JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 227–233

Received August 26, 2005
Accepted November 8, 2005
DOI 10.1211/jpp.58.2.0010
ISSN 0022-3573

Mechanism underlying mitochondrial protection of asiatic acid against hepatotoxicity in mice

Jing Gao, Jin Chen, Xinhui Tang, Liya Pan, Feng Fang, Lizhi Xu, Xiaoning Zhao and Qiang Xu

Abstract

Asiatic acid (AA) is one of the triterpenoid components of *Terminalia catappa* L., which has antioxidative, anti-inflammatory and hepatoprotective activity. This research focused on the mitochondrial protection of AA against acute liver injury induced by lipopolysaccharide (LPS) and D-galactosamine (D-GalN) in mice. It was found that pretreatment with 25, 50 or 100 mg kg^{-1} AA significantly blocked the LPS + D-GalN-induced increase in both serum aspartate aminotransferase (sAST) and serum alanine aminotransferase (sALT) levels, which was confirmed by ultrastructural observation under an electron microscope, showing improved nuclear condensation, ameliorated mitochondrion proliferation and less lipid deposition. Meanwhile, different doses of AA could decrease both the transcription and the translation level of voltage-dependent anion channels (VDACs), the most important mitochondrial PTP component protein, and block the translocation of cytochrome c from mitochondria to cytosol. On the other hand, pre-incubation with 25, 50 and 100 μg mL^{-1} AA inhibited the Ca^{2+}-induced mitochondrial permeability transition (MPT), including mitochondrial swelling, membrane potential dissipation and releasing of matrix Ca^{2+} in liver mitochondria separated from normal mice, indicating the direct role of AA on mitochondria. Collectively, the above data suggest that AA could protect liver from damage and the mechanism might be related to up-regulating mitochondrial VDACs and inhibiting the process of MPT.

Institute of Materia Medica, School of Medicine, Nanjing University, Nanjing 210093, P. R. China

Jing Gao, Liya Pan, Lizhi Xu, Xiaoning Zhao

State Key Laboratory of Pharmaceutical Biotechnology, School of Life Science, Nanjing University, Nanjing 210093, P. R. China

Jing Gao, Jin Chen, Feng Fang, Qiang Xu

Department of Biology, Yancheng Teachers College, Yancheng, P. R. China

Xinhui Tang

Correspondence: J. Gao, School of Medicine, Nanjing University, 22 Hankou Road, Nanjing 210093, China. E-mail: jinggao@nju.edu.cn

Acknowledgments and funding: This work was financially supported by the Natural Science Fund of Jiangsu Province of China (No.BK99051) and the Center of Modern Analysis, Nanjing University. We also would like to thank Professor Zu Xuan Zhang, School of Medicine, Nanjing University, for his support and assistance during this study.

Introduction

It is generally accepted that cell death is the most crucial step in the development of all kinds of liver injury (Itokazu et al 2000; Neuman 2001; Jaeschke et al 2004). More and more evidence suggests that the major function of mitochondria is not only to provide ATP by oxidative phosphorylation but to play many other roles, such as the modulation of intracellular Ca^{2+} homoeostasis and induction of cell death (Newmeyer & Ferguson-Miller 2003; Orrenius 2004).

Mitochondrial permeability transition (MPT), the initiation of death signalling, occurred in livers from ischaemia–reperfusion, D-galactosamine- or carbon tetrachloride-damaged animals following excessive opening of the mitochondrial permeability transition pore (PTP). Some hepatoprotective drugs were found to protect hepatocytes against injury by inhibiting PTP opening (Kim et al 2000; Elimadi et al 2003), but the mechanisms in detail remain unknown.

Recently, the mitochondrial PTP, composed of a voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT), etc., has been recognized as a major complex in MPT (Kim et al 2003; Godbole et al 2003). The PTP opening is also accompanied by the release of cytochrome *c* (Cyt *c*) (Lemasters et al 1997), a strong activator of caspase-9, apoptotic protease activating factor-1 (Zou et al 1999) and, subsequently, caspase-3, which results in apoptosis (Wang 2001). As a protein providing the pathway for transporting ions, ATP and other critical metabolites, VDACs play a very important role in not only the regulation of mitochondrial basic physiological function, such as energy transduction, substance metabolism and intracellular calcium homoeostasis, but also in cell death control, especially apoptosis initiation. The relationship between pathological cases and mitochondrial VDAC level has not been illustrated.

