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Phytoglycoprotein (24 kDa) inhibits expression of PCNA via PKCα and MAPKs in oxygen radical-stimulated Chang liver cells

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Abstract

The purpose of this study was to investigate the inhibitory effect of 24-kDa glycoprotein isolated from *Zanthoxylum piperitum* DC fruit (ZPDC glycoprotein) on glucose/glucose oxidase (G/GO)- or hypoxanthine/xanthine oxidase (HX/XO)-induced cell proliferation in Chang liver cells. We found that ZPDC glycoprotein has significant scavenging effect on the production of intracellular H_2O_2 without cytotoxicity in G/GO- or HX/XO-treated in Chang liver cells. In the G/GO or HX/XO-stimulated protein kinases activity, ZPDC glycoprotein inhibited translocation of protein kinase C alpha (PKCα) to membrane and phosphorylation of extracellular signal-regulated kinase, p38 MAP kinase and c-Jun N-terminal kinase, respectively. In the G/GO or HX/XO-stimulated transcriptional activity, ZPDC glycoprotein also blocked the DNA binding activities of nuclear factor-kappa B and activator protein-1 and attenuated the activities of p50, p65, c-Jun and c-Fos, respectively. Finally, in the G/GO or HX/XO-stimulated cell proliferation, the activity of proliferating cell nuclear antigen was significantly blocked by treatment with ZPDC glycoprotein as well as protein kinase C inhibitor and mitogen-activated protein kinase inhibitors. On the basis of these results, we speculate that this glycoprotein is one of the natural antioxidants and of the modulators on abnormal activation of cell proliferation-related molecules in Chang liver cells.

Keywords: ZPDC glycoprotein; Hydrogen peroxide; Mitogen-activated protein kinase; Anti-cell proliferation; Chang liver cells

1. Introduction

Reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet-}$) and H_2O_2, have multifactorial effects on regulation of cell growth and proliferation, and eventually contribute to cancer development in liver cells [1–3]. Especially, H_2O_2 is a relatively stable uncharged molecule that plays important roles in regulating redox-related signaling molecules such as protein kinases and transcription factors [4].

Protein kinase C (PKC) is one of sensitive targets for ROS that play key roles in cell proliferation and tumor promotion [5]. Among three groups of PKC isoforms, PKCα (classical group) is activated by a variety of stimuli originating from physiological stresses and translocated from cytosol to specialized cellular compartments [6]. Subsequently, this activation of PKCα also has been known to modulate the mitogen-activated protein kinase (MAPK) pathways, such as extracellular signal-regulated kinase (ERK), p38 MAP kinase and c-Jun N-terminal kinase (JNK) [6].

ROS is also a well-known stimulator of two redox-sensitive transcription factors, nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) [7–9]. NF-κB is a ubiquitously expressed transcriptional regulator that controls the expression of genes involved in immune, inflammatory and tumorigenic responses [8]. The predominant inactive form of NF-κB exists as a trimer that consists of p65, p50 and IκBα (Inhibitor kappa B alpha) subunits in cytoplasm. In response to signals elicited by ROS; however, the IκBα subunits dissociates and the activated NF-κB (p65/p50 dimer) migrates to the nucleus for the DNA-binding activity [8]. Another transcription factor, AP-1 is a redox-sensitive oncogene protein, which controls expression of cell growth mediators, comprises as a heterodimer of Jun and Fos proteins, which are protein products of c-Jun and c-Fos protooncogenes [9].

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Since liver plays pivotal roles in a large number of metabolic and immune processes, the physiological and pathophysiological functions of ROS have attracted numerous investigations [3]. Although there is overwhelming evidence that ROS play a significant role in a number of liver diseases, the detailed molecular mechanisms of ROS involvement among redox-related protein kinases and transcription factors are still unclear. In these contexts, many scientists have tried to obtain bioactive substances having the antioxidative activity against abnormal activation of the ROS-related protein kinases and transcription factors under oxidative stress responses [10,11].

Zanthoxylum piperitum DC (ZPDC) has traditionally been used as a spice to produce a fresh flavor as an ingredient of some spice mixes or to suppress unpleasant fishy and meaty odor [12]. It has been also used for a long time to heal vomiting, diarrhea and abdominal pain in Korea [13]. Recently, it has reported that crude extract isolated from ZPDC leaves or seeds has pharmacological activities such as antioxidative and hepatoprotective effects [14,15]. In these concepts, we assumed that ZPDC might contain functional substance with multiple biological activities. In a preliminary study, we isolated 24-kDa glycoprotein from ZPDC fruit, which consists of carbohydrate content (18%) and protein content (82%), and examined the biological functions. This active substance, designated as ZPDC glycoprotein, has antioxidative effect on ROS production in cell-free systems and hepatoprotective effect on CCl_4-induced acute liver injury in mice, indicating that ZPDC glycoprotein has antioxidative potential in liver [16]. Therefore, the purpose of the present study is to clarify the cellular mechanism of oxygen radical scavenging by ZPDC glycoprotein in Chang liver cells. Nobody has studied the antioxidative effect of ZPDC glycoprotein on ROS-induced cell proliferation via ROS-related signaling molecules such as PKCα, MAPKs, NF-κB and AP-1 in the liver cell line.

In present study, we investigated the antioxidative effect of ZPDC glycoprotein on ROS production in glucose/glucose oxidase (G/GO)- or hypoxanthine/xanthine oxidase (HX/XO)-induced Chang liver cells. Moreover, we further studied whether ZPDC glycoprotein has inhibitory effects on cell proliferation via redox-related protein kinases and transcription factors.

2. Materials and methods

2.1. Chemicals

All the plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ). 2′,7′-Dichlorofluoresin diacetate (D6883), HX (H9377), GO (G8135), penicillin G (H0474), pronase E (P5147), $NaIO_4$, S1878), streptomycin (H0447), sulfuric acid (S1526), trypsin (T4549) and XO (X1875) were obtained from Sigma (St, Louis, MO, 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.

