Silencing of Id2 Alleviates Chronic Neuropathic Pain Following Chronic Constriction Injury

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Abstract Inhibitor of DNA binding/differentiation 2 (Id2) belongs to a helix-loop-helix family of proteins. Recent studies have showed that Id2 plays a pivotal role in neuronal survival and neuroprotection. However, under neuropathic pain conditions, the role of Id2 is still unclear. In this study, we investigated the effect of Id2 on neuropathic pain in a rat chronic constriction injury (CCI) model. Our results demonstrated that Id2 was upregulated in the dorsal root ganglion (DRG) in a CCI rat in a time-dependent manner. Intrathecal short-hairpin RNA (shRNA)-Id2 attenuates mechanical allodynia and thermal hyperalgesia in CCI rats, and inhibits the expression of TNF-α and IL-1β in the DRG in CCI rats. Furthermore, knockdown of Id2 reduces the expression of NF-κB p65 in the DRG of CCI rats. Taken together, our findings suggest that knockdown of Id2 may alleviate neuropathic pain by inhibiting the NF-κB activation to inhibit the production of pro-inflammatory mediators. Therefore, Id2 may provide an important target of neuropathic pain treatment.

Keywords Inhibitor of DNA binding/differentiation 2 (Id2) · Neuropathic pain · Chronic constriction injury (CCI) · NF-κB

Introduction

Chronic neuropathic pain is characterized by allodynia in response to nonpainful stimuli and hyperalgesia in response to noxious stimuli resulting from damage or abnormal function of the nervous system (Treede et al. 2008). In recent years, it has become a notable public health problem that affects a broader population worldwide (Neville et al. 2008). In addition, neuropathic pain has been suggested to be an underestimated problem in patients after thoracic surgery (Maguire et al. 2006; Rogers and Duffy 2000). However, neuropathic pain remains a prevalent and persistent clinical challenge as its pathogenesis is unknown. Therefore, there is a considerable need to explore novel therapeutics for neuropathic pain management.

Inhibitor of DNA binding/differentiation 2 (Id2), which belongs to a helix-loop-helix family of proteins, act as dominant-negative inhibitors of basic helix-loop-helix transcription factors by forming heterodimers. It plays important roles in regulating cell proliferation, differentiation, and apoptosis (Israel et al. 1999). For example, high Id2 decreases the metastatic potential of hepatocellular carcinoma by inhibiting vascular endothelial growth factor (VEGF) expression through destabilization of HIF1α protein (Tsunedomi et al. 2008). In addition, Id2 has been implicated in lymphocyte development. Knockdown of Id2 by shRNA in hematopoietic progenitor cells promoted B cell differentiation and induced the expression of B cell lineage-specific genes (Ji et al. 2008). Recently, one study showed that Id2 was upregulated in neuronal cells following hypoxia/ischemia (H/I), and knockdown of Id2 attenuated H/I-induced neuronal apoptosis in vitro, improved neurological deficits, and reduced the volume of ischemic infarction in a rat model of middle cerebral artery occlusion (MCAO) (Guo et al. 2015). However, under neuropathic pain conditions, the role of Id2 is still unclear. In this study, we investigated the effect of Id2 on neuropathic pain in a rat chronic constriction injury (CCI) model.
Materials and Methods

Animal Care

Adult male Sprague-Dawley rats, weighting 200–220 g, were purchased from the Laboratory Animal Center of the First Affiliated Hospital of Wenzhou Medical University (China). The rats were housed in separated cages in a room kept at 24 ± 1 °C and 50–60 % humidity, under a 12:12 light-dark cycle and with free access to food and water ad libitum. Animal experiments conformed to the guidelines issued by the First Affiliated Hospital of Wenzhou Medical University. The present study was performed with approval from the Animal Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All surgery was performed under trichloroacetaldehyde monohydrate (450 mg/kg, i.p) anesthesia, and the Sprague-Dawley rats for dorsal root ganglion (DRG) neurons were sacrificed by decapitation after being anesthetized by CO2. All efforts were made to minimize suffering.

Chronic Constriction Injury Model

The CCI of the sciatic nerve had been performed in adult male Sprague-Dawley rats as previously described (Bennett and Xie 1988). In brief, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The sciatic nerve was exposed and loosely ligated with sterile 4-0 catgut thread at four consecutive sites with an interval of approximately 1 mm. Meanwhile, a sham surgery was performed with the sciatic nerve exposed but not ligated.

