Protection of S-adenosyl methionine against the toxicity of clivorine on hepatocytes

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In this study, we investigated the protective effects of S-adenosyl-L-methionine (SAM), which is a precursor of cellular reduced glutathione (GSH), against the hepatotoxicity of pyrrolizidine alkaloid clivorine. MTT assay showed that SAM (5 μM) prevented the cytotoxicity of clivorine on human normal liver L-02 cells. DNA fragmentation assay showed that SAM (5 μM) improved clivorine-induced L-02 cell apoptosis, and the results of Western blot showed that SAM (5 μM) decreased clivorine-induced caspase-3 activation. Cellular GSH analysis showed that when L-02 cells were exposed to different concentrations (0, 3, 10, 30, 50 and 100 μM) of clivorine for 48 h, cellular GSH was decreased in a concentration-dependent manner, while SAM (5 μM) enhanced 50 μM clivorine decreased cellular GSH. Further MTT assay showed that 5 mM GSH and 5 mM N-acetyl-L-cysteine (NAC) both had protective effects against clivorine-induced hepatotoxicity. Our results suggest that SAM has protective effects against the hepatotoxicity of clivorine possibly by enhancing cellular GSH level and increasing cellular defensive ability against clivorine-induced cytotoxicity.

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1. Introduction

Pyrrolizidine alkaloids (PAs) are natural hepatotoxins and carcinogens with worldwide distribution in more than 6000 high plants including herbal products and traditional remedies (Roeder, 1995, 2000). Consumption of PAs-containing plants leads to high risk to humans and live stocks (Coulombe, 2003), so hepatotoxic PAs attract worldwide attention. The U.S. Food and Drug Administration has already nominated PA riddelliine for genotoxicity and carcinogenicity testing conducted by the National Toxicology Program (NTP).

Abbreviations: GSH, reduced glutathione; γ-L-glutamyl-L-cysteinyl-glycine; PAs, pyrrolizidine alkaloids; SAM, S-adenosyl-methionine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; VOD, veno-occlusive disease.

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There are mainly two types of hepatotoxic PAs, namely retronecine- and otonecine-type (Lin et al., 1998), in which characteristically the eight-membered heterocyclic necine base is bicyclic and monocyclic, respectively. Otonecine-type PA clivorine (Fig. 1A) is the most abundant ingredient of traditional Chinese medicine Ligularia hodgsonii Hook., which has traditionally been used for cough, hepatitis and inflammation. Previous studies have demonstrated that clivorine had potential carcinogenic and mutagenic effects (Yamanaka et al., 1979; Kuhara et al., 1980). There are reports that clivorine is metabolic activated in liver to the reactive pyrrolic ester, which can further crosslink with vital cellular macromolecules, thus leading to liver damage (Cui and Lin, 2000; Lin et al., 2000; Xia et al., 2004). Our previous studies have found that clivorine inhibited human normal liver L-02 cell growth and induced cell apoptosis (Ji et al., 2002, 2005). But up to now there is nearly no report about the detoxification of clivorine or any other PAs.

GSH (γ-L-glutamyl-L-cysteinyl-glycine, reduced glutathione) is a ubiquitous reducing sulphhydril tripeptide, which is the most abundant thiol antioxidant in mammalian cells. It reacts directly with reactive oxygen species (ROS) and keeps sulphhydril groups of cytosolic proteins in reduced form by maintaining thiol redox potential in cells (Hall, 1999; Chandra et al., 2000). S-adenosyl methionine (SAM) is a precursor of cellular GSH in the liver and
also a principal biological methyl donor. It is reported that SAM prevents the hepatotoxicity produced by various hepatotoxins (Mato et al., 1997; Lieber, 1999). In the present study, we investigated the protective function of SAM against the toxic effects of clivorine on human normal liver L-02 cells.

2. Materials and methods

2.1. Chemicals and reagents

Clivorine, isolated from Ligularia hodgsonii Hook. (Ji et al., 2002) and identified by IR, NMR and MS with purity of 99.5%, was dissolved in phosphate-buffered saline (PBS) as 10 mM stock solution. RPMI1640, fetal bovine serum (FBS), penicillin G and streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA). SAM, 5,5′-dithio-bis (2-nitrobenzoic acid), glutathione reductase, N-acetyl-L-cysteine (NAC) and other reagents unless indicated were from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit polyclonal antibody against caspase-3 was purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibody against actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-rabbit IgG(H+L) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Nitrocellulose membranes and pre-stained protein marker were purchased from Bio-Rad (Hercules, CA, USA) and enhanced chemiluminescence detection system was obtained from Amersham Life Science (Buckinghamshire, UK). GSH, GSSG and NADPH were purchased from Roche (Switzerland).

