



## Glycoprotein (116 kD) isolated from *Ulmus davidiana* Nakai protects from injury of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-treated BNL CL.2 cells

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### Abstract:

*Ulmus davidiana* Nakai (UDN) has been used for a long time to cure inflammation in oriental medicine. To evaluate the cytoprotective effects of the UDN glycoprotein, we measured cytotoxicity, the level of intracellular reactive oxygen species (ROS), activity of nuclear factor- $\kappa$ B (NF- $\kappa$ B), nitric oxide (NO) production, and thiobarbituric acid-reactive substances (TBARS) formation in 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-treated BNL CL.2 cells. In TPA-treated BNL CL.2 cells, the results showed that UDN glycoprotein has dose-dependent blocking activities against TPA-induced cytotoxicity and NF- $\kappa$ B activation. In cytotoxic-related events, UDN glycoprotein (200  $\mu$ g/ml) has an inhibitory effect on intracellular ROS production, NO production, and TBARS formation, without any toxic effects in the BNL CL.2 cells. These results suggest that UDN glycoprotein has cytoprotective abilities against TPA-induced oxidative cell injury.

### Key words:

UDN glycoprotein, intracellular ROS, NO production, NF- $\kappa$ B

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**Abbreviations:** H<sub>2</sub>DCF-DA – 2',7'-dichlorodihydrofluorescein diacetate, NF- $\kappa$ B – nuclear factor- $\kappa$ B, NO – nitric oxide, ROS – reactive oxygen species, TBARS – thiobarbituric acid-reactive substances, TPA – 12-*O*-tetradecanoylphorbol 13-acetate, UDN – *Ulmus davidiana* Nakai

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### Introduction

Oxidative stress, induced by reactive oxygen species (ROS), is believed to be a primary factor in various degenerative diseases as well as in the normal process of aging [10]. The ROS including superoxide anion (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical

(OH), and singlet oxygen (<sup>1</sup>O<sub>2</sub>) are continuously generated during normal metabolism or from exogenous factors and agents in aerobic organisms. The ROS are scavenged by endogenous antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) that are abundant in the liver tissue [27]. Under pathological conditions, however, they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. A number of pro-oxidant drugs and other chemicals have been implicated in the oxidative stress and cell injury resulting from the intracellular production of injurious ROS [10].

TPA can exert different cell cycle-dependent effects on cell growth and division. Namely, it can not only act as a mitogen in the resting cell, but also in-

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hibit the cell cycle progression in various cell types [17]. It also induces production of reactive oxidants, such as hydrogen peroxide, and superoxide anion [28, 24], thus causing lipid peroxidation and DNA damage in hepatic and brain tissue [2]. Furthermore, it is also reported that TPA can promote the expression of inducible NO synthase (iNOS) in activated macrophages and hepatocytes [14]. The ability of TPA to induce Ca<sup>2+</sup>-independent iNOS have been taken as a model to search for potential naturally occurring agents which may inhibit NO generation in biological system.

Nitric oxide (NO) is a reactive free radical gas and a messenger molecule with many physiological functions. Derived from L-arginine, it is produced by NO synthase (NOS) enzymes. Among of NOS, iNOS is Ca<sup>2+</sup>-independent and inducible by inflammatory cytokines, and endo- or exogenous toxic agents in various cell types [16]. Once iNOS is induced, NO is produced for prolonged periods, and high amounts of NO are involved in immunoregulatory and cytotoxic actions, either by themselves or through the formation of peroxynitrite with superoxide anion. Namely, NO becomes a highly reactive species through peroxynitrite, which is generated in a diffusion-limited reaction of NO with superoxide anion, thus acting as a toxic factor [3]. This nitrogen intermediate is a powerful oxidant capable of initiating lipid peroxidation and oxidizing lipid-soluble antioxidants [13, 26].

