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Protective effect of glycoprotein isolated from *Ulmus davidiana* Nakai on carbon tetrachloride-induced mouse liver injury

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Abstract

This study was carried out to evaluate the hepatoprotective activity of glycoprotein isolated from the stems of *Ulmus davidiana* Nakai (UDN), which has been used as an anti-inflammatory agent in folk medicine. We evaluated lipid peroxidation in glucose/glucose oxidase (G/GO)-induced BNL CL.2 cells and measured thiobarbituric acid reactive substances (TBARS), lactate dehydrogenase (LDH), nitric oxide (NO), antioxidant enzyme (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)), activity of cytotoxic-related signals (hepatic cytochrome *c*, nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1)) and levels of plasma lipids (triglyceride (TG) and total cholesterol (TC)) in carbon tetrachloride (CCl_4, 1.0 mL kg^{-1})-induced A/J mouse. The results in G/GO-induced BNL CL.2 cells showed that UDN glycoprotein had a dose-dependent inhibitory effect on lipid peroxidation. The results in carbon tetrachloride (CCl_4, 1.0 mL kg^{-1})-induced A/J mouse indicated that treatment with UDN glycoprotein (40 mg kg^{-1}) lowered LDH activity and TBARS formation, and increased NO production and antioxidant enzymes activity, compared with control. Also, our finding from CCl_4-treated mice after pretreatment with UDN glycoprotein demonstrated that the activity of cytotoxic-related signals decreased but the levels of plasma lipids increased, compared with CCl_4 treatment alone. Here, we speculate that UDN glycoprotein has a protective character to CCl_4-induced mouse liver injury.

Introduction

Reactive oxygen species (ROS) cause extensive damage to DNA, proteins and lipids and have been implicated in the initiation of various liver pathological processes, such as fibrogenesis, cirrhosis and steatosis (Cesaratto et al 2004). The main sources of ROS generation in the liver are represented by mitochondria and cytochrome P450 system in hepatocytes, Kupffer cells and neutrophils. Although ROS production cannot be avoided in the metabolic pathway, there is an antioxidant defence system for detoxifying oxygen radicals. This antioxidant defence system, consisting of enzymatic (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) and non-enzymatic (ascorbic acid, glutathione and α-tocopherol) compounds, can maintain the balance between ROS generation and protection from damage by ROS (Jaeschke et al 2002; Cesaratto et al 2004).

Carbon tetrachloride (CCl_4) is widely used as a hepatotoxic compound for screening the anti-hepatotoxic/hepatoprotective activity of drugs in experimental model systems, because CCl_4-induced hepatotoxicity is regarded as an analogue of liver injury caused by a variety of hepatotoxins in man. It has been generally reported and accepted that CCl_4-induced hepatotoxicity results from its hepatotoxic metabolites and trichloromethyl free radical ($\bullet CCl_3$) (Recknagel & Glende 1973). This free radical can react with sulfhydryl groups, such as glutathione (GSH) and thiol-groups in the protein side chain. Also it covalently binds with cell proteins, and then initiates the lipid peroxidation process in the cellular membrane, which eventually leads to various liver pathological processes (Connor et al 1990; Williams & Burk 1990). Therefore, one of the therapeutic strategies against liver injury and disease is to find antioxidant compounds that are able to block liver injury through scavenging of trichloromethyl free radical generated by CCl_4.

CCl_4 is one of the well-known promoters of nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) in the apoptotic response of hepatocytes, inflammatory response of Kupffer

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cells and fibrogenetic response of stellate cells on liver injury (Zawaski et al 1993; Camandola et al 1997; Wu & Zern 1999; Cesaratto et al 2004). NF-κB, a redox-sensitive transcription factor, is closely related to up-regulation of the expression of Fas ligands, chemokines and proinflammatory cytokines (interleukin (IL)-1, IL-6, tumour necrosis factor-α (TNF-α), etc.,) in acute injury and fibrosis of the liver (Wu & Zern 1999). It has been reported that NF-κB exists as either a heterodimeric or a homodimeric complex, but it is classically composed of p50 and Rel A /p65 subunits (May & Ghosh 1998). Like NF-κB, AP-1 is a redox-sensitive transcription factor that comprises members of the basic leucine zipper proteins, including Jun (c-Jun, JunB and JunD) and Fos (c-Fos, Fos B, Fra-1 and Fra-2) families. Recent studies have suggested that c-Jun activation has the most important role in the regulation of cell growth, differentiation and apoptosis (Wang et al 2000). A variety of stimuli can activate the AP-1 pathway, including oxidative stress and lipid peroxidation end-product (4-hydroxy-2,3-nonenal, HNE) in CCl_4-induced liver injury (Camandola et al 1997; Jaeschke et al 2002; Cesaratto et al 2004).

For the past three decades, many polysaccharide–protein complexes (glycoproteins) have been isolated from mushrooms, fungi, yeasts, algae, lichens and plants. Recently, we have found a glycoprotein with an approximate molecular mass of 116 kDa, isolated from *Ulmus davidiana* Nakai (UDN), which consists of a carbohydrate content of 78.65% and a protein content of 21.35% (Lee et al 2004). UDN glycoprotein has pharmacological activity, such as anti-apoptotic and anti-oxidative effects. From this, we speculate that the UDN glycoprotein might protect against liver injury that is caused by trichloromethyl free radical produced from CCl_4, because it has a strong antioxidant character. Nobody has studied the hepatoprotective effect of UDN glycoprotein on CCl_4-induced mouse liver injury. Therefore, we investigated whether the UDN glycoprotein modulates lipid peroxidation, the activity of antioxidant enzymes and cytotoxic related factors and plasma lipids levels in CCl_4-treated mouse liver.

