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Effects of nanoparticulate saponin-platinum conjugates on 2,4-dinitrofluorobenzene-induced macrophage inflammatory protein-2 gene expression via reactive oxygen species production in RAW 264.7 cells

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Nanoparticulate platinum (II) (nano Pt) is a powerful antioxidant that is widely used to scavenge reactive oxygen species (ROS). The antioxidant activity of nano Pt has gained attention as a potentially useful therapeutic for a variety of diseases including cancer and aging. In the present study, we prepared nanoparticulate saponin-Pt (II) (nano saponin-Pt) conjugates using the ethanol reduction method to enhance the permeability and retention effect of Pt. The nano saponin-Pt conjugates were found to restore the viability of approximately 40% of 2,4-dinitrofluorobenzene (DNFB)-treated RAW 264.7 cells. In addition, we found that nano saponin-Pt conjugates acted as a potent antioxidant that reduced the production of ROS and inhibited activation of the MAP kinase pathway and MIP-2 gene expression in response to DNFB. These results provide insight into the potential usefulness of nano saponin-Pt conjugates as a treatment for contact hypersensitivity. [BMB reports 2009; 42(5): 304-309]

INTRODUCTION

It is well known that the antioxidant activity of metal ions can be clinically useful for the treatment of cancers (1). Reactive oxygen species (ROS) produced in metabolic pathways can oxidize biological macromolecules such as DNA, proteins, and lipids, which can lead to the induction of cancer, aging, and inflammatory diseases including atopic dermatitis (2-5). Accordingly, several studies have evaluated antioxidant defense systems in search of effective therapeutics for cancers, aging, and inflammatory diseases.

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Platinum complexes are powerful antioxidants that are widely used to scavenge ROS (1, 6), and the antioxidant activity of platinum complexes has recently gained attention due to their potential for the treatment of a variety of cancers. Cisplatin, *cis*-diamminedichloro-platinum (II), which is a typical example of a potent antioxidant, is the most widely used anticancer drug (1, 7, 8). Additionally, several studies have been conducted to design platinum (II) polymeric conjugates that enhance the circulation time, targeting, and release of platinum (9-11).

The skin sensitizer, 2,4-dinitrofluorobenzene (DNFB), provokes delayed hypersensitivity responses as a result of topical application to the skin (12). DNFB stimulation results in the expression of major histocompatibility (MHC) class II molecules and costimulatory molecules such as CD40, CD54, CD83, and CD86 in dendritic cells (13-15). In addition, the exposure of skin-derived dendritic cells to DNFB induces activation of the p38 MAP kinase and ERK1/2 in the regulation of CD40 expression (5). In a previous study, we found that the production of ROS was associated with activation of the MIP-2 gene promoter by DNFB, and that this occurred via activation of the p38 MAP kinase and ERK1/2 pathways (16, 17).

In the present study, we prepared nanoparticulate saponin extracts-Pt (II) (nano saponin-Pt) conjugates using the ethanol reduction method to enhance the permeability and retention effect of Pt. Because nano Pt conjugates are known to act as reductive catalysts, Pt can be used as an antioxidant to reduce ROS in living cells. Here, we show that nano saponin-Pt conjugates acted as potent antioxidants that scavenged ROS and reduced MIP-2 gene expression in DNFB-stimulated RAW 264.7 cells.

RESULTS

Effect of nano saponin-Pt conjugates on DNFB-induced MIP-2 gene expression

Saponin extracts of ginseng are capable of stimulating the immune response such as enhancement of phagocytic capacity and

induction of cytokines (18). We first identified induction of the endogenous MIP-2 gene expression in RAW 264.7 macrophage cell line by saponin extracts treatment. As shown in Fig. 1, saponin extracts greatly enhanced MIP-2 mRNA expression in a time (Fig. 1A) and dose-dependent (Fig. 1B) manner. However, treatment of nano saponin-Pt conjugates at a concentration lower than 5 μg/ml failed to induce MIP-2 mRNA expression (Fig. 1A). When we treated the cells with nano saponin-Pt conjugates at a concentration of 2.5 μg/ml for longer time up to 12 h, expression of MIP-2 was not induced (Fig. 1B).

