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ACTIVATING TRANSCRIPTION FACTOR 4, A MEDIATOR OF THE INTEGRATED STRESS RESPONSE, IS INCREASED IN THE DORSAL ROOT GANGLIA FOLLOWING PAINFUL FACET JOINT DISTRACTION

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Abstract—Chronic neck pain is one of the most common musculoskeletal disorders in the US. Although biomechanical and clinical studies have implicated the facet joint as a primary source of neck pain, specific cellular mechanisms still remain speculative. The purpose of this study was to investigate whether a mediator (activating transcription factor; 4ATF4) of the integrated stress response (ISR) is involved in facet-mediated pain. Holtzman rats underwent C6/C7 facet joint loading that produces either painful (n=16) or nonpainful (n=8) responses. A sham group (n=9) was also included as surgical controls. Behavioral sensitivity was measured and the C6 dorsal root ganglia (DRGs) were harvested on day 7 to evaluate the total and neuronal ATF4 expression. In separate groups, an intra-articular ketorolac injection was administered either immediately (D0 ketorolac) or 1 day (D1 ketorolac) after painful facet joint loading. Allodynia was measured at days 1 and 7 after injury to assess the effects on behavioral responses. ATF4 and BiP (an indicator of ISR activation) were separately quantified at day 7. Facet joint loading sufficient to elicit behavioral hypersensitivity produced a threefold increase in total and neuronal ATF4 expression in the DRG. After ketorolac treatment at the time of injury, ATF4 expression was significantly (P<0.01) reduced despite not producing any attenuation of behavioral responses. Interestingly, ketorolac treatment at day 1 significantly (P<0.001) alleviated behavioral sensitivity at day 7, but did not modify ATF4 expression. BiP expression was unchanged after either intervention time. Results suggest that ATF4-dependent activation of the ISR does not directly contribute to persistent pain, but it may sensitize neurons responsible for pain initiation. These behavioral and immunohistochemical findings imply that facet-mediated pain may be sustained through other pathways of the ISR.

Key words: ISR, ATF4, facet joint, pain, ketorolac, BiP.

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Abbreviations: ATF4, activating transcription factor 4; DRG, dorsal root ganglion; ISR, integrated stress response; MAP, microtubule-associated protein; NSAID, nonsteroidal anti-inflammatory drug; PERK, PKR-like endoplasmic reticulum stress kinase; PKR, double-stranded RNA-activated protein kinase.

Neck pain affects up to 70% of individuals in their life span, and it is one of the most commonly reported origins of musculoskeletal pain in the general population (Côté et al., 1998; Natvig et al., 2010). In particular, the facet joint is one of the most common sources of pain in the cervical spine (Barnsley et al., 1995; Lord et al., 1996; Manchikanti, 1999). The facet joint capsule contains not only mechanoreceptors for proprioception but also nociceptive fibers that provide a means for transmitting pain signals (Cavanaugh et al., 1996; Inami et al., 2001; McLain, 1994). Mechanical loading of the facet joint, in particular stretch of its capsule, has been demonstrated as a primary mechanism of pain generation in biomechanical and *in vivo* studies (Cavanaugh et al., 1996; Cusick et al., 2001; Deng et al., 2000; Gore et al., 1987; Grauer et al., 1997; Ito et al., 2004; Lee et al., 2004a; Ono et al., 1997; Panjabi et al., 2004; Pearson et al., 2004; Siegmund et al., 2001). Indeed, local anesthetic blocks to the nerves of the facet joint can alleviate, or even abolish, pain in up to 62% of chronic pain cases from mechanical neck injury (Aprill and Bogduk, 1992; Barnsley et al., 1993; Yoganandan et al., 1998). Despite the strong biomechanical and clinical data implicating the facet joint and its capsule's involvement in pain, the cellular mechanisms related to pain from injury to this joint still remain speculative.

Inflammatory processes contribute to persistent pain through a variety of mediators (Kawakami and Weinstein, 1986; McMahon et al., 2005; Millan, 1999). Several different animal models of painful joint inflammation have reported cytokine upregulation and glial activation in the dorsal root ganglion (DRG) and spinal cord (Fenzi et al., 2001; Lee et al., 2008; Miyagi et al., 2006). Glial activation can alter neuronal signaling and can also cause excessive glutamate release (Kawakami and Weinstein, 1986). A number of *in vitro* and *in situ* studies have shown that the release of glutamate and cytokines can directly induce the integrated stress response (ISR) in neurons and other cells, which is critical for cell development and function (Cardozo et al., 2005; Kharroubi et al., 2004; Oyadomari et al., 2001; Shim et al., 2004). Despite mounting evidence linking inflammatory responses to activation of the ISR and the known role of inflammation in pain (Hartwig et al., 2003; Inglis et al., 2005; Lee et al., 2008; Markowitz et al., 2007), there is still very limited information on the role of the ISR in facet- or joint-mediated pain.

