Increased Prostaglandin E₂ Release and Activated Akt/β-Catenin Signaling Pathway Occur after Opioid Withdrawal in Rat Spinal Cord

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Background: Prostaglandin E₂ is an important spinal modulator of nociception. However, the effects of chronic opioid administration and withdrawal on prostaglandin E₂ release and associated signaling pathways in the spinal cord are generally unknown.

Methods: This study sought to examine these effects using a spinal microdialysis technique in a model of chronic morphine administration and withdrawal in the rat.

Results: The authors found that spinal prostaglandin E₂ release was unaffected by chronic morphine treatment but was significantly increased during withdrawal. Recurrent withdrawal did not further enhance this release. The authors also found up-regulation of cyclooxygenase-2 expression and phosphorylation of protein kinase Akt at Ser-473 in response to opioid withdrawal. In addition, they demonstrated that β-catenin, a transcription factor downstream of Akt, was induced during morphine withdrawal, particularly during recurrent withdrawal.

Conclusions: These results suggest that opioid withdrawal activates signaling pathways associated with neuronal survival and transcriptional control, two processes implicated in neuronal development and synaptic plasticity.

OPIOIDS are used extensively for chronic pain as analgesics. It is well established that chronic opioid administration leads to tolerance, and withdrawal induces hyperalgesia in humans and animals.¹,² A number of animal studies have shown that chronic administration of opioid also induces hyperalgesia, although similar opioid-induced pain in humans is still controversial.²⁻⁵ In addition to being modulated by specific regions of the brain such as supraspinal loci, the periaqueductal gray, and the rostral ventromedial medulla,⁶ these potentially antinociceptive effects also seem to be spinally mediated, as evidenced by the ability of a variety of agents to block the phenomena when administered directly to the spinal cord.³⁻⁶

Cyclooxygenase is the rate-limiting enzyme in the formation of prostaglandins including prostaglandin E₂ (PGE₂).⁷,⁸ Substantial evidence suggests that prostaglandins act as modulators of spinal nociception. Cyclooxygenase, in particular cyclooxygenase 2, is present in the spinal cord.⁷,⁹ Previously, we have demonstrated that spinal administration of ibuprofen, a nonselective cyclooxygenase inhibitor, blocks the hyperalgesia associated with opioid withdrawal in a stereospecific and dose-dependent manner,⁴ indicating that spinal PGE₂ may be involved in withdrawal-mediated hyperalgesia.

The Akt (protein kinase B) pathway has critical functions in the regulation of cell survival, cell cycle, and glycogen synthesis.¹⁰,¹¹ In vitro studies have shown that the phosphoinositide 3-kinase pathway involving Akt enhances the μ-opioid receptor desensitization after chronic treatment with agonist.¹² Stimulation of the μ-opioid receptor has been reported to promote neuronal survival in a phosphoinositide 3-kinase and Akt-dependent signaling cascade.¹³,¹⁴ A recent study has demonstrated that Akt is activated in the rat nucleus accumbens and caudate putamen in response to acute and chronic morphine administration.¹⁵ All of these studies implicate a role for Akt in opioid-mediated effects.

To date, the effect of opioid withdrawal, in vivo, on the regulation of PGE₂ release, Akt activity, and the associated downstream effectors such as β-catenin has not been reported. In this study, we used a microdialysis technique to measure spinal PGE₂ release after withdrawal in rats chronically treated with morphine. A similar treatment model has previously been shown to induce hyperalgesia in rats.³,¹⁶ We further examined the associated signaling pathways using Western analysis. This is the first report showing that the expression of β-catenin is induced in response to withdrawal, especially recurrent withdrawal, during chronic morphine administration.

Materials and Methods

Animals

Approval for this study was obtained from the Institutional Animal Care and Use Committee of Baystate Medical Center, Springfield, Massachusetts. Male Sprague-Dawley rats (approximately 350 g) were housed in individual cages with free access to food and water on a 12-h light and dark cycle. All procedures and tests were performed during the light cycle.

