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Protective Mechanisms of *N*-Acetyl-Cysteine Against Pyrrolizidine Alkaloid Clivorine-Induced Hepatotoxicity

Lili Ji,[1,2] Tianyu Liu,[1] Ying Chen,[1] and Zhengtao Wang[1,2*]

[1] *Key Laboratory of Standardization of Chinese Medicines of Ministry of Education, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, PR China*

[2] *Shanghai R&D Centre for Standardization of Traditional Chinese Medicines, Shanghai 201203, PR China*

ABSTRACT

Pyrrolizidine alkaloid (PA) clivorine, isolated from traditional Chinese medicinal plant *Ligularia hodgsonii* Hook, has been shown to induce apoptosis in hepatocytes via mitochondrial-mediated apoptotic pathway in our previous research. The present study was designed to observe the protection of *N*-acetyl-cysteine (NAC) on clivorine-induced hepatocytes apoptosis. Our results showed that 5 mM NAC significantly reversed clivorine-induced cytotoxicity via MTT and Trypan Blue staining assay. DNA apoptotic fragmentation analysis and Western-blot results showed that NAC decreased clivorine-induced apoptotic DNA ladder and caspase-3 activation. Further results showed that NAC inhibited clivorine-induced Bcl-xL decrease, mitochondrial cytochrome *c* release and caspase-9 activation. Intracellular glutathione (GSH) is an important ubiquitous redox-active reducing sulfhydryl (–SH) tripeptide, and our results showed that clivorine (50 μM) decreased cellular GSH amounts and the ratio of GSH/GSSG in the time-dependent manner, while 5 mM NAC obviously reversed this depletion. Further results showed that GSH synthesis inhibitor BSO augmented clivorine-induced cytotoxicity, while exogenous GSH reversed its cytotoxicity on hepatocytes. Clivorine (50 μM) significantly induced cellular reactive oxygen species (ROS) generation. Further results showed that 50 μM Clivorine decreased glutathione peroxidase (GPx) activity and increased glutathione *S* transferase (GST) activity, which are both GSH-related antioxidant enzymes. Thioredoxin-1 (Trx) is also a ubiquitous redox-active reducing (–SH) protein, and clivorine (50 μM) decreased cellular expression of Trx in a time-dependent manner, while 5 mM NAC reversed this decrease. Taken together, our results demonstrate that the protection of NAC is major via maintaining cellular reduced environment and thus prevents clivorine-induced mitochondrial-mediated hepatocytes apoptosis. J. Cell. Biochem. 108: 424–432, 2009.

KEY WORDS: PYRROLIZIDINE ALKALOIDS; CLIVORINE; NAC; APOPTOSIS; CASPASE; GSH; THIOREDOXIN

Pyrrolizidine alkaloids (PAs) are widely distributed natural hepatotoxins found in various plant species of different families worldwide [Roeder, 1995, 2000]. There are many poisoning reports of PAs on humans and livestocks in Europe, South/North America, Japan, China, India via consumption of PAs-containing plants, milk, honey, etc. [Prakash et al., 1999; Coulombe, 2003]. The toxic effects on mammals of PAs have been the subject of extensive investigation. Based on its possible hazards to mammalian, the U.S. Food and Drug Administration and British Medicines Healthcare Products Regulatory Agency (MHRA) both proposed a series of research programs, instructions and standards to alert people pay attention to the toxicity of PAs-containing herbs. Otonecine-type PA clivorine is abundant in *Ligularia hodgsonii* Hook and *Ligularia dentat* Hara, which have been used for cough, hepatitis, and

Abbreviations used : PA, pyrrolizidine alkaloid; NAC, *N*-acetyl-cysteine; –SH, sulfhydryl; GPx, glutathione peroxidase; GST, glutathione *S* transferase; Trx, thioredoxin-1; MHRA, British Medicines Healthcare Products Regulatory Agency; GSH, glutathione; MTT, 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; CDNB, 1-chloro-2,4-dinitrophenol; BSO, L-buthionine (S,R)-sulfoximine; HVOD, hepatic veno-occlusive disease; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun amino-terminal kinase; H_2DCFDA, 2′-7′-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species.

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*Correspondence to: Prof. Dr. Zhengtao Wang, Key Laboratory of Standardization of Chinese Medicines of Ministry of Education, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Shanghai 201203, PR China. E-mail: wangzht@hotmail.com

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inflammation traditionally in Chinese medicine [Kuhara et al., 1980; Ji et al., 2002]. Our previous study has showed that mitochondrial-mediated apoptotic pathway plays important roles in clivorine induced-hepatocytes apoptosis [Ji et al., 2008].

Apoptosis, or programmed cell death is critical for the development and homeostasis of all multicellular organisms. Apoptosis has been implicated in hepatocytes cell death after exposure to many toxins such as carbon tetrachloride [Shi et al., 1998], ethanol [Nanji, 1998], acetaminophen [Kon et al., 2007], galactosamine [Medline et al., 1970], etc. There are two major signal pathways involved in apoptosis that have been demonstrated so far, one is the intrinsic pathway, in which mitochondria plays the central role, and the other is extrinsic pathways, which starts from the ligation of cell plasma membrane death receptors [Huang, 2002]. In the intrinsic pathway, mitochondria membrane permeability is changed upon the stimulation of various apoptosis inducing signals, and causes the release of mitchondrial cytochrome *c*, thus leads to the activation of caspases 9 and 3 [Zhang et al., 2003]. The Bcl-2 family of proteins is the key regulator of the mitochondria response to apoptotic stimulation, of which anti-apoptotic Bcl-xL, Bcl-2, etc. play important roles in maintaining the stabilization of mitochondria in the intrinsic pathway [Borner, 2003].

