Acrolein scavengers, cysteamine and *N*-benzylhydroxylamine, reduces the mouse liver damage after acetaminophen overdose

Ryo KOYAMA[1] and Ryushin MIZUTA[1]*

[1]*Research Institute for Biomedical Sciences, Tokyo University of Science, 2669 Yamazaki, Noda, Chiba 278–0022, Japan*

ABSTRACT. Our previous study suggested that the highly toxic α,β-unsaturated aldehyde acrolein, a byproduct of oxidative stress, plays a major role in acetaminophen-induced liver injury. In this study, to determine the involvement of acrolein in the liver injury and to identify novel therapeutic options for the liver damage, we examined two putative acrolein scavengers, a thiol compound cysteamine and a hydroxylamine *N*-benzylhydroxylamine, in cell culture and in mice. Our results showed that cysteamine and *N*-benzylhydroxylamine effectively prevented the cell toxicity of acrolein *in vitro* and acetaminophen-induced liver injury *in vivo*, which suggested that acrolein is involved in the liver damage, and these two drugs can be potential therapeutic options for this condition.
KEY WORDS: acetaminophen, acrolein, cysteamine, *N*-benzylhydroxylamine, oxidative stress

Acetaminophen is an analgesic drug prescribed widely for human patients and sometimes for veterinary patients [3, 5, 6]. Although it is generally safe, an overdose of acetaminophen can cause severe liver failure and significant mortality [5, 6]. Liver injury is believed to be caused by the metabolic conversion of acetaminophen to a highly reactive intermediate *N*-acetyl *p*-benzoquinoneimine that depletes glutathione and causes oxidative stress, and the stress characterized by the production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH), subsequently aggravates the liver injury. [12].

In a previous study, we showed that the highly toxic α, β-unsaturated aldehyde acrolein (CH_2=CHCHO) is generated under oxidative stress in liver and plays a major role in acetaminophen-induced liver injury [1]. Acrolein is produced in various tissues via lipid peroxidation [16], polyamine oxidation [13] and metabolism of drugs, such as the anticancer drug cyclophosphamide [7]. It can form Michael-type addition adducts with cellular components, particularly proteins and DNA, which results in cell toxicity. Acrolein can easily cross the cell membranes and tissues, because of its solubility in water and alcohol; thus, high concentrations of acrolein produced by lipid peroxidation or polyamine oxidation can spread from the dying cells at the primary site of injury, which results in damage and/or death of adjacent cells [10].

Our previous study showed that acrolein adducts are produced in the livers of acetaminophen-treated mice and that 2-mercaptoethanesulfonate (MESNA) and *N*-acetyl-L-

cysteine (NAC), two known acrolein scavengers, ameliorate acetaminophen-mediated liver injury by neutralizing acrolein toxicity [1]. We confirmed the specificity of NAC and MESNA in cell culture, because these agents inhibited acrolein toxicity but not H_2O_2- or ·OH-mediated toxicity.

MESNA, a thiol compound, has been shown to scavenge acrolein both experimentally and clinically. MESNA interacts with acrolein and forms an inactive compound, which can be detected using mass spectrometry [14]. Acrolein, a metabolite of the anticancer drug cyclophosphamide, causes hemorrhagic cystitis. To prevent this side effect during cancer chemotherapy, MESNA is routinely administered to patients and has successively decreased the incidence of hemorrhagic cystitis [14]. Another thiol compound NAC is a glutathione prodrug approved for clinical use and is routinely used to treat acetaminophen-induced liver injury [11]. A recent study showed that NAC directly interacts with acrolein and forms an inactive compound [20].

