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Spinal cord heme oxygenase participates in glutamate-induced pain-related behaviors

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Abstract

Heme oxygenase catalyzes the formation of CO, Fe^{2+} and biliverdin from the substrate heme. In these studies, we attempted to define the roles heme oxygenase play in pain-related behaviors induced by intrathecal injection of the spinal neurotransmitter glutamate. The intrathecal injection of glutamate or the more selective agonists *N*-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) in C57Bl/6 mice lead to caudally directed pain behaviors which were sensitive to the heme oxygenase inhibitors tin protoporphyrin (Sn-protoporphyrin) and chromium mesoporphyrin (Cr-mesoporphyrin). Intrathecal injections of glutamate in heme oxygenase type 2 (HO-2) null-mutant animals resulted in reduced pain-related behaviors when compared with wild type animals. Glutamate, NMDA and AMPA stimulated cGMP accumulation in mouse spinal cord slices, which was blocked by heme oxygenase inhibitors. Glutamate did not stimulate cGMP production in HO-2 null-mutant animals. Our data are consistent with the hypothesis that pain-related behaviors induced by spinal glutamate rely on the activation of HO-2 and subsequent production of cGMP.

Keywords: Heme oxygenase; Guanylate cyclase; cGMP; Glutamate; Null-mutant; Metalloporphyrin

1. Introduction

The release of glutamate from primary afferent nerve terminals in the dorsal horn of the spinal cord is a key event in nociceptive signal transduction. Investigators have provided evidence that at least three classes of excitatory amino acid receptors are involved, *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate. The role of spinal cord excitatory amino acid receptor activation has been further subdivided into the function of causing spontaneous pain-related behaviors like licking, biting and scratching, and hyperalgesia as can be documented using the hot plate, tail flick or mechanical assays (Alvarez-Vega et al., 2000; Brambilla et al., 1996; Chung et al., 2000; Ferreira et al., 1999; Kawamata and Omote, 1999; Masuyama and Shimizu, 1997; Meller et al., 1994).

A great deal of effort has been expended pursuing the mechanism linking excitatory amino acid receptor activation to pain and hyperalgesia. Evidence suggests that the activation of nitric oxide synthase (NOS) which ultimately results in increased cGMP production is involved in these pain pathways. Thus, the intrathecal perfusion of NMDA leads to pain-related behaviors and increases in spinal cGMP levels in rats (Kawamata and Omote, 1999), and intrathecal injections of NMDA and AMPA cause hyperalgesia in mice (Brambilla et al., 1996; Chung et al., 2000; Masuyama and Shimizu, 1997). These changes can be reduced by the coadministration of NOS inhibitors. Still others have measured increases in tissue cGMP content when spinal cord slices were incubated with excitatory amino acid receptor agonists (Morris et al., 1994; Vles et al., 2000). These cGMP increases were blocked with NOS inhibitors again implicating the NOS system in this signaling pathway.

While cGMP-independent signaling pathways can be activated by the NOS enzyme system, various investigators have shown that the intrathecal injection of cGMP analogs can by themselves cause hyperalgesia in rodents (Morris et al., 1994; Vles et al., 2000). It has also been suggested that long-term changes in neuronal sensitivity are initiated by changes in cGMP content (Lewin and Walters, 1999).

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Though still unclear, the cGMP generated in response to the presence of excitatory amino acids may act directly on cGMP-regulated ion channels or act through cGMP-dependent protein kinase to cause its ultimate effects (Tao et al., 2000; Tao and Johns, 2000).

