:500; Cell Signaling Technology, Inc), and mouse anti- β -actin (1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). β -actin was used as a loading control. The proteins were detected by using anti-rabbit, or anti-mouse secondary antibody and visualized with chemiluminescence reagents provided with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to film. The intensity of blots was quantified with densitometry. The blot density from naïve animals (0 d) was set as 100%.

Double-labeling immunofluorescence histochemistry

Double-labeling immunofluorescence histochemistry was carried out as described previously³⁴. Briefly, the rats were deeply anesthetized and perfused transcardially with 100 mL of 0.01 M phosphate-buffered saline (pH 7.4) followed by 300 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After the perfusion, the lumbar enlargement segments were harvested, postfixed at 4°C for 4 h, and cryoprotected in 30% sucrose overnight. The transverse sections were cut on a cryostat at a thickness of 15 μ m. The sections were incubated overnight at 4°C with a mixture of rabbit polyclonal anti-mTOR (1:1000) and mouse monoclonal anti-NR1 (1:200; Chemicon, Temecula, CA) or a mixture of rabbit polyclonal anti-p70S6K (1:1000) and mouse monoclonal anti-NR1 (1:200; Chemicon). The sections were then incubated with a mixture of goat anti-rabbit IgG conjugated with Cy3 (1:300) and monkey anti-mouse IgG conjugated with Cy2 (1:300; Jackson ImmunoResearch) for 1 h at 37°C. Control experiments included pre-absorption of the primary antiserum with an excess of the corresponding antigen (Cell Signaling Technology), substitution of normal rabbit serum for the primary antiserum, and omission of the primary antiserum in parallel as described previously³⁴.

Statistical analysis

The results from the behavioral tests and Western blotting were analyzed with a one-way or two-way analysis of variance (ANOVA). Data are presented as means $\pm$ SEM. When ANOVA showed significant difference, pairwise comparisons between means were tested by the post-hoc Tukey method. Significance was set at $p < 0.05$. The statistical software package SigmaStat (Systat, San Jose, CA) was used to perform all statistical analyses.

Results

Bone cancer pain model produced by PCC injection in rats

Consistent with the previous study³⁹, the animals injected with PCCs exhibited general health comparable to the rats injected with HBSS; they had well groomed coats and normal muscle strength, body temperature, and general sensory functions (e.g., olfactory, auditory, and visual functions). In addition, body weight and locomotor behavior were indistinguishable between the two groups during the 12-day observation period (data not shown). However, by day 12, most rats injected with PCCs had enlargement around the ipsilateral knee.

Tibial PCC injection produced both mechanical allodynia, as evidenced by a significant decrease in paw withdrawal threshold (Fig. 1A), and thermal hyperalgesia, as evidenced by a significant decrease in paw withdrawal latency (Fig. 1B), on the ipsilateral side compared with pre-inoculation baseline values ($n = 6$). These pain hypersensitivities became apparent between 5 and 7 days and remained pronounced for at least 12 days. Some rats displayed substantial guarding behaviors, especially at later time points after PCC injection. No marked changes in paw withdrawal threshold or latency were observed on the contralateral side after PCC injection (Fig. 1A and B). As expected, HBSS injection did not alter basal

paw withdrawal responses to mechanical or thermal stimuli on either the ipsilateral or contralateral side ($n = 6$; Fig. 1A and B).

Radiography revealed significant cortical destruction of the proximal epiphysis on day 12 after PCC injection (Fig. 1C). No radiological change was observed in rats that received HBSS injection (Fig. 1C). Furthermore, histological examination showed that PCCs were densely packed in the narrow cavity and induced the destruction of trabeculae by day 12 after PCC injection (Fig. 1D).

