Letter

Protective effects of hydrogen sulfide anions against acetaminophen-induced hepatotoxicity in mice

Isao Ishii¹, Shotaro Kamata¹, Yoshifumi Hagiya¹, Yumi Abiko², Tadashi Kasahara¹
and Yoshito Kumagai²

¹Department of Biochemistry, Keio University Graduate School of Pharmaceutical Sciences,
1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan

²Graduate School of Comprehensive Human Sciences, University of Tsukuba,
1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan

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ABSTRACT — The key mechanism for hepatotoxicity resulting from acetaminophen (APAP) overdose is cytochrome P450-dependent formation of *N*-acetyl-*p*-benzoquinone imine (NAPQI), a potent electrophilic metabolite that forms protein adducts. The fundamental roles of glutathione in the effective conjugation/clearance of NAPQI have been established, giving a molecular basis for the clinical use of *N*-acetylcysteine as a sole antidote. Recent evidence from *in vitro* experiments suggested that sulfide anions (S^{2-}) to yield hydrogen sulfide anions (HS^-) under physiological pH could effectively react with NAPQI. This study evaluated the protective roles of HS^- against APAP-induced hepatotoxicity in mice. We utilized cystathionine γ -lyase-deficient (*Cth*^{-/-}) mice that are highly sensitive to acetaminophen toxicity. Intraperitoneal injection of acetaminophen (150 mg/kg) into *Cth*^{-/-} mice resulted in highly elevated levels of serum alanine/aspartate aminotransferases and lactate dehydrogenase associated with marked increases in oncotic hepatocytes; all of which were significantly inhibited by intraperitoneal preadministration of sodium hydrosulfide (NaHS). NaHS preadministration significantly suppressed APAP-induced serum malondialdehyde level increases without abrogating APAP-induced rapid depletion of hepatic glutathione. These results suggest that exogenous HS^- protects hepatocytes by directly scavenging reactive NAPQI rather than by increasing cystine uptake and thereby elevating intracellular glutathione levels, which provides a novel therapeutic approach against acute APAP poisoning.

Key words: Acetaminophen, *N*-acetyl-*p*-benzoquinone imine, Glutathione, Hydrogen sulfide, Hepatotoxicity

INTRODUCTION

Acetaminophen (APAP; *N*-acetyl-*p*-aminophenol; also known as paracetamol) is the most popular analgesic/antipyretic drug. It is generally safe and free of adverse effects at recommended doses. However, APAP overdose is a frequent cause of liver failure and the leading cause of death due to deliberate or accidental overdose in developed countries (Bromer and Black, 2003; Lee, 2008). The majority of orally administrated APAP is glucuronidated or sulfated by the phase II metabolic pathway in the liver, leading to urinary excretion, whereas a small proportion (~10%) is metabolized by certain cytochrome P450 isoforms to *N*-acetyl-*p*-benzoquinone imine (NAPQI), a highly reactive electrophile that forms covalent pro-

tein adducts and thereby causes hepatocellular necrosis (Hinson *et al.*, 2010).

Fundamental roles of intracellular glutathione (GSH) in protection against APAP/NAPQI toxicity have been well established; glutathione *S*-transferase is capable of rapidly detoxifying NAPQI by catalyzing conjugation with reduced GSH. Indeed, NAPQI binding to proteins was not observed in hepatocytes until the complete depletion of GSH (Mitchell *et al.*, 1973). In accordance with this, we demonstrated that mice lacking cystathionine γ -lyase (CTH), the final transsulfuration enzyme that is essential for cysteine biosynthesis and thus efficient GSH biosynthesis, were highly susceptible to APAP-induced hepatic injury because of their rapid hepatic GSH depletion upon APAP administration (Hagiya *et al.*, 2015). Mean-