In the previous data (17), we have showed that DNFB-induced intracellular ROS production is necessary for MIP-2 gene expression by DNFB. Platinum nanoparticles, known to be a potent antioxidant, are widely tried as therapeutics such as anticancer drugs (6). To explore the effect of nano saponin-Pt conjugates on DNFB-induced MIP-2 gene expression, we investigated the MIP-2 gene expression in the presence of nano saponin-Pt conjugate by means of RT-PCR. As we previously reported, treatment with DNFB greatly enhanced the MIP-2 mRNA expression in RAW 264.7 cells. The DNFB-induced MIP-2 gene expression was dramatically reduced in the presence of nano saponin-Pt conjugates, whereas the saponin extracts were ineffective in suppressing the gene expression (Fig. 1C). These results indicate that MIP-2 gene expression is regulated by the nano saponin-Pt conjugates in the DNFB-treated RAW 264.7 cells.

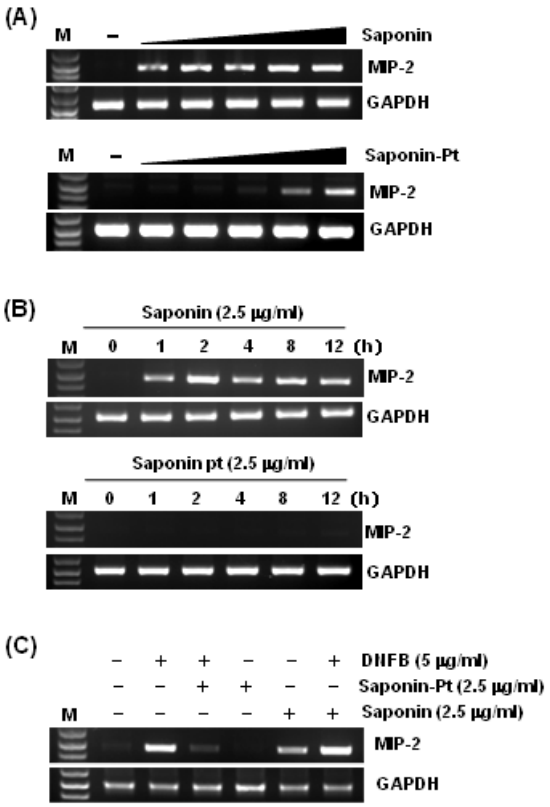

Fig. 1. Effects of nano saponin-Pt conjugates on DNFB-induced MIP-2 expression in RAW 264.7 cells. RAW 264.7 cells were treated with saponin and nano saponin-Pt conjugates (0.1, 0.5, 2.5, 5, 25 μg/ml) for 1 h (A) and the indicated times (B). RAW 264.7 cells were incubated in the absence or presence of saponin (2.5 μg/ml) or nano saponin-Pt conjugates (2.5 μg/ml) for 1 h and then treated with DNFB (5 μg/ml) for 2 h (C). After extraction of the total RNA, the expression of the MIP-2 gene was analyzed by RT-PCR analysis. M denotes DNA standard marker.

Nano saponin-Pt conjugates blockage of DNFB-induced ROS production

To examine whether DNFB-induced ROS production is modulated by nano saponin-Pt conjugates pretreatment, the production of ROS in the cells was measured with the oxidation-sensitive dye dihydrorhodamine 123 by means of confocal image analysis. The ROS was detected in DNFB-treated RAW 264.7 cells and pretreatment with NAC resulted in a significant decrease of dihydrorhodamine fluorescence as described previously (Fig. 2) (17). 2.5 μg/ml of saponin extracts induced ROS production in RAW64.7 cells but nano saponin-Pt conjugates did not. However, pretreatment of the cells with nano saponin-Pt conjugates clearly reduced ROS production in response to DNFB.