Drugs and Chemicals

The drugs and dosages used in this study were morphine implant pellet (75 mg; Murty Pharmaceuticals, Lexington, KY) and naloxone hydrochloride injections...
(0.1 mg/kg; Sigma Chemical Co., St. Louis, MO). Both drugs were administered subcutaneously.

The rabbit polyclonal cyclooxygenase-2, phosphor-Akt (Ser-473), and Akt antibodies were from Cell Signaling (Beverly, MA). The mouse monoclonal β-catenin antibody was from Santa Cruz Biotechnology Inc (Santa Cruz, CA). The mouse monoclonal actin antibody was from Sigma.

**Implantation Surgery**

Each animal underwent implantation of an intrathecal catheter for microdialysis and a subcutaneous catheter for daily drug delivery. The preparation of the catheters was described previously.17 For intrathecal catheter placement, the rat was anesthetized and placed in a stereotaxic head holder. A midline incision was made to expose the atlanto-occipital membrane. The membrane was then pierced, and the loop end of the microdialysis catheter passed intrathecally to a distance of 7 cm, caudal to the level of the thoracolumbar junction. For subcutaneous catheter implantation, the catheter was passed behind the shoulder and placed through the lower end of the incision. A morphine or sham pellet was also placed subcutaneously behind the rat’s shoulder. The incision was then sutured, and the animal was let to recover. Animals showing signs of neurologic deficit after surgery or animals at the end of each experimental paradigm were killed with barbiturate overdose.

**Experimental Paradigms**

Each animal was subjected to one of the following treatments: (1) sham pellet and saline on days 2, 3, and 4 (control group, SSS); (2) morphine pellet and saline on days 2, 3, and 4 (morphine group, MSS); (3) morphine pellet and saline on days 2 and 3 with naloxone on day 4 (acute withdrawal group, MSN); or (4) morphine pellet and naloxone on days 2, 3, and 4 (recurrent withdrawal group, MNN). The morphine dosage and treatment paradigm were chosen based on previous findings that a steady state level of plasma morphine concentration could be maintained until the pellets were removed at 72 h.18 Naloxone, an opioid receptor antagonist, was used to produce morphine withdrawal. In previous studies,3,16 we have demonstrated that by day 4 of the treatment, recurrent withdrawal significantly decreases thermal latencies below controls (an indication of hyperalgesia development) and decreases morphine effectiveness (an indication of tolerance development). Therefore, PGE2 levels and relevant signaling molecules were examined on day 4.

**Microdialysis**

On day 4, the rat was transferred to an individual restrainer. One end of the microdialysis catheter was connected to an inflow tube, which was attached to a syringe pump with a flow rate of 10 µl/min and filled with an artificial cerebrospinal fluid. The fluid consisted of 123 mM NaCl, 3.75 mM KCl, 1.25 mM KH2PO4, 1 mM MgSO4, 2 mM CaCl2, 26 mM NaHCO3, and 10 mM dextrose (pH 7.4). The other end of the microdialysis catheter was connected to an outflow tube for sample collection. After a stabilization period of 30 min, a baseline sample was collected for 10 min. Additional samples were then collected at 10-min intervals for 60 min after an injection of either naloxone or saline. All dialysis samples were kept on ice during collection and were stored at −70°C before analysis.

**Analysis of PGE2 Concentrations**

The PGE2 concentrations were determined with the Correlate-EIA Prostaglandin E2 Enzyme Immunoassay kit (Assay Designs Inc., Ann Arbor, MI) according to the manufacturer’s instructions.