N-acetyl-cysteine (NAC), the acetylated variant of the amino acid L-cysteine, is abundant in the (sulfhydryl, –SH) groups and is a precursor of cellular reduced glutathione (GSH) due to its in vivo converting into metabolites capable of stimulating GSH biosynthesis [Kelly, 1998]. NAC has been widely used in the clinic for the treatment of hepatotoxicity due to GSH deficiency such as acetaminophen overdose [Atkuri et al., 2007]. The objective of our present study was to observe the protection of NAC against clivorine-induced hepatotoxicity and the further involved mechanisms.

MATERIALS AND METHODS

CELLS AND REAGENTS

The L-02 cell line was derived from adult human normal liver [Yeh et al., 1980; Zhang et al., 2007] (Cell Bank, Type Culture Collection of Chinese Academy of Sciences), and cells were cultured in RPMI1640 supplemented with 10% (v/v) fetal bovine serum. Clivorine (Fig. 1A) was isolated from *L. hodgsonii* Hook with the purity $\geq$99.5%.

The following antibodies for caspase-3, caspase-9, cytochrome *c*, Bcl-xL, and Thioredoxin-1 (Trx) were from Cell Signaling Technology (Danvers, MA). The antibody for actin was from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated goat anti-Rabbit IgG(H + L) and peroxidase-conjugated goat anti-mouse IgG(H + L) were from Jackson ImmunoResearch (West Grove, PA). Nitrocellulose membranes and prestained protein marker were from Bio-Rad (Hercules, CA) and enhanced chemiluminescence detection system was from Amersham Life Science (Buckinghamshire, UK). 2′-7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Invitrogen, Inc. (Carlsbad, CA). All other reagents unless indicated were from Sigma Chemical Co.

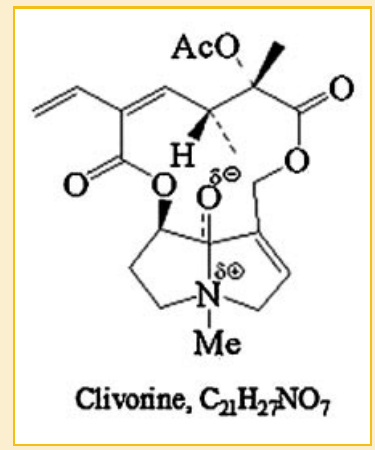

Fig. 1. The chemical structure of clivorine.

MTT ASSAY

Cells were seeded in 96-well microplates and incubated with clivorine for 24 h, and then 5 mM NAC was added and incubated cells in the presence of clivorine for another 24 h. After treatments, cells were incubated with 500 μg/ml 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) for 4 h. The functional mitochondrial succinate dehydrogenases in survival cells can convert MTT to formazan that generates a blue color [Hansen et al., 1989]. At last the formazan was dissolved in 10% SDS–5% isobutanol–0.01 M HCl. The optical density was measured at 570 nm with 630 nm as a reference and cell viability was normalized as a percentage of control.

TRYPAN BLUE STAINING

Cells were seeded in 6-well microplates and incubated with clivorine for 24 h, and then 5 mM NAC was added and incubated cells in the presence of clivorine for another 24 h. After treatment, cells were mixed with 0.4% trypan blue–PBS for 10 min, and the dead cells were stained blue by trypan blue. The number of stained and unstained cells was counted using a hemocytometer and the values were expressed as the percentage of total cells including survival and dead cells.

DNA FRAGMENTATION ASSAY

DNA fragmentation assay was performed as previously described method with minor revision [Zhang et al., 2001]. Briefly, cells were lysed with buffer containing 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 150 mM NaCl, 0.4% SDS, and 100 μg/ml proteinase K and left in 37°C overnight. The fragmented DNA in the lysate was extracted with phenol/chloroform/isopropyl alchol (25:24:1, v/v), and then precipitated for 5 min at liquid nitrogen with chilled 100% ethanol and 3 M sodium acetate. The DNA pellets were saved by centrifuging at 15,000*g* for 15 min and then washed with 70% ethanol and resuspended in Tris–HCl (pH 8.0), with 100 μg/ml RNaseA at 37°C for 1 h. The DNA fragments were separated by 2% agarose gel electrophoresis, stained with ethidium bromide and photographed in UV light.

PREPARATION OF MITOCHONDRIAL AND CYTOSOLIC FRACTIONS

Cells were lysed in lysis buffer containing 10 mM Tris (pH 7.5), 10 mM KCl, 0.15 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 150 mM sucrose for 15 min on ice, then cells were broken with 10 passages through a 26-gauge needle, and the homogenate was centrifuged at 800*g* for 10 min to remove nuclei and unbroken cells. Supernatant was centrifuged at 12,000*g* for 15 min to collect pellets as mitochondrial fraction.

WESTERN-BLOT ANALYSIS

Following the treatments, cells were lysed in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A. Protein concentrations were assayed and normalized to equal protein concentration. Proteins were separated by SDS–PAGE and blots were probed with appropriate combination of primary and HRP-conjugated secondary antibodies. For repeated immunoblotting, membranes were stripped in 62.5 mM Tris (pH 6.7), 20% SDS, and 0.1 M 2-mercaptoethanol for 30 min at 50°C.