Intrathecal Catheter Implantation

Rats were randomly divided into four groups with 10 rats in each group: a sham group, a CCI group, a shRNA-scramble group (CCI + shRNA-scramble), and a shRNA-Id2 group (CCI + shRNA-Id2). Intrathecal catheter implantation was performed according to a method reported previously (Yaksh and Rudy 1976). In brief, rats were anesthetized with 40 mg/kg sodium pentobarbital (i.p.). The sciatic nerve was exposed and loosely ligated with sterile 4–0 catgut thread at four consecutive sites with an interval of approximately 1 mm. Meanwhile, a sham surgery was performed with the sciatic nerve exposed but not ligated.

Evaluation of Thermal Hyperalgesia and Mechanical Allodynia

Thermal hyperalgesia was assessed by the paw withdrawal latency (PWL) to radiant heat according to a method reported previously (Hargreaves et al. 1988). After acclimation, the heat source was positioned under the glass floor directly beneath the hind paw. The intensity of the thermal stimulus was adjusted to achieve an average baseline PWL of approximately 10 s. A digital timer automatically recorded the duration between the start of stimuli and PWL. A 20-s cutoff was used to avoid tissue damage. Each paw was measured alternatively after more than 5 min. Tests were performed 1 day before CCI surgery, and 1, 3, 7, and 14 days after CCI surgery.

Mechanical hyperalgesia was assessed using calibrated von Frey filaments (Chaplan et al. 1994). Rats were placed individually in a transparent plastic box with a mesh floor to allow access to the ventral surface of the hind paw and allowed to acclimate for at least 15 min before testing. Affected hind paws were contacted with Von Frey filaments (Stoelting). Each filament was applied five times to the plantar surface of the right hind paw in an ascending order of force. The withdrawal reflex of at least three of the five applications was defined as appositive response.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated from the DRG using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). 2 μg of total RNA was subjected to reverse transcription using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction (RT-qPCR) was used to analyze the levels of mRNA transcripts using specific primers, along with the manufacturer’s instructions, and analysis was performed using the ABI PRISM 7700 sequence detection system (Perkin-Elmer Biosystems). The following primer pairs were used: Id2, 5′-GGACAGAAACAAACGTCCAG-3′ and 5′-TAAGCTCAGAAGGGAATTCAGAC-3′, and GAPDH, 5′-TGGTGTCCGTCTCGTGA-3′ and 5′-TTGCTGGATGTCGAGGAG-3′. GAPDH was used as a control for normalizing gene expression. Data were analyzed using the formula: $R = 2^{-(\Delta \Delta Ct \text{control} – \Delta \Delta Ct \text{sample})}$.

Western Blot

The proteins were extracted from the DRG of CCI rats and protein concentrations were determined using the Pierce BCA Protein Assay Kit. Equal amounts of
protein were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked in 5 % nonfat milk in tris-buffered saline with Tween 20 (TBST) buffer for 1 h at room temperature before hybridization with primary antibodies against Id2, NF-κB p65 overnight at 4 °C, followed by three washes for 5 min with TBST. After incubation with proper HRP-conjugated secondary antibodies for 1 h at room temperature and three washes with TBST, the resultant protein bands were visualized by ECL reagents according to the manufacturer’s instruction. The relative protein expression levels of Id2 and NF-κB p65 were quantified using Image-Pro Plus 6.0 software and normalized to GAPDH.

**ELISA**

The DRG was harvested 24 h after the last injection of shRNA-Id2 for pro-inflammatory cytokine analysis. Supernatants of the DRG were assayed by the rat TNF-α and IL-1β ELISA kits (Jingmei Biotech Co., Ltd., Shenzhen, China) according to the manufacturer’s instructions.

**Fig. 2** Intrathecal administration of shRNA-Id2 decreased Id2 mRNA and protein expression in the DRG of CCI rats. **a** The representative images of relative mRNA level of Id2 treated with shRNA-scramble and shRNA-Id2 at postoperative day 7; GAPDH was used as a control for normalizing gene expression; **b** the protein expression of Id2 in the DRG of CCI rats at postoperative day 7. The relative protein expression level of Id2 was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. At least three independent experiments were performed. Data were shown as mean ± SD. *P < 0.05 vs the sham group, #P < 0.05 vs the shRNA-scramble group.