2.2. Cell culture

The human normal liver 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). Cells were cultured in RPMI1640 supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine.

2.3. Assessment of cell viability

After various treatments, cells were incubated with 500 µg/ml 3-(4,5-dimethylthiazol-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 100% DMSO–5% iso-butanol–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 percent-age of control.

2.4. 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 at 37 °C overnight. The fragmented DNA in the lysate was extracted with phenol/chloroform/isopropyl alcohol (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.

2.5. Western blot analysis

After pretreatment with or without 5 mM SAM for 2 h, cells were incubated with 50 µM clivorine for 48 h. 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 glycine (pH 2.2) containing 50 mM Tris (pH 7.5), 2% SDS and 0.1 M 2-mercaptoethanol for 30 min at 50 °C.

2.6. Measurement of cellular GSH

Intracellular GSH was determined by the 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) assay according to previous reported method (Sies and Akerboom, 1984) with a minor modification. Briefly, L-02 cells were incubated with or without various concentrations of clivorine for 48 h and harvested in metaphosphoric acid (5%) buffer. The reaction mixture contained 1 mM EDTA, NADPH (0.24 mM), glutathione reductase (0.06 units), DTNB (86 mM) 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 were calculated based on cellular protein concentration.

2.7. Statistical analysis

All values were expressed as means ± standard error of the mean (S.E.M.). Differences between groups were assessed by one-way analysis of variance using the SPSS software package for windows. Post hoc multiple comparison tests was performed for inter-group comparisons using the least-significant difference (LSD) test. P < 0.05 was considered as statistically significant difference.

3. Results

3.1. The protective effects of SAM against clivorine-induced cytotoxicity

The chemical structure of otonecine-type PA clivorine was showed in Fig. 1A. Our previous studies have showed that clivorine had direct toxicity on human normal liver L-02 cells (Ji et al., 2002). In the present study, L-02 cells were pretreated with or without 5 mM SAM for 2 h, and then exposed to various concentrations of clivorine for 48 h. As shown in Fig. 1B, the cell viability of 25, 50, 100 µM clivorine-treated cells was 66.6, 34.0 and 33.2%, respectively, and after pretreated with 5 mM SAM the cell viability of 25, 50, 100 µM clivorine-treated cells was 96.5, 56.4 and 49.6%, respectively. Our results showed that clivorine-induced cytotoxicity in a concentration-dependent manner, while SAM significantly rescued this toxicity.
3.2. SAM rescued clivorine-induced cell apoptosis

Our previous study showed that the toxicity of clivorine on L-02 cells was due to inducing apoptosis (Ji et al., 2005). As shown in Fig. 2A, 50 μM clivorine with 48 h treatment-induced apoptotic DNA ladder in L-02 cells, which is considered a biochemical hallmark for apoptosis (De Maria et al., 1997), while 5 μM SAM inhibited clivorine-induced apoptosis. Caspase-3 is an important executioner caspase and synthesized as an inactive zymogen, whose cleavage represents its activation (Boyce et al., 2004). As shown in Fig. 2B, 50 μM clivorine decreased pro-caspase-3 and increased expression of cleaved caspase-3, while 5 μM SAM improved clivorine-induced caspase-3 activation. These results indicate that SAM inhibits clivorine-induced apoptosis in L-02 cells.

3.3. SAM enhanced clivorine-decreased cellular GSH

As SAM is a precursor of cellular GSH, we further observed the effects of clivorine on cellular GSH amounts. Fig. 3A shows the concentration-dependent effects of clivorine on cellular GSH in L-02 cells. After 48 h treatment, clivorine (100 μM) depleted cellular GSH at all, and clivorine (50 μM) decreased cellular GSH to about 20.3% of control.

In Fig. 3B, we observed the effects of SAM on clivorine-decreased cellular GSH. The results showed that clivorine (50 μM) decreased cellular GSH to about 22.5% of control, while pretreatment with SAM (5 μM) for 2 h significantly rescued clivorine-decreased cellular GSH to 81.0% of control.

3.4. Effects of thiol-containing compounds on clivorine-induced cytotoxicity

We further observed thiol-containing compounds such as NAC and GSH on the hepatotoxicity of clivorine. L-02 cells were pretreated with or without GSH (5 mM) for 2 h, and then 50 μM clivorine was added, after 24 h incubation, 5 mM NAC was added and incubated with cells for another 24 h. As shown in Fig. 4, NAC significantly rescued 50 and 25 μM clivorine decreased cell viability. GSH also rescued 25 μM clivorine decreased cell viability, but had weak protective effects against the hepatotoxicity of 50 μM clivorine.