Materials and Methods

Chemicals

All the plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA). L-Ascorbic acid (A4544), ammonium thiocyanate (A7149), catalase (C40), dextran sulfate (D8906), ethylenediaminetetraacetate (EDTA, E5134), ethylene glycol bis(2-aminoethyl ether)-N, N, N′N′-tetraacetic acid (EGTA, E4378), ferrous chloride (F2130), glucose oxidase (G8135), glutathione peroxidase (GPx, G1637), linoleic acid (L8134) neutral red (N7005), N-1-naphthylethylenediamine dihydrochloride (N5889), β-nicotinamide adenine dinucleotide (β-NADH, N6005), nonidet P-40 (NP-40, N0896), olive oil (O1514), penicillin G (H0474), phenylmethanesulfonyl fluoride (PMSF, P7626), silica gel (28–200 mesh, 22 Å, S4883), streptomycin (H0447), sucrose (S8501), sulfanilamide (S9251), superoxide dismutase (S2515), 1,1,1,3-tetraethoxypropane (T9889), trichloroacetic acid (TCA, T9159), trypsin (T4549) and 2-thiobarbituric acid (TBA, T5500) were obtained from Sigma (St Louis, MO, USA), and carbon tetrachloride (CCl_4, 289116) was obtained from Aldrich (Milwaukee, WI, USA). Dulbecco's modified essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other chemicals and reagents were of the highest quality available.

Preparation of UDN glycoprotein

Samples of *Ulmus davidiana* Nakai were obtained in October 2000 from the Naju Traditional Market in the Chonnam province of South Korea. They were identified by Dr H. T. Lim (Chonnam National University) and the seeds of *U. davidiana* Nakai were chopped into small portions and soaked in water for several months in a dark basement. The water extract was filtered through Whatman filter paper (no. 2) and concentrated with a rotary evaporator (B465; Bunchi, Switzerland). The concentrated solution was dried with a freeze-dryer (SFDS06; Sam won, Seoul, Korea). Five grams of dried crude water extract, dissolved in distilled water, was applied to the silica gel column (4×28 cm, 28~200 mesh, 22 Å). An ordered elution was performed using distilled water, 70% ethanol, absolute ethanol and 5% acetic acid. Only the 70% ethanol eluted solution was collected and lyophilized to 2.5 g (50% of the initial amount), because it had a biological function in trials before the experiment. The dried powder was dissolved again with distilled water. The solution was precipitated with 80% ammonium sulfate and then dialysed with a dialysis membrane (MW cut-off 6000–8000; Spectra/por, CA) against 20 mM Tris-Cl (pH 7.4) at 4°C overnight. After dialysis, the solution was centrifuged at 3000 *g* for 15 min at 25°C using microcon concentrators (MW cut-off 100000) according to the manufacture's protocol (Amicon Inc., MA, USA) and the supernatant was dried with a freeze-dryer and stored at −70°C. After electrophoresis, the UDN glycoprotein was eluted with an electro-eluter (Mini Whole Gel Eluter; Bio-Rad, CA, USA). The final amount of UDN glycoprotein was 5 mg (0.1%) from the initial UDN sample. This glycoprotein was reproducible (in range of amounts: 0.1 ± 0.01%) and its quality was acceptable. After verification of the pure quality, we determined that UDN glycoprotein had a carbohydrate content of 78.65% and a protein content of 21.35% as described previously (Lee et al 2004; Ko et al 2005).

Animals and cell culture

Male mice (A/J), aged 5 weeks, were purchased from Daehan Lab. (Animal Research Center Co. Ltd, DaeJeon, Korea) and housed according to the animal care guidelines approved by the American Society of Mammalogists Care and Use Committee (1998) at the Experimental Animal Room of Veterinary College of Chonnam National University (CNU). All mice had free access to a commercial diet and water and were kept for at least 1 week before the experiments. After stabilization for experimental conditions, the mice were used for study of Fe/ascorbic acid-induced lipid peroxidation assay and a CCl_4-induced liver injury. On the other hand, BNL CL.2 cells (murine embryonic liver cell line) were incubated in DMEM containing 10% FBS, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37°C under a 5% CO_2 atmosphere. The medium was renewed twice per week. Cells (1×10^6 mL^{-1})

were divided into 96-well flat-bottom plates. Oxidative stress was induced by the glucose/glucose oxidase (G/GO) system (50 mU mL^{-1} glucose oxidase in DMEM containing 0.5% D-glucose for 6 h), which involved the generation of hydrogen peroxide (H_2O_2) in BNL CL.2 cells (Rollet-Labelle et al 1998).

Lipid peroxidation assays

Antioxidant activity of UDN glycoprotein against lipid peroxidation in linoleic acid emulsion system was measured as described previously (Lee et al 2005b). The inhibitory effect of UDN glycoprotein against lipid peroxidation was measured at 500 nm and calculated as follows: inhibitory effect (%) = $[(A_{500\ control} - A_{500\ sample})/A_{500\ control}] \times 100$. The inhibitory effect of UDN glycoprotein against Fe^{2+}/ascorbic acid-induced lipid peroxidation in liver homogenate of normal mouse (A/J) was determined according to the methods of Kimura (1997) and Wong et al (1987). Briefly, one gram of mouse liver tissue was sliced and then homogenized with 10 mL of 150 mM KCl–Tris-HCl buffer (pH 7.2). The reaction mixture consisted of 0.5 mL of liver homogenate, 0.1 mL of Tris-HCl buffer (pH 7.2), 0.05 mL of 0.1 mM ascorbic acid, 0.05 mL of 4 mM $FeCl_2$ and 0.05 mL of various concentrations of UDN glycoprotein (25–200 μg mL^{-1}). After incubation for 1 h at 37°C, 0.9 mL of distilled water and 2 mL of 0.6% TBA were added into the reaction buffer and then shaken vigorously. The mixture was heated for 30 min at 100°C in a water bath. After cooling, 5 mL of *n*-butanol was added and the mixture was then shaken vigorously. The *n*-butanol layer was separated by centrifugation at 1500 *g* for 10 min and the supernatant was collected. The inhibitory effect of UDN glycoprotein against lipid peroxidation was measured at 532 nm and calculated as follows: inhibitory effect (%) = $[(A_{532\ control} - A_{532\ sample})/A_{532\ control}] \times 100$.

