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Cyclophilin A Is a Damage-Associated Molecular Pattern Molecule That Mediates Acetaminophen-Induced Liver Injury

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The immune system is alerted to cell death by molecules known as damage-associated molecular patterns (DAMPs). These molecules partly mediate acetaminophen-induced liver injury, an archetypal experimental model of sterile cell death and the commonest cause of acute liver failure in the western world. Cyclophilin A (CypA) is an intracellular protein that is proinflammatory when released by cells. We hypothesized that CypA is released from necrotic liver cells and acts as a DAMP to mediate acetaminophen-induced liver injury. Our data demonstrated that mice lacking CypA (Ppia−/−) were resistant to acetaminophen toxicity. Antagonism of the extracellular receptor for CypA (CD147) also reduced acetaminophen-induced liver injury. When injected into a wild-type mouse, necrotic liver from Ppia−/− mice induced less of an inflammatory response than did wild-type liver. Conversely, the host inflammatory response was increased when CypA was injected with necrotic liver. Antagonism of CD147 also reduced the inflammatory response to necrotic liver. In humans, urinary CypA concentration was significantly increased in patients with acetaminophen-induced liver injury. In summary, CypA is a DAMP that mediates acetaminophen poisoning. This mechanistic insight presents an opportunity for a new therapeutic approach to a disease that currently has inadequate treatment options. The Journal of Immunology, 2011, 187: 000–000.

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Materials and Methods

**Animals**

Ppia<sup>−/−</sup> mice were purchased as cryopreserved embryos from The Jackson Laboratory (Bar Harbor, ME) (strain name: 129.Cg-Ppiatm1Lubn/J; stock number 5320). All mice were genotyped by PCR using tissue from ear clips. The genotyping results were validated by Western blotting (Ab: rabbit anti-CypA; Cell Signaling Technology, Beverly, MA). In separate studies, commercial C57BL/6 mice (Harlan) were used. All investigations conformed with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 82-23, revised 1996) and were performed with appropriate Home Office (U.K.) licenses.

**Model of acetaminophen poisoning**

After a 6-h fast, mice were injected i.p. with acetaminophen dissolved in sterile saline (350 mg/kg) or sterile saline vehicle control. After 6–24 h, blood was collected by cardiac puncture, and tissue was collected for histology and glutathione assay. Histology was assessed by a reviewer blinded to the mouse genotype. Glutathione was measured by colorimetric assay, per the manufacturer’s instructions (Sigma-Aldrich, Gillingham, U.K.). In some studies, C57BL/6 mice were treated with a rat anti-CD147 Ab or isotype control Ab (25 μg, i.p.) 2 h prior to acetaminophen injection.

**FIGURE 1.** Serum ALT concentrations were lower in Ppia<sup>−/−</sup> mice (KO) lacking the gene for CypA than in littermate controls (WT) 24 h after acetaminophen injection. Serum ALT concentration after vehicle injection is also presented. A. These data are presented as individual mice. B. Liver glutathione concentration (GSH) is reduced 6 h after acetaminophen injection in both KO and WT mice compared with after vehicle injection (n = 5 per group; graphs represent mean ± SEM). C. Pretreatment with an anti-CD147 Ab significantly reduced serum ALT 24 h after acetaminophen injection compared with an isotype Ab (n = 10 per group; graphs represent mean ± SEM). *p < 0.05, compared with WT or control.

**FIGURE 2.** Histological liver injury was less in Ppia<sup>−/−</sup> mice (KO; lower panel) than in sex-matched littermate controls (WT; upper panel) 24 h after acetaminophen injection. Cell vacuolation is reduced in the KO mouse. H&E staining. Scale bar, 200 μm.

**FIGURE 3.** A. Serum IL-6 concentrations were lower in Ppia<sup>−/−</sup> mice (KO) than in littermate controls (WT) 24 h after acetaminophen injection. B. Necrotic liver cells from Ppia<sup>−/−</sup> mice (WT) lacking the gene for CypA induced a lower serum IL-6 concentration than did WT liver cells when injected into WT mice. (n = 10 mice/group.) Bar graphs represent mean ± SEM. *p < 0.05.
The anti-CD147 Ab (from RL73.2 hybridoma) and isotype control Ab were kind gifts from Dr. Stephanie Constant (George Washington University, Washington, D.C.). Serum alanine transaminase (ALT) was measured on a Cobas Fara centrifugal analyzer (Roche Diagnostics, Welwyn Garden City, U.K.), and serum IL-6 concentration was measured by ELISA (R&D Systems, Minneapolis, MN).