**Spinal Cord Lysate Preparation and Immunoblotting**

Spinal cords were harvested at the end of the experiments by a pressure ejection method described by the group of Alain Beaudet, M.D., Ph.D. (Professor, Montreal Neurologic Institute, McGill University, Montreal, Québec, Canada). After homogenizing spinal cords, lysates were obtained by incubating at 4°C for 30 min followed by centrifuging at 12,000 rpm for 20 min in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 100 mM NaF, 10 mM MgCl2, and protease inhibitor cocktail (Sigma). Protein content was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Lysates (40 µg protein) were separated on a 10% polyacrylamide gel in the presence of 0.1% SDS with 15 µl prestained protein standards (0.1 mg/ml of each protein; Cell Signaling). After transfer, the membrane was blocked and then probed with antibodies against the proteins of interest as suggested by the manufacturers. The membrane was then incubated with a peroxidase-conjugated secondary antibody. Immunoreactive bands were developed with a chemiluminescence reagent from Amersham (Arlington Heights, IL). All blots were probed with antiactin to normalize for loading differences. Quantification of gels was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

**Statistical Analysis**

The level of PGE2 was presented as means ± SEM in pmol/µl. For Western analysis, in addition to a representative blot, the normalized changes of examined proteins were summarized as mean ± SEM. The differences in PGE2 levels and protein expressions between treatments were analyzed by one-way analysis of variance followed by Newman-Keuls multiple comparison test. The significant P values were given under specific figure legends.
Results

Increased PGE2 Release after Morphine Withdrawal

As shown in figure 1, baseline PGE2 release was not significantly different across all treatment groups, suggesting that chronic morphine administration, implantation, and the infusion procedure had no significant effect on PGE2 release. Compared with controls (SSS), levels of PGE2 in the morphine-treated group (MSS) did not show any significant changes. However, the PGE2 levels in animals subjected to either acute (MSN) or recurrent (MNN) withdrawal increased significantly at 10, 20, and 30 min after naloxone injection. The peak PGE2 levels at 20 min was $935 \pm 1100$ and $1030 \pm 1000$ pmol/l in the MSN and MNN groups, respectively. There were no significant differences in the PGE2 levels between these two groups at all time points tested.

Increased Cyclooxygenase-2 Expression after Morphine Withdrawal

We then investigated whether cyclooxygenase-2 expression was changed in response to morphine treatment and withdrawal. Using a rabbit polyclonal antibody against endogenous levels of total cyclooxygenase 2, we detected a protein band around 74 kd, as suggested by the manufacturer. A representative blot is shown in figure 2A; the summary of fold changes in cyclooxygenase-2 expression after normalized to actin is shown in figure 2B. Compared with the control group, cyclooxygenase-2 expression was increased approximately by 1.89 $\pm$ 0.27- and 2.08 $\pm$ 0.17-fold in the MSN and MNN groups, respectively. The increase of cyclooxygenase-2 protein in the MSN or MNN group was also significantly higher than that in the MSS group (fig. 2B).

Increased Activation of Akt after Morphine Withdrawal

Next, we examined the activation of Akt in the spinal cords of rats after morphine treatment and withdrawal. Akt is activated by phosphorylation at Thr-308 and Ser-473.10 The expression of phosphor-Akt at Ser-473 (p-Akt), which represents endogenous levels of Akt1, Akt2, and Akt3 when phosphorylated at the corresponding residues, was used as an indicator of Akt activation. The total Akt antibody used detects endogenous levels of total Akt1, Akt2, and Akt3 protein. Both phosphor-Akt and total Akt are around 60 kd (fig. 3A). The relative expression of phosphor-Akt was normalized to total Akt protein, and the result is shown in figure 3B. In response to morphine treatment alone, the activated Akt was decreased by approximately 20% when compared with controls. During withdrawal, the normalized Akt phosphorylation was increased by a respective 2.25 $\pm$ 0.29- and 2.09 $\pm$ 0.17-fold in the MSN and MNN groups. Activation of Akt after either acute or recurrent withdrawal was also significant when compared with the MSS group (fig. 3B).
Increased Induction of β-Catenin after Morphine Withdrawal