MEASUREMENT OF CELLULAR GLUTATHIONE

Cellular GSH and oxidative glutathione (GSSG) were determined by the 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) assay according to reported method [Sies and Akerboom, 1984] with a minor modification. Briefly, after treatment cells were harvested in metaphosphoric acid (5%) buffer. The reaction mixture contained 1 mM EDTA, NADPH (0.24 mM), glutathione reductase (0.06 U), DTNB (86 μM), and samples. Yellow 5-thio-2-nitrobenzoic acid (TNB) formation is monitored at 412 nm. GSSG was determined after elimination of GSH with 2-vinylpyridine. The levels of GSH were calculated from the difference between concentrations of total glutathione (GSH + GSSG) and GSSG. The intracellular levels of GSH and GSSG were calculated based on cellular protein concentration.

MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS)

Intracellular ROS were measured according to reported method [Mathew et al., 2008] with a minor modification. Cells were incubated with 20 μM H_2DCFDA and 50 μM clivorine for indicated time. After treatment, cells were washed with PBS buffer and immediately were lysed in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% NP-40, 10% glycerol. The whole cell lysates were centrifuged (10,000*g*, 5 min, 4°C) and 100 μl of lysate was transferred to a Black wall with clear bottom 96-well plate. Fluorescence was immediately read at excitation 485 ± 20 nm, emission 525 ± 20 nm using a Biotech FL600 spectrophotometer (Biotech Instruments, Winooski, VT). The protein concentrations in supernatant were assayed, and all the results were calculated as units of fluorescence per microgram of protein. The data are reported as DCF fluorescence per microgram of protein (% of control) corresponding to the increase of fluorescene per microgram of protein associated with clivorine-treated cells compared to that of untreated cells.

ENZYMATIC ACTIVITIES ASSAY

After treatment cells were harvested in cold Phosphate buffer (pH 7.0) and sonicated (2 × 5 s) in ice, and then centrifuged at 4,000*g* for 10 min. The supernatant was used for the enzymatic assays. Glutathione peroxidase (GPx) activity was assayed according to the previous method [Rotruck et al., 1973] using H_2O_2 as the substrate. One unit of enzyme activity results in terms of utilization of 1 μM of GSH/min, and the activity of GPx was calculated based on cellular protein concentration. Glutathione *S* transferase (GST) activity was measured according to the previous reported method [Habig and Jakoby, 1981] using 1-chloro-2,4-dinitrophenol (CDNB) as the substrate. One unit of enzyme activity results in the binding of 1 μM of GSH/min, and GST activity was calculated based on cellular protein. Protein concentrations were determined by the Bradford method [Bradford, 1976] with bovine serum albumin as a standard.

STATISTICAL ANALYSIS

The results were expressed as means ± SE. Differences between groups were assessed by one-way ANOVA using the SPSS software package for windows. $P \leq 0.05$ was considered as statistically significant.

RESULTS

NAC PREVENTED CLIVORINE-INDUCED CYTOTOXICITY ON L-02 CELLS

L-02 cells were incubated with clivorine for 24 h, and then 5 mM NAC was added to incubate cells in the presence of clivorine for another 24 h. Finally cell viability was analyzed by MTT and Trypan blue staining assays. Figure 2A showed that clivorine decreased cell viability in a concentration-dependent manner, and the cell viability of 10, 25, and 50 μM clivorine is 54.7%, 30.5%, and 19.1% of control, respectively, while with 5 mM NAC co-incubation the cell viability of 10, 25, and 50 μM clivorine is enhanced to 95.2%, 78.8%, and 80.1% of control, respectively. As shown in Figure 2B, clivorine (50 μM) decreased cell viability to 64.7% of total cells, while with 5 mM NAC incubation the cell viability was increased to 73.7% of total cells.

NAC DECREASED CLIVORINE-INDUCED DNA APOPTOTIC LADDER AND CASPASE-3 CLEAVAGE

Next we observed whether NAC could prevent clivorine-induced apoptosis in hepatocytes. As shown in Figure 3A, clivorine (50 μM) obviously induced DNA apoptotic ladder, while with 5 mM NAC co-incubation the DNA apoptotic ladder was decreased in clivorine-treated cells. Figure 3B showed that clivorine (50 μM) decreased the expression of pro-caspase-3 and increased the expression of cleaved caspase-3, which represents the activation of caspase-3, while 5 mM NAC inhibited the decrease of pro-caspase-3 and increase of cleaved caspase-3. All these results indicates that NAC protects hepatocytes against the toxicity of clivorine via inhibiting apoptosis.