Asiatic acid (AA), a triterpenoid compound, is one of the active constituents of the anti-hepatotoxic plant, *Terminalia catappa* L. (Gao et al 2004). Previous studies showed that AA has numerous pharmacological actions, including antioxidant, anti-inflammatory, neuroprotective and anti-cancer effects (Lee et al 2000, 2002b; Hsu et al 2005; Park et al 2005).

In this paper, we evaluated the hepatoprotection of AA against liver injury induced by lipopolysaccharide (LPS) and D-galactosamine (D-GalN), and mainly addressed the possible action of AA on mitochondrial PTPs by regulation of VDACs, the most important proteins on the outer membrane of mitochondria, to search for the possible mitochondrial mechanism underlying the hepatoprotective effect of AA.

Materials and Methods

Animals

Male ICR mice, 18–22 g, were obtained from the Experimental Animal Center of Nanjing Medical University. All mice were kept in a temperature-controlled, air-conditioned conventional animal house with a 12-h light–dark cycle; they were allowed free access to food and water. Procedures were performed according to the recommendations of the institutional animal care committee.

Chemicals

AA was purchased from Sigma-Aldrich (purity > 99%). D-GalN, LPS, Fura-2/AM, rhodamine-123 (Rh123), succinate, rotenone, ciclosporin (cyclosporine A, CsA) and anti-VDAC antibody were purchased from Sigma (St Louis, MO, USA). Anti-Cyt *c* antibody was from Trevigen Inc (Gaithersburg, MD, USA). Tripure reagent was from Roche diagnostics corporation (Indianapolis, IN, USA). AMV reverse transcriptase, RNase inhibitor, dNTP, Oligo$(dT)_{15}$ and Taq polymerase were all from Promega (Madison, WI, USA). All other chemicals were of high purity from commercial sources.

LPS + D-GalN-induced hepatotoxicity

Mice were divided into five groups of eight mice each. AA (25, 50 and 100 mg kg^{-1}) was administered intragastrically (i.g.) using an oral gavage once daily for five consecutive days followed by the final treatment of LPS + D-GalN (10 μg kg^{-1}, 600 mg kg^{-1}, i.p., respectively). Two other groups were as follows: a group of non-intoxicated mice, which received relative vehicles (normal group), and a group that received LPS + D-GalN (injury group) following the treatment with saline (20 mL kg^{-1}, i.g.) for five days. Twelve hours after the final treatment, blood was collected and mice were euthanized. Serum was obtained when the blood was clotted at room temperature and centrifuged at 3000 *g* for 20 min. Meanwhile, the whole liver was excised and sections (~ 1 mm wide) were taken and fixed in Fixation solution (3% paraformaldehyde, 2.5% glutaraldehyde) and regularly prepared for examination under an electron microscope (JEM-1200EX). The remaining liver lobes intended for mRNA and protein analyses were frozen immediately and stored in liquid nitrogen before extraction.

Aminotransferase activity determination

Serum alanine aminotransferase (sALT) and aspartate aminotransferase (sAST) levels, markers for hepatotoxicity, were determined with an automatic analyser (Hitachi 7600–020, Japan).

Isolation of liver mitochondria

Mitochondria were prepared from mouse livers according to the method of Apprille et al (1977). In brief, mouse livers were excised, homogenized in isolation buffer (containing in mM: 225 D-mannitol, 75 sucrose, 0.05 EDTA and 10 Tris-HCl, pH 7.4) at 4°C. The homogenates were centrifuged at 600 *g* for 5 min and supernatants were centrifuged at 8800 *g* for 10 min. The pellet was washed twice with the same medium, followed by protein concentration determination using Coomassie Brilliant Blue (Bradford 1976).