It has been reported that iNOS is regulated at the transcriptional level, and its gene promoter has binding sites for multiple transcription factors including NF- $\kappa$ B [12]. A number of studies also showed that excessive generation of ROS caused by exo- and endogenous source led to the activation of the NF- $\kappa$ B [23]. The NF- $\kappa$ B is considered a primary oxidative stress-responsive transcription factor that functions to enhance the expression of a variety of genes, including those for cytokines and growth factors, adhesion molecules, immunoreceptors and acute response proteins. It exists as a heterodimer of p50 and p65 subunits complexed with the inhibitory subunit (I $\kappa$ B) that prevents migration of p50/p65 to the nucleus [1]. Therefore, inhibition of NF- $\kappa$ B activation by an agent is closely related to its antioxidative activity.

One of the plausible ways to prevent the ROS-mediated cellular injuries is to augment or fortify endogenous defense capacity against oxidative stress through dietary or pharmacological intake of antioxidants [21, 25]. *Ulmus davidiana* Nakai (UDN) is a de-

ciduous tree that is widely distributed in Korea. In oriental medicine, the bark and root of UDN have been used traditionally for treating edema, mastitis, gastric cancer, and inflammation [19]. In previous study, we found that glycoprotein isolated from UDN, with an approximate molecular mass of 116 kD, had inhibitory effects on DNA synthesis and cytokine production (IL-4 and IFN- $\gamma$ ) in mouse primary immune cells [18].

Therefore, in this study, we investigated the protective effects of UDN glycoprotein on TPA-induced oxidative injury in BNL CL.2 cells.

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## Materials and Methods

### Chemicals

12-*O*-Tetradecanoylphorbol 13-acetate (TPA, P8139), 2',7'-dichlorofluorescein diacetate (D6883),  $\beta$ -nicotinamide adenine dinucleotide ( $\beta$ -NADH, N6005), penicillin G (H0474), streptomycin (H0447), and trypsin (T4549) were obtained from Sigma (St. Louis, USA). Nitro blue tetrazolium chloride monohydrate (NBT, 74030) was obtained from Biochemika (Buchi, Switzerland). Dulbecco's modified Eagle 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.

### Cell culture

UDN glycoprotein was isolated from *Ulmus davidiana* Nakai, as described previously [18]. BNL CL.2 cells (murine embryonic liver cell) were incubated in DMEM containing 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C and atmosphere containing 5% CO<sub>2</sub>. The medium was renewed two times per week. The cells (1  $\times$  10<sup>6</sup> cells/ml) were distributed into 35 mm culture dishes or 96-well flat bottom plates. The final volumes were 2 ml/dish on the 35 mm culture dishes and 100  $\mu$ l/well on the 96-well flat bottom plates. The cellular cytotoxicity was determined by assessing the release of lactate dehydrogenase (LDH) into the medium according to the method of Bergmeyer and Bernt [4]. Briefly, cells were treated with UDN glycoprotein (200  $\mu$ g/ml) or TPA (200 nM), and co-treated with UDN glycoprotein

(100–200 µg/ml) in the presence of TPA (200 nM) for 12 h. After that, the culture medium (35 µl) was 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 (reduced nicotinamide adenine dinucleotide) and LDH activity was measured as the rate of loss of β-NADH absorption at 340 nm for 2 min. For the total LDH determination, the cells were treated with Triton X-100 (1%) for 30 min and centrifuged at 1,000 × g for 10 min. The resulting supernatants were added to the reaction mixtures and LDH assay was carried out as described above. Results were expressed as relative percentage, compared to the control value.

#### Determination of intracellular ROS

Amount of intracellular ROS was measured by using nonfluorescent 2',7'-dichlorodihydrofluorescein (H<sub>2</sub>DCF-DA). H<sub>2</sub>DCF-DA is a fluorogenic freely permeable tracer specific for ROS assessment. It is deacetylated by intracellular esterases to the non-fluorescent 2',7'-dichlorohydrofluorescein (DCFH), which is oxidized to the fluorescent compound 2',7'-dichlorofluorescein (DCF) by ROS. Cells were pre-incubated with 10 µM H<sub>2</sub>DCF-DA for 30 min at 37°C, and then washed twice with PBS to remove the excess of H<sub>2</sub>DCF-DA. After that, the cells were treated with various concentrations of TPA (10–200 nM), or co-treated with UDN glycoprotein (200 µg/ml) for 12 h. Finally, the fluorescence intensity was measured at excitation wavelength of 485 nm and emission wavelength of 530 nm using fluorescence microplate reader (Dual Scanning SPECTRAMax, Molecular Devices Corporation, Sunnyvale, CA, USA). The values were calculated as relative intensity of DCF fluorescence, compared to the control.