The NOS enzyme system is certainly important, but is not the only enzyme system capable of controlling spinal cord cGMP levels to regulate pain thresholds. Attention is now being focused on the heme oxygenase system which, like NOS, generates a monoxide product (CO) capable of stimulating guanylate cyclase to produce cGMP. Roles for heme oxygenase have been established in mediating inflammatory, incisional and neuropathic pain (Li and Clark, 2000a,b). Additional data demonstrate that the ability of heme oxygenase to facilitate nociceptive signaling relies in part on a spinal mechanism (Meller et al., 1994; Yamamoto and Nozaki-Taguchi, 1995). Using a rat model system, it was recently demonstrated that mechanical hyperalgesia observed in response to the intrathecal injection of NMDA was reduced by the inclusion of heme oxygenase inhibitors in the injectate (Meller et al., 1992). In the present studies, we attempted to delineate the influence the spinal heme oxygenase enzyme system has on pain-related behaviors and cGMP increases stimulated by glutamate.

2. Methods

2.1. Animals

All experimental protocols were reviewed and approved by the VAPAHCS Subcommittee for Animal Studies prior to the initiation of work. All protocols conform to the guidelines for the study of pain in awake animals as established by the International Association for the Study of Pain. Every effort was made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. The mouse strain used for these experiments was the C57BL/6J (Charles River, Hollister, CA). In some experiments, HO-2 null-mutant mice in the C57Bl/6 background were used (Poss et al., 1995). Male mice between 16 and 22 weeks (23–25 g) were used for all experiments. All animals were kept six to eight per cage with a 12:12-h light/dark cycle and food and water ad libitum.

2.2. Intrathecal injection

Substances known to be involved in nociceptive neurotransmission were injected intrathecally (i.t.) into mice according to the method of Hylden and Wilcox (1980). For these injections, a small patch of fur was shaved the day before experimentation over the lumbar area of the mice. To assess behavioral responses, mice were briefly restrained with a gloved hand, and a 30 gauge, 1/2 in. needle was inserted intrathecally at approximately the L5/6 level with appropriate position signaled by advancement of the needle after walking the tip of off the adjacent lamina, and often by a brief twitch of the tail. There, 5 μl of drug solution was injected with a 25-μl microsyringe (Hamilton, Las Vegas, NV). A preliminary series of experiments using motor block with 5% lidocaine as a test of appropriate intrathecal injection documented a success rate of approximately 95%. Mice were then placed on a glass surface in a clear plastic cylinder with a 20-cm diameter for observation of behaviors. Each animal was used for a single experiment.

2.3. Behavioral assays

Animals having undergone intrathecal injection of AMPA, NMDA or glutamate exhibit pain-related behavior characterized by licking, biting and scratching the tail and hind limbs. For both of these substances, the majority (>90%) of pain-related behavior occurs within the first 5 min after intrathecal injection (Brambilla et al., 1996; Masuyama and Shimizu, 1997). Thus, animals having been injected were observed for this pain-related behavior for the first 5 min after injection while measuring the amount of time spent in the behavior with a stopwatch.

2.4. Spinal cord slice preparations

Mouse spinal cord slices were made from adult C57Bl/6 spinal cords as described previously (Li and Clark, 2001b). Briefly, animals were rapidly asphyxiated with CO_2 then underwent intracardiac perfusion with 20 cm^3 slicing buffer: (in mM) KCl 5, $MgSO_4$ 2, $NaHCO_3$ 26, NaH_2PO_4 1.25, D-glucose 10 and sucrose 252 at 4 °C. Spinal cords were next extruded under pressure using the same buffer and placed on a chilled cutting board. Using a #15 scalpel blade, 1-mm slices were made through the lumbar enlargement of the cord, and placed in recovery buffer: (in mM) NaCl 123, KCl 4, NaH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 26, $CaCl_2$ 2 and D-glucose 10, pre-equilibrated with 95% O_2/5% CO_2 at 15 °C. With continuous oxygenation, the slices were left for 30 min. Isobutylmethyl xanthine (IBMX) was added to the recovery buffer for the last 5 min of this incubation for a final concentration of 250 μM. Experimental incubations, which were performed in duplicate, began with the transfer of slices in 1-ml volume of recovery buffer/IBMX to the wells of a tissue culture plate containing the test substances modified to allow continuous O_2/CO_2 superfusion. This plate was mechanically agitated in a 30 °C water bath for 10 min. The accumulation of cGMP was determined to be linear with time during this period. Incubations were terminated by transferring slices to microfuge tubes containing 1-ml 6% trichloroacetic acid at 4 °C.