Determination of mitochondrial swelling

Mitochondrial swelling was assessed by measuring the absorbance of their suspension at 540 nm. Liver mitochondria were prepared in the assay buffer (0.5 mg protein/mL) containing 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 5 μM rotenone, 10 mM HEPES and 5 mM succinate. To induce mitochondrial swelling, 50 μM Ca^{2+} was administered. CsA (5 μM) was used as a positive reference (Elimadi et al 2001). The extent of mitochondrial swelling was assayed by measuring the decrease in absorbance (A) 1–5 min after the addition of 50 μM Ca^{2+} at 30°C and the inhibitory rate of mitochondrial swelling was calculated as follows: $(\Delta A_{Normal} - \Delta A_{drug})/\Delta A_{Normal} \times 100\%$, $\Delta A = A_{0\,min} - A$ (Lee et al 2002a).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi_m$) was evaluated according to Emaus et al (1986) from uptake of the fluorescent dye rhodamine-123 (Rh123), which accumulates electrophoretically into energized mitochondria in response to their negative inside membrane potential.

Liver mitochondria isolated from normal mice were incubated in the assay buffer (0.5 mg protein/mL; containing in mM: 225 D-mannitol, 70 sucrose, 5 HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 5 succinate and 5 rotenone, pH 7.2) at 25°C. AA 25, 50 or 100 μg mL^{-1} was added, and the discharge of Rh123 was induced by 50 μM $CaCl_2$. $\Delta\psi_m$ was assessed spectrophotometrically (Hitachi 850) with excitation at 505 nm and emission at 534 nm after the addition of 0.3 μM Rh123 at 25°C (Varbiro et al 2001). CsA at 5 μM was used as a positive reference (Elimadi et al 2001).

Measurement of mitochondrial free calcium

The intramitochondrial Ca^{2+} level was assayed by Ca^{2+} indicator dye fura-2/AM. Liver mitochondria (0.5 mg protein/

mL) of normal mice were incubated with the fluorescent fura-2/AM for 30 min at 30°C in suspension medium (containing in mM: 125 sucrose, 65 KCl, 5 succinate and 5 HEPES, and 1 μM Fura-2/AM, pH 7.4), and then washed twice with the medium. The final mitochondria pellet (0.5 mg mL^{-1}) was treated with various concentrations of AA for 3 min and followed by Ca^{2+} release induced by 50 μM $CaCl_2$ 3 min later. Fluorescent intensity (F) of Fura-2 loaded mitochondria was recorded on Hitachi 850 fluorescence spectrometer at an excitation 340 nm and an emission 510 nm (Lee et al 2002a). CsA at 5 μM was used as a positive reference (Elimadi et al 2001).

Western blot analysis for cytochrome *c* release

Livers from different groups were isolated as cytosolic and mitochondrial fractions, whereafter two fractions were separated by 15% SDS–polyacrylamide gel electrophoresis (PAGE), and electrotransferred to a polyvinylidene-difluoride (PVDF) membrane (Bio-Rad) (Kobayashi et al 2003; Salvi et al 2003). The membrane was blocked with 5% skimmed milk for 2 h at room temperature and incubated overnight at 4°C with primary cytochrome *c* antibody in PBS + Tween 20 (PBST) (1:1000). After the membrane was washed in PBST, it was incubated in the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:4000) in PBST for 2 h at room temperature, and followed by 5 min exposure in enhanced chemiluminescence (ECL) solution.

Evaluation of VDAC mRNA level by RT-PCR assay

Total RNA was extracted from livers using Tripure and reverse transcription was started with 2 μg of total RNA at 42°C for 60 min in 20 μL reaction mixture containing 20 U RNase inhibitor, 0.25 mM each of dNTP, 0.5 μg Oligo(dT)$_{15}$ and 15 U AMV reverse transcriptase. The reaction was terminated by incubation at 95°C for 5 min. PCR amplification was performed for 30 cycles, including 4 μL cDNA, by adding 5 mM $MgCl_2$, 2.5 U Taq polymerase, 0.25 mM each dNTP, and 5′- and 3′-sequence-specific oligonucleotide primers for VDAC and β-actin in 1 × Taq polymerase reaction buffer, respectively. Each PCR cycle contained 94°C, 50 s; 60°C, 1 min; 72°C, 1 min; 72°C, 8 min. β-actin was used as a control. The amplified fragments were detected by agarose gel electrophoresis and visualized by ethidium bromide staining. The following oligonucleotide primers were used. For VDAC, sense 5′-GGC TAC GGC TTT GGC TTA AT-3′ and anti-sense 5’-CCC TCT TGT ACC CTG TCT TGA-3′ deduced amplification product of 321 bps. While, for β-actin, sense 5′-TGC TAT CCC TGT ACG CCT CT-3′ and anti-sense 5′-GGA GGA GCA ATG ATC TTG A-3′ deduced amplification product of 601 bps.