#### Production of NO

Cells were treated with UDN glycoprotein (200 µg/ml) or TPA (200 nM), and co-treated with UDN glycoprotein (100–200 µg/ml) in the presence of TPA (200 nM) for 12 h in the 96 well multiple plate. Then, cells were centrifuged at 1,000 × g for 10 min and supernatants were collected. NO production was measured as a function of nitrite (NO<sub>2</sub>) concentration by the method of Green et al. [9]. Supernatants (50 µl)

were mixed with 100 µl of 0.1% sulfanilamide and 100 µl of 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid for 5 min. Absorbance was measured at 540 nm with a MicroReader (Hyperion, Inc., USA). Nitrite was quantified by using sodium nitrate as a standard.

#### Thiobarbituric acid reactive substances (TBARS) assay

Cells were treated with UDN glycoprotein (200 µg/ml) or TPA (200 nM), and co-treated with UDN glycoprotein (100–200 µg/ml) in the presence of TPA for 12 h. Lipid peroxidation was estimated according to the presence of TBARS in cell homogenates using the method of Buege and Aust [6]. Briefly, cells were scraped from the plates into ice cold PBS, and homogenized. One volume of sample was mixed thoroughly with two volumes of stock solution containing 15% w/v trichloroacetic acid, 0.375% w/v TBARS and 0.25 M HCl. The mixture was heated for 30 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 3,000 × g for 10 min and the absorbance of the sample was measured at 535 nm. 1,1,1,3-Tetraethoxypropane was used as a standard. Data are expressed as relative activities vs. control.

#### Preparation of nuclear extracts

Cells were treated with TPA (200 nM) or co-treated with UDN glycoprotein (100–200 µg/ml) in the presence of TPA (200 nM) for 12 h. The cells were scraped in 500 µl of hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1.5 mM MgCl<sub>2</sub>, 0.5% NP-40), followed by centrifugation at 3000 × g for 5 min. The pelleted nuclear proteins were then resuspended in 200 µl of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1.5 mM MgCl<sub>2</sub>, 0.5% NP-40) containing a protease inhibitor cocktail (Boehringer, Mannheim) and centrifuged at 12,000 × g for 15 min at 4°C. The amounts of protein were measured using the Lowry method [22]. Samples of protein were stored at –70°C.

## Western blot assay

Nuclear proteins were analyzed on a 10% polyacrylamide mini-gel electrophoresis at 100 V for 2 h at room temperature using a Mini-PROTEIN II electrophoresis cell (Bio-Rad). After transfer onto nitrocellulose membranes (Millipore, Bedford, MA, USA), the membrane was incubated for 1 h at room temperature in TBS-T solution (10 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% Tween-20) containing 5% non-fat dry milk. The membranes were subsequently incubated for 2 h at room temperature with rabbit polyclonal antibody (1:3000; NF- $\kappa$ B, Santa Cruz Biotechnology, CA, USA) in TBS-T solution. After three washes with TBS-T, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10000; Santa Cruz Biotechnology, CA, USA) in TBS-T. The protein bands were visualized by incubation with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma Chemical Co.). The results from Western blot assay were calculated with the use of the Scion imaging software (Scion Image Beta 4.02, Maryland, USA) as the relative intensity.