2.2. Preparation of ZPDC glycoprotein

Fruits of ZPDC were obtained in October 2006 from Kwang-ju in the Chonnam province of South Korea, and glycoprotein was isolated and purified as described previously [16,17]. Briefly, the ZPDC was cut into small pieces and soaked in 99% ethanol at 4°C for 3 months in a dark basement. The extract was filtered through Whatman filter paper (No. 2) and concentrated using a rotary evaporator (B465; Bunchi, Switzerland). The resulting 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 column (size 4×28 cm) containing silica gel (particle size 28–200 mesh, pore size 22Å). 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 g (40% of the initial amounts of dried-crude ZPDC extract), 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 dialyzed with a dialysis membrane (Spectra/por, MWCO 6,000–8,000, Rancho Dominguez, CA, USA) against 20 mM Tris-Cl (pH 7.4) at 4°C overnight. After dialysis, the solution was concentrated using microcon concentrators according to the manufacturer's protocol (Amicon, Bedford, MA, USA) and the supernatant was dried with a freeze-dryer and stored at −70°C. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with the sample of protein (100 mg/ml) containing 0.1% SDS using a 18% polyacrylamide mini-gel and a Mini-PROTEIN II electrophoresis cell (Bio-Rad) at 110 V, 30 mA for 2.5 h. The gels were stained with Schiff's reagent for the glycoprotein [18]. After staining, the ZPDC glycoprotein (24 kDa) was eluted with an electro-eluter (Mini Whole Gel Eluter; Bio-Rad, Hercules, CA, USA), and the ZPDC glycoprotein solution was further dried with a freeze-dryer and stored at −70°C. The final amount of ZPDC glycoprotein was 3.5 mg (0.07%) from the initial of dried crude ZPDC extract. After verification of high purity (approximately more than 95%) of glycoprotein, the carbohydrate and protein content of the ZPDC glycoprotein was determined as described previously [19]. ZPDC glycoprotein consists of carbohydrate content (18%) and protein content (82%), respectively.

2.3. Cell culture

Chang liver cells (human hepatocyte derivate cell line) were kindly donated by Prof. Lee KY (Department of Biochemistry, College of Medical School, Chonnam National University of Korea). The cells were incubated in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2 atmosphere. The medium was renewed two times per week. The number

of cells (1×10^6 cells/ml) was divided into 35 mm culture dishes or 96-well flat bottom plates. The final volumes were 2 ml on the 35-mm culture dishes and were 100 μl on the 96-well flat bottom plates. Oxidative signal transduction in Chang liver cells was induced either by the G/GO system (40 mU/ml GO in DMEM containing 10 mM G for 4 h), which stimulates the generation of H_2O_2 or by the HX/XO system (10 mU/ml XO in DMEM containing 1 mM HX for 4 h), which stimulates the production of $O_2^{\bullet-}$ or H_2O_2 [20].

2.4. Measurement of intracellular H_2O_2

The production of H_2O_2 was measured by using nonfluorescent 2′,7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is deacetylated by intracellular esterases to the nonfluorescent DCFH, which is oxidized to the fluorescent compound DCF (2′,7′-dichlorofluorescein) by intracellular H_2O_2 [21]. The cells were pre-incubated with DCFH-DA (10 μM) for 30 min at 37°C, and then the cells washed twice with phosphate-buffered saline (PBS) to remove the excess DCFH-DA. After that, the cells were cotreated with ZPDC glycoprotein (50-200 μg/ml) in presence of either G/GO or HX/XO. Finally, the fluorescence intensity was measured either at excitation wavelength of 485 nm and emission wavelength of 530 nm for DCF using fluorescent microplate reader (Dual Scanning SPECTRAmax, Molecular Devices, Sunnyvale, CA, USA). The values were calculated as a relative of the DCF fluorescence intensities, compared to the control.

2.5. Treatments of pronase E and $NaIO_4$

For verification of anti-activity of ZPDC glycoprotein, ZPDC glycoprotein was treated with either pronase E or $NaIO_4$ to induce the hydrolysis of the protein or the oxidation of the carbohydrate portions of the whole ZPDC glycoprotein, as described previously [19]. After that, the ROS scavenging effect of the ZPDC glycoprotein were tested using DCFH-DA in either G/GO or HX/XO-stimulated Chang liver cells.

2.6. Cytotoxicity

The cellular cytotoxicity induced by the treatment of ZPDC glycoprotein, G/GO system or HX/XO system was confirmed according to the method of Mosmann [22] as described previously [19].

2.7. Preparations of membrane, cytosolic, nucleic and whole protein extracts

For the detection of the translocation of PKCα, membrane and cytosolic extracts were prepared according to the method of Patton et al. [23]. Briefly, the Chang liver cells were rinsed twice with PBS after removing the medium and scraped in 300 μl of buffer A [20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 2 mM EGTA] containing a protease inhibitor cocktail (Boehringer, Mannheim). The cells were briefly sonicated and centrifuged at 100,000*g* for 1 h to sediment all membranes and the insoluble cytoskeletal components. The supernatant was designated as a cytosolic fraction. The membrane proteins in the pellet were extracted with buffer B [20 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA] containing a protease inhibitor cocktail (Boehringer, Mannheim) on ice for 30 min and centrifuged at 100,000*g* for 15 min at 4°C. The supernatant was saved as a detergent-soluble membrane fraction. On the other hand, either nucleic protein extract for immunoblotting of NF-κB (p50 and p65), AP-1 (c-Jun and c-Fos) and proliferating cell nuclear antigen (PCNA) or whole cellular protein extract for immunoblotting of p44/42 (ERK1/2), stress-activated protein kinase (SAPK)/JNK and p38 MAP kinase, was isolated from Chang liver cells as described previously [17,19]. The amount of protein was measured by the method of Lowry et al. [24] and the cellular proteins were stored at −70°C prior to use.

2.8. Western blot analysis

Cellular proteins (0.3 mg/ml) were separated in a 10% 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) nonfat dry milk. The membranes were subsequently incubated for 2 h at room temperature with rabbit polyclonal antibodies [PCNA, p50, p65, c-Jun and c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ERK1/2, SAPK/JNK, p38 MAP kinase and phospho-ERK1/2, -SAPK/JNK, -p38 MAP kinase (Cell Signaling Technology, Beverly, MA, USA)] in TBS-T solution containing 5% nonfat dry milk. After three washes with TBS-T, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat antirabbit IgG (1:10,000; Cell Signaling Technology) in TBS-T containing 5% nonfat dry milk. The protein bands were visualized by incubation with enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.9. Electrophoretic mobility shift assay

