Mechanism for Increased Expression of UGT2B in the Liver of Mice with Neuropathic Pain

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Approximately 30% of patients with cancer pain experience concurrent neuropathic pain. Since these patients are not sufficiently responsive to morphine, the development of an effective method of pain relief is urgently needed. Decreased function of the μ opioid receptor, which binds to the active metabolite of morphine M-6-G in the brain, has been proposed as a mechanism for morphine resistance. Previously, we pharmacokinetically examined morphine resistance in mice with neuropathic pain, and demonstrated that the brain morphine concentration was decreased, expression level of P-glycoprotein (P-gp) in the small intestine was increased, and expression level and activity of uridine diphosphate glucuronosyltransferase (UGT2B) in the liver were increased. In order to clarify the mechanism of the increased expression of UGT2B, we examined the phase of neuropathic pain during which UGT2B expression in the liver begins to increase, and whether this increased expression is nuclear receptor-mediated. The results of this study revealed that the increased expression of UGT2B in the liver occurred during the maintenance phase of neuropathic pain, suggesting that it may be caused by transcriptional regulation which was not accompanied by increased nuclear import of pregnane X receptor (PXR).

Key words  morphine; neuropathic pain; nuclear receptor

It has been reported that the onset of neuropathic pain is closely associated with the immune response around the damaged nerve. When a nerve is injured, histamine, inflammatory cytokines, and chemokines are released from mast cells located in the damaged nerve, inducing the activation and accumulation of neutrophils and macrophages to the nerve site. Subsequently, inflammatory cytokines and chemokines released from the accumulated cells sensitize the primary sensory nerves, increasing the release of pain transmitters from the primary sensory nerve endings, resulting in an unusual enhancement of the effect of the pain transmitters on secondary sensory nerves. The effect of pain transmitters on secondary sensory nerves is further augmented by the activation of microglia, a type of glia cell, in the spinal cord. This accelerates the release of inflammatory cytokines and other factors. It has also been reported that expression of the ligand–gated ion channel pregnane 2 receptor (P2X) receptor is increased in activated microglia. This induces the release of brain-derived neurotrophic factor (BDNF) from microglia, which binds to the tyrosine kinase receptor B (TrkB) receptor on secondary sensory nerves, downregulating the expression of K-Cl cotransporter 2 (KCC2). The reduced expression of KCC2 disinhibits GABAergic neurons, which suppress the sensation of pain under normal conditions, resulting in pain enhancement. The expression of P2X receptor and brain-derived neurotrophic factor is also increased by interferon regulatory factor 8 (IRF8), a transcription factor belonging to the interferon regulatory factor family.

A recent report states that the activation of astrocytes, another type of glia cell, is also associated with the onset of neuropathic pain. Microglia and astrocytes play different roles in pain development. The activity of microglia begins to increase immediately after nerve injury occurs, reaching a peak after 1 week, and then gradually declining. In a mouse neuropathic pain model, treatment with a microglia activation inhibitor starting immediately after model preparation suppressed the onset of neuropathic pain. In contrast, astrocytes became activated 1 week after nerve injury and were maintained in an activated state for at least 12 weeks. In a mouse neuropathic pain model, long-term treatment with an astrocyte activation inhibitor starting immediately after model preparation reduced pain 1 week after nerve injury and later. These findings suggest that microglia are involved in the induction of neuropathic pain and that the activation of astrocytes is closely associated with the maintenance of the disease; however, the detailed mechanism of neuropathic pain development remains unclear.