Cytotoxicity of UDN glycoprotein

Cellular cytotoxicity induced by the G/GO system in the presence of UDN glycoprotein was measured using neutral red assay (Wadsworth & Koop 1999) according to our previous paper (Lee et al 2005a).

Experimental design

Mice were divided into five groups with six mice per group (control, CCl_4 or UDN glycoprotein treatment groups). Mice were administered orally with 20 or 40 mg kg^{-1} of UDN glycoprotein once a day for 3 days and the control group was administered with 100 μL of phosphate-buffered saline (PBS). The final administration of UDN glycoprotein was performed 3 h before the CCl_4 injection. Here, CCl_4 was dissolved in olive oil and made up to a final concentration of 10% as a stock solution. After that, the mice were intraperitoneally injected with CCl_4 at a dose of 1.0 mL kg^{-1} diluted in olive oil in a total volume 10 mL kg^{-1}. Twenty-four hours after the CCl_4 injection, mouse blood was collected by cardiac puncture and then centrifuged at 1500 *g* for 20 min at 4°C. The supernatant was separated and stored at −70°C to measure formation of thiobarbituric acid reactive substances (TBARS), activity of lactate dehydrogenase (LDH), production of nitric oxide (NO) and amount of lipid. To determine the toxicity of UDN glycoprotein and CCl_4, relative values of body and liver weights were calculated as shown in equations 1 and 2.

$$\text{Relative value of BW} = [\text{BWB (g)} - \text{BWA (g)}]/\text{BWB (g)} \times 100 \quad (1)$$

$$\text{Relative value of LW} = \text{LW (g)}/\text{BW (g)} \times 100 \quad (2)$$

where BWB is the body weight before administration, BWA is the body weight after administration, LW is the liver weight and BW is the body weight.

Thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation was evaluated as the amount of TBARS in serum according to the method of Buege & Aust (1978). One volume of plasma sample was mixed thoroughly with two volumes of stock solution, which consisted of 15% trichloroacetic acid, 0.375% TBA and 0.25 N HCl. The mixture was heated for 30 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 *g* for 10 min and the absorbance was measured at 535 nm. TBARS were quantified by using 1,1,1,3-tetraethoxypropane as a standard curve.

Lactate dehydrogenase (LDH) assay

The LDH level in mouse plasma was measured according to the method of Bergmeyer & Bernt (1974). Plasma samples (35 μL) were mixed with reaction mixture containing 0.6 mM pyruvate in 48 mM potassium phosphate buffer (pH 7.5), and the final volume of the reaction mixture was brought up to 3.15 mL. The reaction was initiated by the addition of 0.18 mM β-NADH and LDH activity was measured as the rate of loss of β-NADH absorption at 340 nm for 2 min. Data were represented as relative percentage, compared with the control value.

Determination of nitric oxide (NO)

Plasma samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 mg mL^{-1}) to a final concentration of 15 mg mL^{-1}. After centrifugation at 10000 *g* for 5 min at room temperature, 100 μL supernatant was collected. Inducible NO production was measured as a form of nitrite (NO_2^-) concentration by the method of Green et al (1982). Supernatants (100 μL) were mixed with 100 μL of Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid) at room temperature for 5 min. Absorbance was measured at 540 nm with a MicroReader (Hyperion, Inc., USA). NO_2^- amounts were quantified by using $NaNO_2$ as a standard curve.

Measurement of antioxidant enzyme activity

The antioxidant enzyme activity in A/J mice liver was measured according to the methods of Beauchamp & Fridovich (1971), Thomson et al (1978) and Paglia & Valentine (1967). One gram of liver tissue was homogenized with 5 mL of a

0.25 M sucrose buffer (pH 7.5) containing 10 mM EDTA. The homogenates were then centrifuged at 600 *g* for 10 min at 4°C to remove nuclear fractions, and the remaining separated supernatant was re-centrifuged at 10000 *g* for 20 min at 4°C to collect the mitochondrial fraction (pellet) for the catalase (CAT) assay. The supernatant was ultra-centrifuged at 100 000 *g* for 1 h at 4 °C to isolate the cytosolic fraction for the superoxide dismutase (SOD) and GPx assays. The amount of protein was measured using the Lowry method (Lowry et al 1951) and the proteins were stored at –70 °C for further experimental use. One unit of antioxidant enzymes was defined as the amount of enzyme required to reduce the NBT (50%) for SOD at 560 nm, to reduce 1 μM of H_2O_2 min^{-1} for CAT at 220 nm, and to oxidize 1 μM of NADPH min^{-1} for GPx at 340 nm, respectively, and the values were calculated as a percentage of the control value.

Preparation of the mitochondrial extracts

A/J mice were administered with CCl_4 in the presence or absence of UDN glycoprotein treatments (20 and 40 mg kg^{-1}). After sacrifice of mice, the liver tissue (0.1 g) was excised and homogenized with 1 mL buffer A (composition (in mM): 20 HEPES-KOH (pH 7.5), 10 KCl, 1 EDTA, 1 EGTA, 0.1 PMSF, 1.5 $MgCl_2$, 1.0 DTT and 250 sucrose) containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany) using a Dounce homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min at 4°C to remove the nuclei. The supernatants were centrifuged at 10 000 *g* for 15 min at 4°C to collect mitochondria-enriched heavy membrane pellets. The supernatants were further centrifuged at 100000 *g* for 1 h at 4°C and the final supernatants are referred to as cytosolic fractions.

