Caspase-1 Is Hepatoprotective during Trauma and Hemorrhagic Shock by Reducing Liver Injury and Inflammation

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Adaptive immune responses are induced in liver after major stresses such as hemorrhagic shock (HS) and trauma. There is emerging evidence that the inflammasome, the multiprotein platform that induces caspase-1 activation and promotes interleukin (IL)-1β and IL-18 processing, is activated in response to cellular oxidative stress, such as after hypoxia, ischemia and HS. Additionally, damage-associated molecular patterns, such as those released after injury, have been shown to activate the inflammasome and caspase-1 through the NOD-like receptor (NLR) NLRP3. However, the role of the inflammasome in organ injury after HS and trauma is unknown. We therefore investigated inflammatory responses and end-organ injury in wild-type (WT) and caspase-1–/– mice in our model of HS with bilateral femur fracture (HS/BFF). We found that caspase-1–/– mice had higher levels of systemic inflammatory cytokines than WT mice. This result corresponded to higher levels of liver damage, cell death and neutrophil influx in caspase-1–/– liver compared with WT, although there was no difference in lung damage between experimental groups. To determine if hepatoprotection also depended on NLRP3, we subjected NLRP3–/– mice to HS/BFF, but found inflammatory responses and liver damage in these mice was similar to WT. Hepatoprotection was also not due to caspase-1–dependent cytokines, IL-1β and IL-18. Altogether, these data suggest that caspase-1 is hepatoprotective, in part through regulation of cell death pathways in the liver after major trauma, and that caspase-1 activation after HS/BFF does not depend on NLRP3. These findings may have implications for the treatment of trauma patients and may lead to progress in prevention or treatment of multiple organ failure (MOF).

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these functions of caspase-1 have been shown to be independent of the production of IL-1β and IL-18 through experiments involving double knockout mice in a model of sepsis (12).

Given this growing body of evidence indicating a diverse, multifaceted role for caspase-1 in inflammation, we hypothesized that it would play a significant role in the setting of trauma/posttraumatic inflammation. Therefore, we subjected wild-type (WT) and caspase-1–/– mice to HS with bilateral femur fracture (HS/BFF) with the hypothesis that caspase-1 deficiency would attenuate the proinflammatory response with decreased levels of inflammatory cytokines, therefore decreasing remote organ damage after severe trauma. However, in contradiction to our hypothesis, we found an increased proinflammatory cytokine profile, increased hepatocellular death and cellular damage in caspase-1–/– mice compared with WT mice after HS/BFF. Our data therefore suggest that caspase-1 balances the posttraumatic inflammatory response and is an important component of hepatocellular survival. We also show that caspase-1 activation after severe trauma is unlikely to occur through activation of the NLRP3 inflammasome, since we did not see increased inflammation or increased liver damage in NLRP3−/− mice after HS/BFF compared with WT mice. These findings shed new light on the role of caspase-1 in the setting of severe trauma and may lead to new therapeutic approaches for severely injured patients who are prone to multiple organ failure.

MATERIALS AND METHODS

Animal Care

All experimental protocols were approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh. Experimental procedures were carried out in accordance with all regulations regarding the care and use of experimental animals, as published by the National Institutes of Health. Male C57BL/6 (WT) mice (Charles River Laboratories International, Wilmington, MA), caspase-1−/− mice (a gift from Richard Flavell, Yale University, New Haven, CT, USA [13]), and NLRP3 (NALP3, pyrin-1)−/− mice (Millennium Pharmaceuticals, Boston, MA, USA) aged 7–11 weeks, weighing 21–30 g, were used in experiments. Additionally, IL-18−/−, IL-18R−/− and IL-1R1−/− mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were allowed access to rodent chow and water ad libitum.

Genotyping of Caspase-1−/− Mice

Mice were genotyped by reverse transcriptase–polymerase chain reaction (PCR) of digested tail tissue using the following primers: caspase-1 forward: GAGACATATAAGGGAGAGG; caspase-1 reverse: ATGGCACACACAGA TATCGG; and caspase-1 neo: TGCTA AAGGCCATGCTCCAGACTG. PCR conditions used were as follows: 94°C for 3 min; then 35 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s; and then held at 72°C for 5 min before cooling to 4°C until run on 2% agarose gel. Bands were visualized using ethidium bromide. WT mice were identified by a single band at 500 bp. Caspase-1−/− mice were identified by a single band at 300 bp. Heterozygous mice had both bands visible.