**Fig. 1** Id2 was upregulated in the DRG in CCI rats. **a** Id2 mRNA was detected by RT-qPCR at days 1, 3, 7, and 14 after CCI; GAPDH was used as a control for normalizing gene expression; **b** Id2 protein was detected by Western blot at days 1, 3, 7, and 14 after CCI. The relative protein expression level of Id2 was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. At least three independent experiments were performed. Data were shown as mean ± SD. *P < 0.05 vs the sham group.
Statistical Analysis

Data are expressed as mean ± SD. The behavioral data were analyzed by two-way ANOVA followed by the Turkey’s test for post hoc analysis. The data from the biochemical tests and Western blot were analyzed with one-way ANOVA followed by LSD post hoc test. A value of \( P < 0.05 \) was considered statistically significant.

Results

Id2 Was Upregulated in the DRG in CCI Rats

First, we evaluated the expression of Id2 in the DRG in response to CCI using RT-qPCR and Western blot analysis. Id2 levels were measured at 1, 3, 7, and 14 days in the CCI and sham groups. The results showed that both Id2 mRNA and protein levels significantly increased, as compared with that from sham-operated rats at postoperative days 1, 3, 7, and 14 (\( P < 0.05 \); Fig. 1a, 1b). These data suggested that Id2 might play an important role in regulating neuropathic pain.

Knockdown of Id2 Attenuates Mechanical Allodynia and Thermal Hyperalgesia in CCI Rats

To investigate the effect of Id2 on neuropathic pain, the CCI rats were subjected to intrathecal injection of shRNA-Id2 or scramble-Id2. As shown in Fig. 2a, shRNA-Id2 obviously decreased the expression of Id2 mRNA, as compared with shRNA-scramble group at postoperative day 7 (\( P < 0.05 \); Fig. 2a). Western blot results showed that the expression of Id2 was significantly decreased in shRNA-Id2 group (\( P < 0.05 \); Fig. 2b). Then, the paw withdrawal threshold (PWT) and PWL were measured 2 h after each treatment. As shown in Fig. 3a, 3b, intrathecal treatment with shRNA-Id2 dramatically increased PWT and PWL as compared to the shRNA-scramble groups (\( P < 0.05 \)). There was no significant difference between the CCI group and the shRNA-scramble group (\( P > 0.05 \)).

Knockdown of Id2 Inhibited Pro-inflammatory Cytokines Expression in the DRG of CCI Rats

To further explore the effect of Id2 on neuropathic pain development, we analyzed the expression of pro-inflammatory cytokines in the DRG of CCI rats. As
shown in Fig. 4a, b, the levels of TNF-α and IL-1β were significantly increased in the DRG of CCI rats, whereas intrathecal administration of shRNA-Id2 obviously attenuated the levels of TNF-α and IL-1β ($P < 0.05$). These data suggested that knockdown of Id2 may alleviate the pain behaviors by inhibiting TNF-α and IL-1β in the DRG of CCI rats.

Knockdown of Id2 Reduced the Expression of NF-κB p65 in the DRG of CCI Rats

NF-κB is a key transcription factor complex that controls the expression of pro-inflammatory and pain mediators (Niederberger and Geisslinger 2008). To investigate the mechanism of Id2 in CCI-induced neuropathic pain, we investigated the expression of NF-κB p65 in the DRG of CCI rats. As shown in Fig. 5, compared with the sham group, the CCI group showed significantly higher levels of NF-κB p65. However, knockdown of Id2 markedly decreased the expression of NF-κB p65 protein in the DRG of CCI rats ($P < 0.05$).