2.5. cGMP assays

The cGMP content of spinal cord slices was measured as recently described (Li and Clark, 2001b). Briefly, slices in trichloroacetic acid were homogenized with 3 × 5-s bursts

of a sonicator. Microfuge tubes were then centrifuged for 15 min at 12,000 × *g*. Supernatant (250 μl) was transferred to a clean tube and extracted four times with 1-ml water-saturated diethyl ether. The aqueous phase was then collected and frozen until assay. The cGMP assay was done using a colorimetric immunoassay for acetylated samples according to the manufacturer's directions (Amersham Pharmacia, Piscataway, NJ). A standard curve for cGMP was constructed using provided standards.

2.6. Drugs

The alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA, up to 100 ng i.t.) , *N*-methyl-D-aspartate (NMDA, up to 100 ng i.t.), sodium glutamate (up to 50 μg i.t. or 1 mM for slice experiments), 6-(phenylamino)-5,8-quinolinedione (LY-83,583) (up to 0.25 μg i.t. or 10 μM for slice experiments) and isobutyl methyl xanthine (IBMX, 250 μM) used for these experiments were purchased from Sigma (St. Louis, MO). The metalloporphyrins Sn-protoporphyrin, up to 0.75 μg i.t. or 5 μM for slice experiments) and chromium mesoporphyrin (Cr-mesoporphyrin 0.65 μg i.t.) were purchased from Porphyrin Products (Ogden, UT). These were made in 0.9% saline with the pH titrated to 7.0–7.4 prior to injections.

2.7. Statistical analysis

Analysis of repeated measures such as dose–response data was accomplished using an one-way analysis of variance (ANOVA) for repeated measures with post-hoc Dunnett's testing. For simple comparisons of two means, a Student's *t*-test was used. $*P<0.05$, $**P<0.01$. Data are expressed as mean ± S.E.M.

3. Results

3.1. Pain-related behavior after intrathecal administration of glutamate agonists

Glutamate as well as the more selective glutamate receptor agonists NMDA and AMPA elicited pain-related behaviors when injected intrathecally in mice. These behaviors were significantly reduced with the intrathecal co-administration of the heme oxygenase inhibitor Sn-protoporphyrin (Fig. 1A). Preliminary experiments were undertaken to derive doses of glutamate, NMDA and AMPA which caused similar degrees of licking behavior. Unfortunately, AMPA tended to cause excessive extensor posturing and even transient spasm when injected intrathecally at doses greater than 15 ng, so doses no higher than this were used. The more selective (Appleton et al., 1999) heme oxygenase inhibitor Cr-mesoporphyrin was also used to demonstrate a dose-dependent reduction of glutamate-induced behavior (Fig. 1B). Neither the systemic nor intrathecal injection of metalloporphyrins has been observed to cause significant sedation or neuromuscular deficits which would confound interpretation of our results (Li and Clark, 2000a). The injection of saline alone or metalloporphyrin at the doses used caused less than 4-s licking behavior on average.