Effect of i.th. rapamycin on the development of PCC-induced bone cancer pain

To evaluate the role of spinal mTOR in the development of bone cancer pain, we administered rapamycin i.th. once daily for 7 days after injection of HBSS or PCCs into the tibia. To examine its specificity, we used ascomycin, which does not inhibit mTOR activity^{15, 17}, as a control. I.th. administration of 10 μg rapamycin ($n = 5$), but not 10 μg ascomycin ($n = 5$), abolished PCC-induced mechanical allodynia and thermal hyperalgesia (Fig. 2A and B). The effects of rapamycin were dose dependent (Fig. 2C and D). On day 7 after PCC inoculation, the 10- μg dose of rapamycin increased paw withdrawal threshold by 4.84 fold ($p < 0.01$), the 1- μg dose by 3.74 fold ($n = 5$, $p < 0.05$), and the 0.1- μg dose by 1.98 fold ($n = 5$, $p > 0.05$) compared to the corresponding PCC-injected group treated with vehicle (50% DMSO; $n = 5$, Fig. 2C). Similarly, on day 7 after PCC injection, the 10- μg dose of rapamycin increased paw withdrawal latency by 1.35 fold ($n = 5$, $p < 0.01$), the 1- μg dose by 1.22 fold ($n = 5$, $p < 0.05$), and the 0.1- μg dose by 1.04 fold ($n = 5$, $p > 0.05$) compared to the corresponding PCC-injected group treated with vehicle ($n = 5$, Fig. 2D). These effects are reversible because PCC-induced mechanical allodynia and thermal hyperalgesia were observed on day 10 after cessation of i.th. administration of 10 μg rapamycin on day 7 post-PCC injection (data not shown). Neither the vehicle, nor 10 μg of rapamycin or ascomycin significantly altered basal paw withdrawal responses to mechanical or thermal stimuli applied to the contralateral hind paw (data not shown). Likewise, 10 μg rapamycin alone did not affect basal paw withdrawal responses to mechanical or thermal stimuli in HBSS-treated rats ($n = 5$, Fig. 2A and B).

Effect of i.th. rapamycin on the maintenance of PCC-induced bone cancer pain

To further examine the role of spinal mTOR in the maintenance of bone cancer pain, we administered rapamycin, ascomycin, or vehicle i.th. once daily for 5 days beginning on day 7 after rats were injected with HBSS or PCCs. Ten micrograms of rapamycin ($n = 6$), but not ascomycin ($n = 6$), significantly reduced PCC-induced mechanical allodynia and thermal hyperalgesia during the maintenance period (Fig. 3A and B). The effects of rapamycin were dose-dependent (Fig. 3C and D). On day 12 after PCC injection, the 10- μg dose of rapamycin increased paw withdrawal threshold by 5.2 fold ($p < 0.01$) and the 1- μg dose increased paw withdrawal threshold by 2.34 fold ($n = 6$, $p < 0.05$) compared to the corresponding PCC-injected group treated vehicle ($n = 6$, Fig. 3C). The 0.1- μg dose of rapamycin had no effect on paw withdrawal threshold ($n = 6$, $p > 0.05$). Similarly, on day 12 after PCC injection, the 10- μg dose of rapamycin increased paw withdrawal latency by 1.32 fold ($n = 6$, $p < 0.01$) and the 1- μg dose increased paw withdrawal latency by 1.13-fold ($n = 6$, $p < 0.05$), but the 0.1- μg dose had no effect ($n = 6$, $p > 0.05$) compared to the corresponding PCC-injected group treated with vehicle ($n = 6$; Fig. 3D). As expected, vehicle, rapamycin, and ascomycin did not alter basal paw withdrawal responses to mechanical and thermal stimuli applied to the contralateral hind paw during the maintenance period (Data not shown). Rapamycin alone did not affect basal paw withdrawal responses to mechanical or thermal stimuli in HBSS-treated rats during the maintenance period ($n = 6$, Fig. 3A and B).

Effect of i.th. rapamycin on locomotor function

To exclude the possibility that the effect of i.th. rapamycin on PCC-induced pain behaviors was caused by impaired locomotor functions (or reflexes), we examined locomotor functions of experimental animals. As shown in Table 1, the rats treated with either rapamycin or ascomycin exhibited normal locomotor functions, including placing, grasping, and righting reflexes. Convulsions and hypermobility were not observed in any of the treated rats. In addition, we did not observe any significant difference in general behaviors, including spontaneous activity, between the vehicle-treated and the drug-treated rats.