Western blot analysis for VDACs

Liver samples were homogenized in ice-cold lysis buffer. Homogenates were centrifuged at 12000 *g* for 10 min and the supernatants were collected and the protein concentration was determined using Coomassie Brilliant Blue. The samples (40 μg per lane) were dissolved in sample buffer and separated by 12% SDS–PAGE gels and electrophoretically transferred onto a PVDF membrane (Bio-Rad). The membrane was incubated with VDAC primary antibody (1:4000) and β-actin antibody (1:80000). The membrane was then exposed to the ECL solution.

Statistical analysis

Differences among all groups were analysed by one-way analysis of variance, followed by SNK-q-test using SPSS10 software. $P < 0.05$ was accepted as statistically significant.

Results

Protective effect of AA on LPS + D-GalN-induced liver injury in mice

Inhibition of serum enzyme level elevation

Marked elevation in sALT and sAST activity was observed as compared with the normal level (Figure 1A), while 25, 50 and 100 mg kg^{-1} AA significantly blocked the above increase, especially 100 mg kg^{-1} AA treatment, which maintained the sAST almost at normal level.

Protection on the ultrastructure of hepatocytes

Compared with the normal group (Figure 1B), obvious ultrastructural changes in mouse livers were found, showing mitochondrial proliferation, lipid deposition and nuclear condensation. However, the structure of hepatocytes of mice treated with 50 mg kg^{-1} AA was improved to some extent, and in the 100 mg kg^{-1} AA group the hepatocytes were almost similar to normal cells.

Inhibitory effect of AA on cytochrome c *redistribution from the mitochondria to the cytosol*

The regulation of AA on the translocation of cytochrome *c* in the mitochondrial and cytosolic fractions was determined by Western blot (Figure 2). In the cytosolic fraction, normal mice showed only a weak signal for cytochrome *c*, which was increased when mice were treated with LPS + D-GalN alone. In contrast, after pretreatment with AA, lower cytosolic and higher mitochondrial levels of cytochrome *c* were found dose dependently 6 h after LPS + D-GalN administration compared with levels in the LPS + D-GalN group.

Prevention against reduction of liver VDAC expression

The effect of AA on VDAC transcription was examined by RT-PCR. As shown in Figure 3A, the expression of VDAC mRNA was detected in the normal group, but a lower level of VDAC mRNA was detected when mice were stimulated with LPS + D-GalN (10 μg kg^{-1}, 600 mg kg^{-1}, i.p., respectively). Furthermore, AA (25, 50 and 100 mg kg^{-1}) significantly blocked the LPS + D-GalN-stimulated VDAC mRNA reduction.

AA up-regulation on VDAC protein expression was further corroborated by Western blot (Figure 3B). Normal mouse livers showed a strong signal for VDAC, and mice receiving LPS + D-GalN alone showed a significant decrease. In contrast,