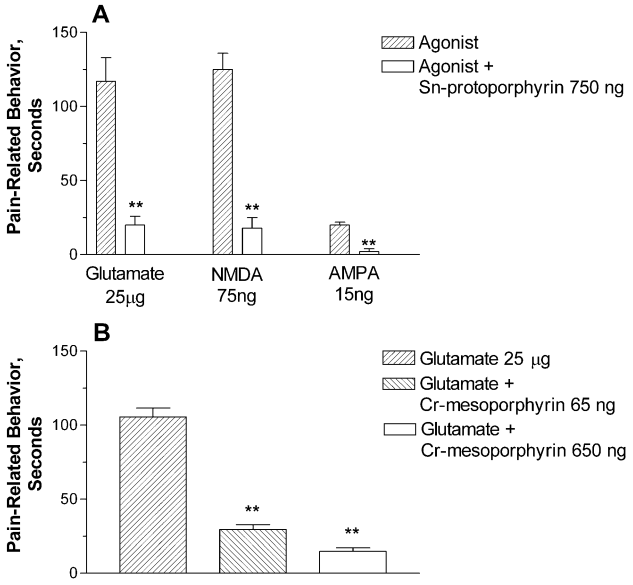

Fig. 1. Intrathecal glutamate receptor agonists elicit pain-related behaviors. In these experiments, c57Bl/6 mice were injected intrathecally with 5 μl of saline solution containing the named components. Pain-related behaviors consisting of caudally directed licking, biting and scratching were quantified in the 5 min following injection. Data represented by each bar are from five to eight animals. $*P<0.05$, $**P<0.01$.

3.2. The role of cGMP in mediating glutamate-induced pain-related behavior

We hypothesized that the production of cGMP stimulated by glutamate facilitated the pain-related behaviors. We therefore compared the intrathecal injection of glutamate alone to glutamate with the guanylate cyclase inhibitor LY-83,583. This inhibitor reduced the glutamate-induced pain-related behaviors in a dose-dependent fashion (Fig. 2A). In an additional series of experiments, we reproduced our observation of the inhibition of pain-related behaviors with Sn-protoporphyrin, and went on to show that the inclusion of the cGMP analog 8-Br cGMP in the intrathecal injectate along with glutamate and Sn-protoporphyrin nearly fully reconstituted the pain-related behaviors (Fig. 2B). Neither LY-83,583 or 8-Br cGMP induced licking behaviors in mice when injected alone.

3.3. Pain-related behaviors in HO-2 null-mutants

Because of the possibility of pharmacologically non-specific effects of the metalloporphyrins Sn-protoporphyrin and Cr-P, we turned our attention to HO-2 null-mutant mice. HO-2 is the predominant form of heme oxygenase

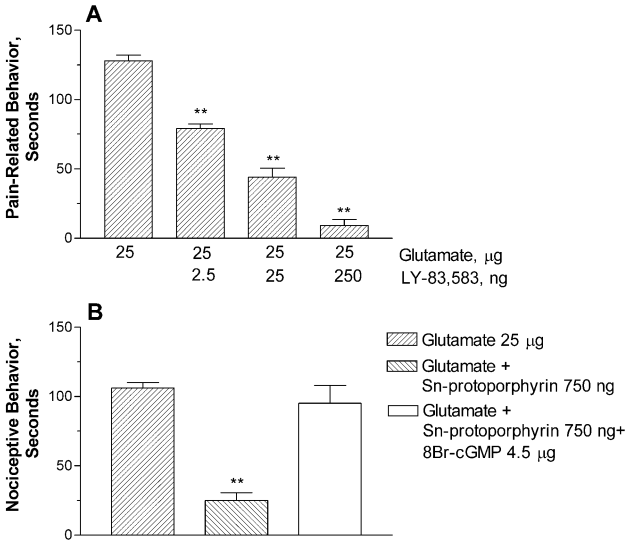

Fig. 2. The role of cGMP in glutamate-induced pain-related behavior. The data presented in panel A represent the total times spent in pain-related behaviors for 5 min after the intrathecal injection of 25 μg glutamate with various doses of the guanylate cyclase inhibitor LY-83,583. In panel B, mice were stimulated with the intrathecal injection of glutamate or glutamate with additional test substances. Data represented by each bar are from five to six animals. $*P<0.05$, $**P<0.01$.

expressed in the spinal cord (Dwyer et al., 1995). We had previously used the HO-2 null mutants in pain-related experiments and found them to have disrupted nociceptive responses in the formalin assay of acute inflammatory pain (Li and Clark, 2000a). In these experiments, we observed that HO-2 null-mutants had significantly reduced amounts of pain-related behavior observed after the intrathecal injection of glutamate (Fig. 3).