Correspondence: Isao Ishii (E-mail: isao-ishii@umin.ac.jp)

while, CTH is also known as a hydrogen sulfide (H_2S)/reactive persulfides (polysulfides)-producing enzyme (Kimura, 2015; Ono *et al.*, 2014; Ida *et al.*, 2014). H_2S could exert its cytoprotective effects by increasing cellular cyst(e)ine uptake and activating glutathione synthesis (Kimura *et al.*, 2010) whereas aqueous sulfide anions (S^{2-}) or polysulfides (sodium polysulfides as well as GSH polysulfides) could trap NAPQI by forming sulfur adducts (Trettin *et al.*, 2014; Abiko *et al.*, 2015). Therefore, the increased susceptibility of *Cth*^{-/-} mice to APAP hepatotoxicity could be attributable, at least in part, to their decreased production of such endogenous H_2S /reactive persulfide (polysulfide) species. In this study, we examined whether the simplest H_2S anion donor (NaHS) could confer cytoprotective effects against APAP-induced severe hepatic injury in mice independent of their hepatic GSH level increase.

MATERIALS AND METHODS

Animals

CTH hemizygous knockout (*Cth*^{+/-}) male or female mice on a C57BL/6J inbred strain (CLEA Japan, Tokyo, Japan) were bred to obtain *Cth*^{-/-} female mice (Ishii *et al.*, 2010). Mice were housed in an air-conditioned room kept on a 12-hr dark/light (8 pm–8 am) cycle, and allowed free access to a standard dry rodent diet and water. All animal procedures conformed to the *Guide for the Care and Use of Laboratory Animals, Eighth Edition* published by the US National Research Council, and were approved by the Animal Care Committee of Keio University (No. 09187-(4)).

APAP and NaHS injection

Adult (8–10-week-old) female mice were fasted for 20 hr (3 pm–11 am) and then injected intraperitoneally with APAP (150 mg/kg; Sigma A7302) dissolved in saline (Hagiya *et al.*, 2015). After 4 hr, mice were anesthetized with isoflurane and blood/liver samples were collected. In some experiments, mice were preadministered intraperitoneally with NaHS (18.7 mg/kg; Sigma 161257) dissolved in saline 2 hr prior to APAP injection.

Serum biochemical analysis

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were measured using a Dri-Chem 7000i biochemistry analyzer (Fujifilm, Tokyo, Japan). Serum levels of malondialdehyde (MDA) were measured using a TBARS (thiobarbituric acid-reactive substances) assay kit (Cayman Chemical, Ann Arbor, MI, USA) (Yamada *et*

al., 2012).

Histochemistry

Livers were quickly dissected out, fixed in 10% formalin, dehydrated in an ascending ethanol series (from 75% to 100%) and then xylene, and embedded in paraffin. Three-micrometer sections were cut, deparaffinized, and stained using an Apop Tag Peroxidase *In Situ* Apoptosis Detection kit (EMD Millipore, Billerica, MA, USA) for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Hagiya *et al.*, 2015). The slides were examined using a BZ-9000 microscope (Keyence, Osaka, Japan) fitted with Plan Apo 4 × and 10 × objective lenses (Nikon, Tokyo, Japan).

Hepatic total glutathione measurement

Liver aliquots were homogenized in 5% 5-sulfosalicylic acid and the homogenates were centrifuged at 4°C for 10 min at 10,000 g. Then the supernatants were centrifuged at 4°C for 10 min at 10,000 g. Levels of total GSH (GSH + oxidized glutathione [GSSG]) in the final supernatant were measured using a GSSG/GSH Quantification kit (Dojindo, Kumamoto, Japan) (Hagiya *et al.*, 2015).

Statistical analysis

Data are means ± S.D. (*n* as indicated). Statistical analyses were performed using two-tailed unpaired Student's *t*-test, where *P* values of 0.05 or less were considered significant.