Activation of mTOR and p70S6K in spinal dorsal horn and dorsal root ganglion after PCC injection

The pharmacological studies described above suggest that mTOR and its downstream effectors are activated at the spinal cord level after PCC injection. To address our conclusion, we examined the activity of mTOR and p70S6K by assessing their phosphorylation levels in L₄₋₅ spinal dorsal horns and DRGs. We found that PCC injection produced time-dependent increases in the levels of phosphorylated (p-) mTOR and p-p70S6K in the ipsilateral dorsal horn when compared with those of naïve rats (0 d) (n = 5/ time point, Fig. 4A and B). Significant increases began at day 3 and were maintained for at least 12 days after PCC injection (Fig. 4A and B). In contrast, p-mTOR and p-p70S6K expression was significantly increased in the ipsilateral DRGs only at 3 days post-injection (Fig. 5A and B). As expected, the expression levels of p-mTOR and p-p70S6K in the contralateral dorsal horn (Figs. 4C and D) and DRG (data not shown) were not altered during the observation period. HBSS injection did not change the basal level of p-mTOR or p-p70S6K in the dorsal horn or DRG on either side (data not shown). These results indicate that dorsal horn mTOR and its downstream effector p70S6K are activated during the development and maintenance of bone cancer pain. Quantitative Western blot analysis showed that PCC injection did not lead to significant changes in the levels of total mTOR or p70S6K in the ipsilateral or contralateral L₄₋₅ dorsal horn or DRG within the 12-day period (Figs. 4 and 5), indicating that PCC-induced bone cancer pain input alters the phosphorylation status of mTOR and p70S6K rather than total protein expression.

Role of spinal NMDA receptors in PCC-induced activation of mTOR and p70S6K in spinal cord

Finally, we investigated how mTOR and its downstream pathway are activated in dorsal horn after PCC injection. Preclinical and clinical research has shown that blocking NMDA receptors produces a significant analgesic effect on bone cancer pain^{20, 21, 26, 32}, suggesting that NMDA receptors are required for the induction of bone cancer-evoked pain hypersensitivities. To test whether spinal NMDA receptors are involved in PCC-induced activation of dorsal horn mTOR and its downstream pathway, the rats were infused i.th. with saline or DL-AP5, a selective NMDA receptor antagonist, at a constant flow rate of 2 µg/µL/h via syringe pump. One hour after infusion, HBSS or PCCs were injected into the tibia. Behavioral testing was carried out 1 day before drug infusion and on days 3, 5, and 7 after PCC or HBSS injection. Consistent with previous reports^{26, 32}, DL-AP5 abolished PCC-induced mechanical allodynia and thermal hyperalgesia (Fig. 6A and B). DL-AP5 alone did not alter basal paw withdrawal responses of either hind paw to mechanical and thermal stimuli (Fig. 6). After behavioral testing on day 7, ipsilateral L₄₋₅ dorsal horn was collected for Western blot analysis. PCC injection significantly increased the levels of p-mTOR ($203 \pm 0.84\%$, n = 5, $p < 0.01$) and p-p70S6K ($357 \pm 1.81\%$, n = 5, $p < 0.01$) in the ipsilateral dorsal horn of rats pretreated with saline, but not in those pretreated with DL-AP5 (n = 5, Fig. 6C and D). DL-AP5 alone did not significantly affect basal levels of p-mTOR and p-p70S6K in dorsal horn of HBSS-injected rats (n = 5, Fig. 6C and D).

Our double-labeling studies showed that mTOR and p70S6K colocalized with the NMDA receptor subunit NR1 in dorsal horn neurons (Fig. 7). Approximately 62.5% of mTOR-positive neurons and 40% of p70S6K-positive neurons in dorsal horn were positive for NR1 ($n = 3$). We were unable to obtain cellular distributions of p-mTOR and p-70S6K in dorsal horn from Copenhagen rats, although we have previously found these proteins to be localized in dorsal horn of Sprague-Dawley rats using same antibodies³⁴.

Taken together, these findings suggest that NMDA receptors mediate PCC-induced activation of mTOR and its downstream effectors in dorsal horn under bone cancer pain conditions.