**Model of the inflammatory response to necrotic liver**

To determine whether CypA acts as a DAMP, we followed the protocol used by Chen et al. (24). Livers were removed from wild-type (WT) and Ppia−/− mice. After homogenization in sterile saline and centrifugation, the resulting supernatant was freeze/thawed five times. Mice were then injected i.p. with the necrotic liver cells (36 mg protein). After 6 h, the peritoneal cavity was lavaged with 5 ml warmed sterile saline, and blood was taken by cardiac puncture. In a second study, necrotic liver from C57BL/6 mice (36 mg protein) was injected i.p. into C57BL/6 mice that had been treated 2 h before with either anti-CD147 Ab or isotype control Ab (25 μg i.p.). After 6 h, the peritoneal cavity was lavaged with 5 ml warmed sterile saline. In a third study, necrotic liver from C57BL/6 mice (20 mg protein) was injected i.p. into C57BL/6 mice together with 100 μg purified CypA (a kind gift from Prof. M. Walkinshaw, Edinburgh University) or vehicle (saline). After 6 h, the peritoneal cavity was lavaged with 5 ml warmed sterile saline.

Concentrations of myeloperoxidase (MPO) and keratinocyte-derived chemokine (KC) were measured in the lavage fluid by ELISA (Calbiochem, La Jolla, CA), whereas IL-6 concentration was measured in blood by ELISA (R&D Systems).

**Human studies**

Urine samples were collected from patients at the Royal Infirmary of Edinburgh (Edinburgh, U.K.). The study was prospectively approved by the Scotland ‘A’ Research Ethics Committee. Informed consent or assent was obtained from all patients or the patient’s nominated next of kin before study inclusion. The inclusion criterion for this study was an adult patient with a clear history of excess acetaminophen ingestion. Exclusion criteria were patients detained under the Mental Health Act; patients with known permanent cognitive impairment, an acute life-threatening illness, or an unreliable history of overdose; and patients who take anticoagulants (e.g., warfarin) therapeutically or have taken an overdose of anticoagulants. Patients were classified into four groups based on routine blood results: not-detected (ND) group, acetaminophen not detected in the blood 4 h after ingestion; below-line group, acetaminophen was detected in the blood, but the concentration was below the treatment line on the Rumack–Matthew nomogram; above-line group, acetaminophen concentration was above the treatment line, but no liver injury occurred subsequent to the overdose; and organ-injury group, ALT 10× the upper limit of normal (>500 U/l) and a clear history of acetaminophen ingestion. All urine samples were collected within the first 24 h of hospital admission and were stored at −80°C.

The urinary CypA concentration was determined by Western blotting, as previously described (22). The team member performing the Western blotting was blinded to which of the four groups the urine sample belonged. Each gel contained at least one sample from each patient group and a positive control (purified human CypA). The primary Ab was a rabbit anti-human CypA Ab (USBiological, Swampscott, MA), and the secondary Ab was an HRP-conjugated anti-rabbit Ab (Santa Cruz Biotechnology, Santa Cruz, CA). After visualization of the secondary Ab’s binding using ECL, the photographic films were scanned using the VersaDoc imaging system (Bio-Rad, Hercules, CA), and relative band densities were measured using Quantity One software (Bio-Rad). The relative band densities were normalized by urinary creatinine concentration.

In a substudy, one urine sample was separated into the exosomal and nonexosomal fraction to determine the localization of urinary CypA. The sample was centrifuged at 15,000 × g for 10 min to pellet any shed cells, large membrane fragments, and other debris. The supernatant was then centrifuged at 200,000 × g for 1 h to pellet the exosomes (25). The pellet and supernatant were probed by Western blotting for CypA and the urine exosome marker CD24 (26) (anti-CD24 Ab, a kind gift of Dr. P. Altevogt, German Cancer Research Center, Heidelberg, Germany).

**Statistical analysis**

Pilot studies with WT mice demonstrated that, 24 h after acetaminophen injection, the mean serum ALT concentration was ∼4000 U/l, with an SD ∼2500 U/l. Therefore, detection of a 2000-U/l difference between groups, with 80% power at a two-sided 5% significance level, required ∼50 mice.

**FIGURE 4.** A and B, In WT mice, peritoneal lavage fluid concentrations of KC and MPO were lower 6 h after injection of necrotic liver from Ppia−/− mice (KO) lacking the gene for CypA than 6 h after injection of WT liver. C and D, In WT mice 6 h after injection of WT necrotic liver, peritoneal lavage fluid concentrations of KC and MPO were lower following pretreatment with an anti-CD147 Ab compared with isotype control. (n = 10 mice/group.) Bar graphs represent mean ± SEM. *p < 0.05.

**FIGURE 5.** The peritoneal lavage fluid concentration of KC (A) and MPO (B) was increased following i.p. injection of necrotic liver from WT mice (20 mg) plus CypA (100 μg) compared with vehicle (saline) plus necrotic liver. The lavage fluid concentration of KC and MPO after vehicle and CypA injection without liver is also presented. (n = 10 mice/group.) Bar graphs represent mean ± SEM. *p < 0.05, versus vehicle plus liver.
The significance of differences between experimental groups was determined by the Student t test. A nominal significance of \( p < 0.05 \) was considered significant.