RESULTS AND DISCUSSION

APAP injection (150 mg/kg) dramatically elevated serum levels of ALT (~30,000 IU/L), AST (~40,000 IU/L), and LDH (~100,000 IU/L) in *Cth*^{-/-} mice but much less (30, 159, 335 IU/L, respectively) in wild-type mice within 4 hr (Fig. 1A–C), as we reported previously with high serum ALT levels in APAP-injected *Cth*^{-/-} (not wild-type) mice (Hagiya *et al.*, 2015). Administration of NaHS (18.7 mg/kg; 500 μM estimate in body fluid) alone did not affect serum ALT/AST/LDH levels in wild-type or *Cth*^{-/-} mice; however, it suppressed APAP-induced ALT/AST/LDH elevation in *Cth*^{-/-} mice (Fig. 1A–C) although not significantly as for LDH (Fig. 1C). Administration of NaHS slightly elevated levels of serum ALT (but not those of AST/LDH significantly) in APAP-injected WT mice (Fig. 1A–C); the reason for this is unknown. APAP overdose is known to cause oncotic necrosis (oncosis) that is distinguishable from caspase 3-mediated apoptosis but is detectable by TUNEL staining; TUNEL staining also detects oncotic nuclei that are randomly fragment-

HS⁻ anions ameliorate acetaminophen hepatotoxicity in mice

Fig. 1. Protective effects of sodium hydrosulfide (NaHS) on acetaminophen (APAP)-induced hepatic injury. Wild-type (WT) and *Cth*^{-/-} mice were fasted for 18 hr and then injected with NaHS (18.7 mg/kg) or saline. Two hours later, the mice were injected with APAP (150 mg/kg) or saline. After 4 hr, the mice were anesthetized and blood samples were collected. Serum levels of ALT (A), AST (B), and LDH (C) were measured. Data are means $\pm$ S.D. (*n*: sample number); **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 by *t*-test. Some data (bars and error bars) are not seen for APAP-non-injected *Cth*^{-/-} mouse samples (a-f) because of their much lower levels compared to those for APAP-injected *Cth*^{-/-} mouse samples; actual values are 13.2 $\pm$ 5.5 for a, 15.4 $\pm$ 6.1 for b, 114 $\pm$ 95 for c, 98 $\pm$ 52 for d, 360 $\pm$ 209 for e, and 281 $\pm$ 127 for f (all in IU/L).

ed by Ca²⁺-dependent/caspase-independent endonucleases (Hagiya *et al.*, 2015; Jaeschke *et al.*, 2012). TUNEL staining of hepatic sections identified chromatin-clumped nuclei as well as the cytoplasm of oncotic hepatocytes around the centrilobular regions (both brown-colored) (Hagiya *et al.* 2015) in both APAP- and APAP/NaHS-injected *Cth*^{-/-} mice, but the TUNEL-positive areas were much smaller when mice were preadministrated with NaHS (Fig. 2A-C). In representative hepatic sections, TUNEL-positive areas were 56.6 $\pm$ 6.5% (*n* = 3) of whole section areas in APAP/NaHS-injected *Cth*^{-/-} mice, whereas 67.5 $\pm$ 3.9% (*n* = 3) in APAP-injected *Cth*^{-/-} mice (*P* = 0.082 by *t*-test). In contrast, TUNEL-positive cells were not found in hepatic sections from APAP alone-injected (Fig. 2D) or APAP/NaHS-injected (Fig. 2E) wild-type mice.

Several working hypotheses have been proposed for the cytoprotective actions of H₂S/polysulfides (Kimura, 2015). H₂S may somehow potentiate the activity of the cystine/glutamate antiporter as well as the cysteine transporter and enhance the activity of glutamate-cysteine ligase, a rate-limiting enzyme of GSH synthesis; both of which could contribute for increased intracellular GSH levels to protect cells from oxidative toxicity (Kimura *et al.*, 2010). On the other hand, polysulfides (but not H₂S itself probably) may sulphydrate particular cysteine residues in various functional proteins, thereby regulat-