To make the double stranded oligonucleotide, each strand of oligonucleotides for NF-κB (5′-AGTTGAGGGGACTTTCCC AGGC-3′) and AP-1 (5′-TTCCGGCTGACTCATCAAGCG-3′) were annealed in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) by heating at 94°C for 10 min. For probing of NF-κB and AP-1 double stranded oligonucleotides, they were labeled with [γ-^{32}P]dATP (0.25 mCi, Amersham Pharmacia Biotech, Buckinghamshire, UK) by T4 polynucleotide kinase and purified on a QIAquick Nucleotide Removal Kit according to the manufacturer's protocol (LRS Laboratory, QIAGEN Distributor, Seoul, Korea). The DNA–protein binding reaction was performed by incubation of the NF-κB and AP-1 probes, and 10 μg of

nuclear protein extracts and 0.5 μg/ml poly dI/dC (Sigma Chemical) in a binding buffer [0.2 M dithiothreitol, 20 mg/ml bovine serum albumin, buffer C (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40), buffer D (20% ficoll 400, 100 mM HEPES, 300 mM KCl)] at room temperature for 1–2 h. The DNA–protein complexes were resolved by applying 6% nondenaturing polyacrylamide gel in 0.5×tris-borate-EDTA (45 mM Tris-borate, 1 mM EDTA). Electrophoresis was carried out at 200 V for 3 h in a cold room. Gels were then dried on 3M blotting paper (Whatman) and exposed to X-ray film at −70°C overnight.

2.10. Statistical analysis

All data from experiments were obtained from triplicates and represented as means±S.D. The significant differences between treatments were determined by the Student's *t* test and the Duncan test in one-way analysis of variance (SPSS program, v. 12.0).

3. Results

3.1. Inhibitory effect of ZPDC glycoprotein on the production of intracellular H_2O_2 in G/GO- or HX/XO-stimulated Chang liver cells

When the cells were treated with 10 mM G/40 mU/ml GO or 1 mM HX/10 mU/ml XO, levels of H_2O_2 production significantly increased by 0.6, 0.8 and 0.9 or 0.4, 0.7 and 0.8 for 2, 4 and 6 h, respectively, compared to the control (Fig. 1A). The efficient production of H_2O_2 in the G/GO or HX/XO system was observed at 4 h. By contrast, when the cells were cotreated with ZPDC glycoprotein in presence of the G/GO or HX/XO system, the levels of H_2O_2 production significantly diminished by 0.6 and 0.9 in G/GO system or by 0.5 and 0.8 in HX/XO system for 4 h at 100 and 200 μg/ml of ZPDC glycoprotein, respectively, compared to the G/GO or HX/XO treatment alone, not the case of treatment group of ZPDC glycoprotein (50 μg/ml) (Fig. 1B). However, when ZPDC glycoprotein was treated with either pronase E to induce the hydrolysis of the protein or $NaIO_4$ to induce the oxidation of the carbohydrate, the H_2O_2 scavenging activity of ZPDC glycoprotein were markedly reduced (Fig. 1C). For instance, there were no significant differences in the production of H_2O_2 between G/GO or HX/XO treatment alone and G/GO or HX/XO treatment in presence of ZPDC glycoprotein, which was treated with either Pronase E or $NaIO_4$. No cellular cytotoxic effect due to ZPDC glycoprotein, G/GO and HX/XO alone was observed for 4 h at the abovementioned concentrations (Fig. 1D).

3.2. Inhibitory effect of ZPDC glycoprotein on the translocation of PKCα in G/GO- or HX/XO-stimulated Chang liver cells

Results in western blot analysis indicated that ZPDC glycoprotein was shown to have inhibitory effect on the translocation of the PKCα protein from the cytosol to the membrane in G/GO- or HX/XO-stimulated Chang liver cells (Fig. 2). After exposure to G/GO or HX/XO systems for 4 h, the intensity of PKCα band decreased at the cytosolic extract, whereas it increased at membrane extract, indicating that G/GO or HX/XO systems cause the translocation of PKCα to the membrane. However, ZPDC glycoprotein (200 μg/ml) treatment showed the remarkable blocking effect of this PKCα translocation in G/GO- or HX/XO-stimulated Chang liver cells.

3.3. Inhibitory effect of ZPDC glycoprotein on the phosphorylation of MAPKs in G/GO- or HX/XO-stimulated Chang liver cells

We further studied whether or not ZPDC glycoprotein has antioxidative property to inhibit the activation of ERK 1/2, p38 MAP kinase and JNK in G/GO- or HX/XO-stimulated Chang liver cells. The results revealed that the phosphorylation of all MAPKs was significantly increased by treatment with G/GO or HX/XO, whereas the total protein expressions of ERK 1/2, p38 MAPK and JNK were not changed in Chang liver cells, compared to the control (Fig 3, Lanes 2 and 6). However, when the cells were cotreated with ZPDC glycoprotein (100 and 200 μg/ml) in presence of the G/GO or HX/XO system, the phosphorylation of all MAPKs was markedly inhibited without change of total protein expressions in dose-dependent manner.

3.4. Inhibitory effect of ZPDC glycoprotein on the activation of NF-κb and AP-1 in G/GO- or HX/XO-stimulated Chang liver cells

When the cells were exposure to G/GO or HX/XO systems, the DNA-binding activity NF-κB complex and the level of its subunits (p50 and p65) in nuclear protein extract were markedly up-regulated, respectively, compared to the control (Fig. 4A, Lanes 3 and 6; Fig. 4B, Lanes 2 and 5). However, these transcriptional activities of NF-κB were down-regulated after treatment with ZPDC glycoprotein, respectively, compared to the G/GO or HX/XO treatment alone (Fig. 4A, Lanes 4 and 7; Fig. 4B, Lanes 3 and 6). Similarly, the DNA-binding activity of AP-1 complex was higher in the cells treated with G/GO or HX/XO alone (Fig. 4C, Lanes 3 and 6) than in the control (Fig. 4C, Lanes 2 and 5). In addition, the band intensities of AP-1 subunits (c-Jun and c-Fos) were much stronger in the cells, treated with G/GO or HX/XO alone (Fig. 4D, Lanes 2 and 5) than in the control (Fig. 4D, Lanes 1 and 4). By contrast, ZPDC glycoprotein (200 μg/ml) treatment exhibited lower activities of AP-1 complex and its subunits (Fig. 4C, Lanes 4 and 7; Fig. 4D, Lanes 3 and 6) than in the cells treated with G/GO or HX/XO alone, indicating that ZPDC glycoprotein suppresses the transcriptional activity of NF-κB and AP-1 in G/GO- or HX/XO-stimulated Chang liver cells.