Inhibition of DNFB-induced ERK1/2 activation, p38 MAP kinase activation by nano saponin-Pt conjugates

In our previous results, MIP-2 gene expression appears to be regulated by the ERK1/2 and p38 kinase pathways which are dependent on the formation of ROS in the DNFB-stimulated RAW 264.7 cells (16, 17). To explore whether DNFB-induced ERK1/2 and p38 MAP kinase activation is modulated by pretreatment with nano saponin-Pt conjugates, we examined p38 kinase and ERK1/2 phosphorylation by means of Western blotting analysis. The exposure of the RAW 264.7 cells to 5 μg/ml of DNFB resulted in phosphorylation of ERK1/2 and p38 MAP kinase at 30 min and 1 h post-treatment, respectively. Pretreatment with NAC resulted in a significant decrease of ERK1/2 and p38 MAP kinase as described previously (Fig. 3) (17). 2.5 μg/ml of saponin extracts alone induced activation of ERK1/2 and p38 MAP kinase in RAW64.7 cells, but nano saponin-Pt conjugates did not influence on MAP kinase activation. However, when the cells were treated with DNFB in the presence of nano saponin-Pt conjugates, the phosphorylation of ERK1/2 and p38 MAP kinase in response to DNFB was reduced (Fig. 3).

Effect of nano saponin-Pt conjugates on DNFB-induced cell death

DNFB induces cell death via activation of caspase-3 in a dendritic cell line derived from mouse skin (19). Therefore, we investigated the effect of nano saponin-Pt conjugates on DNFB-induced cell death. We analyzed the effect of nano saponin-Pt conjugates on viability of DNFB-treated RAW 264.7 cells using