As described above, the pathogenesis of neuropathic pain and the effect of the disease on higher brain function have been widely investigated, however, the effect of neuropathic pain on peripheral organs remains unclear. We previously demonstrated that increased expression of uridine diphosphate glucuronosyltransferase (UGT)2B in the liver was a major cause of morphine resistance in neuropathic pain. The present study focused on nuclear receptors, which have been reported to regulate the expression of UGT2B in the liver. UGT2B expression in the liver is regulated primarily by the constitutive androstane receptor (CAR), a nuclear receptor and conjugating enzyme most highly expressed in the liver; this receptor is also found in the small intestine. In addition to conjugating enzymes such as UGT2B, metabolizing enzymes such as CYP2B and transporters such as multidrug resistance-associated protein 2 (MRP2) are known to target genes of CAR. Previous reports have indicated that another nuclear receptor, pregnane X receptor (PXR), also regulates the expression of UGT2B in the liver. In HepG2
human hepatocellular carcinoma cells, the addition of a ligand of PXR, aflatoxin B1, nearly doubled the mRNA expression level of UGT2B7, which is involved in morphine metabolism in humans, and nearly doubled the mRNA expression level of CYP3A4, a target gene of PXR. PXR shows the highest expression in the liver, with some expression also observed in the small and large intestines.\(^{40}\) The known target genes of PXR are conjugating enzymes such as UGT2B, metabolizing enzymes of the CYP3A subfamily,\(^{1,42}\) and transporters such as P-glycoprotein (P-gp).\(^{43-45}\) PXR shows low ligand specificity and binds to a wide range of chemicals and biological substances. The objective of the present study was to evaluate the effect of neuropathic pain on peripheral organs, in a mouse neuropathic pain model, by examining the phase of the disease in which the expression of UGT2B in the liver increases, and whether this increased expression is nuclear receptor-mediated.

**MATERIALS AND METHODS**

**Animal Handling** Male ICR mice (20–25g) were purchased from Japan SLC, Inc. (Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan). Mice were kept at room temperature (24±1°C) and 55±5% humidity with 12 h of light (artificial illumination; 08:00–20:00). Food and water were available and ad libitum. Each animal was used only once. The present study was conducted in accordance with the Guiding Principles for the Care and use of Laboratory Animals, as adopted by the Committee on Animal Research at Hoshi University.

**Neuropathic Pain Model** The mice were anesthetized with 3% isoflurane. We produced a partial sciatic nerve injury by tying a tight ligature with a 8-0 silk suture around approximately one-third to one-half the diameter of the sciatic nerve on the right side under a light microscope (SD30, Olympus, Tokyo). In sham-operated mice, the nerve was not ligated. Pain hypersensitivity to a heat stimulus was evaluated in mice with neuropathic pain using a paw flick test or tail flick test (Fruhstorfer et al., 1982; Kallina and Grau, 1995; Keizer et al., 2001; Keizer et al., 2008; Keizer et al., 2007; Mulder and Pritchett, 2004; Pitcher et al., 1999; Yashpal et al., 1982).

Antinociception induced by morphine was determined by tail-flick test or tail flick test (Fruhstorfer et al., 2001; Keizer et al., 2001; Keizer et al., 2007; Grau, 1995; Keizer, 2008; Keizer et al., 2007; Mulder and Pritchett, 2004; Pitcher et al., 1999). Pain hypersensitivity to a heat stimulus was measured by the von Frey test (Fruhstorfer et al., 2001; Keizer et al., 2007; Pitcher et al., 1999).

**RNA Preparation from the Tissue Samples** RNA was extracted from approximately 15 mg of frozen small intestine and brain using the TRI reagent (Life Technologies, U.S.A.). The resulting solution was diluted 50-fold using TE buffer, and the purity and concentration (µg/mL) of the RNA were calculated by measuring absorbance at 260 and 280 nm using a U-2800 spectrophotometer (Hitachi High Technologies, Tokyo, Japan).

**Quantitative-PCR** A high-capacity cDNA synthesis kit was used to synthesize cDNA from 1 µg of RNA. Tris-ethylenediaminetetraacetic acid (TE) buffer was used to dilute the cDNA 20-fold for the cDNA TE buffer solution. To each well of a 96-well PCR plate, 25 µL of iQ SYBR green supermix, 3 µL of forward primer of the target gene (5 pmol/µL), 3 µL of reverse primer (5 pmol/µL), 4 µL of cDNA TE buffer solution, and 15 µL of RNase-free water were added. The denaturation temperature was set at 95°C for 15 s, the annealing temperature was set at 56°C for 30 s, and the elongation temperature was set at 72°C for 30 s. The fluorescence intensity of the amplification process was monitored using the My iQ™ single color real-time reverse transcription (RT)-PCR detection system (Bio-Rad Laboratories). The mRNA expression levels were normalised using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1).