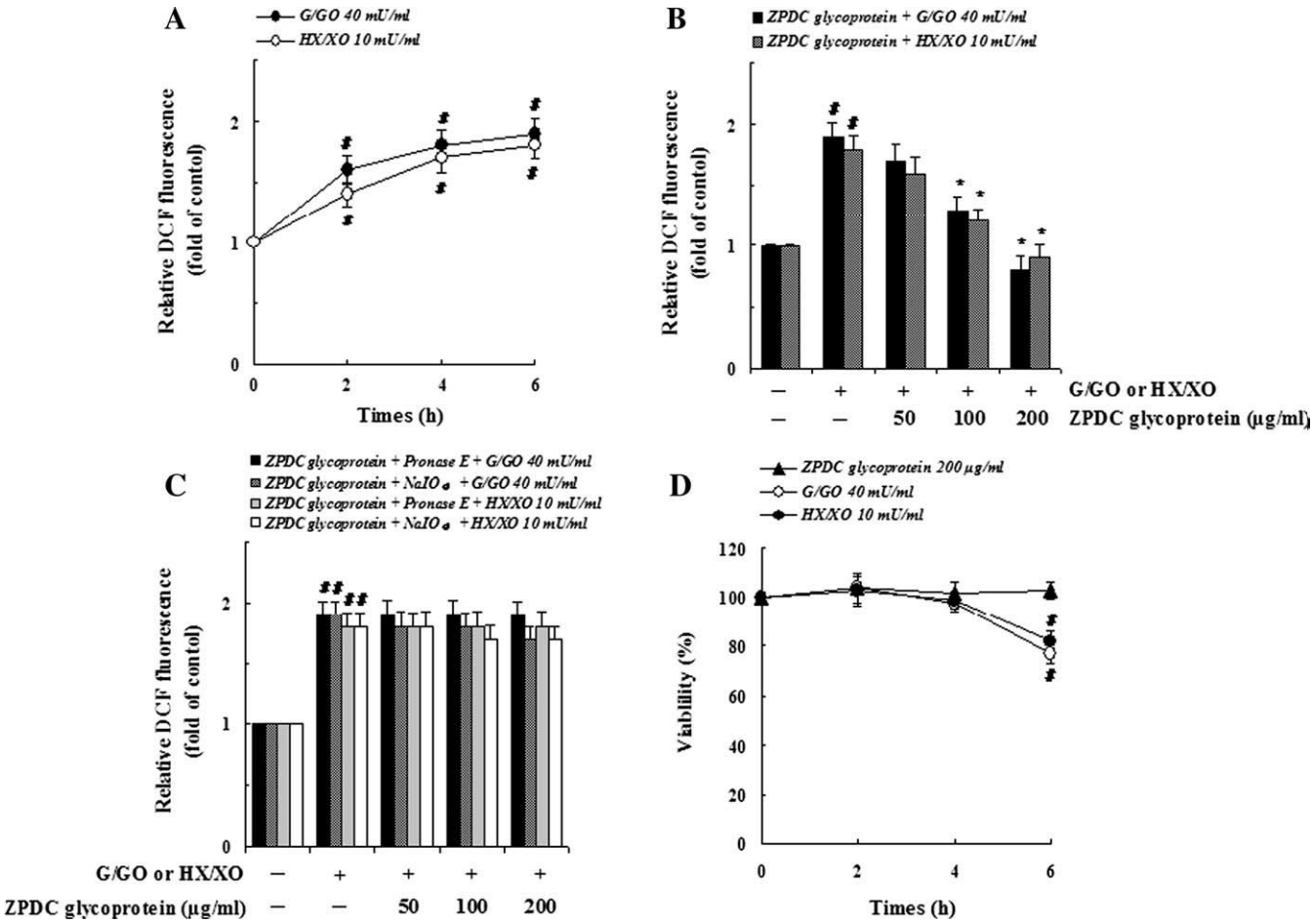

Fig. 1. Inhibitory effect of ZPDC glycoprotein on the production of intracellular H_2O_2 in G/GO- or HX/XO-stimulated Chang liver cells. Chang liver cells were treated with either G/GO (40 mU/ml) alone or HX/XO (10 mU/ml) alone for various times (0–6 h) (A). The cells were also exposed to the G/GO or HX/XO system for 4 h in the absence or presence of various concentrations of ZPDC glycoprotein (50–200 μg/ml) (B) or ZPDC glycoprotein, which was treated with Pronase E or $NaIO_4$ (C). The production of intracellular H_2O_2 was measured at an excitation wave length (485 nm) and an emission wave length (530 nm) using a fluorescent microplate reader. The values were calculated as relative intensities of DCF fluorescence, compared to the control. Chang liver cells were treated with G/GO (40 mU/ml), HX/XO (10 mU/ml) or ZPDC glycoprotein (200 μg/ml) alone for 4 h, respectively (D). Viabilities of the cells were evaluated by MTT assay. The values are expressed as a percentage of the control value. Each bar is the means±S.D. of triplicate experiments. #P<.01, significant difference between control and G/GO or HX/XO treatment alone; *P<.05 and **P<.01, significant differences between G/GO or HX/XO treatment alone and G/GO or HX/XO treatment in presence of ZPDC glycoprotein treatments, respectively.

3.5. Inhibitory effect of ZPDC glycoprotein on the activation of PCNA in G/GO- or HX/XO-stimulated Chang liver cells

Finally, we investigated whether or not ZPDC glycoprotein modulates the abnormal cell proliferation in G/GO- or HX/XO-stimulated Chang liver cells. As shown in Fig. 5, the activity of PCNA protein (as a cell proliferation maker) in nuclear protein extracts was elevated after treatment with either G/GO or HX/XO (Fig. 5A and B, lane 2), compared to the control, whereas it was obviously abrogated by treatment with ZPDC glycoprotein (200 μg/ml) (Fig. 5A and B, Lane 3), respectively, compared to the G/GO or HX/XO treatment alone. To convince whether the activation of PKCα, ERK 1/2, p38 MAPK and JNK are essential to the G/GO- or HX/XO-stimulated cell proliferation, we further performed Western blot assay using the inhibitors of PKCα (staurosporine, 0.5 μM), ERK 1/2 (PD98059, 1 μM), p38 MAP kinase (SKF86002, 1 μM) and JNK (SP600125, 1 μM), respectively. Under these concentrations, the antioxidative activities of the above inhibitors were not observed (data not shown). When the cells were exposed to G/GO or HX/XO for 4 h after pretreatment with above inhibitors for 0.5 h, the band intensities of PCNA protein were gradually attenuated in G/GO- or HX/XO-stimulated Chang liver cells, respectively, compared to the G/GO or HX/XO treatment alone, suggesting that the both PKCα and MAPK-dependent signaling pathway are involved in the G/GO- or HX/XO-stimulated cell proliferation pathway in Chang liver cells.