Experimental Groups

Mice from each mouse strain were assigned to three groups: control (no manipulation, n = 2–3 per strain), sham (femoral artery cannulation only, n = 3–5 per strain) and HS/BFF (1.5 h HS + 4.5 h fluid resuscitation and bilateral femur fracture, n = 4–6 per strain). HS/BFF group mice were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg; Ovation Pharmaceuticals, Deerfield, IL, USA) and inhaled isoflurane (Abbott Labs, Chicago, IL, USA). After bilateral groin dissections, both femoral arteries were cannulated using PE-10 tubing flushed with 2 units of heparin per animal (Pharmacia & Upjohn, Kalamazoo, MI, USA). Bilateral femur fractures were manually induced using hemostats. Baseline mean arterial pressure was determined with the catheter connected to a blood pressure transducer (Micro-Med, Tustin, CA, USA). Then, using the second catheter, hemorrhage was induced to a mean arterial pressure of 25 mmHg for 1.5 h followed by fluid resuscitation with Ringer’s solution (3x the volume of shed blood) through the catheter. Mice were kept under anesthesia for an additional 4.5 h. Sham group mice underwent initial cannulation procedures and anesthesia only. Control mice were sacrificed without any procedures performed to obtain physiological baseline levels. All animals were sacrificed after a total time course of 6 h.

Reagents

Antibodies for Western blot analysis: caspase-1 was from Upstate (Millipore, Billerica, MA, USA); β-actin was from Biovision (San Francisco, CA, USA); and caspase 8, 9 and 12 and cleaved poly(ADP-ribose) polymerase (PARP) were from Cell Signaling Technology (Danvers, MA, USA). Receptor interacting protein-1 (RIP-1) was from LifeSpan Biosciences (Seattle, WA, USA), and Bel2 was from Abcam (Cambridge, MA, USA).

Western blot analysis was performed as previously published (14). Western gel images were quantified by densitometry using Image J software (National Institutes of Health, Bethesda, MD, USA).

Blood and Tissue Collection and Plasma Analysis

Anesthetized mice were euthanized by cardiac puncture and blood withdrawal. Immediately after cardiac puncture, the liver and the lungs were harvested and snap-frozen in liquid nitrogen and then stored at −80°C. The collected heparinized blood samples were centrifuged at 2,300g for 10 min, and plasma was aliquoted and stored at −80°C. Immediately, one sample was used for quantification of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Dry-Chem Veterinary Chemistry Analyzer, HESKA, Loveland, CO, USA; slides from Fujifilm Corporation, Asaka-shi Saitama, Japan). Plasma cytokine levels were determined...
either by enzyme-linked immunosorbent assay (ELISA) for IL-18 (MBL, Naka-ku, Japan), IL-6 and IL-10 (R&D, Minneapolis, MN) or using Luminex™ multiplexing bead array platform (MiraiBio, Alameda, CA, USA) using a mouse cytokine bead sets for IL-1α, IL-1β, IL-6, IL-10, IL-12, keratinocyte-derived chemokine (KC) and monocyte chemoattractant protein (MCP)-1 (Invitrogen, San Diego, CA, USA).

**Lung Myeloperoxidase Activity**

Myeloperoxidase (MPO) activity was determined by ELISA of tissue lysates from lungs. The assay was performed according to the manufacturer’s instructions (Mouse MPO ELISA kit; Cell Sciences, Canton, MA, USA). Briefly, 10 mg frozen lung tissue was homogenized in lysis buffer with a Tissue Tearor™ machine (Biospec Products, Bartlesville, OK, USA). After centrifugation at 1,500 g for 15 min, supernatants were collected and diluted five-fold. Subsequently, MPO activity of each sample was determined and then standardized to its respective protein concentration (BCA Protein Assay Kit; Pierce, Rockford, IL, USA).