NAC PREVENTED CLIVORINE-INDUCED MITOCHONDRIAL-MEDIATED APOPTOTIC SIGNALS

Our previous study has showed that clivorine induced the degradation of anti-apoptotic Bcl-xL, mitochondrial cytochrome

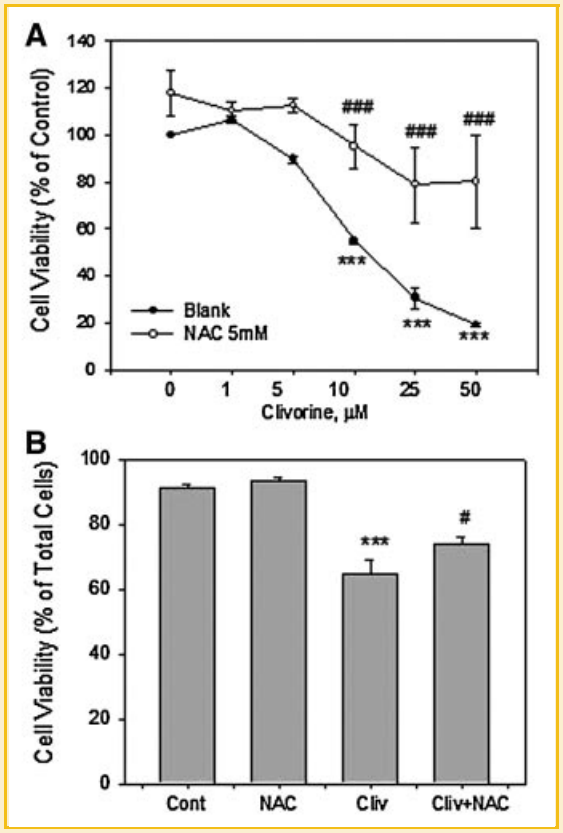

Fig. 2. NAC inhibited clivorine-induced cytotoxicity on L-02 cells. A: Cells were incubated with various concentrations of clivorine for 24 h, and then incubated with 5 mM NAC for another 24 h in the presence of clivorine. After treatment, the survival cells were determined by MTT assay. The results are expressed in percent of control and presented as the means ± SE (n = 6). B: Cells were incubated with various concentrations of clivorine for 24 h, and then incubated with 5 mM NAC for another 24 h in the presence of clivorine. After treatment, the survival cells were determined by Trypan blue staining assay. The results are expressed in percent of total cells and presented as the means ± SE (n = 6), $^{***}P \leq 0.001$ versus control, $^{\#}P \leq 0.05$, $^{\#\#\#}P \leq 0.001$ versus clivorine.

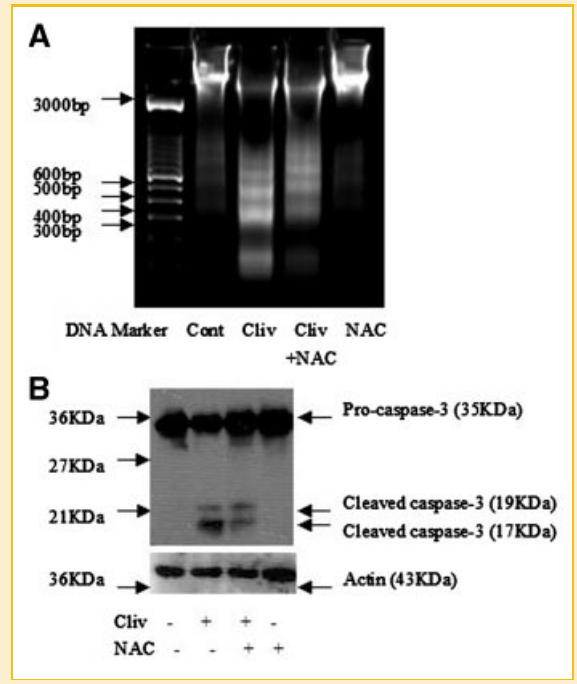

Fig. 3. NAC prevented clivorine-induced apoptosis. A: Cells were incubated with 50 μM clivorine for 24 h, and then incubated with 5 mM NAC for another 24 h in the presence of clivorine. After treatment DNA was extracted (described in Materials and Methods Section) and electrophoresed on 2% agarose gel containing ethidium bromide. B: Cells were incubated with 50 μM clivorine for 24 h, and then incubated with 5 mM NAC for another 24 h in the presence of clivorine. After treatment, pro-caspase-3 and cleaved caspase-3 were determined by Western-blot analysis, and actin was used for loading control. Each blot represents one of three independent experiments.

c release, and caspase-9 activation, thus leading to caspase-3 activation and initiating cell apoptosis [Ji et al., 2008]. So in the present study we further observe whether NAC prevent clivorine-induced mitochondrial-mediated apoptotic signals. The results of Western-blot (Fig. 4) showed that 50 μM clivorine significantly decreased Bcl-xL expression, induced mitochondrial cytochrome *c* release and caspase-9 cleavage, while 5 mM NAC reversed all these effects induced by clivorine. Our results indicate that NAC prevents clivorine-induced mitochondrial-mediated hepatocytes apoptosis.

NAC RESCUED CLIVORINE-DEPLETED CELLULAR GSH

NAC is a well-known exogenous precursor of cellular GSH, so in the further study we observed the effects of clivorine on cellular GSH and the protective effects of NAC. As shown in Figure 5A, 50 μM clivorine firstly enhanced cellular GSH in the early time of 24 and 32 h, and then decreased cellular GSH in the later 40 and 48 h. Figure 5B showed that 50 μM clivorine decreased the ratio of GSH/GSSG in the time-dependent manner. These results indicate that cellular GSH plays an important role in regulating the toxicity of clivorine. Next we observed the protection of NAC on clivorine-depleted cellular GSH. As shown in Figure 5C,D, 5 mM NAC significantly rescued cellular GSH and GSH/GSSG after cells were treated with 50 μM clivorine for 48 h.