4. Discussion

Our results in the present study indicated that 24 kDa of ZPDC glycoprotein has inhibitory effect on abnormal

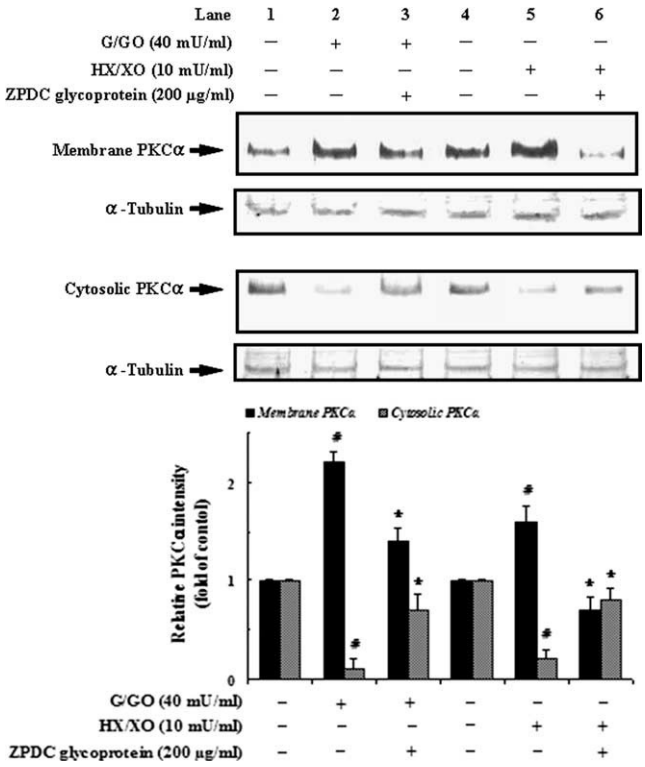

Fig. 2. Inhibitory effect of ZPDC glycoprotein on the translocation of PKCα in G/GO- or HX/XO-stimulated Chang liver cells. Chang liver cells were exposed to the G/GO or HX/XO system for 4 h in the absence or presence of ZPDC glycoprotein (200 μg/ml). Membrane and cytosolic protein extracts (0.3 mg/ml) were loaded to perform western blotting using an anti-PKCα polyclonal antibodies. (Lane 1) Control for G/GO. (Lane 2) G/GO alone. (Lane 3) 200 μg/ml ZPDC glycoprotein+G/GO. (Lane 4) Control for HX/XO. (Lane 5) HX/XO alone. (Lane 6) 200 μg/ml ZPDC glycoprotein +HX/XO. The relative intensity of PKCα protein bands was quantified using Scion Imaging Software (Scion Image Beta 4.02, MD, USA), compared to control. Each bar is the means±S.D. of triplicate experiments. $^{\#}P<.01$, a significant difference between control and G/GO or HX/XO treatment alone; $^{*}P<.05$, significant differences between G/GO or HX/XO treatment alone and G/GO or HX/XO treatment in presence of ZPDC glycoprotein treatments.

activation of cell proliferation-related molecules, possibly through its antioxidative ability in G/GO- or HX/XO-treated Chang liver cells. The result in Fig. 1 showed that ZPDC glycoprotein has a dose-dependent blocking effect on the production of H_2O_2 in G/GO- or HX/XO-treated Chang liver cells. H_2O_2 is a freely diffusible small molecule, has been shown to act as a second messenger and modulate multiple gene expressions, like Ca^{2+}, diacylglycerol or cyclic adenosine monophosphate (cAMP) [25]. Although H_2O_2 exerts its cytotoxic effect via the formation of $^{\bullet}OH$ in metal-catalyzed Fenton or Haber Weiss reactions on the cellular injury [20,26], our results indicated the H_2O_2 produced by G/GO and HX/XO systems does not have cytotoxic effect in Chang liver cells. Regarding the concentration of H_2O_2 in the culture media, it has been reported that the fibroblastic cytotoxicity and mitochondrial DNA injury were induced by treatment with 50 μM H_2O_2 for 1 h in fibroblast cells [27], whereas hepatic cytotoxicity and apoptosis were induced by treatment with 200 μM H_2O_2 for 24–36 h in liver cells [28]. This means that H_2O_2 to induce cytotoxicity is generally required higher concentration in liver cells than that in fibroblast cells. One of possible reasons why H_2O_2 induces cytotoxicity at higher concentration in liver cells is that it already exists many antioxidative enzymes to scavenge oxygen free radicals and Phase II detoxifying enzymes to inhibit the production of electrophilic xenobiotic metabolites. Thus, we assumed that the H_2O_2 in presents study might enter the intracellular space of the cells to participate the events such as cell proliferation and tumor promotion as a signaling molecule but not as cytotoxic molecule in G/GO or HX/XO-treated Chang liver cells, even though either G/GO or HX/XO to induce cell proliferation is required higher concentration. Interestingly, after treatment with either Pronase E for the hydrolysis of protein or $NaIO_4$ for the oxidation of carbohydrate, the H_2O_2 scavenging activity of ZPDC glycoprotein was markedly reduced, suggesting that it absolutely needs to have two parts (carbohydrate and

