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EVIDENCE FOR A ROLE OF HEAT SHOCK PROTEIN-90 IN TOLL LIKE RECEPTOR 4 MEDIATED PAIN ENHANCEMENT IN RATS

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Abstract—Spinal cord microglial toll-like receptor 4 (TLR4) has been implicated in enhancing neuropathic pain and opposing morphine analgesia. The present study was initiated to explore TLR4-mediated pain modulation by intrathecal lipopolysaccharide, a classic TLR4 agonist. However, our initial study revealed that intrathecal lipopolysaccharide failed to induce low-threshold mechanical allodynia in naive rats, suggestive that TLR4 agonism may be insufficient to enhance pain. These studies explore the possibility that a second signal is required; namely, heat shock protein-90 (HSP90). This candidate was chosen for study given its known importance as a regulator of TLR4 signaling. A combination of *in vitro* TLR4 cell signaling and *in vivo* behavioral studies of pain modulation suggest that TLR4-enhancement of neuropathic pain and TLR4-suppression of morphine analgesia each likely require HSP90 as a cofactor for the effects observed. *In vitro* studies revealed that dimethyl sulfoxide (DMSO) enhances HSP90 release, suggestive that this may be a means by which DMSO enhances TLR4 signaling. While 2 and 100 μg lipopolysaccharide intrathecally did not induce mechanical allodynia across the time course tested, co-administration of 1 μg lipopolysaccharide with a drug that enhances HSP90-mediated TLR4 signaling now induced robust allodynia. In support of this allodynia being mediated via a TLR4/HSP90 pathway, it was prevented or reversed by intrathecal co-administration of a HSP90 inhibitor, a TLR4 inhibitor, a microglia/monocyte activation inhibitor (as monocyte-derived cells are the predominant cell type expressing TLR4), and interleukin-1 receptor antagonist (as this proinflammatory cytokine is a downstream consequence of TLR4 activation). Together, these results suggest for the first time that TLR4 activation is necessary but not sufficient to induce spinally mediated pain enhancement. Rather, the data suggest that TLR4-dependent pain phenomena may require contributions by multiple components of the TLR4 receptor complex.

Key words: HEK293-TLR4, lipopolysaccharide, chronic constriction injury, morphine, mechanical allodynia, analgesia.

Peripheral nerve injury activates spinal microglia and astrocytes, as reflected by activation marker upregulation (Watkins et al., 2005). Microglial activation is generally thought to occur prior to astrocyte activation (Raghavendra et al., 2003a). Such activation causes the release of proinflammatory products including interleukin-1, which enhances pain (Kwon et al., 2005). Indeed, blockade of microglial activation with minocycline prevents neuropathic pain development (Mika, 2008) and blockade of proinflammatory mediators such as interleukin-1 reverses neuropathic pain (Watkins et al., 2005).

While glia are important for the development and maintenance of neuropathic pain, how peripheral nerve injury triggers spinal glial activation is not clear. Numerous candidate neuron-to-glia signals have been proposed for initiating glial activation, including neurotransmitters, neuromodulators, and neuronally derived chemokines such as fractalkine and monocyte chemotactic protein-1 (Watkins et al., 2007; White et al., 2007).

Recently, a very intriguing mechanism has been proposed for spinal microglial activation in response to peripheral nerve injury; that is, activation of toll-like receptor 4 (TLR4) (Tanga et al., 2005; Hutchinson et al., 2008b). Within the CNS, TLR4 is expressed predominantly by microglia and by resident and recruited macrophages (Olson and Miller, 2004). To our knowledge, there are no known reports of TLR4 expression by neurons within the spinal cord, the anatomical region of focus here. TLR4 activation leads to the production of proinflammatory mediators implicated in neuropathic pain, including interleukin-1 (Olson and Miller, 2004). While TLR4 is classically thought of as the receptor activated by endotoxin (lipopolysaccharide (LPS) from gram negative bacteria), TLR4 has recently been recognized as also becoming activated in response to "endogenous danger signals" (Osterloh and Breloer, 2008). These are substances released by host cells by cellular stress or damage. In neuropathic pain, these could arise, for example, from neuronal components such as heat shock proteins (Costigan et al., 1998) or degraded cell membrane components (Osterloh and Breloer, 2008) released by stressed or dying sensory afferents, by alterations in the blood–brain barrier (Gordh et al., 2006) al-

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Abbreviations: %MPE, percent maximal possible effect; aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; BL, baseline; CCI, chronic constriction injury; CD14, cluster determinant 14; DMAG, 17-dimethylaminoethylamino-17-desmethoxygeldanamycin; DMSO, dimethyl sulfoxide; HEK293, human embryonic kidney-293; HSP, heat shock protein; IL-1, interleukin-1; IL-1RA, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; MD2, myeloid differentiation factor 2; PE, polyethylene; SEAP, secreted alkaline phosphatase; TLR, toll like receptor.

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lowing entry into the neuropil of blood components normally prevented from doing so (Ward et al., 2006), or other such sources. While the identity of the endogenous danger signal(s) activating TLR4 in response to peripheral nerve damage remains unknown, what is clear is that: (a) peripheral nerve injury upregulates spinal TLR4 mRNA (Tanga et al., 2005; Hutchinson et al., 2008b), (b) development of neuropathic pain (mechanical allodynia) is suppressed in TLR4 knockout/knockdown mice (Tanga et al., 2005), and (c) established neuropathic pain (mechanical allodynia) is reversed by intrathecal classical LPS-derived TLR4 antagonists or the recently discovered novel TLR4 antagonists (+)- or (−)-naloxone (Hutchinson et al., 2008b).