4. Discussion

PA-containing plants have worldwide distribution, and there are many reports about the poison of them to livestock or human in various countries (Stegelmeier et al., 1999). In China it is also reported that PAs-induced clinical hepatic veno-occlusive disease (HVOD) in liver. Clivorine is the major PA in traditional Chinese medicine Ligularia hodgsonii Hook, and our previous reports have showed its direct toxicity on human normal liver L-02 cells (Ji et al., 2002). In the present study, the results of MTT assay showed that SAM (5 μM) pretreatment prevented clivorine-induced cytotoxicity in L-02 cells (Fig. 1B). SAM has already been reported to alleviate liver injury in...
many studies including hepatotoxin-induced injury (Corrales et al., 1991; Wu et al., 1996; Lieber, 2002; Song et al., 2003; Bailey et al., 2006; Wang and Cederbaum, 2006). Our results suggest that the hepatotoxicity of clivorine can also be prevented by SAM.

Our previous study has showed that clivorine induced L-02 cells apoptosis as detected by FACS analysis of sub-G1 peak and Hoechst 33258 staining (Ji et al., 2005). DNA fragmentation visualized by agarose gel electrophoresis is considered a biochemical hallmark for apoptosis (De Maria et al., 1997). The present study showed that SAM inhibited clivorine-induced apoptotic DNA ladder. Caspase-3 is an important downstream executor in apoptosis, and several substrates such as PARP, lamina are cleaved by activated caspase-3 (Cohen, 1997; Lee et al., 2002). Our results showed that SAM inhibited clivorine-induced caspase-3 activation.

The involved mechanism may play a role in the protective ability of SAM to clivorine. SAM is a precursor of GSH through its conversion to cysteine via the trans-sulfuration pathway (Cantoni, 1975). It is well known that intracellular GSH is an important endogenous antioxidant in hepatocytes, which conjugates toxic substances and predominantly participates in the detoxification of carcinogens, free radicals and peroxides, ultimately protecting cells and organs against carcinogen-induced toxicity (DeLeve and Kaplowitz, 1991). In the present study we found that SAM significantly enhanced clivorine-depleted cellular GSH (Fig. 3), hence that effects on cellular GSH level may play the important role in the toxicity of clivorine and the protection by SAM. Meanwhile, there is already reported that GSH plays important role in PA monocrotaline-induced HVOD (DeLeve et al., 1996). All these studies indicate that cellular GSH may play important role in the toxicity of PAs.

As SAM inhibited clivorine-induced DNA apoptotic ladder and caspase-3 activation, which indicates that SAM may prevent clivorine-induced L-02 apoptosis. There are many reports that cellular GSH antioxidant system is involved in cell apoptotic process (Rumora et al., 2007; Santra et al., 2007), and what is the concrete relationship between intracellular GSH and apoptotic signal pathway in clivorine-induced apoptosis needs further investigation.

Given that intracellular GSH depletion may be relevant to cytoxicity of clivorine on L-02 cells, we further examined whether thiol-containing compounds such as exogenous NAC and GSH would exert a protective effect against clivorine-induced cytotoxicity. NAC, an aminothiol and synthetic precursor of intracellular cysteine and GSH, is converted in the body into metabolites capable of increasing GSH synthesis and prevent the toxicity of many toxins (Kelly, 1998; Menor et al., 2004). GSH is a thiol-containing compound, which can abrogate the hepatotoxicity induced by some exogenous substances (Kim et al., 2003). In the present study, we found that 5 mM NAC had significant protective effects against the toxicity of clivorine (Fig. 4), as NAC is also the precursor of intracellular GSH, so NAC may prevent the hepatotoxicity of clivorine via enhancing cellular GSH just like SAM. Exogenous GSH (5 mM) also had protective effects against 25 μM clivorine-induced cytotoxicity, while had weak protective effects against 50 μM clivorine-induced cytotoxicity (Fig. 4). All these results demonstrate that increasing cellular GSH have protective effects on the hepatotoxicity of clivorine.

In conclusion, the present study suggests that SAM may increase cellular GSH level and then detoxify the hepatotoxicity of clivorine. The present study is the first one of its kind to report the detoxification of clivorine and studies are in progress to identify the molecular mechanisms responsible for such a protective function. This study is most helpful for the clinical detoxification of clivorine, and also provides a helpful direction for the searching of the alexipharmic substances against clivorine or other PAs.

Conflict of interest statement

None.

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