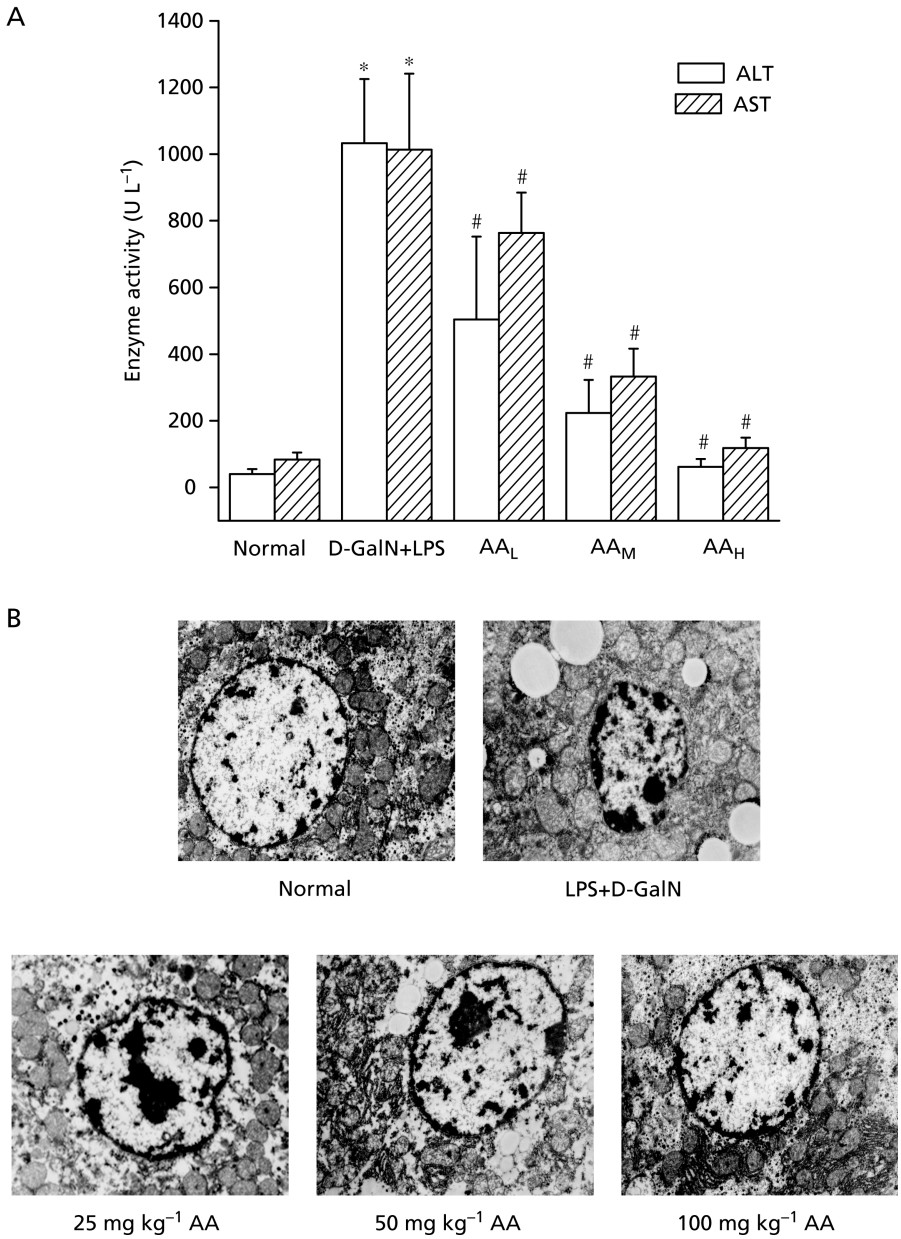

Figure 1 Protective effect of AA on hepatic injury induced by LPS + D-GalN. Mice were divided into 5 groups as follows: Normal, LPS + D-GalN, AA_L, AA_M, AA_H (25, 50, 100 mg kg^{-1}). LPS + D-GalN and different AA groups were orally treated with saline or various concentrations of AA for 5 days before the intraperitoneal injection of LPS (10 μg kg^{-1}) and D-GalN (600 mg kg^{-1}). A. Effects of AA on the elevation of serum AST and ALT activity in LPS + D-GalN-insulted mice. The blood samples were obtained 12 h later. Each value represents mean ± s.d. of 8 mice. *$P < 0.01$, vs Normal; #$P < 0.01$, vs LPS + D-GalN group. B. Effect of AA on the ultrastructure of hepatocytes insulted by LPS + D-GalN. Specimens were taken 12 h later and regularly prepared for the examination under an electron microscope (magnification ×5000).

in mice pre-administered with AA, a stronger VDAC protein band occurred 6 h following LPS + D-GalN treatment compared with mice treated with LPS + D-GalN alone.

Direct protection of AA on liver mitochondria

Inhibitory effects on Ca^{2+}-induced mitochondrial swelling

There was obvious swelling of liver mitochondria after the addition of 50 μM Ca^{2+} (Figure 4A). AA at various concentrations exerted a dose-dependent effect against the swelling. The inhibitory rates of 25, 50 and 100 μg mL^{-1} AA at 5 min reached 28.0%, 49.6% and 69.6%, respectively.