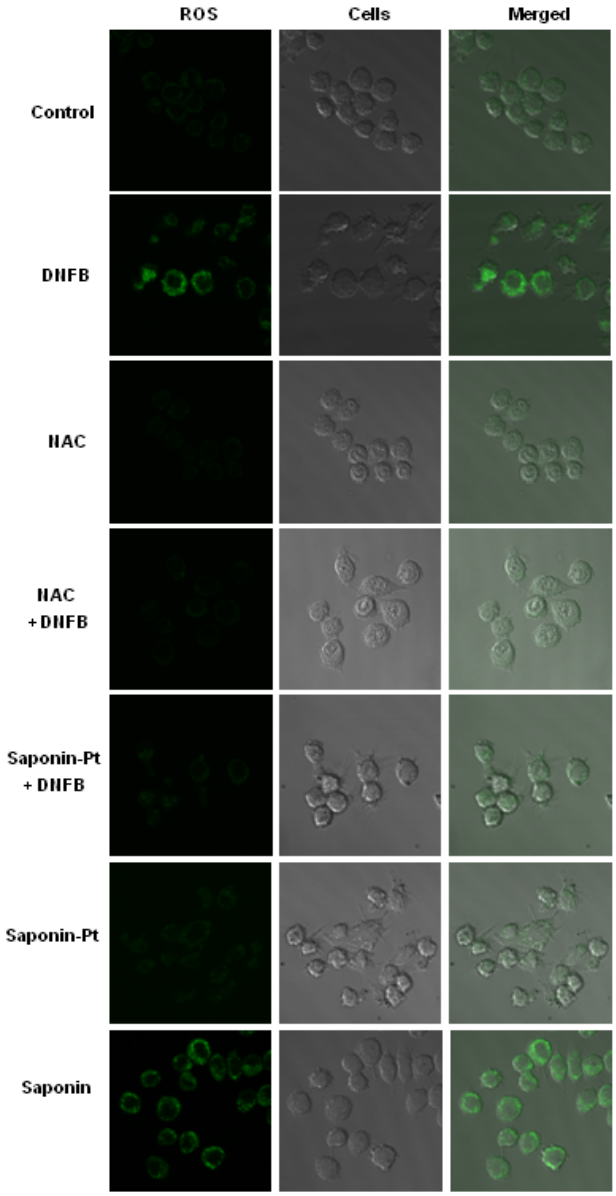

Fig. 2. Effects of nano saponin-Pt conjugates on DNFB-induced intracellular ROS production in RAW 264.7 cells. RAW 264.7 cells were incubated in the absence or presence of saponin (2.5 μg/ml), nano saponin-Pt conjugates (2.5 μg/ml), or NAC (20 mM) for 1 h and then treated with DNFB (5 μg/ml) for 10 min. The intracellular ROS levels were measured after staining with DCF-DA.

MTT assay. As shown in Fig. 4A, the viability of the cells significantly decreased in a time-dependent manner by DNFB treatment. However, the viability of the cells was restored about 40% in the DNFB-treated RAW 264.7 cells in the presence of nano saponin-Pt conjugates. Additionally, we investigated DNFB-induced DNA cleavage in RAW 264.7 cells by TUNEL

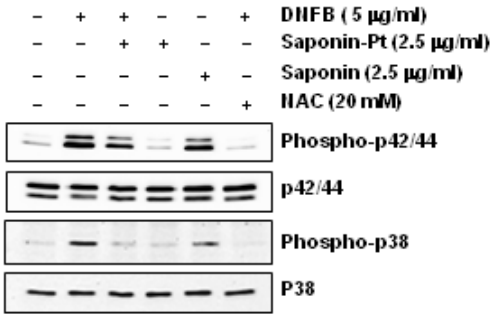

Fig. 3. Effects of nano saponin-Pt conjugates on DNFB-induced activation of ERK1/2 and p38 kinase. RAW 264.7 cells were incubated in the absence or presence of saponin (2.5 μg/ml), nano saponin-Pt conjugates (2.5 μg/ml), or NAC (20 mM) for 1 h and then treated with DNFB (5 μg/ml) to induce the activation of ERK1/2 and p38 MAP kinase at 30 min and 1 h post-treatment, respectively. The cell lysates were analyzed by Western blotting with phospho-specific antibodies to ERK1/2 (Thr202/Tyr204) or p38 (Thr180/Tyr182). The amounts of ERK1/2 and p38 are shown in the bottom panel for each pair.

staining using fluorescence microscopy within 12 h after DNFB treatment. As shown in Fig. 4B, TUNEL-positive cells were detected in the DNFB-treated RAW 264.7 cells. When the cells were treated with DNFB in the presence of nano saponin-Pt conjugates, TUNEL-positive cells were reduced as detected in NAC-pretreated cells. These results suggest that nano saponin-Pt conjugates acted as a potent antioxidant which reduced ROS in the DNFB-stimulated RAW 264.7 cells.

DISCUSSION

DNFB is an electrophilic compound that belongs to a large group of dinitrohalobenzenes in which the chloride can be replaced by fluoride, chloride, or iodide to form DNFB, DNCB, or DNIB, respectively, each of which exerts similar biological effects. Specifically, when dinitrohalobenzene compounds are topically applied to the skin they preferentially induce a Th2 response that leads to increased levels of IL-4, IL-10, and IL-13 and relatively low amounts of Th1-type cytokines such as IFN-γ (20, 21). As a result, dinitrohalobenzene compounds are often used as model haptens to evoke contact hypersensitivity reactions in a well established atopic dermatitis (AD) mouse model.

DNFB induces the production of ROS, which acts as an intracellular second messenger that leads to biological responses (5, 17). In a previous study, we examined the effects of modification of DNP-proteins on skin and the DNFB-treated macrophage cell line RAW 264.7. The results of that study suggested that the DNP-modification of cellular proteins is a key reaction in DNFB-induced MIP-2 expression. Furthermore, the results of that study indicated that the production of ROS is associated with activation of the MIP-2 gene promoter by DNFB, which occurs via activation of the p38 MAP kinase and ERK1/2 pathways (17).