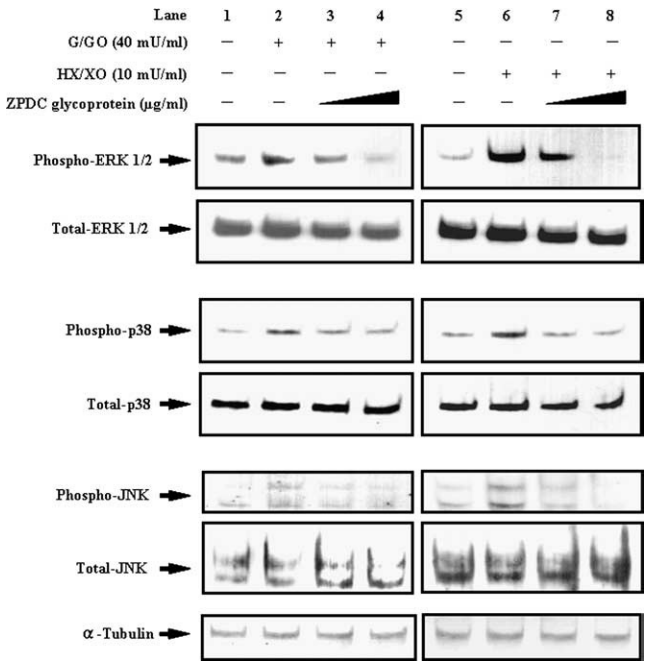

Fig. 3. Inhibitory effect of ZPDC glycoprotein on the phosphorylation of MAPKs in G/GO- or HX/XO-stimulated Chang liver cells. Chang liver cells were exposed to the G/GO or HX/XO system for 4 h in the absence or presence of ZPDC glycoprotein (100 and 200 μg/ml). Whole protein extracts (0.3 mg/ml) were loaded to perform Western blotting using an anti-ERK1/2, -SAPK/JNK, -p38 MAP kinase polyclonal antibodies and anti-phospho-ERK1/2, -SAPK/JNK, -p38 MAPK polyclonal antibodies. (Lane 1) Control for G/GO. (Lane 2) G/GO alone. (Lane 3) 100 μg/ml ZPDC glycoprotein +G/GO. (Lane 4) 200 μg/ml ZPDC glycoprotein+G/GO. (Lane 5) Control for HX/XO. (Lane 6) HX/XO alone. (Lane 7) 100 μg/ml ZPDC glycoprotein +HX/XO. (Lane 8) 200 μg/ml ZPDC glycoprotein+HX/XO. Each bar is the means±S.D. of triplicate experiments. $^{\#}P<.01$, significant difference between control and G/GO or HX/XO treatment alone. $^{*}P<.05$, significant differences between G/GO or HX/XO treatment alone and G/GO or HX/XO treatment in presence of ZPDC glycoprotein treatments.