**Immunofluorescence and Confocal Microscopy**

Portions of harvested livers were fixed in 2% paraformaldehyde for 2 h followed by cryopreservation. Apoptotic cells in liver sections were identified using terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL) staining following the manufacturer’s protocol (Promega Corporation, Madison, WI). Nuclei were counterstained with Hoechst nuclear stain (Invitrogen). TUNEL-positive cells were imaged using a Nikon microscope (Nikon, Melville, NY, USA) and quantitated using a Metamorph™ image acquisition and analysis system (Universal Imaging, West Chester, PA, USA). TUNEL-positive cells from six random fields per section were counted blindly and expressed as a percentage of the total cell number for those fields. Immunofluorescence was used to determine the number of Ly-6G-positive neutrophils in liver sections. Liver sections (5 μm) were incubated with 2% bovine serum albumin (BSA) in PBS for 1 h, followed by five washes with PBS + 0.5% BSA (PBB). The samples were then incubated with rat Ly-6G primary antibody (1:100; BD Pharmingen, San Diego, CA, USA) for 1 h at 37°C. Samples were washed 5× with PBB followed by incubation in Cy3 secondary antibody diluted in PBB (1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Samples were washed 3× with PBB, followed by a single wash with PBS before a 30-s incubation with Hoeschst nuclear stain. Nuclear stain was removed and samples were washed with PBS before being cover-slipped with Gelvatol (23 g polyvinyl alcohol 2000, 50 mL glycerol, 0.1% sodium azide to 100 mL PBS). Positively stained cells in six random fields were imaged on a Fluoview 1000 confocal scanning microscope (Olympus, Melville, NY, USA). Imaging conditions were maintained at identical settings with original gating performed using the negative control (no primary antibody).

**Figure 1.** Caspase-1 is activated after HS/BFF. (A) Western blot images showing caspase-1 is activated (cleaved) in liver of WT (C57BL/6) mice at 6 h after HS/BFF. (B) Caspase-1−/− mice did not express IL18 at baseline or after HS/BFF but NLRP3−/− mice expressed higher levels of IL18 than WT mice after HS/BFF measured by ELISA. (C) Western blot images with densitometry showing caspase-1 is activated (cleaved) in liver of NLRP3−/− mice at 6 h after HS/BFF. n = 3–6/group. Data show mean ± SEM. *P < 0.05, control versus HS/BFF; #P < 0.05 NLRP3−/− HS/BFF versus WT HS/BFF. C, control; ND, not detected; S, sham.
Caspase-1 Is Hepatoprotective in Trauma

Caspase-1 Is Activated during HS/BFF in Mice

To assess the role of the inflammasome and caspase-1 in the inflammatory response and end-organ injury that occurs after trauma, we compared the response of WT, caspase-1–/– and NLRP3–/– mice subjected to HS/BFF (Figure 2). As shown in Figure 1B, IL-18 was detectable at baseline in WT mice and also in sham-treated WT mice at low levels. IL-18 levels were significantly increased in WT mice at 6 h after HS/BFF compared with control or sham mice (Figure 1B). As expected, caspase-1–/– mice did not express any detectable IL-18 even after HS/BFF. We also investigated IL-18 levels in NLRP3–/– mice after HS/BFF, to determine whether this is the main inflammasome activated during trauma. Interestingly, IL-18 levels in NLRP3–/– mice were significantly higher than in WT mice (Figure 1B), and there was similar activation of caspase-1 in NLRP3–/– liver after HS/BFF compared with WT (Figure 1C). These data provide evidence for activation of caspase-1 within the initial 6 h after HS/BFF, but also suggest that NLRP3 is not the main inflammasome involved in this activation. Although IL-1β is also processed by caspase-1, this cytokine has not been proven as a reliable marker for caspase-1 activation, since there are multiple other pathways that can cleave pro–IL-1β to the active form (15). We detected low levels of IL-1β in plasma of both WT (21.2 ± 4.6 pg/mL) and caspase-1–/– (23.9 ± 12.1 pg/mL) mice at 6 h after HS/BFF.

Caspase-1–/– Mice Produce Increased Levels of Inflammatory Cytokines after Trauma

We next assessed the magnitude of the systemic inflammatory response by measuring circulating levels of several cytokines and chemokines known to be elevated after trauma. As expected, levels of IL-6, IL-12, KC and MCP-1 were all increased after HS/BFF in WT mice, with the greatest increases in IL-6 and KC (Figure 2). Unexpectedly, even greater increases in IL-6, IL-12 and MCP-1 were seen in caspase-1–/– mice after HS/BFF (Figure 2), with no differences between WT and NLRP3–/– circulating cytokine levels (data not shown). These data suggest that the absence of caspase-1 leads to a greater initial systemic inflammatory response that does not depend on NLRP3 inflammasome activation. Interestingly, KC levels were not different between WT and caspase-1–/– mice after HS/BFF at 6 h.