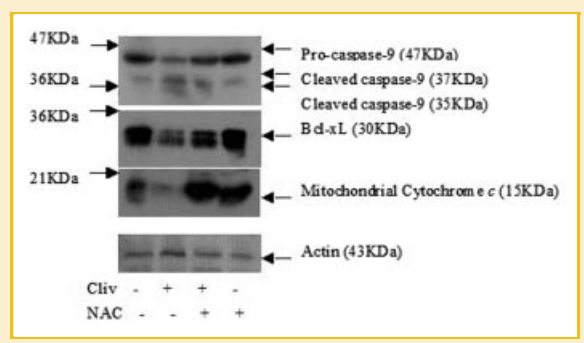

Fig. 4. NAC inhibited clivorine-induced mitochondrial-dependent apoptotic signals. Cells were incubated with 50 μM clivorine for 24 h, and then incubated with 5 mM NAC for another 24 h in the presence of clivorine. After treatment, pro-caspase-9, cleaved caspase-9, Bcl-xL, and cytochrome c were determined by Western-blot analysis, and actin was used for loading control. Each blot represents one of three independent experiments.

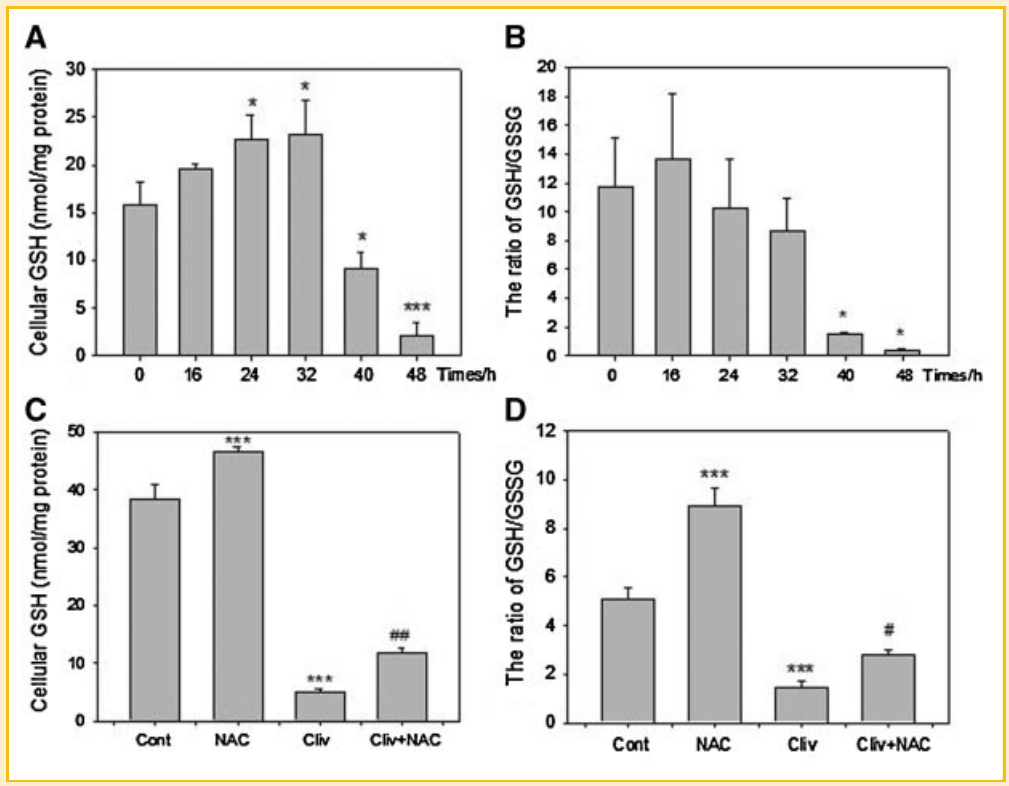

Fig. 5. NAC rescued clivorine-decreased cellular GSH. Cells were incubated with 50 μM clivorine for the indicated times, and cellular GSH (A) and GSH/GSSG (B) were determined according to the Materials and Methods Section. All values are means ± SE (n = 6), $^{*}P \leq 0.05$, $^{***}P \leq 0.001$ versus control. Cells were incubated with 50 μM clivorine for 24 h, and then incubated with 5 mM NAC for another 24 h in the presence of clivorine. After treatment, cellular GSH (C) and GSH/GSSG (D) were determined according to the Materials and Methods Section. All values are means ≤ SE (n = 6), $^{***}P \leq 0.001$ versus control, $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ versus clivorine.

CELLULAR GSH REGULATED CLIVORINE-INDUCED HEPATOTOXICITY

L-Buthionine (S,R)-sulfoximine (BSO) is a well-known inhibitor of cellular GSH synthesis, and in our study we found that cellular GSH was totally depleted after L-02 cells were treated with 10 μM BSO for 24 h, while MTT assay showed that 10 μM BSO had no significant cytotoxicity on L-02 cells after 48 h treatment (data not shown). Figure 6A showed that 10 μM BSO significantly augmented the cytotoxicity of clivorine on L-02 cells. We further observed the protection of exogenous GSH on clivorine-induced cytotoxicity on L-02 cells. As shown in Figure 6B, 5 mM GSH co-incubation obviously increased the cell viability compared to clivorine-treated cells.