Inhibition of Ca^{2+}-induced dissipation of mitochondrial membrane potential

When 50 μM Ca^{2+} was added to the isolated mitochondria, a progressive increase in the discharge of Rh123 from the mitochondria occurred, which indicated a loss of mitochondrial membrane potential. Its fluorescent intensity (F) value of the

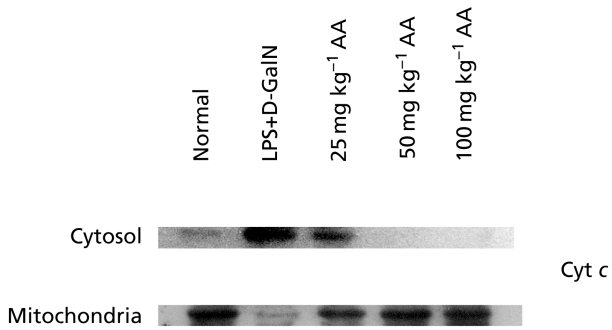

Figure 2 Inhibitive effects of AA on Cyt *c* translocation from mitochondria to cytosol induced by LPS + D-GalN. Livers were taken 12 h after LPS (10 μg kg^{-1}) and D-GalN (600 mg kg^{-1}) intraperitoneal injection, and mitochondria were prepared in the buffer (1.0 mg protein/mL). The protein samples were isolated as cytosolic and mitochondrial fractions and analysed by Western blot.

mitochondrial suspension was significantly increased 46.0% 3 min after the addition of Ca^{2+}. When AA was added into the isolated mitochondria 3 min before the addition of Ca^{2+}, a dose-dependent inhibition of mitochondrial membrane potential loss was observed (Figure 4B).

Decrease in Ca^{2+}-induced intramitochondrial free Ca^{2+} release

Addition of 50 μM Ca^{2+} caused an obvious decrease (37.8%) in the fluorescent intensity of the mitochondrial suspension (Figure 4C), which indicated intramitochondrial free Ca^{2+} release. Against the control, pretreatment with AA blocked the mitochondrial Ca^{2+} release in a dose-dependent manner and the inhibitory rates of 20, 50 and 100 μg mL^{-1} AA on Ca^{2+} release were 36.1%, 48.2% and 63.3%, respectively.

Discussion

LPS + D-GalN-induced tissue injury has been used as a model of fulminant hepatitis and hepatic shock (Bohlinger et al 1996; Schumann & Tiegs 1999). Liver injury induced by LPS + D-GalN is a characterized system of acute hepatic failure and usually used for screening of anti-hepatotoxic or hepatoprotective activity of drugs (Nowak et al 2000). sAST and sALT detection are the most sensitive tests for diagnosis of liver injury. This study revealed a significant increase in the activity of sALT and sAST within 12 h following exposure to LPS + D-GalN, indicating considerable hepatocellular injury, which could be inhibited by the oral administration of AA at doses of 25, 50 and 100 mg kg^{-1}, demonstrating its hepatoprotective effects. At the same time, the effects of AA on sAST also suggest its possible roles on mitochondria, because 80% of sAST was released from mitochondria.

Previous studies have shown that after LPS + D-GalN administration, the transition of cytochrome *c* from the mitochondria into the cytosol was observed, accompanied by gradual and continuous increases in AST and ALT levels in the plasma, and the opening of the pores on the mitochondrial surfaces in the process of mitochondrial permeability transition (Tafani et al 2000). The Fas/Fas ligand pathway might also be involved in the above toxic effect, but AA didn't act on it (Hsu et al 2005). This paper just focused on the mitochondrial pathway and the results indicated that AA could inhibit the release of cytochrome *c* from mitochondria in a dose-dependent manner, and protected mitochondria against injury induced by LPS + D-GalN. AA-induced mitochondrial apoptosis in human breast cancer cells was only reported recently (Hsu et al 2005); the opposite effects might be due to different species of cell types. On the other hand, hepatocellular Ca^{2+} overload could activate the mitochondrial Ca^{2+} uniporter in the mitochondrial inner membrane, and induce a mitochondrial Ca^{2+} influx. However, the excessive intramitochondrial Ca^{2+} can lead to the opening of mitochondrial PTP and finally damage mitochondria and induce apoptotic or necrotic cell death (Schild et al 2001; Jambrina et al 2003). Thus, Ca^{2+}-induced liver MPT has become a widely used model for evaluating the effects of drugs or other substances on mitochondria. Our data have revealed that AA could act on mitochondria PTP directly against Ca^{2+}-induced mitochondrial swelling, membrane potential rupture and release of matrix Ca^{2+}, which suggests that AA could protect mitochondria against liver injury induced by LPS + D-GalN. Indeed, it has been believed that inhibition of mitochondrial PTP opening might constitute a relevant pharmacological approach to