Caspase-1–/– Mice Have Worse Organ Damage after Severe Trauma Compared with WT Mice

As markers of liver damage, we measured plasma levels of AST and ALT in WT, caspase-1–/– and NLRP3–/– mice after HS/BFF. AST and ALT levels were significantly increased in both WT and caspase-1–/– mice after HS/BFF compared with baseline levels (Figure 3A). However, the levels of both AST and ALT were significantly higher in plasma from caspase-1–/– mice compared with WT mice (Figure 3A). Again, there was no difference in AST and ALT levels in NLRP3–/– mice compared with WT mice at 6 h after HS/BFF (Figure 3A). These data further suggest that caspase-1 activation independent of NLRP3 inflammasome is protective during trauma, with increased liver damage corresponding well with increased cytokine levels in caspase-1–/– mice.

To further investigate the extent of increased liver damage in caspase-1–/– mice after HS/BFF, we assessed necrosis in the liver by hematoxylin and eosin (H&E) staining of liver sections as well as by RIP-1 expression in liver tissue. We found evidence of increased necrotic areas of liver in caspase-1–/– mice compared with WT mice by H&E staining, but again NLRP3–/– liver showed similar necrosis to WT liver at 6 h after HS/BFF (Figure 3B). Necrosis was mainly centrilobular and much more extensive in caspase-1–/– mice (Figure 3B). We also detected increased expression of RIP-1, a marker of necrosis, in caspase-1–/– whole liver lysates by Western blot compared with WT mice (Figure 3C). Even sham injury resulted in increased RIP-1 levels in caspase-1–/– liver compared with WT. Altogether, these data show evidence of increased cell death in the liver of caspase-1–/– mice after HS/BFF.

Caspase-1-dependent cytokines, IL-1β and IL-18, have been shown in some...
studies of inflammation to be protective in certain models. We therefore wanted to determine whether a lack of IL-18 or IL-1β signaling was responsible for the increased liver damage seen in caspase-1<sup>-/-</sup> mice after HS/BFF. We used commercially available IL-18ko, IL-18Rko and IL-1R1ko mice and subjected them to HS/BFF similarly to caspase-1<sup>-/-</sup> mice. There were no statistical differences in either AST or ALT levels between these strains of mice and WT (C57BL/6) mice either after sham injury or after HS/BFF (Figure 4). IL-1R1ko mice trended toward lower levels of AST and ALT than WT or IL-18 signaling-deficient mice, but this was not statistically significant.

These data suggest that neither IL-1<sub>β</sub> nor IL-18 signaling is protective during HS/BFF.

**Increased Neutrophil Influx in Caspase-1<sup>-/-</sup> Liver Compared with WT**

We also determined neutrophil influx into both lung and liver as further markers of organ inflammation (Figure 5).

Liver Ly-6G levels were significantly increased in caspase-1<sup>-/-</sup> mice compared with WT mice after HS/BFF (Figure 5B). However, pulmonary MPO levels were similar between WT and caspase-1<sup>-/-</sup> mice despite being significantly increased compared with sham and baseline (Figure 5A). These data may suggest caspase-1 regulates proinflammatory pathways preferentially in the liver, or that at the time point measured, any early or late differences in neutrophil accumulation in the lung may not be present at 6 h.

**Caspase-1<sup>-/-</sup> Mice Have Increased Apoptosis in Liver after Trauma Compared with WT Mice**

As caspase-1<sup>-/-</sup> mice showed increased proinflammatory cytokine production and also increased indication of liver damage after trauma compared with WT mice, we wanted to determine whether these differences were secondary to changes in apoptosis in the liver.

Caspase-1 is known to have regulatory effects on apoptosis, as well as regulating another form of inflammatory cell death, pyroptosis (16). Both apoptosis and pyroptosis increase TUNEL staining in cells. We therefore determined the number of TUNEL-positive cells in liver sections from WT and caspase-1<sup>-/-</sup> mice after sham surgery and after HS/BFF, compared with unmanipulated controls. TUNEL staining increased significantly in livers of both WT and caspase-1<sup>-/-</sup> mice after sham surgery alone compared with controls (Figure 6A). Levels of TUNEL staining were significantly increased again in caspase-1<sup>-/-</sup> mice after HS/BFF, but this increase did not occur in WT mice after HS/BFF (Figures 6A, B).