Preparation of the nuclear extract

For determining the activity of NF-κB and AP-1, nuclear extract was prepared from frozen liver tissue by modification of the method of Deryckere & Gannon (1994). Briefly, the liver tissue (0.1 g) was homogenized in 1 mL buffer B (0.6% NP-40, 150 mM NaCl, 10 mM HEPES (pH 7.9), 1 mM EDTA, and 0.5 mM PMSF) using a Dounce homogenizer. The homogenate was centrifuged at 200 *g* for 30 s at 4°C. The supernatant was transferred to a clean tube, incubated on ice for 5 min and then centrifuged again at 5000 *g* for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 100 μL buffer B and then centrifuged at 2700 *g* for 3 min at 4°C. The supernatant was discarded and the pellet was resuspended in 100 μL buffer C (420 mM NaCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.5 mM PMSF, 1.2 mM $MgCl_2$, 0.5 mM DTT and 25% glycerol) containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany), incubated on ice for 1 h and then centrifuged at 11 000 *g* for 10 min at 4°C. The amount of protein in the supernatant was evaluated by the Lowry method (Lowry et al 1951) and stored at –70°C before use.

Western blot analysis

The mitochondrial and nuclear extracts for immunoblotting of cytochrome *c*, AP-1 (c-Jun) and NF-κB (p50) were isolated from liver tissue. These sample proteins were separated on a 12% polyacrylamide mini-gel at 100 V for 2 h at room temperature using a Mini-PROTEIN II electrophoresis cell (Bio-Rad). After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The transferred membranes were incubated for 1 h at room temperature in TBS-T solution (10 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.1% (v/v) Tween-20) containing 5% (w/v) non-fat dry milk. The membranes were subsequently incubated for 2 h at room temperature with rabbit polyclonal antibody (1:3000; cytochrome *c*, c-Jun and NF-κB (p50); Santa Cruz Biotechnology, CA, USA) in TBS-T solution containing 5% non-fat dry milk. After three washes with TBS-T, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10 000; Santa Cruz Biotechnology, CA) in TBS-T containing 5% non-fat dry milk. The protein bands were visualized by incubation with nitrobluetetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma Chemical Co.).

Cholesterol assay

The levels of triglyceride and total cholesterol in the CCl_4-induced mouse plasma were measured as described previously (Lee et al 2005b) according to the methods of Warnick et al (1982) and Tercyak (1991).

Statistical analysis

All data from in-vivo (n = 6) and in-vitro (n = 9) experiments were represented as means ± s.d. A one-way analysis of variance and the Duncan test were used for multiple comparisons (SPSS program, ver 10.0).

Results

Inhibitory effect of UDN glycoprotein against lipid peroxidation

We examined the scavenging activity of UDN glycoprotein on lipid peroxidation in both the linoleic acid emulsion system (ammonium thiocyanate assay) and Fe^{2+}/ascorbic acid system (mouse liver homogenates) (Table 1). The results showed that UDN glycoprotein had a dose-dependent scavenging ability on the lipid peroxyl radicals generated by the oxidation of linoleic acid; the values of lipid peroxyl radical scavenging activity were 35 and 62% at 100 and 200 μg mL^{-1} of UDN glycoprotein, respectively. In normal A/J mouse liver homogenates, the values based on the Fe^{2+}/ascorbic acid system were 61 and 84% at 100 and 200 μg mL^{-1} of UDN glycoprotein, respectively.

Cytoprotective activity of UDN glycoprotein in BNL CL.2 cells

When the UDN glycoprotein (25–200 μg mL^{-1}) alone was added to the culture medium of BNL CL.2 cells (murine embryonic liver cell line), the cell viabilities showed that there was no significant difference, compared with the control

Table 1 Antioxidative effect of UDN glycoprotein on lipid peroxidation

Concn (μg mL^{-1})	Inhibitory effect on lipid peroxidation (%)	
	Linoleic acid emulsion system	Fe^{2+}/ascorbic acid system
Control	0	0
UDN glycoprotein		
25	11	18
50	23	31
100	35*	61**
200	62**	84**

The antioxidative activity of UDN glycoprotein is represented as relative absorbance (%) compared with the control as the standard. *$P < 0.05$ and **$P < 0.01$, compared with control. Each experiment was performed three times in triplicate (n = 9) and the values of the absorbance are represented as means ± s.d.

(Figure 1A). In contrast, when the cells were exposed to various concentrations of G/GO for suitable incubation times, the cell viability decreased gradually. In G/GO-dependent cytotoxicity, the viability values were respectively diminished by 54, 70 and 82% at 50, 100 and 200 mU mL^{-1} GO for 6 h, compared with control (Figure 1B). In the time-dependent cytotoxicity, they were diminished by 19, 54, 71 and 83% for 3, 6, 12 and 24 h at 50 mU mL^{-1} of GO, compared with the control (Figure 1C). Therefore, this condition (40 mU mL^{-1} GO, 6 h) was chosen to study the cytoprotective activity of UDN glycoprotein against the G/GO system, because cell viability was less than 50% under this experimental condition. On the other hand, when cells were co-treated with UDN glycoprotein in the G/GO system, the cell viability values significantly increased, compared with the G/GO alone (Figure 1D). For instance, the cell viability values were augmented by 21, 30 and 43% at 50, 100 and 200 μg mL^{-1} of UDN glycoprotein, respectively, compared with the G/GO alone.

Effect of UDN glycoprotein on TBARS formation, LDH activity and NO production in CCl_4-treated mouse serum

As shown in Table 2, there was no significant difference in the levels of TBARS formation, LDH activity or NO production after treatment with UDN glycoprotein (40 mg kg^{-1})