Discussion

Metastasis to bone is the most common cause of cancer-related pain. Although opioids and nonsteroidal anti-inflammatory drugs have been used in cancer pain treatment, they have limited efficacy and produce serious side effects. Uncovering the mechanisms that underlie pain hypersensitivity in cancer-related pain may lead to novel therapeutic strategies for prevention and/or treatment of this disorder. The injection of cancer cells into bone produces persistent pain hypersensitivities in rodent models that mimic the clinical pain of patients with bone metastases. Herein, we report that spinal cord mTOR may be required for the induction and maintenance of PCC-induced bone cancer pain, making it a potential new target for the treatment of cancer-related pain.

Several bone cancer pain models have been developed. Schwei et al.²⁷ first reported a mouse model in which osteolytic sarcoma cells derived from a spontaneous connective tissue cancer were injected into the femur of syngeneic C3H mice. A female rat model was subsequently developed in which MRMT-1 rat mammary gland carcinoma cells were injected into the tibia²¹. Because rats are more frequently used in studies than mice, and sexual hormones have profound effects on pain⁸, a male rat model was recently established in which AT-3.1 PCCs are injected into the tibia³⁹. Consistent with previous studies^{38, 39}, we observed that PCC injection produced significant and persistent mechanical allodynia and thermal hyperalgesia on the ipsilateral side. The degree of mechanical allodynia and the time at which mechanical allodynia and thermal hyperalgesia occurred differed somewhat from those in previous reports^{38, 39}. These discrepancies may be related to differences in concentrations of the injected PCCs and locations of von Frey filament application in hind paw (plantar side vs lateral edge).

In our study, mTOR and its downstream effectors in dorsal horn and DRG were activated under PCC-induced cancer pain conditions. We found that phosphorylation of mTOR and p70S6K in the ipsilateral L₄₋₅ dorsal horns was significantly increased on days 3, 5, 7, and 12 after PCC injection. It is likely that these increases occurred in neurons, as PCC injection does not change the levels of total mTOR and p70S6K in dorsal horn and mTOR and p70S6K are detected in dorsal horn neurons, but not in dorsal horn glial cells, in naïve rats³⁴ and in the rats with PCC injection (data not shown). This conclusion is also supported by the evidence that blocking NMDA receptors (that are expressed predominantly in dorsal horn neurons) abolished PCC-induced increases in dorsal horn p-mTOR and p-p70S6K. To further rule out the possibility that the increased phosphorylation occurs in dorsal horn glial cells, the double labeling of p-mTOR and p-p70S6K with the markers of dorsal horn astrocytes (e.g., GFAP) and microglia (e.g., OX-42) will be performed when the antibodies against p-mTOR and p-p70S6K are available for immunostaining in the tissues from Copenhagen rats.

It is worth noting that the increases in p-mTOR and p-p70S6K in dorsal horn did not coincide temporally with induction of mechanical allodynia and thermal hyperalgesia. This inconsistency may be related to the fact that protein translation and neuronal plastic changes take some time to occur after mTOR and its downstream effectors become activated. Given that neuronal plastic changes in dorsal horn are considered to underlie the mechanisms of cancer-related pain development^{6, 27, 29}, PCC-induced pain hypersensitivities develop at a time point after mTOR activation. In the ipsilateral L₄₋₅ DRGs, PCC-induced increases in p-mTOR and p-p70S6K were observed only on day 3 after PCC injection. The significance of this transient increase in the DRG is unclear, but might be related to cancer-induced neuronal plastic changes in the DRG.