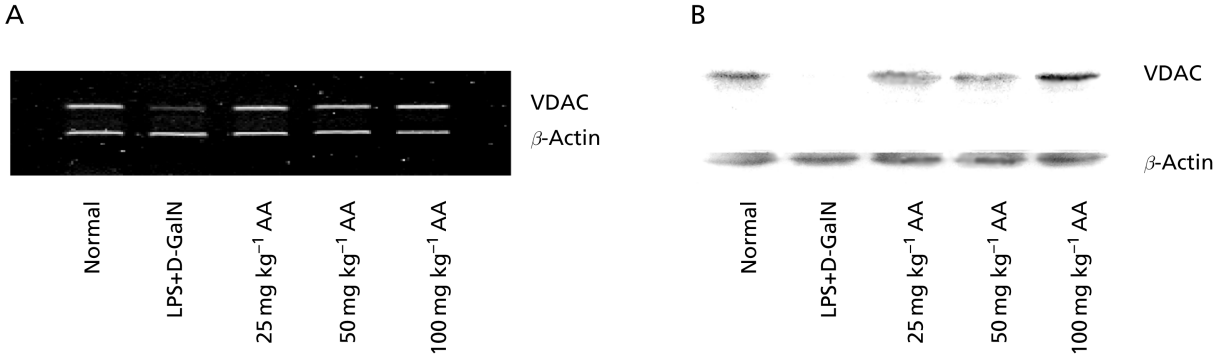

Figure 3 Effect of AA on mitochondrial VDAC expression in LPS + D-GalN-insulted mouse livers. Livers from various groups were taken 6 h following LPS (10 μg kg^{-1}) and D-GalN (600 mg kg^{-1}) intraperitoneal injection. A. Inhibitory effect of AA on the decrease in VDAC mRNA level induced by LPS + D-GalN, which was analysed by RT-PCR. B. Inhibitory effect of AA on the decrease in VDAC protein level induced by LPS + D-GalN, which was analysed by Western blot.