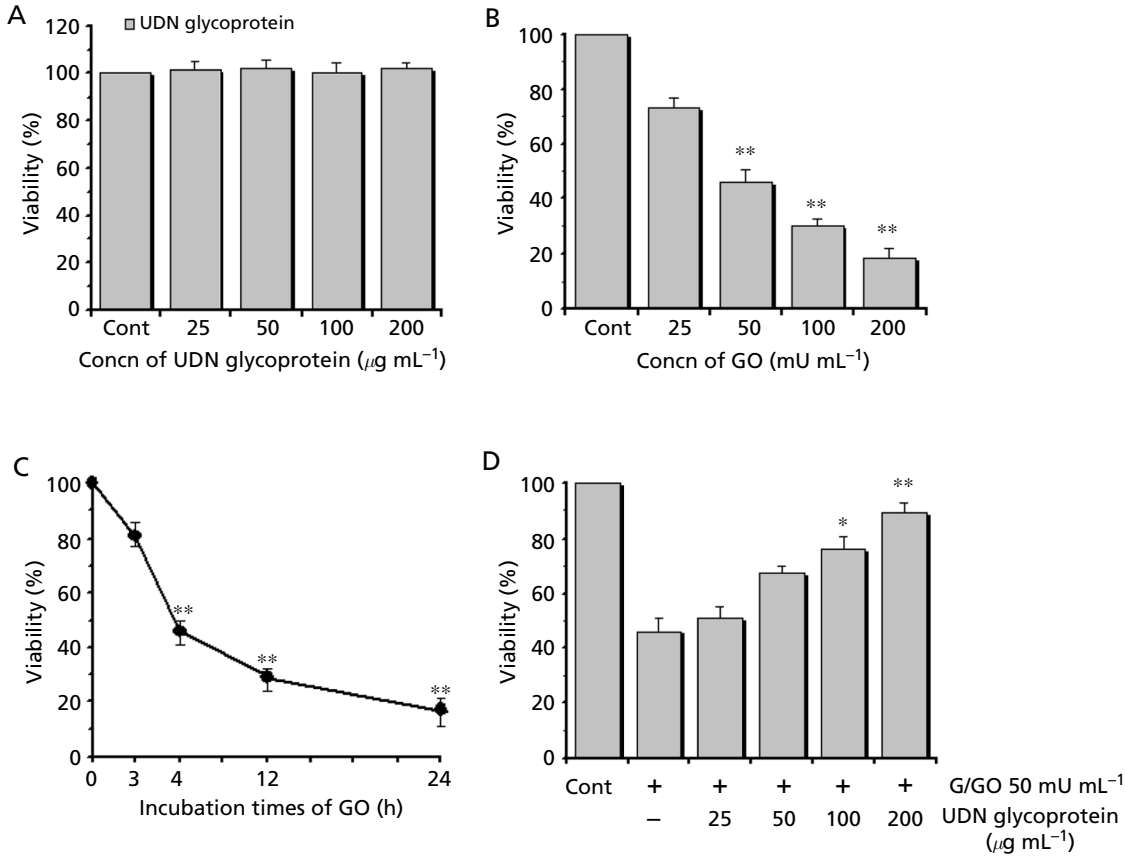

Figure 1 Cytoprotective activity of UDN glycoprotein in BNL CL.2 murine embryonic cells. Viability of the cells was evaluated by neural red assay. The values are expressed as a percentage of the control value. Each bar represents the means ± s.d. from three triplicate experiments (n = 9). UDN glycoprotein was added to the cells alone (A) or with various concentrations of G/GO (B); cells were incubated with GO alone for various times (C) and with G/GO and UDN glycoprotein at various concentrations (D). B. **$P < 0.01$, G/GO treatment compared with control. C. **$P < 0.01$, G/GO (50 mU mL^{-1}) treatment times compared with control. D. *$P < 0.05$, **$P < 0.01$, UDN glycoprotein treatment compared with G/GO (50 mU mL^{-1}) alone.

Table 2 Effect of UDN glycoprotein on TBARS formation, LDH activity and NO production in CCl_4-treated mouse serum

	Control	UDN glycoprotein 40 mg kg^{-1}	CCl_4 (1.0 mL kg^{-1}) +UDN glycoprotein (mg kg^{-1})		
			0	20	40
TBARS (μmol L^{-1})	0.5	0.4	1.1##	0.8*	0.6**
LDH (%)	100	83	176##	131**	110**
NO (μmol)	28.7	26.2	16.1##	21.4*	27.8**

TBARS formation, levels of LDH activity and NO production after treatment with UDN glycoprotein in mice serum were measured. Each bar represents the means ± s.d., n = 6. ##$P < 0.01$, CCl_4 treatment compared with control; *$P < 0.05$ and **$P < 0.01$, treatment with CCl_4 following pretreatment with UDN glycoprotein compared with CCl_4 treatment alone.

alone, compared with control. After treatment with CCl_4 (1.0 mL kg^{-1}) for 24 h, the level of TBARS formation and LDH activity in mouse serum was augmented by 0.6 μmol L^{-1} and 76%, compared with the control. However, when the mice were pretreated with UDN glycoprotein (20 and 40 mg kg^{-1}) for 3 days, the level was diminished by 0.3 and 0.5 μmol L^{-1} in TBARS formation and 45 and 66% in LDH activity, compared with the CCl_4 treatment alone. By contrast, the level of NO production was decreased by 12.6 μmol after treatment with CCl_4 (1.0 mL kg^{-1}) alone, compared with control. Interestingly, when the mice were injected with CCl_4 (1.0 mL kg^{-1}) after pretreatment with UDN glycoprotein (20 and 40 mg kg^{-1}) for 3 days, the production of NO was augmented by 5.3 and 11.7 μmol, compared with the CCl_4 treatment alone.

Effect of UDN glycoprotein on activity of hepatic antioxidant enzymes in CCl_4-treated mouse liver

The results in the antioxidant enzyme assays showed that the activity of SOD, CAT and GPx in liver tissue was augmented by 14, 13 and 21%, respectively, after treatment with UDN glycoprotein (40 mg kg^{-1}) for 3 days, compared with the control (Figure 2). After treatment with CCl_4 (1.0 mL kg^{-1}) for 24 h, however, it was significantly diminished by 27, 30 and 43%, respectively, for SOD, CAT, and GPx, compared with the control. On the other hand, when the mice were treated with CCl_4 after pretreatment with UDN glycoprotein (20 and 40 mg kg^{-1}), the activity of the antioxidant enzymes increased. For instance, the values of the antioxidants enzyme activities were augmented by 5 and 14% for SOD, 9 and 19% for CAT and 28 and 39% for GPx at 20 and 40 mg kg^{-1} UDN glycoprotein. Among all three hepatic antioxidant enzymes, the activity of GPx in this study showed the most susceptible dependence on treatment with both UDN glycoprotein and CCl_4.