Results

CypA/CD147 mediates acetaminophen toxicity

Acetaminophen injection (350 mg, i.p.) produced a significant increase in serum ALT 24 h postinjection in mice (Fig. 1). Compared with sex-matched, littermate WT controls, Ppia\(^{-/-}\) mice had significantly lower serum ALT concentrations postacetaminophen injection (Fig. 1). Six of 50 mice studied died within 24 h of acetaminophen injection and, therefore, were not included in the analysis of serum ALT concentration. All of the dead mice were of WT genotype, consistent with Ppia\(^{-/-}\) mice being comparatively resistant to acetaminophen poisoning. Liver glutathione concentration was reduced by acetaminophen in both Ppia\(^{-/-}\) mice and WT controls (Fig. 1). Pretreatment with an anti-CD147 Ab significantly reduced the serum ALT value compared with a control isotype Ab (Fig. 1). Acetaminophen injection produced liver cell vacuolation and necrosis that was greater in WT mice than in Ppia\(^{-/-}\) mice (Fig. 2).

Extracellular CypA signals necrosis and initiates an inflammatory response

CypA stimulates inflammatory cells to release IL-6 (data not shown). Postacetaminophen injection, serum IL-6 concentrations were significantly lower in Ppia\(^{-/-}\) mice than in controls (Fig. 3). To investigate whether this is due to CypA being a DAMP, we injected necrotic liver from Ppia\(^{-/-}\) mice and littermate controls into WT mice. Necrotic liver from Ppia\(^{-/-}\) mice induced significantly lower serum IL-6 concentrations 6 h after injection compared with WT cells (Fig. 3). Published studies demonstrated that CypA induces neutrophil chemotaxis (11). To explore whether CypA released from liver cells is chemotactic for neutrophils in vivo, we measured the MPO and KC concentration in peritoneal lavage fluid 6 h after injection of necrotic liver. Both MPO and KC concentrations were reduced in the peritoneal lavage fluid when Ppia\(^{-/-}\) necrotic liver cells were injected (compared with necrotic liver cells from littermate WT controls) and with anti-CD147 Ab pretreatment (compared with isotype Ab pretreatment) (Fig. 4).

The concentration of KC in necrotic cells was similar in WT and Ppia\(^{-/-}\) mice (11 and 15 ng/g protein, respectively). Conversely, the concentration of MPO and KC was increased in the lavage fluid when CypA was coinjected with necrotic liver cells (Fig. 5).

CypA is released in humans with acetaminophen-induced liver injury

Using Western blot, we could not detect CypA in blood from patients with acetaminophen-induced liver injury (data not shown). However, urinary CypA concentration was significantly elevated in patients with acetaminophen-induced liver injury (organ-injury group) compared with the other three patient groups (Fig. 6). This finding remained statistically significant when the CypA concentration was normalized for differences in urinary concentration (urinary creatinine). Human urine contains exosomes, protein-rich lipid vesicles that contain CypA (27). We separated the exosomal fraction from the soluble fraction to determine the location of the CypA released into the urine with acetaminophen poisoning (Fig. 7). CypA was present in both the soluble fraction and exosomes. Western blotting of the exosomes revealed CypA and additional immunoreactive proteins not identified in whole urine or the soluble fraction. These may represent other cyclophilins that are known to be contained in human urinary exosomes (27).

Discussion

This study demonstrated that CypA is a key mediator of acetaminophen-induced liver injury and suggests that it acts as a DAMP. Consistent with this, in humans, CypA is released from cells following acetaminophen-induced liver injury. Inflammation plays an important, but incompletely defined, role in the pathophysiology of acetaminophen-induced liver injury. It is well-established that hepatotoxicity is dependent on the intracellular formation of the reactive acetaminophen metabolite
N-acetyl-p-benzo-quinone imine, with subsequent mitochondrial injury being particularly important in the disease pathogenesis (28). Necrotic cell death releases DAMPs that activate an innate immune response (2), at least in part via activation of the TLR system (29). The nature and magnitude of this inflammatory response influence whether the drug-induced liver injury resolves or worsens. Studies demonstrated that proinflammatory mediators, such as TNF-α (30), IL-1 (31), and IFN-γ (32), worsen tissue injury, whereas other cytokines have opposite, protective roles [e.g., IL-4 and IL-10 (33)]. However, the exact role of inflammation is still an area of controversy [e.g., there is conflicting evidence on the importance of neutrophil infiltration into the liver in acetaminophen poisoning (34, 35)]. From our data, it seems that CypA is an important mediator of acetaminophen-induced liver injury, as demonstrated by the fact that mice lacking this protein are resistant to liver injury compared with littermate WT controls. The absence of CypA does not seem to affect the production of N-acetyl-p-benzo-quinone imine in the liver, because glutathione consumption is unaffected when this gene is deleted. Extracellular CypA was demonstrated to be proinflammatory (14, 21) in a number of disease models, and these effects are secondary to binding to CD147. We found that CD147 is also an important mediator of acetaminophen-induced liver injury, suggesting that CypA binding to CD147 may induce an inflammatory response that worsens the drug-induced liver injury.

