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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 $L_{4\text{-}5}$ dorsal horn and transiently in $L_{4\text{-}5}$ 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.*

Key words: *mTOR, NMDA receptors, activation, dorsal horn, cancer pain.*

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

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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, 2 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 horn.[3] 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 to 225 g (Harlan, Frederick, MD) were housed on a standard 12-hour 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 to 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-amno-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 .05% trypsin and .02% EDTA. Before being injected into the rats, the detached cells were first collected by centrifuging 10 mL of medium for 3 minutes at 1,200 rpm. The resulting pellet was washed twice with 10 mL of calcium- and magnesium-free HBSS and centrifuged again for 3 minutes 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 μL 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 $L_{4\text{-}5}$ 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 to 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 administrated intrathecally in a 10-μL volume followed by 12 μL 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 μg/μL) were continuously infused for the experimental period at a rate of 1 μL/hour.