Effect of UDN glycoprotein on the activity of cytochrome *c*, NF-κB and AP-1 in CCl_4-treated mouse liver

After treatment with CCl_4 (1.0 mL kg^{-1}) for 24 h, the cytotoxic related proteins (cytochrome *c*, NF-κB (p50) and AP-1 (c-Jun)) were obviously activated (Figure 3). It should be noted that the band intensity of cytochrome c in cytosol was strong at activation. For example, the cytochrome *c* protein was released from the mitochondria into the cytosol after treatment with CCl_4 (1.0 mL kg^{-1}) for 24 h (Figure 3A, lane 2). After pretreatment with the UDN glycoprotein (20 and 40 mg kg^{-1}), however, the band intensities of cytochrome *c* in cytosol were gradually decreased (Figure 3A, lanes 3 and 4). For the NF-κB (p50) and AP-1 (c-Jun) proteins, the intensity of their bands in nuclear extract fractions was increased by the addition of CCl_4 (1.0 mL kg^{-1}) for 24 h (Figure 3B, lane 2). In contrast, when the mice were treated with CCl_4 after pretreatment with UDN glycoprotein (20 and 40 mg kg^{-1}), the intensity of the bands for NF-κB (p50) and AP-1 (c-Jun) was obviously weakened in a dose-dependent manner (Figure 3B, lanes 3 and 4).

Effect of UDN glycoprotein on plasma lipid levels in CCl_4-treated mouse

The level of triglyceride and total cholesterol for the period of oral administration of UDN glycoprotein alone was not changed, compared with control (Table 3). After treatment with CCl_4 (1.0 mL kg^{-1}) for 24 h, however, it was significantly diminished by 22.2 and 49.2 mg 100 mL^{-1} for triglycerides and total cholesterol, respectively, compared with the control. In contrast, when the mice were treated with CCl_4 after pretreatment with UDN glycoprotein (40 mg kg^{-1}), the levels of plasma lipoproteins were augmented by 18.4 and 40.0 mg 100 mL^{-1} for triglycerides and total cholesterol, compared with the CCl_4 treatment alone. During the experiment, there was no significant difference in relative body weights between the control mice and those treated with either CCl_4, UDN glycoprotein or a combination (CCl_4 and UDN glycoprotein). Interestingly, the relative liver weights were augmented by 0.9% after treatment with CCl_4 (1.0 mL kg^{-1}) alone. In contrast, the mice injected with CCl_4 after pretreatment with UDN glycoprotein (40 mg kg^{-1}) had significantly reduced relative liver weights (reduced by 0.8%) compared with those receiving CCl_4 treatment alone.

Discussion

Many glycoproteins isolated from plants have been investigated with a view to their application as host defence potentiators

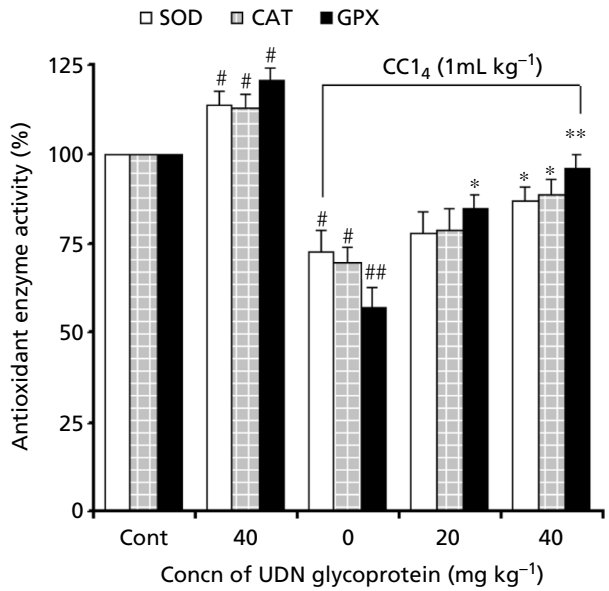

Figure 2 Effect of UDN glycoprotein on activity of hepatic antioxidant enzymes (SOD, CAT and GPx) in CCl_4-treated mouse liver. Results are represented as U (mg protein)$^{-1}$ in each supernatant and the values were calculated as a relative percentage of the control value. Each bar represents the mean ± s.d., n = 6. #$P < 0.05$, activity of antioxidant enzymes after UDN treatment compared with control; ##$P < 0.01$, CCl_4 (1.0 mL kg^{-1}) treatment alone compared with control; *$P < 0.05$, **$P < 0.01$, CCl_4 (1.0 mL kg^{-1}) treatment alone compared with CCl_4 (1.0 mL kg^{-1}) treatment following pretreatment with UDN glycoprotein.

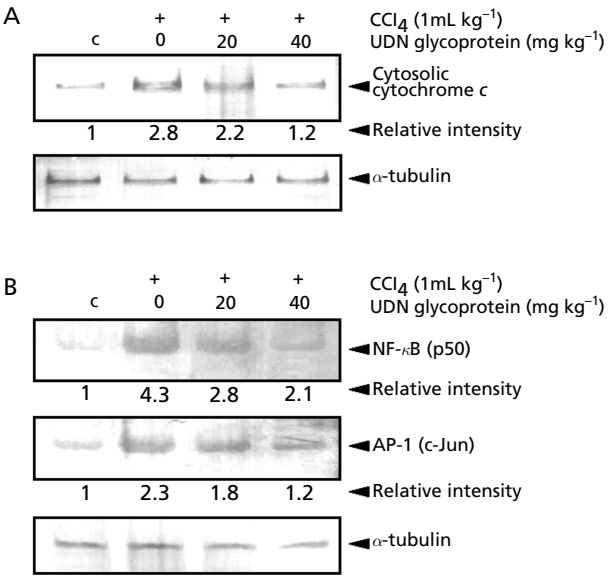

Figure 3 Effect of UDN glycoprotein on activity of cytochrome *c*, NF-κB and AP-1 in CCl_4-treated mouse liver. Detection of cytochrome *c*, NF-κB (p50) and AP-1 (c-Jun) proteins was performed by western blotting using anti-cytochrome *c*, -NF-κB (p50) and -AP-1 (c-Jun) polyclonal antibodies. The relative intensities of cytochrome *c*, NF-κB (p50) and AP-1 (c-Jun) bands were quantified using Scion Imaging Software (Scion Image Beta 4.02, MD), compared with control. C, Control; lane 2, 1.0 mL kg^{-1} CCl_4 alone; lanes 3, and 4, 20 mg kg^{-1} and 40 mg kg^{-1} of UDN glycoprotein in the presence of CCl_4 treatment. α-Tubulin was used as an internal control.