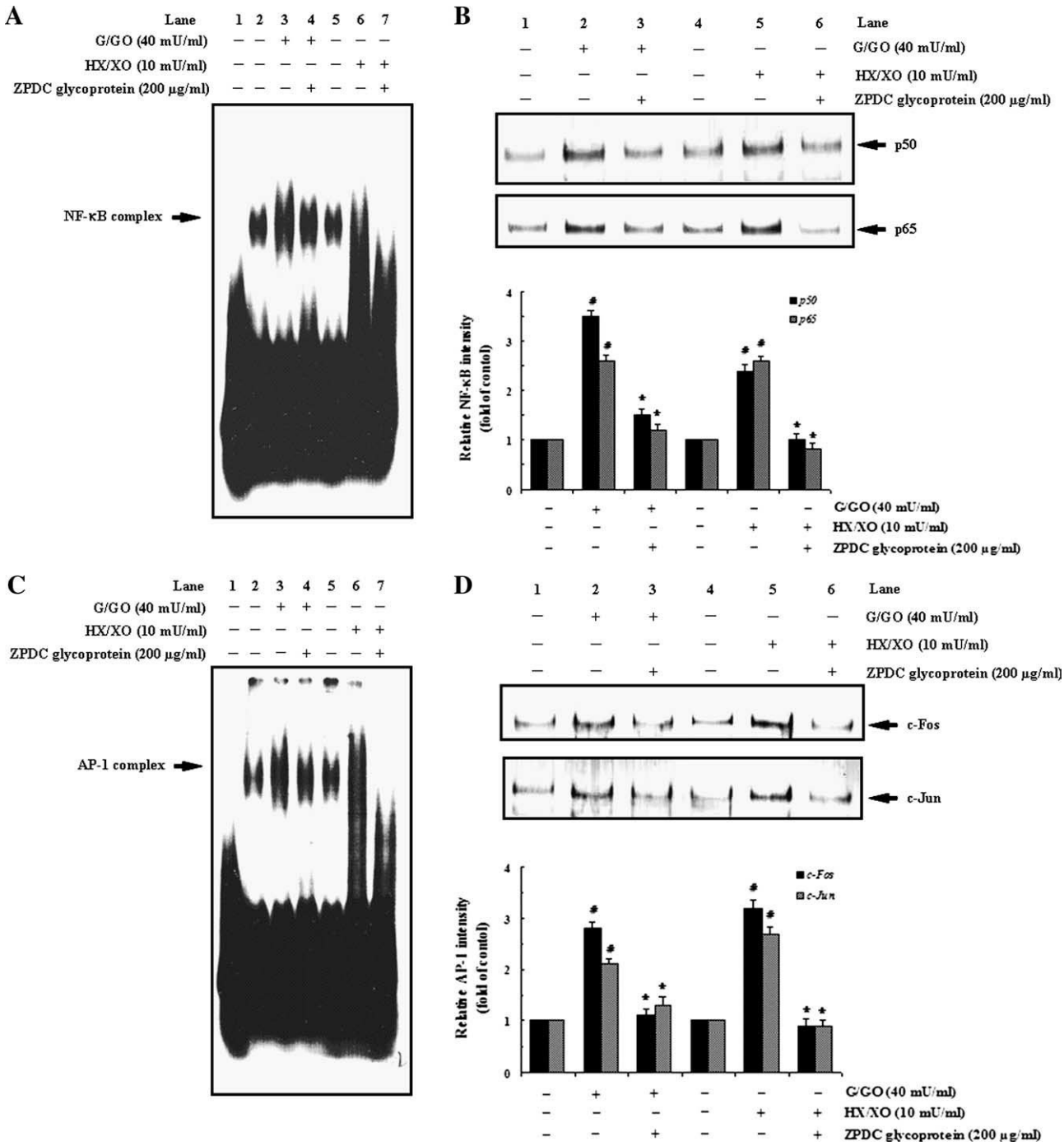

Fig. 4. Inhibitory effect of ZPDC glycoprotein on the activation of NF-κB and AP-1 in G/GO- or HX/XO-stimulated Chang liver cells. Chang liver cells were exposed to the G/GO or HX/XO system for 4 h in the absence or presence of ZPDC glycoprotein (200 μg/ml). Detections of NF-κB (A) and AP-1 (C) DNA binding activity in nucleic protein extract were performed by electrophoretic mobility shift assay using the NF-κB and AP-1 oligonucleotide. Lane 1, free probe alone (no nuclear extracts); Lane 2, control for G/GO; Lane 3, G/GO alone; Lane 4, 200 μg/ml ZPDC glycoprotein + G/GO; Lane 5, control for HX/XO; Lane 6, HX/XO alone; Lane 4, 200 μg/ml ZPDC glycoprotein + HX/XO. Nucleic protein extracts (0.3 mg/ml) were loaded to detect NF-κB subunits (p50 and p65) (B) and AP-1 subunits (c-Fos and c-Jun) (D) and western blotting was performed using an anti-p50, -p65, -c-Fos and -c-Jun polyclonal antibodies. (Lane 1) Control for G/GO. (Lane 2) G/GO alone. (Lane 3) 200 μg/ml ZPDC glycoprotein+G/GO. (Lane 4) Control for HX/XO. (Lane 5) HX/XO alone. (Lane 6) 200 μg/ml ZPDC glycoprotein+HX/XO. The relative intensities of p50, p65, c-Fos and c-Jun bands were quantified using Scion Imaging Software (Scion Image Beta 4.02), compared to the control. Each bar is the means±S.D. of triplicate experiments. $^{\#}P<.01$, significant difference between control and G/GO or HX/XO treatment alone. $^{*}P<.05$, significant differences between G/GO or HX/XO treatment alone and G/GO or HX/XO treatment in presence of ZPDC glycoprotein treatments.

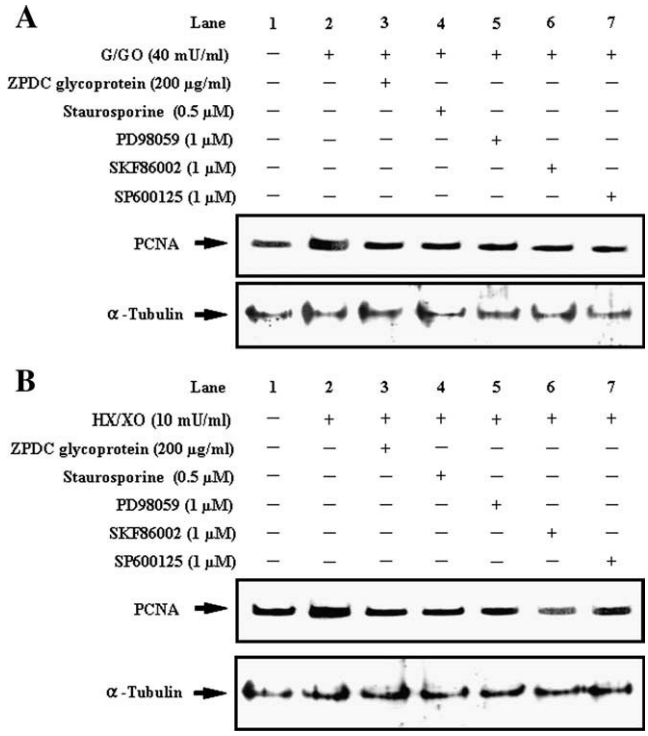

Fig. 5. Inhibitory effect of ZPDC glycoprotein on the activation of PCNA in G/GO- or HX/XO-stimulated Chang liver cells. Chang liver cells were pretreated with inhibitors of PKCα (staurosporine, 0.5 μM), ERK 1/2 (PD98059, 1 μM), p38 MAPK (SKF86002, 1 μM) and JNK (SP600125, 1 μM) for 0.5 h and then exposed to the G/GO (A) or HX/XO (B) system for 4 h in the absence or presence of ZPDC glycoprotein (200 μg/ml). Nucleic protein extracts (0.3 mg/ml) were loaded to perform western blotting using an anti-PCNA polyclonal antibodies. (Lane 1) Control for G/GO or HX/XO. (Lane 2) G/GO or HX/XO alone. (Lane 3) 200 μg/ml ZPDC glycoprotein +G/GO or HX/XO. (Lane 4) 0.5 μM staurosporine+G/GO or HX/XO. (Lane 5) 1 μM PD98059+G/GO or HX/XO. (Lane 6) 1 μM SKF86002 + G/GO or HX/XO. (Lane 5) 1 μM SP600125 + G/GO or HX/XO. α-Tubulin was used as an internal control. The relative intensity of PCNA protein bands was quantified using Scion Imaging Software (Scion Image Beta 4.02), compared to control. Each bar is the means±S.D. of triplicate experiments. $^{\#}P<.01$. significant difference between control and G/GO or HX/XO treatment alone. $^{*}P<.05$, significant differences between G/GO or HX/XO treatment alone and G/GO or HX/XO treatment in presence of ZPDC glycoprotein, PKC inhibitor or MAPKs inhibitors.

protein) for its maximal antioxidative activity. Considering the cell-proliferative effect of $O_2^{\bullet-}$ in HX/XO system, we speculated that ZPDC glycoprotein might block the intracellular cell proliferative signals by scavenging the H_2O_2 production rather than $O_2^{\bullet-}$ in the cells because $O_2^{\bullet-}$ was trapped in the cell membrane and could only pass via a bilayer after production in the extracellular medium [29].

To further understand the antioxidative mechanisms of ZPDC glycoprotein on abnormal cell proliferation, we investigated whether or not ZPDC glycoprotein has inhibitory effects on activity of protein kinases and transcription factors in G/GO- or HX/XO-treated Chang liver cells. It has been reported that PKCα contains unique structural features that are susceptible to oxidative modification by H_2O_2, and the overexpression of PKCα enhances mitogenesis and tumor promotion via modulation of MAPKs activity [5,6]. The result in Fig. 2 showed that the G/GO or HX/XO system induces the translocation of cytosolic PKCα to the membrane, whereas ZPDC glycoprotein remarkably blocks the activation of PKCα in the system, indicating ZPDC glycoprotein has inhibitory effect on G/GO- or HX/XO-induced PKCα activation, possibly through its extracellular H_2O_2 scavenging effect prior to enter into the Chang liver cells. In addition, ZPDC glycoprotein has ability to inhibit the phosphorylation of ERK 1/2, p38 MAPK and JNK in G/GO- or HX/XO-treated Chang liver cells. In general, ERK 1/2 is mainly related to anabolic processes, such as cell division, proliferation and differentiation, whereas p38 MAP kinase and JNK are mostly associated with various cellular stress responses, such as growth arrest and apoptosis [30,31]. However, the regulation and function of MAPKs are still a controversial issue, although it has been extensively studied for MAPK activation during oxidative stress. For instance, it has been reported that momentary activation of p38 MAP kinase and JNK induced by tumor necrosis factor-α can induce cell survival, while the continued activation of these MAPKs leads to cell death [32], and momentary activation of ERK 1/2 is a cell proliferation signal, while continued induction is a growth arrest signal [33]. Furthermore, MAPK activation is highly divergent and is largely dependent on the cell types [34]. In the present study, ERK 1/2, p38 MAPK and JNK were activated in G/GO- or HX/XO-treated Chang liver cells, suggesting that H_2O_2 produced by G/GO and HX/XO systems can induce the all MAPKs without cytotoxic effect. By contrast, ZPDC glycoprotein significantly suppressed these phosporylation of the MAPKs, indicating that blockage of ERK 1/2, p38 MAPK and JNK activation is one indirect antioxidative mechanism of ZPDC glycoprotein on G/GO- or HX/XO-stimulated H_2O_2 production in Chang liver cells.