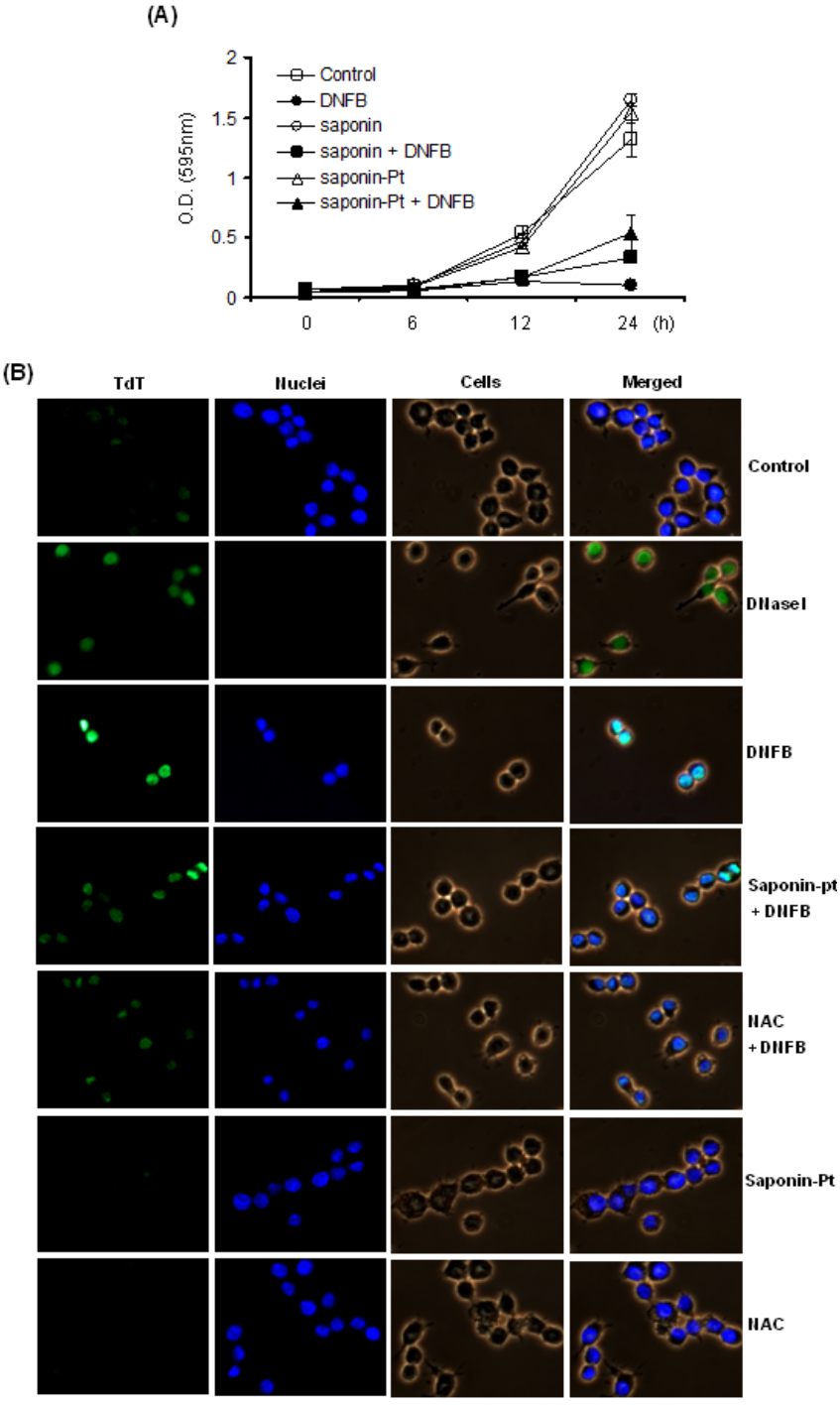

Fig. 4. Effects of nano saponin-Pt conjugates on the cell viability and cell death of DNFB-treated RAW 264.7 cells. (A) RAW 264.7 cells were incubated in the absence or presence of saponin (2.5 μg/ml) or nano saponin-Pt conjugates (2.5 μg/ml) for 1 h and then treated with DNFB (5 μg/ml) for the indicated time periods. The cell viability was then measured by MTT after the indicated time periods. (B) The cells were incubated in the absence or presence of saponin (2.5 μg/ml) or nano saponin-Pt conjugates (2.5 μg/ml) for 1 h and then treated with DNFB (5 μg/ml) for 12 h. TUNEL assays were performed as described in the Materials and Methods. The DNase I treatment served as a control of TUNEL-positive cells.

In the present study, we demonstrated that MIP-2 gene expression is regulated by nano saponin-Pt pretreatment in DNFB-treated RAW 264.7 cells. We also observed a significant reduction of ROS production and ERK1/2 and p38 MAP kinase activation in cells that were pretreated with nano saponin-Pt conjugates. Furthermore, the viability of the DNFB-treated cells was restored to approximately 40% by pretreatment with the nano saponin-Pt conjugates. Taken togeth-

er, these results suggest that the nano saponin-Pt conjugates acted as potent antioxidants for the reduction of ROS in DNFB-stimulated RAW 264.7 cells and provide insight into the potential for the use of nano saponin-Pt conjugates to treat contact hypersensitivity.

MATERIALS AND METHODS

Reagents

DNFB and N-acetyl-cysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). When the NAC was used, the cells were preincubated with the chemicals for 1 h before treatment with DNFB. Antibodies to p38, phosphor-p38 (Thr180/Tyr182), ERK1/2, and phosphor-ERK1/2 (Thr202/ Tyr204) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Preparation of nanoparticulate saponin-Pt conjugates