or biological response modifiers for inhibiting abnormal metabolic process in living organisms (Ooi & Liu 2000). In this study, we investigated whether UDN glycoprotein (116 kDa) can protect against hepatic injury generated by CCl_4 in mouse. The results from lipid peroxidation and cytotoxicity assays indicated that UDN glycoprotein has the potential to reduce lipid peroxyl radical (LOO•)-induced linoleic hydroperoxide formation and Fe^{2+}/ascorbic acid-induced lipid peroxidation in mouse liver microsomes or mitochondria, providing protection from hydrogen peroxide-induced hepatocyte damage.

Such results mean that UDN glycoprotein exerts a therapeutic effect on toxic metabolite-induced liver injury, possibly through its antioxidant function because the liver is an important target for the toxicity of drugs, xenobiotics and oxidative stress as a major organ of the antioxidant defence system (Jaeschke et al 2002).

To confirm the antioxidative potential of UDN glycoprotein in liver, we further investigated the glycoprotein's ability to effectively protect against CCl_4-induced acute liver injury. Our results showed that UDN glycoprotein has hepatoprotective effects, which are deduced from it blocking TBARS formation and LDH activity in CCl_4-treated mice serum. The mechanism underlying the CCl_4-stimulated TBARS formation and LDH activity is thought to result from its reductive dehalogenation by the NADPH-cytochrome P-450 system into the trichloromethyl free radical (•CCl_3), because the radical can remove hydrogen atoms from unsaturated fatty acid and create carbon-centered lipid radicals (Recknagel & Glende 1973; Connor et al 1990; Williams & Burk 1990; Simeonova et al 2001). After that, the lipid peroxyl radicals (LOO•), in turn, remove hydrogen atoms from another unsaturated fatty acid of lipid in the cell membrane and then the propagated lipid peroxidation leads to hepatic mitochondrial dysfunction, fibrogenesis, cirrhosis and steatosis (Recknagel et al 1989; Jaeschke et al 2002; Cesaratto et al 2004). In addition, the CCl_4-induced lipid peroxidation is also closely related to serum LDH activity, since the release of LDH, including aminotransferases enzymes (AST and ALT), into the serum is a sensitive marker of liver and other tissues damage (Naziroglu et al 1999). Based on these theories, we figured out that UDN glycoprotein reduced the levels of the TBARS formation and LDH activity by preventing the lipid peroxidation process in CCl_4-induced liver injury. Interestingly, our study also showed that the NO production was significantly reduced in CCl_4-induced mouse serum, whereas it was increased after injection of CCl_4 following pretreatment with UDN glycoprotein (40 mg kg^{-1}). Recently, it was reported that a possible reason for the lower NO level after CCl_4 treatment was that NO could be consumed to terminate the lipid peroxidation by scavenging •CCl_3, CCl_3OO• and LOO•, and could deplete the co-substrate NADPH by interacting with CCl_4 mainly in hepatocytes (Zhu & Fung 2000). Thus, the up-regulation of NO production by UDN glycoprotein may be a useful therapeutic strategy to protect against CCl_4-induced liver damage by blocking the lipid peroxidation process.

It has been reported that several liver diseases, such as fibrogenesis, cirrhosis and steatosis, can be caused by an imbalance in redox status (Cesaratto et al 2004; Pessayre et al

Table 3 Effect of UDN glycoprotein on triglyceride (TG) and total cholesterol (TC) levels in CCl_4-treated mouse serum

	Control	UDN glycoprotein 40 mg kg^{-1}	CCl_4 (1.0 mL kg^{-1}) +UDN glycoprotein (mg kg^{-1})		
			0	20	40
TG (mg 100 mL^{-1})	68.7 ± 7.6	66.0 ± 4.2	46.5 ± 6.4#	51.6 ± 1.7	64.9 ± 6.8*
TC (mg 100mL^{-1})	170.6 ± 6.1	165.1 ± 8.7	121.4 ± 3.3#	133.1 ± 6.5	161.4 ± 3.3*
Relative weight (%)					
Body	100.0 ± 0.2	99.8 ± 0.8	97.9 ± 0.7	99.1 ± 0.8	98.9 ± 0.7
Liver	5.6 ± 0.5	5.7 ± 0.9	6.5 ± 0.6#	6.1 ± 0.3	5.7 ± 0.6*

Results are expressed as means ± s.d., n = 6. #$P < 0.05$, CCl_4 treatment compared with control; *$P < 0.05$, CCl_4 treatment following pretreatment with UDN glycoprotein compared with CCl_4 treatment alone.