CLIVORINE INDUCED INTRACELLULAR ROS AND ON THE ACTIVITIES OF GSH-RELATED ANTIOXIDANT ENZYMES

Next we observed whether clivorine induced intracellular ROS, which has been involved in hepatotoxicity induced by various hepatotoxins such as alcohol, chromium, etc. [Bagchi et al., 2002; Conde de la Rosa et al., 2008]. H_2DCFDA is a cell-permeable probe that enters the cells and is deacetylated to a nonfluorescent product, 2′-7′-dichlorodihydrofluorescein (H_2DCF) by cellular esterase, and then is oxidized by cellular ROS to a fluorescent product, 2′-7′-dichlorofluorescein (DCF). As shown in Figure 7A, clivorine (50 μM) significantly increased the generation of intracellular ROS in L-02 cells. GSH maintains intracellular proteins in the reduced and active form by either the reduction of toxic peroxides via selenium-containing GPx enzyme or the thiol-disulfide exchange reactions mediated by GST. In the further study, we observed the effects of clivorine on cellular GPx and GST enzymes activities. As shown in Figure 7B, cellular GPx activity was significantly decreased after cells were treated with 50 μM clivorine for 48 h, which may be due to the destruction of cells by clivorine. Figure 7C showed the significant higher activity of GST by about 400% in cells with 50 μM clivorine treatment for 48 h than in untreated cells, which indicates that GST may be critical for regulating the toxicity of clivorine on hepatocytes.

CLIVORINE ON CELLULAR TRX EXPRESSION

Trx is a small redox-active protein and abundant in cytosol, which complements cellular GSH system in protection against oxidative stress. As shown in Figure 8A, clivorine (50 μM) significantly decreased the expression of Trx, indicating that clivorine also destroys the balance of cellular Trx system. Further we observed whether NAC could reverse this imbalance. Figure 8B showed that 5 mM NAC rescued the decrease of Trx, which indicates that keeping cellular GSH balance is helpful for prevent the destroy of clivorine on cellular Trx system.

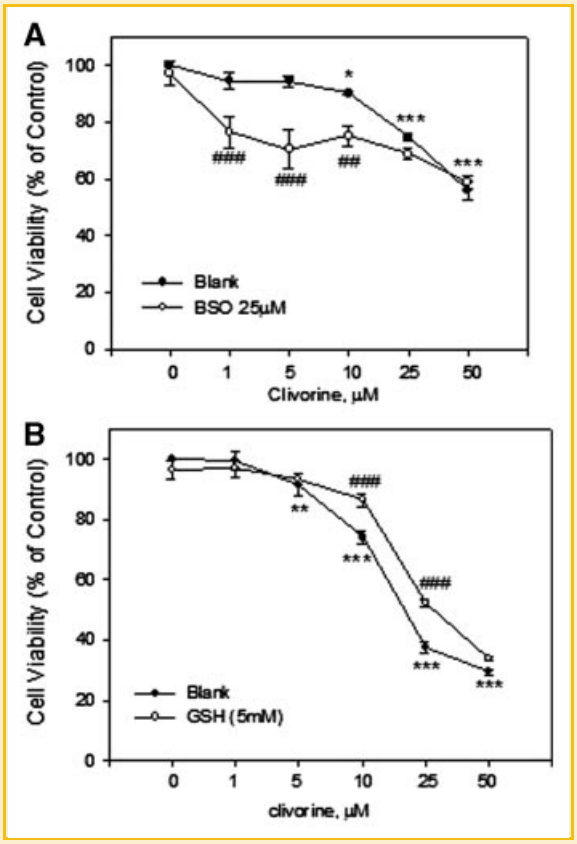

Fig. 6. Cellular GSH regulated clivorine-induced cytotoxicity on L-02 cells. A: Cells were pretreated with 10 μM BSO for 24 h, and then incubated with various concentrations of clivorine for 48 h. After treatment, the survival cells were determined by MTT assay. The results are expressed in percent of control and presented as the means ± SE (n = 6). B: Cells were pretreated with 5 mM GSH for 2 h, and then incubated with various concentrations of clivorine for 48 h. After treatment, the survival cells were determined by MTT assay. The results are expressed in percent of control and presented as the means ± SE (n = 6), $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus control, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P < 0.001$ versus clivorine.

DISCUSSION

PAs are worldwide-distributed natural hepatotoxins and its consumption is responsible for the cause of hepatic sinusoidal-obstruction syndrome that is also known as hepatic veno-occlusive disease (HVOD) [Chojkier, 2003; Coulombe, 2003; Walsh and Dingwell, 2007]. Generally it is assumed that the hepatotoxicity of PAs is irreversibly and there is no report about the detoxification of PAs up to now. Clivorine is an otonecine-type hepatotoxic PA abundant in traditional Chinese Medicinal plants *L. hodgsonii* Hook and *L. dentat* Hara. Our previous study has already showed the hepatotoxicity induced by clivorine [Ji et al., 2002, 2005]. In the present study, we showed a marked enhancement of cell viability and improvement of apoptosis by NAC co-incubation in clivorine-treated hepatocytes. Cell viability analysis by MTT and Trypan blue staining showed that 5 mM NAC markedly enhanced the cell viability in clivorine-treated L-02 hepatocytes (Fig. 2). The improvement of clivorine-induced apoptosis was evidenced by the decreased amounts of apoptotic DNA ladder and caspase-3 activation in NAC-treated hepatocytes (Fig. 3). The hepatoprotective function of NAC has been widely demonstrated [Higuchi et al., 2001; Zaragoza et al., 2001; Dambach et al., 2006], and our results showed that NAC also prevented PA clivorine-induced hepatotoxicity.