These data suggest an increase in apoptosis in caspase-1<sup>-/-</sup> mice after HS/BFF because pyroptosis is considered to be caspase-1 and IL-1β dependent (16).
We therefore investigated levels of other apoptosis markers in liver of caspase-1–/– and WT mice by Western blot. There were increased levels of proapoptotic caspase-12 and cleaved PARP (cPARP) at baseline in caspase-1–/– liver compared with WT (Figures 6C, D). Although neither caspase-12 nor cPARP levels increased in caspase-1–/– mice after HS/BFF, levels remained statistically significantly higher in caspase-1–/– liver than in WT after HS/BFF. Caspase-8 and caspase-9 levels were significantly higher at baseline in WT liver compared with caspase-1–/– liver (Figures 6C, D). Caspase-8 levels significantly increased in caspase-1–/– liver after both sham injury and HS/BFF and were then significantly higher than corresponding WT levels. WT levels of caspase-9 decreased significantly.
cantly after HS/BFF, but caspase-9 levels in caspase-1−/− liver remained unchanged.

Changes in the antiapoptotic protein, Bcl2, were also seen in caspase-1−/− liver. At baseline, Bcl2 levels were similar between WT and caspase-1−/− mice (Figure 6C). However, Bcl2 levels were significantly decreased in caspase-1−/− liver after HS/BFF and even after minor sham injury. However, Bcl2 levels did not change in WT liver. Altogether these data show an increase in overall levels of proapoptotic proteins and a concomitant decrease in the antiapoptotic protein, Bcl2. Therefore, these findings support a regulatory role for caspase-1 in cell death pathways in the liver, which may result in hepatoprotection after HS/BFF.

DISCUSSION

Posttraumatic systemic inflammation plays a pivotal role in the development of subsequent remote organ damage (17,18). We have shown in this study that activation of caspase-1 after severe trauma may be protective in the liver and that this protection may be partly through the regulation of cell-death pathways. Caspase-1 activation (cleavage) is known to occur through the formation of the inflammasome, a signaling platform involving NLRs and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (19). Cleavage of caspase-1 then classically leads to the cleavage of pro-IL-1β, and pro-IL-18 with the subsequent release of active proinflammatory cytokines into systemic circulation (18,20,21). Blocking inflammasome formation and activation may therefore be a useful strategy in modifying the inflammatory response, especially in diseases where IL-1β plays a main role in inflammation, including during the inflammatory response to trauma (22,23).

We therefore expected that in mice lacking the main inflammasome effector protein, caspase-1, there would also be a reduction in the level of the inflammatory response associated with a severe trauma model that includes bilateral femur fracture and tissue hypoxia secondary to damage secondary to increases in circulating proinflammatory cytokines, such as the increases seen in IL-6 and IL-12 in caspase-1−/− mice after HS/BFF. Reducing cytokine-induced liver damage may therefore be one mechanism through which caspase-1 is protective in our model. However, it is also unclear at present if the higher cytokine levels in the caspase-1−/− mice contribute to the increased liver damage, or if higher cytokine levels are the result of greater organ injury.

Another important finding from our study is that activation of caspase-1 does not seem to occur primarily through the NLRP3 inflammasome. There are many NLRPs that have been identified as being expressed in different cell types, but at present, only a handful have been shown to form an inflammasome and activate caspase-1, including NLRP1, NLRP3, absent in melanoma-2 (AIM2) and ice protease activating factor (IPAF) (29). Our findings are particularly interesting because, to date, almost all DAMPs, including reactive oxygen species, activate the NLRP3 inflammasome. However, AIM2 may also be important in our model, since it has been shown to be activated by DNA, which can be released as a result of tissue damage and subsequent cell death. It is also possible that DAMPs can activate another inflammasome, such as NLRP1, or lead the formation of the ASC inflammasome without the need for an NLR, as has been shown in some infection models (30,31). Further studies are needed to fully identify the main activators of caspase-1 after trauma.

We have shown evidence that caspase-1 deficiency may also predispose to an increased tendency to the induction of apoptosis during trauma. Caspase-1 has been shown to regulate apoptosis pathways through binding to antiapoptotic proteins, Bcl2 and BclXL (32). We show here that caspase-1−/− mice significantly downregulate liver expression of Bcl2 after trauma, and this may have profound effects on apoptosis. A recent report also identified another regulator of apoptosis, PARP, as a cleavage target for caspase-1 itself (28). Our results support an effect of absence of caspase-1 leading
CASPASE-1 IS HEPATOPROTECTIVE IN TRAUMA