The immune response to cell death is central to a variety of diseases and has been the focus of a significant amount of research interest (2). A variety of molecular species alerts the immune system to cell death; our data support CypA being one such molecular entity. The key experimental finding that supports this claim is that the peritonal and systemic inflammatory response is reduced when CypA is absent from the injected necrotic liver, whereas it is increased when additional CypA is injected with liver. Our findings are consistent with the published work describing extracellular CypA as a powerful chemotactic agent for neutrophils, eosinophils, and T lymphocytes (14, 15, 21, 36). For example, in the vasculature, deletion of the gene for CypA significantly reduces inflammatory cell migration into the blood vessel wall following ligation of an artery (16). CD147 is an extracellular receptor for CypA, and inhibition of this receptor abolishes CypA chemotaxis in vitro (15). We found that an anti-CD147 Ab reduced the inflammatory response to necrotic liver, an effect similar to that produced by deletion of the gene for CypA. This suggested that CypA acts as a DAMP via binding to CD147. However, there are other ligands for CD147 (24, 25), and we cannot fully exclude that these, in addition to CypA, are activating CD147 in our experimental models.

Rodent models often do not faithfully reflect human disease, and this can lead to blocks in the translation of experimental findings to improved patient care. We found that CypA was significantly elevated in the urine of patients with acetaminophen-induced liver injury but not in our control groups. CypA is an intracellular protein; the increase in the urine is likely to reflect either release from necrotic cells injured directly by acetaminophen or release from activated inflammatory cells reacting to the drug-induced cell death. The actual cells that are releasing CypA into the urine may be kidney tubular cells injured directly by acetaminophen (37). Urinary CypA was increased in patients with liver injury but normal kidney function (data not shown), suggesting that CypA may be detecting subclinical kidney injury. An alternative explanation is that the urine reflects an increase in the blood CypA concentration. However, we could not detect CypA in blood samples from patients with acetaminophen-induced liver injury. Future studies will focus on the development of more sensitive assays to determine whether CypA can be accurately and reproducibly measured in blood and further develop this protein as a potential clinical biomarker. The key point is that these human data complement our mouse results by demonstrating that, with liver injury, CypA is released into the extracellular space (a prerequisite for a DAMP). The increase in urinary CypA is due to an increased amount of soluble protein and CypA contained within exosomes. Previous studies reported that human urinary exosomes contain CypA (27). The increase in CypA in both the soluble and exosomal compartments of the urine is consistent with the release of intracellular exosomes and cytoplasmic proteins secondary to necrotic cell death.

Cyclosporine binds CypA to prevent its intracellular and extracellular actions (9). Analogs of cyclosporine that do not cross the cell membrane promise to further define the contribution of extracellular CypA to disease (38). However, in the context of acetaminophen poisoning, intracellular cyclosporine provides an additional pathway to prevent injury because it binds to cyclophilin D (CypD) in mitochondria. CypD is a key regulator of the mitochondrial permeability transition pore, the opening of which leads to mitochondrial disruption and cell death (39, 40). The direct cellular toxicity induced by acetaminophen is largely due to mitochondrial permeability transition pore opening (28), with a significant role for CypD (41). Consistent with this, cyclosporine was demonstrated to prevent acetaminophen injury (42). Therefore, there are two mechanisms by which cyclosporine may prevent acetaminophen-induced liver injury in humans: the prevention of mitochondrial injury inside the cell and inhibition of the actions of CypA outside the cell. Both of these mechanisms are independent of the well-established immunosuppressive action of cyclosporine. Nonimmunosuppressive cyclophilin inhibitors that bind to CypA and CypD are in human trials as treatments for hepatitis C infection (43). Future studies will be needed to confirm that these agents inhibit acetaminophen-induced liver injury, but the absence of immunosuppression has the potential to improve the drug’s risk/benefit profile by reducing the risk for sepsis in these critically ill patients.

In summary, our data demonstrated that CypA is a DAMP that mediates acetaminophen poisoning. A proof-of-concept safety and efficacy study of cyclosporine in patients with acetaminophen-induced liver injury is now warranted.

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Disclosures
The authors have no financial conflicts of interest.

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