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The redox state of the alarmin HMGB1 is a pivotal factor in neuroinflammatory and microglial priming: A role for the NLRP3 inflammasome

Matthew G. Frank*, Michael D. Weber, Laura K. Fonken, Sarah A. Hershman, Linda R. Watkins, Steven F. Maier

Department of Psychology and Neuroscience, Center for Neuroscience, University of Colorado Boulder, CO, USA

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ABSTRACT

The alarmin high mobility group box-1 (HMGB1) has been implicated as a key factor mediating neuroinflammatory processes. Recent findings suggest that the redox state of HMGB1 is a critical molecular feature of HMGB1 such that the reduced form (fr-HMGB1) is chemotactic, while the disulfide form (ds-HMGB1) is pro-inflammatory. The present study examined the neuroinflammatory effects of these molecular forms as well as the ability of these forms to prime the neuroinflammatory and microglial response to an immune challenge. To examine the neuroinflammatory effects of these molecular forms in vivo, animals were administered intra-cisterna magna (ICM) a single dose of fr-HMGB1 (10 μg), ds-HMGB1 (10 μg) or vehicle and basal pro-inflammatory effects were measured 2 and 24 h post-injection in hippocampus. Results of this initial experiment demonstrated that ds-HMGB1 increased hippocampal pro-inflammatory mediators at 2 h (NF-κBIα mRNA, NLRP3 mRNA and IL-1β protein) and 24 h (NF-κBIα mRNA, TNFα mRNA, and NLRP3 protein) after injection. fr-HMGB1 had no effect on these mediators. These neuroinflammatory effects of ds-HMGB1 suggested that ds-HMGB1 may function to prime the neuroinflammatory response to a subsequent immune challenge. To assess the neuroinflammatory priming effects of these molecular forms, animals were administered ICM a single dose of fr-HMGB1 (10 μg), ds-HMGB1 (10 μg) or vehicle and 24 h after injection, animals were challenged with LPS (10 μg/kg IP) or vehicle. Neuroinflammatory mediators and the sickness response (3, 8 and 24 h after injection) were measured 2 h after immune challenge. We found that ds-HMGB1 potentiated the neuroinflammatory (NF-κBIα mRNA, TNFα mRNA, IL-1β mRNA, IL-6 mRNA, NLRP3 mRNA and IL-1β protein) and sickness response (reduced social exploration) to LPS challenge. fr-HMGB1 failed to potentiate the neuroinflammatory response to LPS. To examine whether these molecular forms of HMGB1 directly induce neuroinflammatory effects in isolated microglia, whole brain microglia were isolated and treated with fr-HMGB1 (0, 1, 10, 100, or 1000 ng/ml) or ds-HMGB1 (0, 1, 10, 100, or 1000 ng/ml) for 4 h and pro-inflammatory mediators measured. To assess the effects of these molecular forms on microglia priming, whole brain microglia were pre-exposed to these forms of HMGB1 (0, 1, 10, 100, or 1000 ng/ml) and subsequently challenged with LPS (10 ng/ml). We found that ds-HMGB1 increased expression of NF-κBIα mRNA and NLRP3 mRNA in isolated microglia, and potentiated the microglial pro-inflammatory response (TNFα mRNA, IL-1β mRNA and IL-1β protein) to LPS. fr-HMGB1 failed to potentiate the microglial pro-inflammatory response to LPS. Consistent with prior reports, the present findings demonstrate that the disulfide form of HMGB1 not only potentiates the neuroinflammatory response to a subsequent immune challenge in vivo, but also potentiates the sickness response to that challenge. Moreover, the present findings demonstrate for the first time that ds-HMGB1 directly potentiates the microglia pro-inflammatory response to an immune challenge, a finding that parallels the effects of ds-HMGB1 in vivo. In addition, ds-HMGB1 induced expression of NLRP3 and NF-κBIα in vivo and in vitro suggesting that the NLRP3 inflammasome may play role in the priming effects of ds-HMGB1. Taken together, the present results

* Corresponding author at: Department of Psychology and Neuroscience, Center for Neuroscience, Campus Box 345, University of Colorado Boulder, Boulder, CO 80309-0345, USA.

E-mail address: matt.frank@colorado.edu (M.G. Frank).

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suggest that the redox state of HMGB1 is a critical determinant of the priming properties of HMGB1 such that the disulfide form of HMGB1 induces a primed immunophenotype in the CNS, which may result in an exacerbated neuroinflammatory response upon exposure to a subsequent pro-inflammatory stimulus.

1. Introduction

The alarmin high mobility group box-1 (HMGB1) has been implicated as a key factor mediating neuroinflammatory processes in several pathophysiological conditions including seizure (Maroso et al., 2010), ischemia (Kim et al., 2006), chronic pain (Agalave et al., 2014) and alcohol-induced neuroinflammation (Zou and Crews, 2014). Recently, we have shown that HMGB1 is also a critical mediator of stress-induced priming of the microglial pro-inflammatory response to a subsequent immune challenge (Weber et al., 2015). However, the mechanism(s) by which HMGB1 exerts these neuroinflammatory effects has not been clarified.