NMDA receptors play a critical role in the activation of dorsal horn mTOR and p70S6K following PCC injection. Our pharmacologic study demonstrated that PCC-induced increases in dorsal horn p-mTOR and p-p70S6K were completely blocked by an NMDA receptor antagonist. Morphologic evidence further demonstrated colocalization of NMDA receptor subunit NR1 with mTOR and p70S6K in dorsal horn neurons. These *in vivo* findings suggest that NMDA receptors mediate PCC-induced activation of dorsal horn mTOR and p70S6K (Fig. 8). This conclusion is further supported by *in vitro* experiments in which brief glutamate/NMDA stimulation activates the mTOR signaling pathway in brain neurons¹⁹. How NMDA receptor activation triggers activation of dorsal horn mTOR and its downstream signals is unclear. An increase in $[Ca^{2+}]_i$ following NMDA receptor activation might activate the PI3K/Akt pathway⁴⁰. Phosphorylated Akt can activate mTOR in central neurons¹⁶, and peripheral inflammation initiates spinal activation of the PI3K/Akt/mTOR signaling pathway³⁵. It is very likely that PI3K/Akt participates in NMDA receptor-triggered dorsal horn activation of mTOR and its downstream effectors (Fig. 8). Increased $[Ca^{2+}]_i$ may also result from an inflow of extracellular Ca^{2+} through voltage-dependent calcium channels and Ca^{2+} -permeable AMPA/kainate receptors and from the mobilization of intracellular stores through the activation of group 1 metabotropic glutamate receptors. It is noteworthy that group 1 metabotropic glutamate receptors are coupled to the PI3K/Akt/mTOR pathway in brain neurons^{15, 24}. Whether these events are involved in PCC-induced dorsal horn activation of mTOR and its downstream effectors will be determined in our future studies.

In conclusion, we demonstrated that PCC-induced dorsal horn activation of the mTOR pathway was mediated by NMDA receptors under cancer pain conditions. We also showed that i.th. rapamycin dose-dependently attenuated PCC-induced mechanical allodynia and thermal hyperalgesia during both development and maintenance of cancer-related pain. Moreover, i.th. rapamycin did not affect acute basal nociceptive responses or locomotor functions. Our recent work has also indicated that i.th. rapamycin does not produce significant systemic side effects such as immunosuppression (data not shown). Given that mTOR inhibitors are FDA-approved drugs that have been used clinically for organ transplantation⁹ and that phase II clinical trial of mTOR inhibitors demonstrates significant effects on cancer progression in patients^{1, 11, 28, 36}, local spinal mTOR inhibition may represent a promising novel strategy for treating cancer-related pain.

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Fig. 1.

The establishment of a cancer-related pain model in male rats. (A and B) Injection of AT-3.1 prostate cancer cells (4.5×10^5 cells/15 μ L dissolved in Hanks' balanced salt solution) into the tibia induced mechanical allodynia (A) and thermal hyperalgesia (B) on the ipsilateral (ipsi), but not contralateral (contra), side. Hanks' balanced salt solution injection, used as a control, had no effect on ipsilateral or contralateral paw withdrawal responses. $n = 6/\text{group}$. ** $p < 0.01$ vs the corresponding Hnaks-injected side. (C) Radiographs of tibiae 12 days after cancer cell or Hanks injection. The arrowhead shows the injection site. Arrows indicate structural destruction of the proximal cortical bone. (D) Hematoxylin & eosin (HE) staining of the proximal cortical bones 12 days after cancer cell or Hanks injection. Note that tumor cells were densely packed in the narrow cavity and caused destruction of trabeculae. Scale bar: 150 μ m.

Fig. 2.

Effect of intrathecal administration of rapamycin on the development of cancer-induced mechanical allodynia and thermal hyperalgesia. (A and B) Pretreatment with rapamycin (Rap), but not with ascomycin (Asc), completely blocked cancer-induced decreases in ipsilateral paw withdrawal threshold (A) and latency (B). Rapamycin alone did not affect basal responses to mechanical (A) and thermal (B) stimuli on the ipsilateral side in rats injected with Hanks' balanced salt solution. Vehicle (50% DMSO). $n = 5/\text{group}$. $\#p < 0.05$, $\#\#p < 0.01$ vs the corresponding baseline. (C and D) Cancer-induced decreases in ipsilateral paw withdrawal threshold (C) and latency (D) were significantly attenuated by 1- μg and 10- μg doses of rapamycin, but not by the 0.1- μg dose. $n = 5/\text{group}$; $*p < 0.05$, $**p < 0.01$ vs the corresponding vehicle-treated group. $\#\#p < 0.01$ vs the corresponding baseline.

Fig. 3.