3.4. Glutamate receptor agonists and spinal cord slice cGMP levels

Having implicated cGMP produced indirectly by the activity of HO-2 in glutamate-induced pain-related behaviors, we used a recently developed spinal cord slice model for additional studies (Dwyer et al., 1995). In Fig. 4, data are presented which demonstrate that when mouse spinal cord slices are incubated with glutamate or the more selective agonists NMDA and AMPA, dose-dependent increases in tissue cGMP content can be documented thus confirming and extending previously reported observations (Kawamata and Omote, 1999; Vles et al., 2000). Furthermore, the inclusion of the heme oxygenase inhibitor Sn-protoporphyrin in the incubations at a concentration below that which interferes with NOS or guanylate cyclase activity directly (Appleton et al., 1999), nearly completely eliminated agonist-induced cGMP increases. Use of Cr-mesoporphyrin yielded similar results (data not shown). Control spinal cord slices averaged 11-pmol cGMP per mg tissue.

In additional experiments, we were able to demonstrate that the guanylate cyclase inhibitor LY-83,583 blocked glutamate-induced increases in cGMP content in the spinal cord slice model (Fig. 5A). Glutamate (1 mM) was ineffec-

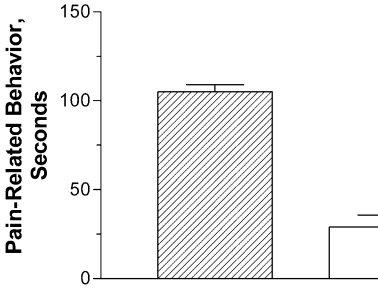

Fig. 3. Pain-related behavior elicited by glutamate in control and HO-2 null-mutant animals. In these experiments, 25-μg glutamate was injected intrathecally in C57Bl/6 wild type or HO-2 null mutant mice with the same background strain. Times spent in pain-related behaviors were measured, $n=6$/group. $*P<0.05$, $**P<0.01$.