Fig. 4 Knockdown of Id2 inhibited pro-inflammatory cytokine expression in the DRG of CCI rats. The DRG was harvested 24 h after the last injection of shRNA-Id2 for pro-inflammatory cytokine analysis. (a and b) Supernatants of the DRG were assayed by the rat TNF-α and IL-1β ELISA kits. At least three independent experiments were performed. Data were shown as mean ± SD. *$P < 0.05$ vs the sham group, $^#P < 0.05$ vs the shRNA-scramble group

Fig. 5 Knockdown of Id2 reduced the expression of NF-κB p65 in the DRG of CCI rats. Rats were anesthetized with sodium pentobarbital 24 h after the last treatment. The proteins were extracted from the DRG using RIPA lysis buffer. a Western blot analysis of NF-κB p65 expression in the DRG of CCI rats. b The relative protein expression level of NF-κB p65 was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. At least three independent experiments were performed. Data were shown as mean ± SD. *$P < 0.05$ vs the sham group, $^#P < 0.05$ vs the shRNA-scramble group
Discussion

The main findings of this study were as follows: (1) Id2 was upregulated in the DRG in CCI rat in a time-dependent manner; (2) Intrathecal shRNA-Id2 attenuated mechanical allodynia and thermal hyperalgesia in CCI rats; (3) Intrathecal shRNA-Id2 administration inhibited the expression of TNF-α and IL-1β in the DRG in CCI rats; (4) Knockdown of Id2 reduced the expression of NF-κB p65 in the DRG of CCI rats.

The chronic constriction injury model is the most commonly employed neuropathic animal model of nerve damage-induced allodynia/hyperalgesia (Bennett and Xie 1988). In this study, the CCI model was used to investigate the effect of Id2 on neuropathic pain. Our results showed that Id2 was upregulated in the DRG in CCI rat in a time-dependent manner. These results suggest for the first time that Id2 plays an important role in chronic neuropathic pain.

Nerve injury leads to a release of pain-related mediators, such as TNF-α, IL-1β, IL-6, and prostaglandins, resulting in inflammatory and immune responses, sensitization of the central nervous system, and facilitation of pain processing (Moalem and Tracey 2006). Previous studies showed that TNF-α expression was significantly increased in DRG after CCI (Schäfers et al. 2002); neutralizing antibodies against TNF-α reduced thermal hyperalgesia and mechanical allodynia in the CCI model of neuropathic pain (Sommer et al. 1998). IL-1β is also a potent pro-inflammatory cytokine involved in neuropathic pain (Apkarian et al. 2006; Nadeau et al. 2011; Zelenka et al. 2005). Intrathecal injection of IL-1β induces mechanical allodynia, and IL-1 receptor antagonist can inhibit hyperalgesic responses to IL-1β (Baamonde et al. 2007). In the present study, we found that intrathecal administration of shRNA-Id2 obviously attenuated the levels of TNF-α and IL-1β. These data suggest that knockdown of Id2 may alleviate the pain behaviors by inhibiting TNF-α and IL-1β in the DRG of CCI rats.

A growing body of evidence indicates the NF-κB can control a variety of genes encoding inflammatory and nociceptive mediators and play an important role in the development of central pain sensitization (Fu et al. 2010; Lee et al. 2004; Sakaue et al. 2001). It has been reported that the expression and activation of NF-κB p65 is upregulated in the DRG neurons after partial sciatic nerve injury (Ma and Bisby 1998). Small interfering RNA-mediated knockdown of NF-κB p65 obviously inhibited the expression of pro-inflammatory factors (TNF-α, IL-1β and IL-6) and alleviated mechanical allodynia and thermal hyperalgesia in a CCI model of rats (Sun et al. 2012). Another study reported that pretreatment with NF-κB inhibitor (PDTC) improved mechanical allodynia and downregulated the over-expression of TNF-α and tumor necrosis factor receptor 1 (TNFR1) induced by peri-sciatic administration of TNF (Wei et al. 2007). The deletion of inhibitor kappa B kinase beta (IKKβ) in DRG neurons also reduced nerve injury-induced NF-κB in the DRG and was associated with reduced upregulation of IL-1β, monocyte chemoattractant protein-1/chemokine (CC motif) ligand 2 (MCP-1/CCL2), and TNF-α in the DRG (Kanggiesser et al. 2012). In the present study, we found that intrathecal administration of shRNA-Id2 significantly decreased the expression of NF-κB p65 induced by CCI. Therefore, knockdown of Id2 may alleviate neuropathic pain in the DRG by inhibiting the NF-κB activation to inhibit the production of pro-inflammatory mediators.

In summary, our study suggests that intrathecal shRNA-Id2 attenuates neuropathic pain in CCI rats. Therefore, Id2 may provide an important target of neuropathic pain treatment.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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