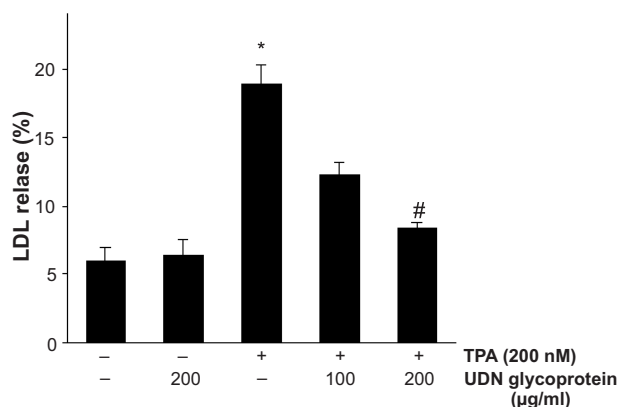
## Statistical analysis

All experiments were done in triplicate, and data were expressed as the means  $\pm$  SD. A one-way analysis of variance (ANOVA) and Duncan test were used to detect significant differences by multiple comparisons (SPSS program, ver. 10.0).

## Results

### Protective effect of UDN glycoprotein against TPA-induced cytotoxicity

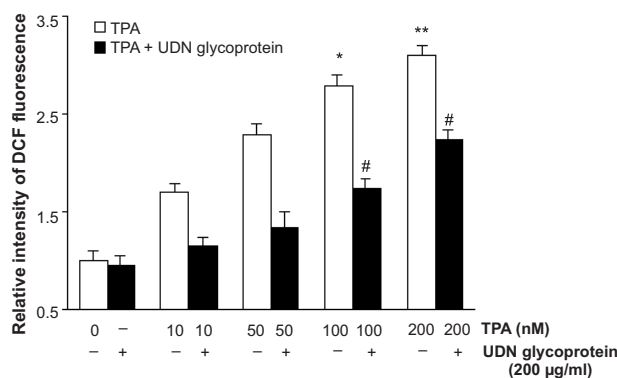
To investigate the protective effects of UDN glycoprotein against the TPA-induced cytotoxic injury, we measured the LDH activity in cell culture medium, as an index of cytotoxicity [7]. As shown in Figure 1, when the cells were exposed to 200 nM TPA, value of cytotoxicity was 18.9%. However, the LDH activity was diminished by 6.7 and 10.6% on the increasing concentrations of UDN glycoprotein (100 and 200  $\mu$ g/ml) in the presence of TPA (200 nM), compared to the TPA treatment alone.



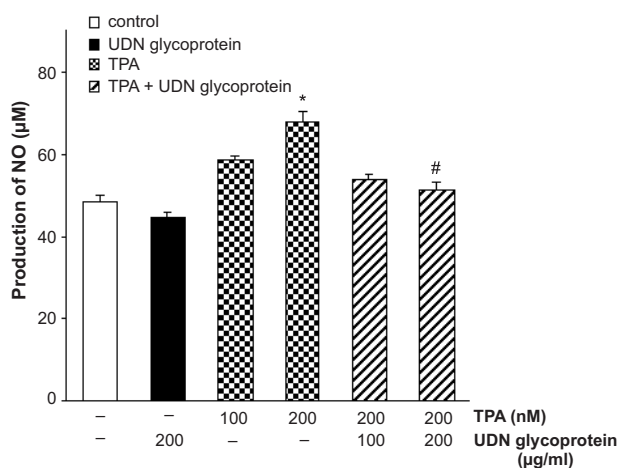
**Fig. 1.** Cytotoxicity of UDN glycoprotein. Data were presented as the means  $\pm$  SD from triplicates ( $n = 3$ ). \* represents significant difference compared with the control,  $p < 0.01$ ; # represents a significant difference between UDN glycoprotein + 200 nM TPA and 200 nM TPA alone,  $p < 0.05$

## Determination of intracellular ROS

As shown in Figure 2, the relative content of intracellular ROS, calculated by monitoring dichlorofluorescein (DCF) fluorescence, was gradually increasing in TPA-treated BNL CL.2 cells. Namely, intracellular ROS levels were significantly dose-dependently increased upon treatment with TPA. For instance, the values of relative fluorescence intensity for ROS were 1.70, 2.30, 2.80, and 3.10 at 10, 50, 100, and 200 nM of TPA, respectively. However, increased intracellular ROS levels were considerably diminished by addition of UDN glycoprotein, compared to the TPA treatment alone. For example, the relative intensities for ROS