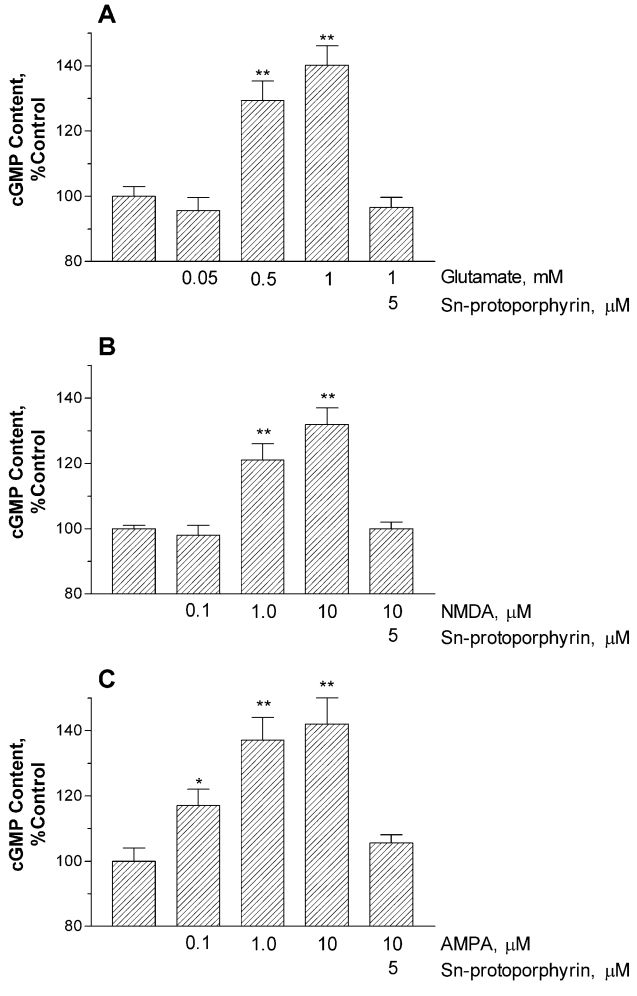

Fig. 4. Glutamate receptor agonists elicit cGMP accumulation in mouse spinal cord slices. In these experiments glutamate (panel A), NMDA (panel B), and AMPA (panel C) were used. Slices were incubated at 30 °C for 10 min prior to cGMP assay. The data represent the results of two or more slices analyzed in duplicate from three separate experiments for each condition. $*P<0.05$, $**P<0.01$.

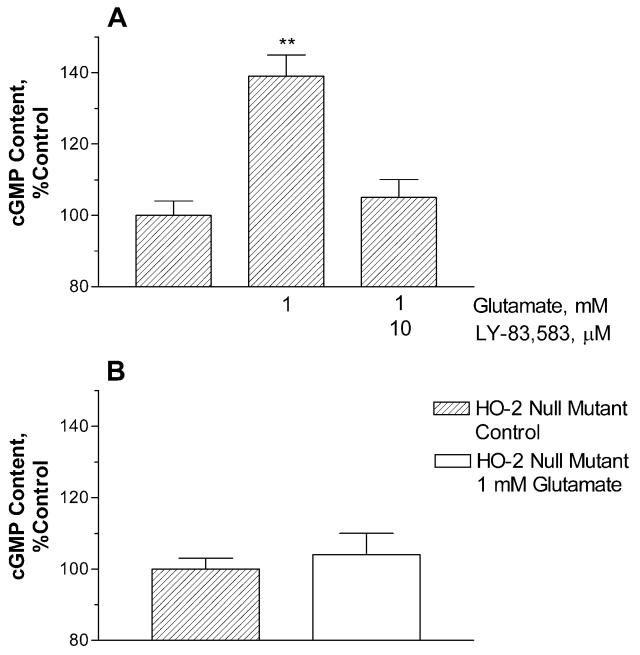

Fig. 5. The roles of guanylate cyclase and HO-2 in glutamate-induced cGMP accumulation. In panel A, spinal cord slices were incubated in saline or glutamate with or without LY-83,583. In panel B, spinal cord slices from HO-2 null-mutant animals were incubated in saline or saline containing 1-mM glutamate. The data represent the results of two or more slices analyzed in duplicate from three separate experiments for each condition. $*P<0.05$, $**P<0.01$.

tive at stimulating cGMP production in slices from HO-2 null-mutant animals (Fig. 5B).

4. Discussion

In planning these studies, we had hypothesized that on the behavioral level spinal glutamate would elicit pain-related behaviors which were dependent on the activity of HO-2, an enzyme expressed in spinal cord tissue known to be important to nociceptive signal transduction. Furthermore, since the only signaling pathway identified at this point in which HO-2 participates is one involving the production of CO from heme and the subsequent activation of guanylate cyclase to produce cGMP, we hypothesized that cGMP was a signaling molecule critical to the manifestation of pain-related behaviors in response to glutamate. On the biochemical level, we hypothesized that the increase in cGMP content of spinal cord slices in response to glutamate and more selective agonists was dependent to some extent on the activity of HO-2. Both pharmacological and genetic tools were utilized to examine these hypotheses.