The integrated stress response, also known as the endoplasmic reticulum (ER) stress response, is a common cellular response to disruption of homeostasis in

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injury or disease states (Dong et al., 2008; Harding and Ron, 2002; Katayama et al., 2004; Rao and Bredesen, 2004). The ISR is a tripartite pathway initiated by three ER-localized proteins, double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum stress kinase (PERK), inositol-requiring enzyme (IRE1a), and activating transcription factor 6. Activation of the ISR culminates in increased expression of the ISR binding protein (BiP), which plays a major role in the repair of unfolded and misfolded proteins (Schröder and Kaufman, 2005). In addition to BiP, each pathway activates proteins that enhance protein folding, establish homeostasis, and attenuate translation. The latter is a direct consequence of activation of the PERK pathway by phosphorylation and subsequent attenuation of eukaryotic initiation factor 2α (eIF2α), which promotes translation initiation. Interestingly, phosphorylation of eIF2α favors translation of activating transcription factor 4 (ATF4), which has been shown to promote apoptotic cell death via transactivation of C/EBP-homologous protein (CHOP) (Cherasse et al., 2007; Ohoka et al., 2005; Yamauchi et al., 2007). In neurons, ATF4 can contribute to long-term synaptic plasticity in mice (Chen et al., 2003). Sustained modification of spinal neurons has also been observed in our rat model of facet-mediated pain (Quinn et al., 2010). That model also exhibits spinal neuroinflammation and disrupted homeostasis in the injured afferents, as well as increased glutamate activity in the spinal cord and ISR activation in the DRG after facet injury in association with sustained behavioral sensitivity (Dong et al., 2008; Dong and Winkelstein, 2010; Lee et al., 2008; Quinn et al., 2010). Although we have previously observed increases in neuronal BiP in the DRG after painful facet joint injury, no studies have investigated the extent and pathway of ISR activation in facet-mediated pain.

The objectives of this study were to investigate whether ATF4 in injured afferents is involved in behavioral hypersensitivity that develops after painful facet joint injury. As such, facet capsule stretch was applied in our rat model at magnitudes that do and do not produce sustained behavioral hypersensitivity in the forepaw (Dong et al., 2008; Dong and Winkelstein, 2010; Lee and Winkelstein, 2009) to characterize ATF4 expression in the affected DRG after joint injury and to determine if painful joint loading is associated with the upregulation of ATF4 expression. In addition, to determine whether any changes in ATF4 are related to behavioral sensitivity, additional studies were performed assessing ATF4 and BiP expression after painful loading conditions with a nonsteroidal anti-inflammatory drug (NSAID) treatment. Ketorolac is an NSAID that reduces pain by nonselectively inhibiting cyclooxygenase (COX) activity, which leads to diminished production of prostaglandins (Cassinelli et al., 2008; Dogan et al., 2004; Turner et al., 2011). Specifically, ketorolac injection reduces joint inflammation and postoperative pain in clinical and animal models (Convery et al., 1998; Ng et al., 2006; Swift et al., 1998). Therefore, in this study, ketorolac was administered to the injured joint either immediately after injury at day 0 or at 1 day after painful loading, in separate groups. In those studies, BiP expression was evaluated along with ATF4 to assess the effects of treatment on ISR activation.

EXPERIMENTAL PROCEDURES

Animal care and surgical procedures

All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983). Male Holtzman rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) weighing 350–425 g were housed under USDA- and AAALAC-compliant conditions with free access to food and water and a 12/12 h light–dark cycle.

All surgical procedures were performed under inhalation anesthesia (4% isoflurane for induction, 2.5% for maintenance) and have been previously described (Dong et al., 2008; Dong and Winkelstein, 2010; Lee et al., 2004b). Briefly, an incision was made from the base of the skull to the second thoracic vertebra and the bilateral C6/C7 facet joints were exposed by removing the surrounding soft tissue and musculature. The C6 and C7 laminae were rigidly attached to microforceps of a customized loading device that imposed a controlled joint injury by displacing the C6 vertebra rostrally, holding it for 30 s and returning to its initial unloading position, while the C7 vertebra remained stationary. Two different C6/C7 facet joint distractions were applied separately to either induce (0.5 mm; painful $n=16$) or not induce (0.2 mm; nonpainful $n=8$) behavioral hypersensitivity, based on previous studies (Dong et al., 2008; Dong and Winkelstein, 2010). Sham procedures were also performed as a surgical control with no applied joint distraction (0 mm; sham $n=9$) but all other surgical procedures. The magnitude of the injury severity was measured by quantifying vertebral and joint capsule distractions during joint loading. Polystyrene particles were affixed to the C6 and C7 laminae and joint capsule for motion tracking. Joint distraction was defined as the maximum displacement of C6 laminae relative to C7, and the capsule distraction was defined as the average resultant displacement of the rostral edge of its capsule relative to the caudal edge. As an additional biomechanical measure of injury severity, maximum principal strain for the capsule was calculated using engineering software (LS-DYNA) (Dong and Winkelstein, 2010; Lee et al., 2004b; Weisshaar et al., 2010; Winkelstein et al., 2000). Joint displacements and capsule strains were compared between painful and nonpainful groups using an unpaired *t*-test.

A subset of rats from the painful group was randomly selected to receive intra-articular administration of ketorolac (12 μg in 10 μl saline) either immediately (D0 ketorolac; $n=4$) or at 1 day (D1 ketorolac; $n=5$) after painful joint loading. Joint injection procedures were performed under inhalation anesthesia (2.5% isoflurane), and ketorolac (Sigma-Aldrich; St. Louis, MO, USA) was administered in the bilateral C6/C7 facet joints using a 10-μl syringe with a 33G beveled needle (Hamilton; Reno, NA, USA). The needle was gently inserted into the facet joint by piercing through its capsule in the dorsal medial region with the injection bolus delivered slowly.

Behavioral assessment

Behavioral sensitivity was assessed by measuring bilateral mechanical hyperalgesia in the forepaws on days 1, 3, 5, and 7 after painful and nonpainful distraction injury or sham procedures. Prior to surgery, rats were also assessed for hyperalgesia to provide a baseline measurement to serve as an unoperated control response for each rat. Methods to measure hyperalgesia were adopted from Chaplan's up/down method and have been previously reported and validated (Chaplan et al., 1994; Decosterd and