ing their functions (Kimura, 2015; Ono *et al.*, 2014; Ida *et al.*, 2014). We demonstrated previously that APAP induced rapid depletion of hepatic GSH levels and elevation of serum MDA levels in *Cth*^{-/-} mice but not in wild-type mice (Hagiya *et al.*, 2015). In this study, NaHS preadministration failed to abolish APAP-induced rapid depletion of hepatic GSH levels (Fig. 3A) but significantly reduced APAP-induced serum MDA levels (Fig. 3B). These results suggested that exogenous HS⁻ anions directly scavenged reactive NAPQI without increasing cystine uptake and intracellular GSH levels. This is supported by Trettin *et al.* who detected *in vitro* formation of NAPQI sulfur adducts in dog liver lysates mixed with APAP/Na₂S as well as liver lysates of APAP-injected mice mixed with Na₂S (Trettin *et al.*, 2014), or by our group that detected *in vivo* formation of NAPQI sulfur adducts in the liver/serum/urine of APAP-injected mice as well as *in vitro* formation of NAPQIH₂-SH, NAPQIH₂-S-NAPQIH₂ and NAPQIH₂-SS-NAPQIH₂ during the reaction between NAPQI and Na₂³²S (or Na₂³⁴S) by LC-MS/MS analysis (Abiko *et al.*, 2015). All these results suggested that NAPQI sulfur adducts would be produced in mice given APAP and NaHS.

A previous study mentioned the curative effects of NaHS (3 mg/kg) against APAP (650 mg/kg)-induced mild hepatotoxicity (ALT: from ~325 to ~70 in IU/L) in outbred Swiss male mice in which NaHS administra-

Fig. 2. Protective effects of sodium hydrosulfide (NaHS) on acetaminophen (APAP)-induced hepatic injury. *Cth*^{−/−} (A–C) and wild-type (WT; D and E) mice were fasted for 18 hr and then injected with saline (A and D) or NaHS (18.7 mg/kg; C and E). Two hours later, all mice were injected with APAP (150 mg/kg). Paraffin-embedded hepatic sections were stained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). B is the microscopic magnification of a part of A. Bars: 500 μ m (black) or 100 μ m (red). CV, central vein; PV, portal vein.

Fig. 3. Effects of sodium hydrosulfide (NaHS) on acetaminophen (APAP)-induced hepatic glutathione (GSH) depletion and serum malondialdehyde (MDA) level increase. Wild-type (WT) and *Cth*^{−/−} mice were fasted for 18 hr and then injected with NaHS (18.7 mg/kg) or saline. Two hours later, the mice were injected with APAP (150 mg/kg) or saline. After 4 hr, the mice were anesthetized and liver/blood samples were collected. Hepatic GSH levels (A) and serum MDA levels (B) were measured. Data are means $\pm$ S.D. (*n* as indicated), and effects of APAP or NaHS are significant at **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 by *t*-test.

tion significantly increased hepatic GSH levels (Morsy *et al.*, 2010). Therefore, the sites of HS[−] action may alter depending on the levels of APAP toxicity. Obviously, our experiments are more related to the clinical setting of (occasionally lethal) severe acute liver failure due to APAP overdose. Although *N*-acetylcysteine has been used for the treatment with APAP overdose, early treatment is recommended because it requires the hepatic activities of glutamate-cysteine ligase and glutathione synthase for its conversion to GSH. Our study suggests that H₂S/polysulfide treatment could be the final remedy for terminal patients with APAP overdose by capturing NAPQI formed from APAP, resulting in its sulfur adducts such as NAPQIH₂-cysteine trisulfide adduct and NAPQIH₂-GSH persulfide adduct (Abiko *et al.*, 2015). We unfortunately failed to obtain effective hepatoprotection using NaHS postadministration after APAP injection in our *Cth*^{−/−} mice (data not shown), but H₂S/polysulfide postadministration with *N*-acetylcysteine could be beneficial. Further trials using newly developed several polysulfide donors are also awaited.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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