We also investigated whether morphine treatment and withdrawal had any effects on the expression of β-catenin protein. The monoclonal antibody used detects β-catenin specifically with a molecular weight of 92 kd. Under the conditions used, we consistently observed multiple bands around the molecular weight (fig. 4A). Because it is quite probable that the multiple bands were the result of posttranslational modifications to β-catenin, we included all the bands observed for quantification purposes. In response to withdrawal, the expression of β-catenin in the spinal cord increased 1.55 ± 0.14-fold in the MSN group and 2.81 ± 0.60-fold in the MNN group. The induction of β-catenin by recurrent withdrawal was also significantly increased when compared with the MSS and MSN groups (fig. 4B).

Discussion

In this study, we determined whether spinal PGE₂ levels were altered during chronic opioid administration or during opioid withdrawal. Our results clearly showed that PGE₂ release was increased in response to opioid withdrawal but was unaffected by chronic opioid treatment. The basal spinal PGE₂ levels observed in our study correlate well with previously published reports. In addition, we examined whether recurrent opioid withdrawal was associated with enhanced release of PGE₂, as has been found to occur with spinal excitatory amino acids, specifically glutamate. Our results showed that recurrent withdrawal did not further increase the basal or the precipitated PGE₂ release. Furthermore, we observed that in response to morphine withdrawal, there were up-regulation of spinal cyclooxygenase-2 expression, activation of protein kinase Akt, and induction of β-catenin. Similar changes of these proteins were observed in another set of animals subjected to the same treatments without intrathecal catheter implantation.

Our results demonstrate that withdrawal is a key factor for triggering the release of PGE₂ in the spinal cord during chronic morphine administration. The expression of the cyclooxygenase isoforms is differentially regulated. Basal levels of PGE₂ are normally maintained by cyclooxygenase-1 expression, whereas increased PGE₂
levels are usually generated by cyclooxygenase 2.7 Consistent with increased PGE₂ levels, we observed that cyclooxygenase-2 expression is up-regulated in response to withdrawal. These results provide direct evidence showing the involvement of PGE₂ and cyclooxygenase 2 in opioid withdrawal-mediated phenomena, adding strong support to our previous findings that spinally administered cyclooxygenase inhibitors could significantly attenuate opioid withdrawal hyperalgesia.4 In addition, these results are consistent with previous studies from other groups showing that inhibitors of prostaglandin synthesis reduce signs of opioid withdrawal in rats.22 Spinally administered prostaglandin inhibitors have also been previously reported to inhibit the development of opioid tolerance.23,24

Prostaglandin E₂ produces its effects through the PGE₂ receptor, a G protein–coupled receptor. It has been shown that G protein–coupled receptors are able to activate the phosphoinositide 3-kinase and Akt signal transduction pathway.25 Therefore, we examined the effects of chronic morphine treatment and withdrawal on Akt activation. Our data show that phosphorylation of Akt at Ser-473, an indicator of Akt activation, is increased during morphine withdrawal but not during chronic morphine administration. These results correlate well with our PGE₂ and cyclooxygenase-2 data. They are also in agreement with previous studies showing that Akt is involved in opioid signaling.12–15 The only in vitro study, to date, regarding changes of phosphorylated Akt levels after chronic morphine administration was in the rat nucleus accumbens.15 In line with their observations, our results demonstrate that phosphorylation of Akt and the ratio of phosphorylated Akt to Akt declined in rats subjected to morphine treatment alone. In addition, for the first time, we show that both acute and recurrent withdrawal enhance Akt activation in the rat spinal cord.

β-Catenin is one of the downstream effectors of Akt and plays a dual role in the maintenance and regulation of cell–cell interactions as well as the regulation of gene activity.26 It interacts with adhesion molecules such as cadherins, regulators of the synaptic remodeling process, to influence synaptic size and strength, thus coordinating synaptic plasticity.27,28 Recent studies have also show that β-catenin forms complexes with the tumor suppressor gene product adenomatous polyposis coli and directly interacts with and constitutively activates transcription factors of the T-cell factor/lymphoid enhancer factor gene family.29We demonstrated here for the first time that morphine withdrawal, especially recurrent withdrawal, induced the expression of β-catenin, suggesting that the Akt/β-catenin cascade is involved in modulation of nociception during acute and recurrent opioid withdrawal.