The central component of the apoptotic signaling pathway is a proteolytic system, which involves a family of proteases: the caspases. In mitochondrial-mediated apoptotic signaling pathway the release of cytochrome *c* from mitochondria plays important roles in activating downstream caspases, while anti-apoptotic Bcl-xL protein prevents the release of cytochrome *c* from mitochondria. Our previous study has already showed that clivorine induced hepatocytes apoptosis via mitochondrial-mediated apoptotic signaling pathway [Ji et al., 2008]. The present study in Figure 4 showed that NAC reversed clivorine-induced Bcl-xL decrease, mitochondrial cytochrome *c* release, and caspase-9 activation, which suggest that NAC prevents clivorine-induced mitochondrial-mediated apoptosis.

As the precursor of intracellular GSH, NAC can increase GSH levels in liver cells and replenish GSH stores following the depletion by exogenous hepatotoxins such as acetaminophen [Corcoran and Wong, 1986] or in liver disease such as biliary cirrhosis [Pastor et al., 1997]. We further observed the effects of clivorine on cellular GSH and the protection of NAC. Our results in Figure 5A showed that clivorine (50 μM) increased cellular GSH amounts during the early incubation time of 24, 32 h, which may be due to the self-defense of cells against the toxicity of clivorine, and later cellular GSH was exhausted after long time incubation (40, 48 h). Further results showed that 5 mM NAC rescued the depletion of GSH in clivorine-treated hepatocytes, which indicates that the protection of NAC against clivorine-induced hepatotoxicity may be via increasing cellular GSH biosynthesis. We further observed the effects of cellular GSH synthesis inhibitor BSO and exogenous GSH on clivorine-induced hepatotoxcicity. As shown in Figure 6A, cell viability in clivorine (1, 5, 10, 25 μM)-treated cells with BSO (10 μM) pretreatment for 24 h was significantly decreased as compared to clivorine-treated cells without BSO pretreatment. Figure 6B showed that cell viability in clivorine (5, 10, 25, 50 μM)-treated cells with 5 mM GSH co-incubation was obviously higher than only clivorine-treated cells. All these results indicate that cellular GSH is critical for the regulation of hepatotoxicity of clivorine.

Oxidative stress occurs when the production of ROS exceeds their depletion by antioxidant compounds or enzymes. Cellular excessive ROS are able to react with most cellular macromolecules such as enzymes, DNA, protein, thus result in disruption of cells [Bienert et al., 2006]. H_2DCFDA was generally used as an indicator of intracellular ROS generation, and our results in Figure 7A showed that clivorine significantly induced intracellular ROS production. Cellular defenses against ROS include low-molecular antioxidants such as glutathione, thioredoxin, ascorbate, and related antioxidant enzymes like GPx, GST, catalase, superoxide dismutase [Leonard et al., 2004]. Mammlian GPx and GST both belong to a multifunctional family of detoxification enzymes, and they are both GSH-related antioxidant enzymes. GPx can reduce a large spectrum of hydroperoxides at the expense of cellular thiols,