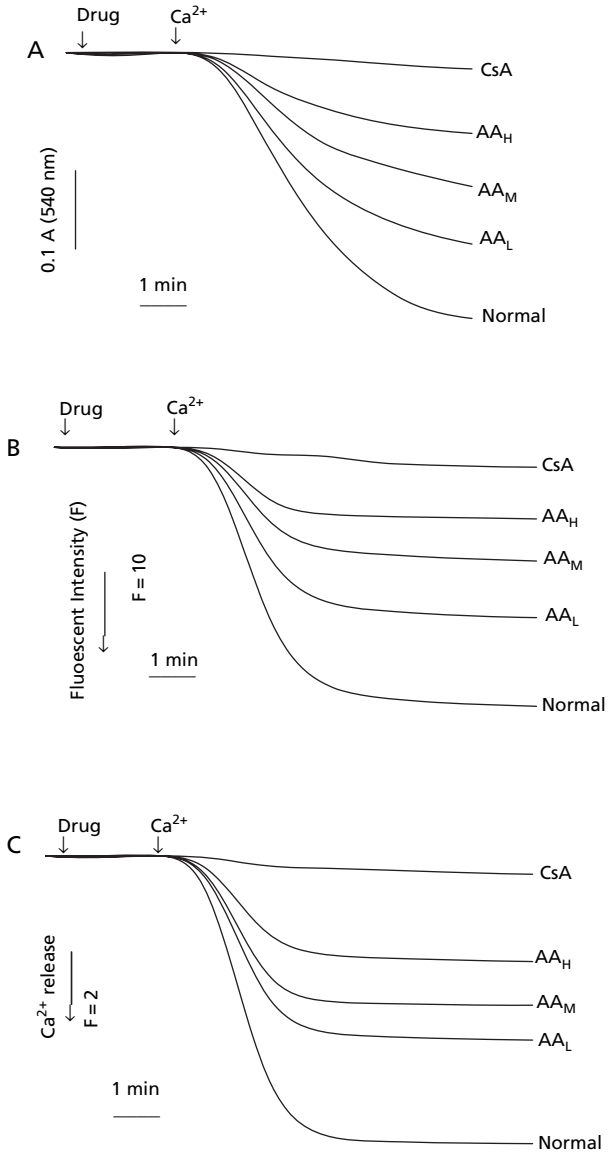

Figure 4 Direct protective effect of AA on isolated mitochondria. Mitochondria from the livers of normal mice were prepared and divided into 5 groups: control, 25, 50, 100 μg mL^{-1} AA (AA_L, AA_M, AA_H) and ciclosporin (cyclosporine A, CsA; 5 μM, as a positive reference). Various concentration of drugs or CsA were added to mitochondria solution (1.0 mg protein/mL) 3 min before the incubation with 50 μM Ca^{2+} at 30°C. The results represented typical recordings from experiments of at least three different mitochondrial preparations. A. Inhibitory effects of AA on Ca^{2+}-induced mitochondrial swelling, which was assayed by the decrease in absorbance at 540 nm. B. Effects of AA on Ca^{2+}-induced mitochondrial membrane potential dissipation, which was evaluated spectrophotometrically using fluorescent dye rhodamine-123 with excitation at 505 nm and emission at 534 nm. C. Effects of AA on Ca^{2+}-induced mitochondrial Ca^{2+} release, which was evaluated using Fura-2 with excitation at 340 nm and emission at 510 nm.

protect cells from death, and the search for novel PTP inhibitors should be an important strategy for the treatment of liver diseases (Morin et al 2002; Elimadi et al 2003).

Considering the exact target of AA on mitochondria, components of PTP proteins, especially VDAC (Kokoszka et al 2004), the only PTP constituent protein on the outer membrane of mitochondria, attracted attention because the permeability of the outer mitochondrial membrane is important in modulating cell death (Green & Kroemer 2004). It is interesting to find that the VDAC level had been decreased significantly after administration of LPS + D-GalN, which was accompanied by the decreased sensitivity of mitochondrial swelling induced by Ca^{2+}, suggesting the decreased modulating ability of PTP. However, pretreatment with AA could dose-dependently inhibit the reduction of both the transcription and the translation level of VDAC in the process of acute liver injury, which could be blocked by AA, suggesting that VDAC level might be another criterion to screen new anti-hepatotoxic drugs.

Cytochrome *c* release is a very important marker of mitochondria-induced cell death (Wang 2001), and the mechanism by which cytochrome *c* transits from mitochondria into the cytosol might be as follows: through a mitochondrial PTP complex composed of VDAC (PTP theory) (Marzo et al 1998); through a non-PTP VDAC-Bax pore in the mitochondrial outer membrane (VDAC-Bax theory) (Shimizu et al 1999); through ion channels in the mitochondrial membrane formed by Bax homodimers (Bax ion channel theory) (Saito et al 2000); or through destruction of the mitochondrial outer membrane (destroyed outer membrane theory) (Vander Heiden et al 1997). Since it has been shown that microinjection of a VDAC-neutralizing antibody inhibits apoptosis (Shimizu et al 2001; Zheng et al 2004), it appears that the release of cytochrome *c* is related to VDAC. However, it is bewildering that we found that the decreased level of VDAC was concomitant with the release of cytochrome *c* in this experiment. On the basis of recent research, we put forward a hypothesis that the function of VDAC might be increased following the conformation alteration with the treatment of LPS + D-GalN and AA might exert a hepatoprotective effect against this functional change, but the authenticity still needs to be confirmed by further experiment, especially in the primary culture of different cells.

Collectively, the above data indicate that AA has hepatoprotective activity and the mechanisms underlying its protective effects may be related to regulation of the VDAC level and its protein conformational change.

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