In fact, when pain-related behaviors were assessed in response to glutamate and the selective agonists NMDA and AMPA, it was found that two potent metalloporphyrin heme oxygenase inhibitors, Sn-protoporphyrin and the more selective Cr-mesoporphyrin eliminated most of the pain-related behavior implicating the heme oxygenase enzyme system. These results parallel the results of Meller et al. (1994) who found that mechanical hyperalgesia induced by the intrathecal injection of NMDA in rats was sensitive to the heme oxygenase inhibitor Sn-protoporphyrin. Also consistent with these results, the HO-2 null mutant mice had significantly reduced glutamate-stimulated behaviors. These mice had previously been shown to exhibit less nociceptive behavior in response to subcutaneous formalin injection (Dwyer et al., 1995). Evidence that cGMP produced as the indirect result of heme oxygenase activity is a second messenger molecule facilitating these behaviors was found in the data demonstrating that the guanylate cyclase inhibitor LY-83,583 dose-dependently reduced glutamate-induced pain-related behavior. More importantly, the cGMP analog 8Br-cGMP was able to reconstitute the behavioral response to glutamate in the presence of Sn-protoporphyrin. It should be recognized that some concern has been raised about the specificity of this guanylate cyclase inhibitor such as the possibility that LY-83,583 may indirectly activate nitric oxide synthase (NOS) (Hobbs, 1997). This particular nonspecific action of LY-83,583 is unlikely to explain our results, however, since it would have the tendency to increase rather than decrease pain behaviors.

When spinal cord tissue was examined in vitro, increases in cGMP content were demonstrable in the presence of glutamate, NMDA and AMPA. Consistent with what was predicted from the behavioral experiments, these cGMP increases were reduced or eliminated when a heme oxygenase inhibitor or a guanylate cyclase inhibitor was present. We failed to measure a significant increase in spinal cord slice cGMP content in tissue from HO-2 null-mutant mice. In a recent report from our laboratory using the same slice model, it was shown that CO containing solutions could increase spinal cord slice cGMP content (Li and Clark, 2001b).

Our results using NMDA and AMPA do not exclude the possibility that glutamate also acts through metabotropic (mGluR) receptors to elicit pain behaviors. Pharmacological evidence has implicated spinal mGluRs as mediating pain in several different models (see, for example (Coderre, 1992; Minami et al., 1994; Yashpal et al., 2001b)). At this point, no evidence exists linking heme oxygenase activation to any particular class of receptors exclusively. In fact, HO-2 is very widely expressed in spinal cord tissue being present in a large fraction of the neurons in most spinal cord laminae (Li and Clark, 2001a).

Mechanistically, our results are consistent with a pathway in which glutamate acting through AMPA and NMDA receptors stimulates HO-2 activity. The resulting CO then goes on to stimulate guanylate cyclase leading to increased cGMP levels. It is unclear how the stimulation of glutamate receptors leads to HO-2 activation, but one recent report suggests that PKC may be able to phosphorylate HO-2 resulting in enzymatic activation (Dore et al., 1999). In fact, PKC activation mediated by excitatory amino acid receptors has been demonstrated in spinal cord tissue for both neuro-

pathic and incisional models of pain (Yashpal et al., 2001a). Our studies did not directly document HO-2 activation, but inferred glutamate receptor-induced activation based on the inhibitory effects of Sn-protoporphyrin, Cr-mesoporphyrin and HO-2 gene deletion on behavioral and biochemical responses. Also unclear is how the resulting cGMP goes on to facilitate the pain-related behavior, but cyclic nucleotide gated ion channels and/or cGMP-dependent protein kinase may be involved (Tao et al., 2000; Tao and Johns, 2000). It should be pointed out that cGMP analogs by themselves generally do not lead to spontaneous pain behaviors at low doses (present study, (Ferreira et al., 1999; Garry et al., 1994)), but in some way facilitate the manifestation of a full behavioral response. Other data have shown that heme oxygenase inhibitors do not alter baseline nociceptive thresholds, but reduce hyperalgesia and allodynia (Li and Clark, 2000a,b; Meller et al., 1994). Thus, the HO-cGMP pathway when activated may provide the conditions for a robust behavioral response, but additional events are required after glutamate receptor activation to cause the actual pain-related behaviors.

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