**Fig. 2.** Changes of intracellular ROS production in TPA-treated BNL CL.2 cells. Data represent the means  $\pm$  SD from triplicate experiments ( $n = 3$ ). \* and \*\* represent significant differences compared with the control,  $p < 0.05$  and  $p < 0.01$ , respectively; # represents a significant difference between UDN glycoprotein (200  $\mu$ g/ml) + TPA and TPA alone,  $p < 0.05$

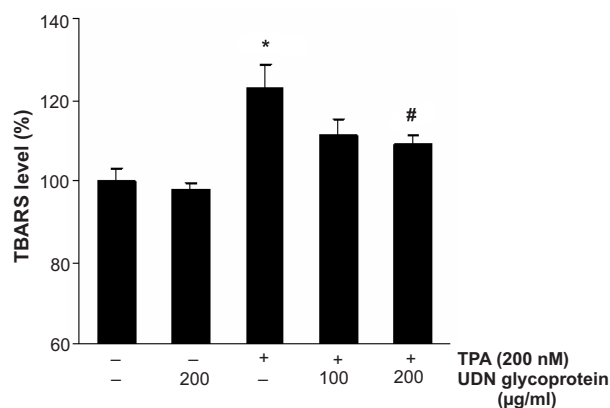


**Fig. 3.** Effect of UDN glycoprotein on NO production in TPA-treated BNL CL.2 cells. Data represent the means  $\pm$  SD from triplicate experiments ( $n = 3$ ). \* represents a significant difference compared with the control,  $p < 0.05$ ; # represents a significant difference between UDN glycoprotein + 200 nM TPA and 200 nM TPA alone,  $p < 0.05$

were diminished by 0.55, 0.95, 1.05, and 0.85 at 10, 50, 100, and 200 nM of TPA in the presence of UDN glycoprotein (200  $\mu\text{g/ml}$ ), respectively.

### Production of NO

The inhibitory effects of UDN glycoprotein on the levels of NO production in TPA-treated BNL CL.2 cells were measured (Fig. 3). There was no significant difference between the level of NO production after treatment with UDN glycoprotein (200  $\mu\text{g/ml}$ ) alone, and the control one. When the cells were treated with



**Fig. 4.** Effect of UDN glycoprotein on lipid peroxidation in TPA-treated BNL CL.2 cells. Data represent the means  $\pm$  SD from triplicate experiments ( $n = 3$ ). \* represents significant differences compared with the control,  $p < 0.01$ ; # represents a significant difference between UDN glycoprotein + 200 nM TPA and 200 nM TPA alone,  $p < 0.05$

100 and 200 nM TPA in the absence of UDN glycoprotein, the levels of NO were gradually increased by 10.2 and 19.6  $\mu\text{M}$ , compared to the control. However, the increased level of NO was significantly decreased by 16.6  $\mu\text{M}$  by the additions of UDN glycoprotein (100 and 200  $\mu\text{g/ml}$ ) in the presence of 200 nM TPA alone, compared to the TPA (200 nM) treatment alone.