The present study was originally initiated to explore TLR4-mediated pain enhancement by intrathecal LPS, a classic TLR4 agonist. However, our initial pilot studies and the data included in Experiment 1 both revealed that a wide range of intrathecal LPS doses failed to induce mechanical allodynia in naive (non-neuropathic) rats. The clear involvement of TLR4 under neuropathic pain conditions, yet failure of TLR4 activation to enhance pain in normal rats, suggests that a pure TLR4 signal may not be sufficient to enhance pain. That is, it suggests that a second signal is required. Hence the present studies explore this issue for TLR4 receptor activation *in vitro* and for enhancing pain *in vivo*.

EXPERIMENTAL PROCEDURES

Subjects

Pathogen-free adult male Sprague–Dawley rats (n=6 rats/group for each experiment; 300–375 g; Harlan Labs, Madison, WI, USA) were used in all experiments. Rats were housed in temperature (23±3 °C) and light (12 h: 12 h light: dark cycle; lights on at 0700) controlled rooms with standard rodent chow and water available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder.

Drugs

(+)-Naloxone was obtained from the National Institute on Drug Abuse (Research Triangle Park, NC and Bethesda, MD, USA). Sterile endotoxin-free isotonic saline (Abbott Laboratories, North Chicago, IL, USA) was its vehicle. Lipopolysaccharide (LPS; *Escherichia coli*; serotype: 0111: B4, Sigma, St. Louis, MO, USA), 17-dimethylaminoethylamino-17-desmethoxygeldanamycin (17-DMAG; Calbiochem, San Diego, CA, USA), geldanamycin (Sigma), dimethyl sulfoxide (DMSO; Sigma), and minocycline (Sigma) were obtained commercially. Morphine was gifted from Mallinkrodt (St. Louis, MO, USA). Interleukin-1 receptor antagonist (IL-1ra) and its vehicle were gifted from Amgen (Thousand Oaks, CA, USA). Where applicable, drugs were prepared and are reported as free base concentrations. Vehicles were administered equivolume to the drugs under test. Stock solutions of each compound (except LPS) were negative for endotoxin contamination using a non-TLR4 dependent endotoxin test (Limulus amebocyte lysate assay).

Behavioral assessment of responsivity to mechanical stimuli and radiant heat

All testing was conducted blind with respect to group assignment according to our previously published procedures.

Von Frey test. The von Frey test (Chaplan et al., 1994) was performed (Experiments 1, 2, 3, 7 and 9) within the sciatic innervation region of the hindpaws as previously described in detail (Chacur et al., 2001; Milligan et al., 2001). Von Frey assessments were made prior to (baseline) and at specific times after experimental manipulations, as detailed in each experiment. The behavioral responses were used to calculate absolute threshold (Harvey, 1986; Treutwein and Strasburger, 1999), as described in detail previously (Milligan et al., 2000, 2001).

Hargreaves test. Thresholds for behavioral response to heat stimuli applied to the tail were assessed in Experiment 4 using a modified Hargreaves test (Hargreaves et al., 1988) as described previously (Hutchinson et al., 2008a (in press)).

Chronic constriction injury (CCI)

Neuropathic pain was induced using the CCI model of partial sciatic nerve injury (Bennett and Xie, 1988). CCI was performed at mid-thigh level of the left hindleg as previously described (Hutchinson et al., 2008a (in press)). Drug testing was delayed until 10–14 days after surgery to ensure establishment of neuropathic pain prior to the initiation of drug delivery.

Catheter implantation and intrathecal drug administration

Acute intrathecal drug administration was based on that described previously. Catheters were preloaded with drugs at the distal end in a total volume of no greater than 25 μl and the drugs were administered over 20–30 s. In studies where drug injection was delayed until after recovery from anesthesia, the guide needle was removed after catheter placement, the catheter was sutured to the superficial musculature of the lower back, and the exterior end led s.c. to exit through a small incision at the nape of the neck. In this case, the catheters were 90 cm in length, allowing remote drug delivery 2 h after catheter placement, without touching or otherwise disturbing the rats during the testing.

TLR4 cell line culture and reporter protein assay

A human embryonic kidney-293 (HEK293) cell line stably transfected to express human TLR4 at high levels was purchased from Invivogen (293-htlr4a-md2cd14; here referred to as HEK-TLR4) and cultured and tested as previously described in detail (Hutchinson et al., 2008b, 2009 (in press)).

Cell fractionation and HSP90 expression quantification

Cultures of HEK-TLR4 cells were processed using a compartmental protein extraction kit (BioChain Institute, Hayward, CA, USA), to separate cytoplasmic proteins from membrane proteins. Additionally, the supernatant from each culture was lyophilized and re-suspended in a small volume of water in order to shift supernatant protein concentrations within a detectable range (at least 2 mg/ml). For each culture, the protein concentration of the cytoplasmic, membrane, and supernatant fraction was measured using the bicinchoninic acid method (Smith et al., 1985). Equivalent amounts of protein were pipetted blind as to group assignment into NuPAGE Bis–Tris (4–12%) gels (Invitrogen, Carlsbad, CA, USA), which were then run at 160 V for 1 h. Following electrophoresis, proteins were transferred to a nitrocellulose membrane electrophoretically at 40 V for 1 h. Non-specific binding sites on the membrane were blocked with 5% non-fat milk in TBS containing 0.5% tween-20. Membranes were incubated overnight at 4 °C with polyclonal primary antibodies against heat shock protein-90 (HSP90) (Cell Signaling, Danvers, MA, USA). After washing, the antibody-protein complexes were probed with appropriate sec-