HMGB1 is a nuclear protein that functions as a danger associated molecular pattern (DAMP), which is released into the extracellular milieu to signal cellular damage, cellular stress, or pathogen insult (Bianchi, 2007). The primary structure of HMGB1 is composed of an A box domain, which functions as an HMGB1 antagonist and a B box domain, which exhibits pro-inflammatory properties (Klune et al., 2008). In addition, HMGB1 can function either as a chemotactic or pro-inflammatory mediator depending on the redox state of three critical cysteines (Venereau et al., 2012). HMGB1 functions as a chemotactic factor if cysteines C23, C45, and C106 remain in a thiol state (fully reduced HMGB1; fr-HMGB1), but in this state lacks pro-inflammatory properties. Alternatively, HMGB1 exerts pro-inflammatory effects if cysteines C23 and C45 become oxidized, while C106 remains in a thiol state. Oxidation of C23 and C45 results in the formation of a disulfide bond (disulfide HMGB1; ds-HMGB1), which is a critical determinant of the cytokine stimulating capacity of HMGB1 (Yang et al., 2012). Further, ds-HMGB1 lacks chemotactic properties, which suggests that fr- and ds-HMGB1 are mutually exclusive molecular forms (Venereau et al., 2012). Notably, oxidation of all three cysteines abrogates both the chemotactic and pro-inflammatory activity of HMGB1 (Yang et al., 2012).

The fr-HMGB1 forms a complex with the chemokine C-X-C motif ligand 12 (CXCL12), which then signals through the chemokine receptor, C-X-C chemokine receptor type 4 (CXCR4), to mediate chemotaxis, while the pro-inflammatory effects of ds-HMGB1 are mediated by the pattern recognition receptor, Toll-like receptor 4 (TLR4) (Lu et al., 2013). TLR4-mediated effects of HMGB1 are dependent upon the thiol state of cysteine C106 (Yang et al., 2012). Interestingly, the box A domain of HMGB1 can by itself function to competitively antagonize the pro-inflammatory effects of HMGB1 (Yang et al., 2004), presumably through TLR4.

Very few studies have investigated the role of HMGB1 redox states in neuroinflammatory processes. Balosso and colleagues found that the ds-HMGB1 increased NMDA-induced neuronal cell death and potentiated kainate-induced seizures (Balosso et al., 2014). A recent study by Liesz and colleagues found that cerebral ischemia induced the release of the fr-HMGB1 from necrotic brain lesions and that HMGB1 in a disulfide redox state was released into serum (Liesz et al., 2015). Recently, we found that box A administered into the CNS blocks stress-induced sensitization of the microglial pro-inflammatory response to lipopolysaccharide (LPS) *ex vivo*, suggesting that stress induces the release of HMGB1 in the CNS, which then functions to sensitize neuroinflammatory processes (Weber et al., 2015). Although it is unclear which form of HMGB1 mediated stress-induced sensitization of microglia, we found that ds-HMGB1 administered into the CNS *in vivo* was sufficient to prime microglia, whereas fr-HMGB1 failed to induce priming. Several key questions arising from this study and addressed here pertain to (1) the ability of ds-HMGB1 to induce neuroinflammatory effects *in vivo* independent of priming, (2) the ability of ds-HMGB1 to prime neuroinflammatory processes *in vivo* to a subsequent immune challenge, (3) whether the neuroinflammatory priming effects of ds-HMGB1 are behaviorally relevant, (4) whether ds-HMGB1 directly acts upon microglia to induce a primed state, and (5) the mechanism by which ds-HMGB1 primes microglia and the neuroinflammatory response to a subsequent immune challenge.

Several lines of evidence raise the possibility that the nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain containing 3 (NLRP3) inflammasome might mediate ds-HMGB1-induced neuroinflammatory priming. The NLRP3 inflammasome is a multiprotein complex that mediates the processing and maturation of the pro-inflammatory cytokine interleukin (IL)-1β (Lamkanfi and Kanneganti, 2010). We and others have shown that stress induces NLRP3 (Pan et al., 2014; Weber et al., 2015) and NLRP3 is considered a sensor of a diverse array of DAMPs (Leemans et al., 2011). Of particular relevance here, formation of the NLRP3 inflammasome requires both a priming step and an activating step for the processing of IL-1β to proceed (Hornung and Latz, 2010). Therefore, the present investigation explored the effects of ds-HMGB1 on NLRP3 *in vivo* and *in vitro* as a mechanistic basis of the neuroinflammatory priming effects of ds-HMGB1.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (60–90 day-old; Harlan Sprague–Dawley, Inc., Indianapolis, IN, USA) were pair-housed with food and water available *ad libitum*. The colony was maintained at 25 °C on a 12-h light/dark cycle (lights on at 07:00 h). All rats were allowed 1 week of acclimatization to the colony rooms before experimentation. All experimental procedures were conducted in accordance with the University of Colorado Institutional Animal Care and Use Committee.

2.2. Reagents

Lyophilized fr-HMGB1 and ds-HMGB1 were obtained from HMGBiotech (Milan, IT), suspended in pyrogen-free sterile water and are certified LPS-free. LPS (*E. coli* serotype O111:B4) was obtained from Sigma (St. Louis, MO).

2.3. Intra-cisterna magna (ICM) injections of fr-HMGB1 and ds-HMGB1

Rats were anesthetized with isoflurane (~3 min). The dorsal aspect of the skull was shaved and swabbed with 70% EtOH, a 27-gauge needle, attached via PE50 tubing to a 25 μl Hamilton syringe, was inserted into the cisterna magna. To verify entry into the cisterna magna, cerebrospinal fluid (CSF) was withdrawn (~2 μl) and visually inspected for the presence of red blood cells. Clear

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