### Effect of UDN glycoprotein on lipid peroxidation

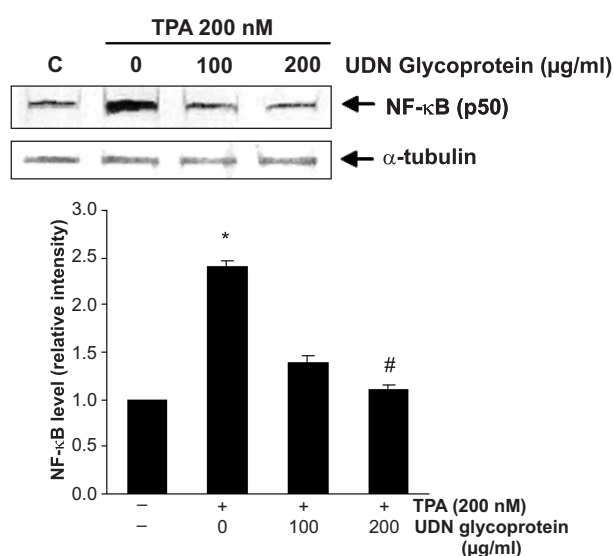
The effects of UDN glycoprotein on lipid peroxidation in TPA-induced BNL CL.2 cells were presented in Figure 4. We measured the levels of TBARS, as a marker of lipid peroxidation, and expressed as relative activities. When the cells were treated with 200 nM TPA, the level of TBARS was 122.7%. It was increased compared to the control. However, with addition of UDN glycoprotein (100 and 200  $\mu\text{g/ml}$ ) in the presence of 200 nM TPA, the TBARS levels were diminished by 10.2 and 13.7%, respectively, compared to the TPA (200 nM) treatment alone.

### Effect of UDN glycoprotein on TPA-induced NF- $\kappa$ B activation

When the cells were exposed to 200 nM TPA alone for 12 h, the NF- $\kappa$ B activity was markedly increased, compared to control (Fig. 5, lane 2). Upon treatment with the UDN glycoprotein (100 and 200  $\mu\text{g/ml}$ ), however, the intensities of bands were obviously weakened in a dose-dependent manner (Fig. 5, lanes 3 and 4). On the other hand, the intensities of bands in Western blot were calculated using Scion Imaging Software. After exposure to TPA (200 nM) alone, the value of relative band intensity was increased by 1.4. In contrast, when the cells were treated with UDN glycoprotein in presence of TPA (200 nM), the values of relative band intensities were diminished by 1.01, and 1.21 at 100 and 200  $\mu\text{g/ml}$ .

## Discussion

Oxidative stress caused by ROS was shown to be linked to liver diseases, such as hepatotoxicity, and other liver pathological conditions. Cells are protected from ROS-induced damage by a variety of endogenous ROS scavenging enzymes, chemical com-



**Fig. 5.** Effect of UDN glycoprotein on NF- $\kappa$ B activity in TPA-treated BNL CL.2 cells.  $\alpha$ -Tubulin was used as an internal control. \* represents significant differences compared with the control,  $p < 0.01$ ; # represents a significant difference between UDN glycoprotein + 200 nM TPA and 200 nM TPA alone,  $p < 0.05$ , C – control

pounds and natural products. Thus, elimination of excessive ROS or suppression of their generation by antioxidants may be effective in preventing oxidative cell damage. Recently, we reported that UDN glycoprotein has anti-apoptotic effects as well as antioxidative effects on hydroxyl radicals, and modulatory effects on redox-sensitive transcription factors in cultured mouse primary thymocytes [18, 20].

This study has evaluated the protective ability of UDN glycoprotein with its inherent antioxidant activity, and has shown UDN glycoprotein to have an effect on the cellular damage caused by TPA-induced oxidative stress in BNL CL.2 cells. In this study, we used TPA, which has been employed as an oxidative stress inducer [15, 28] to induce oxidative hepatotoxicity *in vitro*. The results showed that TPA reduced cell viability in BNL CL.2 cells, as evidenced by the leakage of cytosolic LDH (Fig. 1). LDH is a stable cytoplasmic enzyme present in all cells, and it is rapidly released into the cell culture medium upon damage of the plasma membrane. Such efflux of LDH from cells has been used as a manifestation of cellular injury, and it is correlated with an increase in the permeability of plasma membrane [7]. Therefore, an increase in the number of dead or plasma membrane-damaged cells like TPA-exposed BNL CL.2 cells results in an increase in LDH activity in the culture medium. Sup-

plementation of UDN glycoprotein in the presence of TPA, however, significantly augmented cell viability. Furthermore, the ROS level increased by TPA was considerably attenuated by addition of UDN glycoprotein (200  $\mu$ g/ml), indicating protective effect of UDN glycoprotein on TPA-induced cell death *via* oxidative damage caused by intracellular ROS. This means that UDN glycoprotein has cytoprotective abilities and can protect cells from endogenous oxidant injury induced by TPA and against the associated cell injury. The results have shown that UDN glycoprotein scavenges the intracellular ROS generated by TPA (Fig. 2).