Accumulating evidences have been shown that the activation of NF-κB and AP-1 is modulated by MAPKs [35–37]. In addition, oxidants and tumorigenic agents have been shown to modulate the DNA-binding activity of NF-κB and AP-1 through PKC and/or MAPK pathway [7,38,39]. In the current study, ZPDC glycoprotein markedly suppressed the NF-κB and AP-1 DNA binding activities through suppressions of p50 and p65 subunits and c-Fos and c-Jun subunits in G/GO- or HX/XO-treated Chang liver cells (Fig. 3). Several studies in NF-κB activation have demonstrated that a heterodimeric or homodimeric complex of p50 and p65 appears to have tumorigenic activity, but not homodimeric complex of p50, because p50-p50 homodimer complex may block the activation of some tumor promoter gene [40,41], implying that either p50-p65 heterodimer or p65-p65 homodimer complex can be involved in G/GO- or HX/XO-stimulated cell proliferative and tumorigenic signals. Based on our results for NF-κB subunit, we assume that ZPDC glycoprotein has the ability to inhibit the p50-p65 heterodimer complex on G/GO- or HX/XO-stimulated H_2O_2

production in Chang liver cells. NF-κB also frequently acts together with other transcription factors such as AP-1, which regulates various genes responsible for inflammatory and tumorigenic events [42]. Although the Jun family has the ability to form stable homodimers that bind to AP-1 DNA consensus sequences, Fos family cannot form a stable homodimer. Instead, the Fos family mediates gene expression by forming heterodimers with Jun family. Interestingly, these Jun-Fos heterodimers are more stable and transcriptional active than the Jun homodimers [9]. In these contexts, our results speculate that G/GO or HX/XO system stimulates high transcriptional activity of AP-1 through up-regulation of Jun-Fos heterodimers, whereas these events remarkably inhibited by treatment with ZPDC glycoprotein in G/GO- or HX/XO-treated Chang liver cells. Collectively, the results of the present work indicate that ZPDC glycoprotein scavenges the H_2O_2 production and indirectly suppresses the H_2O_2-related PKC and MAPKs via modulation of NF-κB and AP-1 in G/GO- or HX/XO-treated Chang liver cells.

Also, abnormal activation of NF-κB and AP-1 has been known to cause the uncontrolled cell proliferation and differentiation, leading to malignant transformation [43]. Therefore, agents that inhibit improper cell proliferation are known to have immense potential in chemoprevention and chemotherapy [44]. In the present study, the level of PCNA protein, which is known as a 36-kDa nonhistone nuclear acidic protein expressed in the nuclei of proliferating cells during S-phase [45], was obviously induced by exposure to the G/GO or HX/XO system, whereas such excessive level of cell proliferation was down-regulated by addition of ZPDC glycoprotein in Chang liver cells. This means that ZPDC glycoprotein, which inhibits PCNA activation on G/GO- or HX/XO-mediated H_2O_2 production, might act as a potent inhibitor on abnormal cell proliferation under oxidative stress responses. With respect to the antiproliferative effect of ZPDC glycoprotein in our experiments, we speculate that ZPDC glycoprotein is able to scavenge extracellular H_2O_2 induced by either G/GO or HX/XO system prior to enter into the Chang liver cells, and then the event is responsible for inhibition of PCNA expression by blocking H_2O_2-related signaling transduction cascade. Furthermore, our results in this experiment showed that the G/GO- or HX/XO-induced cell proliferation was remarkable abolished by treatments of the staurosporine, PD98059, SKF86002 and SP600125. These phenomena imply that PKCα-, ERK1/2-, p38MAP kinase- and JNK-dependent signaling pathways are involved in G/GO- or HX/XO-induced cell proliferation. These observations also support the above results that G/GO- or HX/XO-induced H_2O_2 can activate PKCα, ERK1/2, p38 MAPK and JNK, whereas ZPDC glycoprotein blocks this activation of redox-related signaling transduction molecules responsible for cell proliferation in Chang liver cells.

In addition, considering in vivo biofunctional activities of ZPDC glycoprotein in gut, the carbohydrate part of ZPDC glycoprotein could not be well digested in the mammalian small intestine and forms a viscous solution, especially in the ileum, which is also thought to delay the absorption of various chemicals including ROS-generating compounds. Consequently the viscous ZPDC glycoprotein might interfere with ROS-generating compound absorption in the small intestine and subsequently protect or maintain the normal physiological levels in gastrointestinal environment. Furthermore, we recently have reported that administration of ZPDC glycoprotein (20 mg/kg) has inhibitory effect on mouse model of CCl_4-stimulated acute liver injury [16]. This hepatoprotectvie effect of ZPDC glycoprotein was associated with its antioxidative effect on trichloromethyl free radicals ($\bullet CCl_3$)-induced lipid peroxidation or was related to normalize the abnormal levels of lactate dehydrogenase, alanine transaminase and antioxidative enzymes. Thus, we assume that ZPDC has ability to inhibit the liver pathological factors via its direct/indirectly strong antioxidative activity in liver.

In conclusion, ZPDC glycoprotein has strong scavenging activities against H_2O_2 and inhibitory effect on PCNA in G/GO- or HX/XO-treated Chang liver cells. In addition, ZPDC glycoprotein normalized the abnormal activities of PKCα, MAPKs, NF-κB and AP-1 in these cells, suggesting that ZPDC glycoprotein is a natural antioxidant, which can modulate cell proliferation-related molecules in G/GO- or HX/XO-treated Chang liver cells. Further research remains whether ZPDC glycoprotein modulates cell cyclic regulators and tumor suppressor genes during cell proliferation and tumor promotion.

Acknowledgments

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