In this study, we determined the involvement of acrolein and examined two putative acrolein scavengers, a thiol compound cysteamine (CystE) and a hydroxylamine *N*-benzylhydroxylamine (NBHA), to identify novel therapeutic options for liver injury [17, 19]. Thiol and hydroxylamine trap aldehyde and decrease its toxicity [18]. We compared the neutralization activity of CystE and NBHA against acrolein *in vitro*. We cultured the human Burkitt's lymphoma cell line Ramos (5×10^4 cells/m*l*) in RPMI1640 medium supplemented with 50 U/m*l* streptomycin, 100 U/m*l* penicillin G and 5% fetal calf serum (FCS) at 37°C in an atmosphere of 5% CO_2. FCS was heat-inactivated and dialyzed against phosphate-buffered saline at 4°C. We examined the factors that decrease the cell toxicity of acrolein, and they were added after the addition of acrolein in the culture. Acrolein and CystE were purchased from KANTO CHEMICAL Co. (Tokyo, Japan), and NBHA and NAC were from Sigma-Aldrich (St. Louis, MO, U.S.A.). NAC was used as a positive control. After 24 hr, the numbers of viable cells were counted under
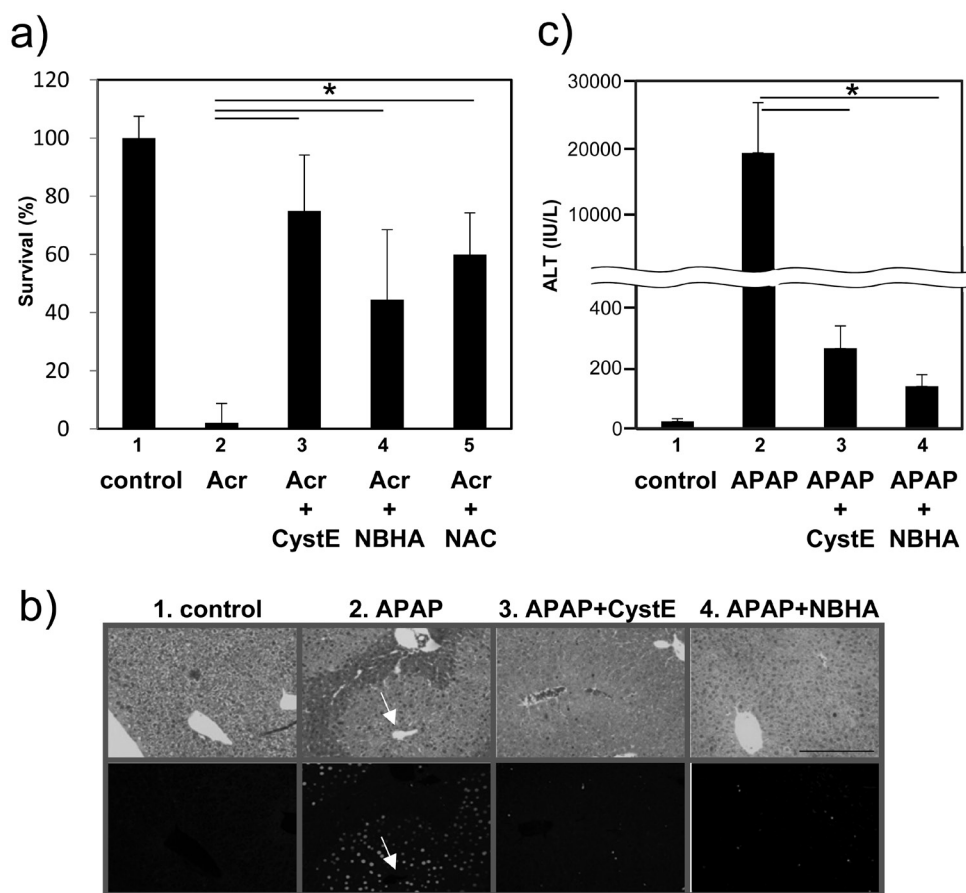
Fig. 1.   Cysteamine (CystE) and *N*-benzylhydroxylamine (NBHA) prevent acrolein toxicity and acetaminophen-induced hepatic injury. (a) CystE and NBHA prevent acrolein toxicity *in vitro*. Ramos cells were incubated with or without acrolein (Acr) and acrolein scavengers. The numbers of viable cells were counted and shown as the percentages relative to that obtained in the medium alone. Samples were treated with the medium alone (lane 1), Acr (lane 2), Acr and CystE (lane 3), Acr and NBHA (lane 4), and Acr and *N*-acetyl-L-cysteine (NAC) (lane 5). Results are presented as the average of more than three independent experiments with standard deviation. Asterisk (*): *P*<0.01. (b) CystE and NBHA prevent acetaminophen (APAP)-induced hepatic injury. Results of hematoxylin and eosin (H&E) staining and TdT-mediated dUTP nick end labeling (TUNEL) assay are shown in the top and bottom panels, respectively. Panel 1: vehicle-treated liver as a control, Panel 2: APAP-treated liver, Panel 3: APAP- and CystE-treated liver, and Panel 4: APAP- and NBHA -treated liver. Arrow: central vein. Scale bar: 500 μm. (c) Serum alanine aminotransferase (ALT) levels in mice treated with vehicle only (lane 1, n=3), APAP alone (lane 2, n=3), APAP and CystE (lane 3, n=3), and APAP and NBHA (lane 4, n=3). Asterisk (*): *P*<0.05.

a microscope in the presence of 0.25% trypan blue, and they were expressed as the percentages relative to that obtained in the medium alone (Fig. 1a). Cellular toxicity caused by 100 μM acrolein (lane 2) was prevented by 100 μM CystE (lane 3) or 100 μM NBHA (lane 4) to the same extent as that by 100 μM NAC (lane 5). The results suggest that both CystE and NBHA rapidly inactivated acrolein and protected the cells from acrolein-mediated toxicity *in vitro*.