Since TPA acts as a promoter of the iNOS expression in activated macrophages and hepatocytes [14], the level of NO production in this study was also dose-dependently increased in TPA-induced BNL CL.2 cells (Fig. 3). However, the exact mechanism of TPA-mediated regulation of the expression of iNOS is unknown. In inflammatory processes of the liver, such as chronic viral hepatitis, hepatocarcinoma and septic shock, NO and superoxide anion react together at a diffusion control rate to yield peroxynitrite, which is known to inflict cellular injury [13]. This generated peroxynitrite is highly toxic, and attenuates antioxidative enzyme activities including Mn- and Fe-containing SOD and aconitase of mitochondria in the liver. Therefore, it appears to induce lipid peroxidation in various models including multiple sclerosis and encephalomyelitis [13, 26]. In general, such reactions are significant only when NO generated by the induction of NOS is high. Thus, the observed inhibition of NO production by UDN glycoprotein suggests that it may be useful in reducing chronic inflammation and restoring depleted antioxidant machinery.

In addition, treatment with UDN glycoprotein checked the reduction in increased TBARS level in TPA-induced BNL CL.2 cells, compared to the TPA treatment alone. Determining lipid peroxidation as TBARS levels provides an indirect measurement of antioxidant deficit. In particular, polyunsaturated fatty acids located in cellular membranes and in blood are highly prone to attack, leading to the generation of lipid peroxides [5]. Such lipid peroxidation is an autocatalytic process, which is a common cause of cell injury. Therefore, inhibition of lipid peroxidation has been used as an important index of antioxidant activity. From our results, it was verified that UDN glycoprotein has a potential to inhibit lipid peroxidation (Fig. 4).

Several studies reported that ROS are important in signal transduction and that an alteration in ROS flux and, in turn, the extracellular redox status can exert various effects on signal transduction pathways responsible for the maintenance and regulation of cellular function [8, 11]. Notably, NF- $\kappa$ B is constitutively existent as an inactive cytoplasmic heterodimeric complex that can be activated by proper stimuli including ROS, and leading to the dissociation of NF- $\kappa$ B from cytoplasmic I $\kappa$ B complex, phosphorylation, and subsequently ubiquitination of I $\kappa$ B upon exposure of the cell to various extracellular stimuli causing rapid degradation of this inhibitory subunit by proteasome [1]. The activated NF- $\kappa$ B is then able to translocate into the nucleus, where it binds to specific DNA sequence, thereby controlling their expression. In this study, TPA-induced NF- $\kappa$ B activation was effectively inhibited upon increasing concentrations of UDN glycoprotein (Fig. 5). This suggests that formation of intracellular ROS by TPA is responsible for the cellular injury. Such ROS not only attack cellular components including DNA, membrane phospholipids and protein SH groups, but also cause NF- $\kappa$ B activation. The mechanism by which NF- $\kappa$ B activation promotes TPA-induced cell injury is unknown. It is maybe due to the fact that NF- $\kappa$ B activation promotes some forms of TPA-induced oxidative stress. One possible explanation of activation of NF- $\kappa$ B is that NO may have elevating ability in this model. Activation of NF- $\kappa$ B regulates iNOS gene expression in the upstream signal pathway, leading to NO production. The increased NO may be detrimental during oxidative stress-induced cell injury because of the ability of NO to be converted to toxic peroxynitrite in the presence of superoxide anion [3]. Therefore, we speculate that inhibition of TPA-induced activation by UDN glycoprotein is associated with its antioxidant activity.

In conclusion, results of this study have shown that UDN glycoprotein protects cells from injury through reduction of intracellular ROS stimulated by TPA, which leads to NO production, lipid peroxidation, and NF- $\kappa$ B activation in BNL CL.2 cells. Further research must be carried out to elucidate the protective mechanism of signal transduction pathway at gene expression level.

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