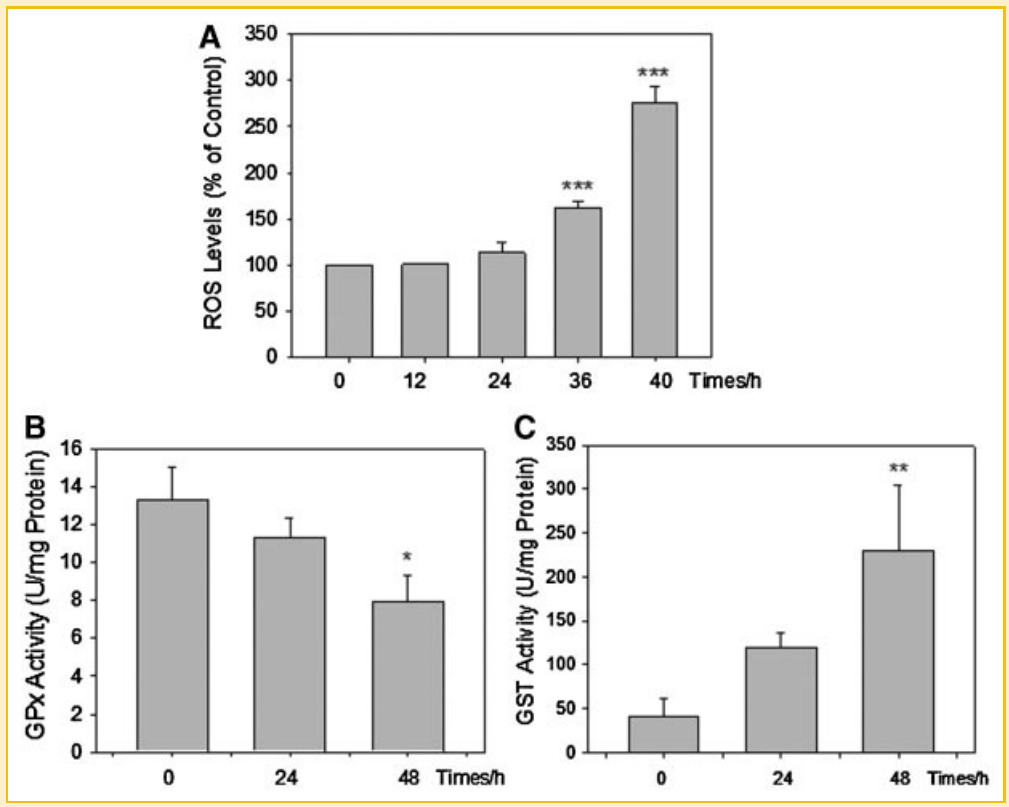

Fig. 7. Clivorine induced intracellular ROS and on the activities of GPx and GST. A: Cells were incubated with 50 μM clivorine for the indicated times, and cellular ROS was detected according to the Materials and Methods Section. B: Cells were incubated with 50 μM clivorine for the indicated times, and cellular GPx activity was analyzed according to the Materials and Methods Section. C: Cells were incubated with 50 μM clivorine for the indicated times, and cellular GST activity was analyzed according to the Materials and Methods Section. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus control.

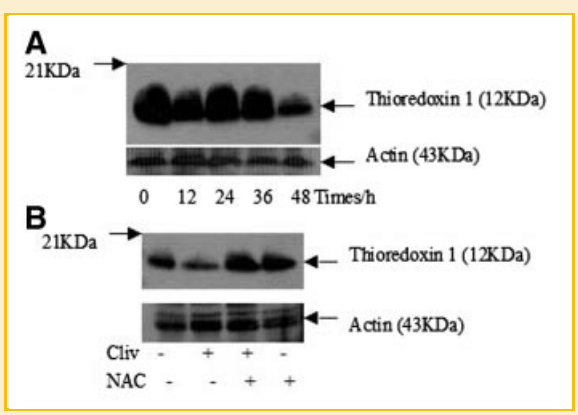

Fig. 8. NAC prevented clivorine-induced decrease of cellular Trx. A: Cells were incubated with 50 μM clivorine for the indicated times, and cellular Trx was determined by Western-blot analysis with actin used for loading control. B: Cells were incubated with 50 μM clivorine for 24 h, and then incubated with 5 mM NAC for another 24 h in the presence of clivorine. After treatment, cellular Trx was determined by Western-blot analysis, and actin was used for loading control. Each blot represents one of three independent experiments.

typically GSH, and functions in protecting cells from oxidative damage [Hou et al., 1996; Sies et al., 1997]. The function of GST is to catalyze the conjugation of electrophilic xenobiotics (or their metabolites) to GSH, thus prevents their potential toxicity [Jakoby, 1978; Awasthi et al., 1994]. Our results in Figure 7B showed that GPx activity decreased after cells were treated with clivorine for 48 h, indicating the deceased capacity against clivorine-induced oxidative damage and which may be due to the destruction of the cells with clivorine treatment. Our further results in Figure 7C showed that clivorine obviously increased GST activity after 48 h treatment, which suggests that cellular GST may play critical roles in regulating clivorine-induced hepatotoxicity. As mammalian cytosolic GSTs are divided into five subfamilies (Alpha, Mu, Pi, Theta, and Zeta) according to the similarity in primary structure [Ikeda et al., 2002], and which GST is critical for the toxicity needs further investigation.

Cellular reducing environment is critical for keeping proteins thiols reduced and maintain their normal activity. Trx is a ubiquitous disulfide oxidoreductase, which along with cellular GSH plays major roles in maintaining the cytoplasm in a reducing environment and protecting against oxidative injury [Watson et al., 2003]. Our present results in Figure 8A,B showed that clivorine decreased the expression of Trx in the time-dependent manner,

while NAC co-incubation obviously reversed this decrease. These results indicate that cellular Trx also participates in clivorine-induced hepatotoxicity, furthermore there is some cross-link between cellular GSH and Trx system.

There are reports that Trx binds to apoptosis signal-regulating kinase 1 (ASK1) forming an inactive complex, once breaking this complex will activate ASK1 and leading to the activation of c-Jun amino-terminal kinase (JNK)/p38 mitogen-activated protein (MAP) kinases, thus initiate apoptosis [Saitoh et al., 1998; Tobiume et al., 2001]. There are reports that JNK is critical for the hepatotoxicity induced by acetaminophen [Gunawan et al., 2006; Latchoumycandane et al., 2007; Bourdi et al., 2008], and it is assumed that Trx-ASK1 association plays important roles in acetaminophen-induced activation of JNK [Nakagawa et al., 2008]. Acetaminophen was also reported to deplete cellular GSH and NAC was widely used for acetaminophen overdose clinically [Zhao et al., 2002; Bajt et al., 2004; Terneus et al., 2008]. Whether clivorine decreased Trx expression will lead to the activation of ASK1-JNK signaling axis like acetaminophen or cause the activation of other signaling pathway needs further investigation.

Modulation of intracellular thiols has been used to protect hepatocytes against the damage by the reactive oxygen intermediates, which is currently suggested as a novel therapeutic approach for different liver diseases. NAC is a thiol (–SH containing) compound and is one of the most extensively studied thiol agent, which has been in clinical use for more than 40 years. Our results showed that NAC protects against PA clivorine-induced hepatotoxicity, and the possible mechanism involves that NAC maintains cellular reduced environment, and thus inhibits clivorine-induced mitochondrial-mediated apoptotic signaling pathway.

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