A number of spinal mechanisms have been proposed for opioid withdrawal mediated phenomena. During withdrawal, there is a spinal release of various neurotransmitters and mobilization of second messengers such as protein kinase A, protein kinase C, and nitric oxide. There is also increased production of spinal dynorphin, substance P, and calcitonin gene–related peptide during extended opioid exposure.1,2,29 It is known that withdrawal is associated with the spinal release of glutamate, and recurrent withdrawal results in sustained increases of glutamate.3,21 Spinal release of glutamate with activation of N-methyl-D-aspartate receptors has been suggested as a principle mechanism of both opioid withdrawal hyperalgesia and tolerance.1,29,30 The mechanism of glutamate elevation after recurrent withdrawal might be caused by increased N-methyl-D-aspartate receptor activity from positive feedback of enhanced protein kinase C activity.21 Correlating well with increased glutamate levels, we observed that β-catenin protein is markedly enhanced by recurrent withdrawal. Because β-catenin, through interactions with cadherins, can regulate the induction of long-term potentiation,31,32 increased β-catenin could result in a persistent enhancement of synaptic transmission. Therefore, increased β-catenin may be another mechanism that underlies the sustained increase in glutamate levels found after recurrent opioid withdrawal.

On the other hand, evidence has suggested that withdrawal-mediated hyperalgesia may occur independently of opioid tolerance. Our previous studies, using a spontaneous periodic abstinence model, showed that hyperalgesia and tolerance may evolve as distinct entities in the same animal.16–18 Other studies also demonstrate that different mechanisms may be responsible for the development of tolerance and the manifestations of withdrawal.34,35 Differential cellular adaptations may underlie this differential regulation of hyperalgesia and tolerance.36,37

It should be pointed out that the μ-opioid receptor, which mediates the analgesic properties of morphine, is also a G protein–coupled receptor. It is possible that increased Akt activity and β-catenin expression are not consequential events of increased PGE₂ release. Instead, they could be the direct result of morphine treatment or withdrawal. Support for this possibility is apparent in the following observations. In response to morphine treatment alone, PGE₂ release is unchanged; cyclooxygenase-2 and β-catenin expression show a trend of increase, whereas Akt activation is slightly decreased. In addition, compared with the morphine treatment group, both acute and recurrent withdrawal further enhance the expression of cyclooxygenase 2 and activation of Akt, but only recurrent withdrawal further induces the β-catenin expression. These results also suggest that the mechanisms underlining the changes of Akt and β-catenin may not be exactly the same. Further studies are needed to define the detailed interactions and relations of the signal transduction pathways involved.

In summary, our data show that PGE₂ release occurs during periods of withdrawal but not as a result of...
chronic opioid administration alone. In addition to increased PGE₂ levels, opioid withdrawal corresponds to an increase in cyclooxygenase-2 expression and protein kinase Akt activity, as well as induction of β-catenin. Substantial evidence has shown that activation of G protein–coupled receptors (opioid or PGE₂ receptors) could lead to Akt phosphorylation, and β-catenin has been reported to be a downstream effector of Akt.

Therefore, we propose that in response to morphine withdrawal, there is increased cyclooxygenase-2 expression and PGE₂ release in the spinal cord. Through G protein–coupled receptors, the increased PGE₂ levels activate the Akt and/or β-catenin cascade, thus modulating nociceptive responses during morphine withdrawal. These results suggest that nonsteroidal antiinflammatory drugs or cyclooxygenase inhibitors, when used as adjunctive agents, may negate the nociceptive effects of withdrawal during chronic opioid therapy.

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