Effect of intrathecal administration of rapamycin on the maintenance of cancer-induced mechanical allodynia and thermal hyperalgesia. (A and B) Post-treatment with rapamycin (Rap), but not with ascomycin (Asc), significantly attenuated cancer-induced decreases in ipsilateral paw withdrawal threshold (A) and latency (B). Rapamycin did not alter basal responses to mechanical (A) and thermal (B) stimuli on the ipsilateral side in rats injected with Hanks' balanced salt solution. V: vehicle (50% DMSO). $n = 6/\text{group}$. Cancer-induced decreases in ipsilateral paw withdrawal threshold (A) and latency (B) were significantly attenuated by 1- μ g and 10- μ g doses of rapamycin, but not by the 0.1- μ g dose. $n = 6/\text{group}$; * $p < 0.05$, ** $p < 0.01$ vs the corresponding vehicle-treated group.

Fig. 4.

Time-dependent activation of mTOR and p70S6K in L₄₋₅ dorsal horn after cancer cell injection. (A and B) The levels of phosphorylated (p-) mTOR (A) and p-p70S6K (B) were significantly increased on the ipsilateral side on days 3, 5, 7, and 12 after cancer cell injection. The levels of neither p-mTOR (C) nor p-p70S6K (D) were changed on the contralateral side after cancer cell injection. Cancer cell injection did not alter expression of total mTOR (A and C) or total p70S6K (B and D) in L₄₋₅ dorsal horns on either side. *n* = 5/group; * *p* < 0.05, ** *p* < 0.01 vs the corresponding naïve rats (0).

Fig. 5.

Transient activation of mTOR and p70S6K in L₄₋₅ DRG after PCC injection. (A and B) The levels of phosphorylated (p-) mTOR (A) and p-p70S6K (B) were significantly increased on the ipsilateral side only on day 3 after cancer cell injection. Cancer cell injection did not alter expression of total mTOR (A) or total p70S6K (B) on the ipsilateral side. n = 5/group. * $p < 0.05$, ** $p < 0.01$ vs the corresponding naïve rats (0).

Fig. 6.

Effect of blocking NMDA receptors on cancer-induced mechanical allodynia and thermal hyperalgesia and on activation of dorsal horn mTOR and p-70S6K. (A and B) Intrathecal infusion of DL-AP5 (AP5) completely blocked cancer-induced decreases in ipsilateral paw withdrawal threshold (A) and latency (B) on the ipsilateral side. DL-AP5 alone did not change basal responses to mechanical (A) or thermal (B) stimuli on the ipsilateral side in rats injected with Hanks' balanced salt solution. $n = 5/\text{group}$. * $p < 0.05$, ** $p < 0.01$ vs the corresponding baseline (0 day). (E and F) Intrathecal infusion of DL-AP5 significantly repressed cancer-induced increases in the levels of p-mTOR (C) and p-p70S6K (D) in dorsal horn on day 7 after cancer cell injection. H, Hanks; S, saline; V, vehicle (50% DMSO); C, cancer cells. $n = 5/\text{group}$. ** $p < 0.01$ vs the Hnaks-injected group that received saline infusion. ## $p < 0.01$ vs the cancer cell-injected group that received saline infusion.

Fig. 7. Colocalization of NMDA receptor subunit NR1 with mTOR and p70S6K in dorsal horn neurons. Immunofluorescent colocalization (arrows) of red reaction products for mTOR and p70S6K and green products for NR1. Scale bar: 30 μ m.

Fig. 8.

Proposed model for spinal cord NMDA receptor (NMDAR)-mediated activation of mTOR and its downstream effectors under cancer pain conditions. Under normal conditions, Mg^{2+} blocks NMDAR activity, and intracellular kinases including mTOR are inactive. Cancer cell-caused peripheral noxious insult triggers the removal of Mg^{2+} and NMDAR activation. Ca^{2+} influx through NMDARs may activate intracellular PI3K/Akt. The latter may phosphorylate mTOR. The active mTOR phosphorylates p70S6K and 4E-BP1, which lead to the initiation of protein translation under cancer pain conditions.

Table 1

Mean (SEM) changes in locomotor function

Treatment	Functional test		
	Placing	Grasping	Righting
Vehicle (50% DMSO)	5 (0)	5 (0)	5 (0)
Rapamycin (10 µg)	5 (0)	5 (0)	5 (0)
Ascomycin (10 µg)	5 (0)	5 (0)	5 (0)

N = 5/group, 5 trials.