To examine the protective role of CystE or NBHA in acetaminophen-induced hepatic injury, we administered CystE or NBHA to acetaminophen-treated mice. C57BL/6 mice were fasted 14~16 hr before acetaminophen injection, but were allowed access to water. Acetaminophen was suspended in 50% Milli-Q water and 50% propylene glycol, and the suspension was intraperitoneally (ip) injected into mice (600 mg/kg body weight). CystE (200 mg/kg) and NBHA

(500 mg/kg) were ip injected immediately after the injection of acetaminophen. The mice were sacrificed at 12 hr after acetaminophen injection to collect tissues and sera. A piece of the liver from each mouse was fixed with formalin and embedded in paraffin. Serial liver sections made from the paraffin samples were used to directly examine the hepatic injury with hematoxylin-eosin (H&E) staining or TdT-mediated dUTP nick end labeling (TUNEL) assay (Fig. 1b). H&E staining of the acetaminophen-treated liver sections showed hepatocyte necrosis in the area surrounding the central veins (Fig. 1b, panel 2, top). DNA damage in the liver sections was assessed using the TUNEL assay. The TUNEL assay was initially developed to determine apoptosis, but in a recent study, we showed that TUNEL-positive signals can be detected even in necrosis [9]. We detected TUNEL-positive signals in the necrotic regions (Fig. 1b, panel 2, bot-

tom). CystE- and NBHA-treated livers did not show severe necrosis in both H&E and TUNEL staining (Fig. 1b, panels 3 and 4). Serum alanine aminotransferase (ALT) levels that are closely correlated with hepatic damage were determined using an automated analyzer (Hitachi 7140; Hitachi Instruments Service Co., Tokyo, Japan) [1]. The ALT levels were high in acetaminophen-treated mice, and treatment with CystE or NBHA markedly decreased these levels (Fig. 1c; compare lanes 2 and 3, or lanes 2 and 4, $P<0.05$ each). These data indicate that administration of CystE or NBHA prevented acetaminophen-induced liver injury.

CystE and NBHA have shown to exert neuroprotective effects in a number of cell lines and in animal models. CystE protects rat primary astroglial cultures from glutamate toxicity [4] and Huntington's disease knock-in murine striatal cells from toxicity induced by 3-nitropropionic acid [8]. CystE has shown neuroprotective effects in the R6/2 murine model of Huntington's disease [2] and in the MPTP murine model of Parkinson's disease [15]. In addition, NBHA has been used successfully *in vivo* and *in vitro* to sequester acrolein and prevent its toxic effects in murine models of neurodegeneration [18] and in cultured mouse mammary carcinoma cells [20].

In this study, we showed that the administration of acrolein scavengers CystE or NBHA prevented acetaminophen-induced liver injury, which suggested that acrolein is involved in liver damage, and both these drugs can be used as novel therapeutic options. In addition to neurodegeneration and acetaminophen-induced liver injury, acrolein may be associated with other diseases. Thus, CystE and NBHA are promising drug candidates that can be used for the prevention and progression of such diseases.

REFERENCES

1. Arai, T., Koyama, R., Yuasa, M., Kitamura, D. and Mizuta, R. 2014. Acrolein, a highly toxic aldehyde generated under oxidative stress in vivo, aggravates the mouse liver damage after acetaminophen overdose. *Biomed. Res.* **35**: 389–395. [Medline] [CrossRef]

2. Dedeoglu, A., Kubilus, J. K., Jeitner, T. M., Matson, S. A., Bogdanov, M., Kowall, N. W., Matson, W. R., Cooper, A. J., Ratan, R. R., Beal, M. F., Hersch, S. M. and Ferrante, R. J. 2002. Therapeutic effects of cystamine in a murine model of Huntington's disease. *J. Neurosci.* **22**: 8942–8950. [Medline]

3. Gaynor, J. S. 2008. Control of cancer pain in veterinary patients. *Vet. Clin. North Am. Small Anim. Pract.* **38**: 1429–1448, viii. [Medline] [CrossRef]

4. Ientile, R., Campisi, A., Raciti, G., Caccamo, D., Currò, M., Cannavò, G., Li Volti, G., Macaione, S. and Vanella, A. 2003. Cystamine inhibits transglutaminase and caspase-3 cleavage in glutamate-exposed astroglial cells. *J. Neurosci. Res.* **74**: 52–59. [Medline] [CrossRef]

5. Ishida, Y., Kondo, T., Kimura, A., Tsuneyama, K., Takayasu, T. and Mukaida, N. 2006. Opposite roles of neutrophils and macrophages in the pathogenesis of acetaminophen-induced acute liver injury. *Eur. J. Immunol.* **36**: 1028–1038. [Medline] [CrossRef]