**Measurement of PXR Transported to the Nucleus in the Liver** Nuclear extracts were prepared using the NE-PER nuclear extraction kit according to the manufacturer’s instructions. CER II reagent was added to 100 mg of liver tissue. The mixture was homogenized with 8 strokes at 1000 rpm at 4°C using a polytron homogenizer as a stirrer (2 cm³, HOM, AS ONE Cooperation, Osaka, Japan), followed by incubation on ice. The homogenate was then mixed with the CER II reagent and centrifuged for 5 min at 16000×g at 4°C. After removing the supernatant, the pellet was suspended in the NER reagent. The suspension was incubated and centrifuged for 10 min at 16000×g at 4°C, and the supernatant was used as the nuclear extract sample.

**Statistical Analysis** The numerical data are expressed as the mean±standard error (S.E.) or standard deviation (S.D.). The significance of the differences were examined by Student’s t-tests for pairs of values. The results with values of \(p<0.05\) were considered significant.

**RESULTS**

A mouse neuropathic pain model was prepared and the
mRNA expression level of UGT2B1 in the liver was measured on days 3, 7, and 28 after model preparation (Fig. 1). Increased expression of UGT2B in the liver was evident only on day 28, but not on days 3 and 7, suggesting that the increased expression of UGT2B in the liver occurred during the maintenance phase and not in the induction phase of neuropathic pain.

Since the expression of UGT2B has been shown to be regulated by the CAR nuclear receptor, CAR activity was analyzed based on the amount transported into the nucleus. On day 28, when a significant increase in the expression level of UGT2B was observed in the neuropathic pain group compared with the control group, there was no difference in the amount of CAR transported to nucleus in the liver between groups (Fig. 2). To confirm the absence of CAR activation, the mRNA expression level of CYP2B10, which is induced by CAR activation, was measured (Fig. 3). The mRNA expression level of CYP2B10 in the liver on day 28 in the neuropathic pain group was relatively higher than that in control mice, although no significant difference was found between groups.

Since it has recently been reported that another nuclear receptor, PXR, induces the expression of UGT2B, we also examined PXR activity (Fig. 4). The amount of PXR transported to the nucleus did not significantly differ between the neuropathic pain group and control group. Interestingly, however, the mRNA expression levels of CYP3A11 and CYP3A25 were nearly doubled via PXR in the neuropathic pain group (Fig. 5).

**DISCUSSION**

Increased expression of UGT in the liver was not observed at 3 and 7 d following the preparation of mice models for neuropathic pain. However, expression did increase after 28 d (Fig. 1). In the area surrounding the nerve damage, chemokines (CCL2, CCL3) are released from inflammatory cytokines (interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-alpha) and neurons of macrophages and mast cells.46,47 It has been reported that downstream of these inflammatory...
cytokines, nuclear factor kappaB (NF-κB) is activated, leading to the inhibition of PXR and RXR-alpha binding and lowered expression of cytochrome P450. Therefore, the decrease in UGT2B expression observed during the induction period appears to have resulted from such inflammatory cytokines. By contrast, the maintenance phase of neuropathic pain is a period during which astrocytes of the spinal cord are activated. An increase in the expression of UGT2B is likely to be associated with the activation of these astrocytes.

If this is the case, why is there an increase in the expression of UGT during the maintenance phase? It is currently known that the main molecules responsible for the control of UGT2B expression are the nuclear receptors CAR and PXR. In our study, we first conducted experiments that were focused on CAR. No change was found in the amount of intranuclear localization of CAR between the control group and the treatment group (Fig. 2). In addition, when the CYP2B10 mRNA expression, which is controlled by CAR, was quantitatively determined, no change was found between the two groups. These results suggest that CAR has no role in increasing the expression of liver UGT2B.

Next, we conducted experiments focusing on PXR. No changes in the amount of intranuclear localization of PXR were noted between the control group and the treatment group (Fig. 3). However, the expression levels of CYP3A11 and CYP3A25 mRNA, which are controlled by PXR, were found to have increased by about two-fold in the treatment group (Fig. 5). Based on these results, it is possible that the increase of UGT is independent of intranuclear localization of PXR, and may have been caused only by transcriptional activation. Histone modification by protein arginine methyltransferase 1 (PRMT1) has recently been reported to play an important role in the binding sequence of PXR (PXRE) during the transcriptional activation of the ligand-dependent CYP3A4 of PXR. It has also been reported that a decrease in histone acetylation (HAT) activity at the time of neuropathic pain is associated with the activation of these astrocytes.

According to the relationship between the activation of astrocytes and enhanced activity of HAT, epigenetic analysis studies may be necessary in future.

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### Conflict of Interest

The authors declare no conflict of interest.

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