2004). Therefore, many scientists have tried to find bioactive compounds having an ability to protect liver against cellular oxidative damage, as well as an ability to enhance the activity of antioxidant enzymes. From this point of view, we speculate that UDN glycoprotein enhances the activity of SOD, CAT and GPx in CCl_4-induced acute liver injury in mice, because UDN glycoprotein has an antioxidative activity, especially to inhibit the lipid peroxidation process. As shown in Figure 2, the activity of SOD, CAT, and GPx increased after treatment with UDN glycoprotein (40 mg kg^{-1}) alone and was reduced in the liver of mice treated with CCl_4 alone, compared with control. However, their activity in the CCl_4-treated mouse liver gradually increased upon concentration of the pretreatment with UDN glycoprotein, compared with CCl_4 treatment alone. One of the possible mechanisms for the reduction in SOD, CAT and GPx activity is that the treatment of mouse liver with CCl_4 concurrently induces both processes of acute injury and regeneration; injury events are dominantly expressed in the early stage but the regeneration process is latent (Taniguchi et al 2004). Therefore, the SOD, CAT and GPx were degraded or saturated to block the CCl_4-induced massive free radical production at an early stage. By contrast, a possible reason why SOD, CAT and GPx activity after UDN glycoprotein treatment was augmented in both the absence of CCl_4 and in the presence of CCl_4 is that UDN glycoprotein possesses not only the capacity to scavenge the small number of ROS that are inevitably generated due to the incomplete reduction of O_2 in electron transfer reactions in normal aerobic metabolisms, but also the capacity to block the CCl_4-induced massive ROS production. The excessive activity of SOD, CAT and GPx and antioxidative activity of UDN glycoprotein can together contribute to the synergistic/additive scavenging activity for radicals, suggesting that the antioxidative potential of UDN glycoprotein seems to stimulate activity of antioxidant enzymes, compared with control. Therefore we speculate that UDN glycoprotein particularly plays on the early stage in CCl_4-induced liver injury. Our results also indicated that the activity of GPx was particularly susceptible to treatment with both UDN glycoprotein and CCl_4. The possible reason for the susceptibility of GPx activity is that GPx confers greater protection against oxidative stress than SOD, CAT, or the combination of SOD and CAT, to convert hydrogen peroxide into harmless water in the stages of initiation and propagation of lipid peroxidation (Toussaint et al 1993; Lomaestro & Malone 1995). From these concepts, our results demonstrated that UDN glycoprotein has hepatoprotective activity by enhancing the antioxidant enzyme activity in CCl_4-induced acute liver injury.

The redox imbalance between oxidant and antioxidant molecules is closely related to cytochrome *c* release from mitochondria in CCl_4-induced acute liver injury, because both CCl_4-induced ROS and lipid peroxidation end-products can block the flow of electrons in the respiratory chain (Pessayre et al 2004). As expected, cytochrome *c* protein was released from the mitochondria into the cytosol after treatment with CCl_4. In contrast, when mice were pretreated with UDN glycoprotein, the release of mitochondrial cytochrome *c* was markedly blocked, suggesting that UDN glycoprotein has the potential to prevent oxidant-induced mitochondrial damage. Regarding transcriptional factors, our results also showed that UDN glycoprotein (40 mg kg^{-1}) decreases the activity of NF-κB (p50) and AP-1(c-Jun) in nuclear extract, compared with their activity in the CCl_4-treated group (Figure 3). From the results, we deduced that the CCl_4 activates redox-sensitive transcription factors (NF-κB and AP-1) before the process of liver regeneration, and then the activated NF-κB and AP-1 can stimulate the expression of the TNF-α gene as an inflammatory response in mainly Kupffer cells (macrophage cells). Following the release of TNF-α from Kupffer cells, the interaction between TNF-α and the TNF-R1 death receptor in hepatocytes stimulates the release of mitochondrial cytochrome *c*, and finally results in apoptosis to remove the CCl_4-damaged hepatocytes (Camandola et al 1997; Menegazzi et al 1997; Pessayre et al 2004; Taniguchi et al 2004). Although we did not determine the interaction between TNF-α and the TNF-R1 death receptor, the UDN glycoprotein firstly results in blockade of the inflammatory process by deactivation of NF-κB and AP-1 in Kupffer cells, and then inhibits the mitochondrial cytochrome *c* efflux in the early stage of CCl_4 damage to hepatocytes.

We further investigated the antioxidant potential of UDN glycoprotein to regulate the levels of plasma lipid in CCl_4-induced mouse. It has been demonstrated that a single administration of CCl_4 results in the accumulation of triglyceride and total cholesterol in liver by inhibiting the secretion of lipoproteins into the blood circulation; the levels of triglyceride

and total cholesterol were subsequently reduced in serum in mice (Seakins & Robinson 1963; Pencil et al 1984), and such impairment of lipid transport in liver significantly increases the relative liver weight, compared with body weight, in the mouse (Seakins & Robinson 1963; Pencil et al 1984). Our results showed that the levels of triglycerides and total cholesterol were significantly reduced in the serum of CCl_4-induced mice, while the lipoproteins levels were increased after injection of CCl_4 following pretreatment with UDN glycoprotein, but not upon concentration of UDN glycoprotein, compared with CCl_4 treatment alone. Also, the results showed that pretreatment with UDN glycoprotein gradually brings about normal liver weight, although it was heavier than control after treatment with CCl_4 alone. A possible reason for the normalization of plasma total cholesterol and triglycerides by UDN glycoprotein is that it can maintain the rate of mitochondrial fatty acid β-oxidation in delivery of electrons to the respiratory chain through inhibition of lipid peroxidation process and mitochondrial cytochrome *c* release in CCl_4-induced mouse liver and eventually leads to normal liver function in lipid metabolism (Pessayre et al 2004). Taken together, UDN glycoprotein firstly acts to scavenge induced radicals generated by CCl_4 and then it consequently increases the levels of total cholesterol and triglycerides to become normalized.

Conclusions

The results of this study indicate that UDN glycoprotein has hepatoprotective potential through inhibition of the lipid peroxidation process, activity of transcription factors (NF-κB, AP-1) and mitochondrial cytochrome *c* release, stimulation of the activity of antioxidant enzymes and NO production and normalization of the plasma lipid levels in CCl_4-induced mouse liver injury. Therefore, we speculate that UDN glycoprotein, as a natural antioxidant, is an important modulator in the biodefensive system. However, further research must be carried out to elucidate the mechanisms of hepatoprotective-related gene expression by UDN glycoprotein at the molecular biological level.

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