6. Kaplowitz, N. 2004. Acetaminophen hepatoxicity: what do we know, what don't we know, and what do we do next? *Hepatology* **40**: 23–26. [Medline] [CrossRef]

7. Kehrer, J. P. and Biswal, S. S. 2000. The molecular effects of acrolein. *Toxicol. Sci.* **57**: 6–15. [Medline] [CrossRef]

8. Mao, Z., Choo, Y. S. and Lesort, M. 2006. Cystamine and cysteamine prevent 3-NP-induced mitochondrial depolarization of Huntington's disease knock-in striatal cells. *Eur. J. Neurosci.* **23**: 1701–1710. [Medline] [CrossRef]

9. Mizuta, R., Araki, S., Furukawa, M., Furukawa, Y., Ebara, S., Shiokawa, D., Hayashi, K., Tanuma, S. and Kitamura, D. 2013. DNase γ is the effector endonuclease for internucleosomal DNA fragmentation in necrosis. *PLoS ONE* **8**: e80223. [Medline] [CrossRef]

10. Mohammad, M. K., Avila, D., Zhang, J., Barve, S., Arteel, G., McClain, C. and Joshi-Barve, S. 2012. Acrolein cytotoxicity in hepatocytes involves endoplasmic reticulum stress, mitochondrial dysfunction and oxidative stress. *Toxicol. Appl. Pharmacol.* **265**: 73–82. [Medline] [CrossRef]

11. Perry, H. E. and Shannon, M. W. 1998. Efficacy of oral versus intravenous N-acetylcysteine in acetaminophen overdose: results of an open-label, clinical trial. *J. Pediatr.* **132**: 149–152. [Medline] [CrossRef]

12. Sener, G., Sehirli, O., Cetinel, S., Yeğen, B. G., Gedik, N. and Ayanoğlu-Dülger, G. 2005. Protective effects of MESNA (2-mercaptoethane sulphonate) against acetaminophen-induced hepatorenal oxidative damage in mice. *J. Appl. Toxicol.* **25**: 20–29. [Medline] [CrossRef]

13. Sharmin, S., Sakata, K., Kashiwagi, K., Ueda, S., Iwasaki, S., Shirahata, A. and Igarashi, K. 2001. Polyamine cytotoxicity in the presence of bovine serum amine oxidase. *Biochem. Biophys. Res. Commun.* **282**: 228–235. [Medline] [CrossRef]

14. Takamoto, S., Sakura, N., Yashiki, M. and Kojima, T. 2003. Inactivation of acrolein by sodium 2-mercaptoethanesulfonate using headspace-solid-phase microextraction gas chromatography and mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **791**: 365–369. [Medline] [CrossRef]

15. Tremblay, M. E., Saint-Pierre, M., Bourhis, E., Lévesque, D., Rouillard, C. and Cicchetti, F. 2006. Neuroprotective effects of cystamine in aged parkinsonian mice. *Neurobiol. Aging* **27**: 862–870. [Medline] [CrossRef]

16. Uchida, K., Kanematsu, M., Morimitsu, Y., Osawa, T., Noguchi, N. and Niki, E. 1998. Acrolein is a product of lipid peroxidation reaction. Formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. *J. Biol. Chem.* **273**: 16058–16066. [Medline] [CrossRef]

17. Vidro-Kotchan, E., Yendluri, B. B., Le-Thai, T. and Tsin, A. 2011. NBHA reduces acrolein-induced changes in ARPE-19 cells: possible involvement of TGFβ. *Curr. Eye Res.* **36**: 370–378. [Medline] [CrossRef]

18. Wood, P. L., Khan, M. A., Kulow, S. R., Mahmood, S. A. and Moskal, J. R. 2006. Neurotoxicity of reactive aldehydes: the concept of "aldehyde load" as demonstrated by neuroprotection with hydroxylamines. *Brain Res.* **1095**: 190–199. [Medline] [CrossRef]

19. Wood, P. L., Khan, M. A. and Moskal, J. R. 2007. Cellular thiol pools are responsible for sequestration of cytotoxic reactive aldehydes: central role of free cysteine and cysteamine. *Brain Res.* **1158**: 158–163. [Medline] [CrossRef]

20. Yoshida, M., Tomitori, H., Machi, Y., Hagihara, M., Higashi, K., Goda, H., Ohya, T., Niitsu, M., Kashiwagi, K. and Igarashi, K. 2009. Acrolein toxicity: Comparison with reactive oxygen species. *Biochem. Biophys. Res. Commun.* **378**: 313–318. [Medline] [CrossRef]