Nanoparticulate saponin-Pt was prepared by the ethanol reduction method using hydrogen hexachloroplatinate (H_2PtCl_6) as described previously (22). Briefly, 0.5 g of saponin extracts (*Ginseng panax* C.A.Meyer) was dissolved in water (1 L) and stirred for 10 min. Then, 0.5 g of H_2PtCl_6 (Sigma) was added and stirred for 10 min before the mixtures were stirred for an additional 1 h in reducing agent ($NaBH_4$). After the mixtures were centrifuged at 12,000 rpm for 30 min, the precipitates containing nano saponin-Pt were resolved in water. In the following experiments, we used this nano saponin-Pt after filtered with 20 μM pore size. The nanoparticulate particle size (2-4 nanometers) was qualified by a transmission electron microscope (JEM-2010).

Cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under a humidified atmosphere of 95% air and 5% CO_2. The cell cultures were maintained until passage 20 and then discarded.

Cell viability assay (MTT assay)

The cell growth and viability against DNFB, saponin extracts, or nano saponin-Pt conjugates on RAW 264.7 cells was determined by MTT assay as described previously (23). RAW 264.7 cells were seeded in 48-well plates and treated with 5 mg/ml DNFB with or without saponin extracts (2.5 μg/ml) or nano saponon-pt conjugates (2.5 μg/ml) for 24 h. After cells were treated with DNFB for the indicated times, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) solution were added to each well, and the plates were incubated for an additional 4 h at 37°C. After removal of the medium, the formazan crystals were solubilized in DMSO. The color development was qualified by a spectrophotometer at 570 nm with a reference wavelength of 650 nm.

RT-PCR analysis

After treating the cells with DNFB and/or saponon extracts, nano saponin-Pt conjugates for the indicated time periods, total RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Six micrograms of total RNA was reverse-transcribed in the first-strand synthesis buffer containing 6 μg/ml of oligo (dT) primer, 50U of reverse transcriptase, 4 mM of dNTP, and 40U of RNase inhibitor. One microliter of the cDNA mixture was subjected to the standard PCR reaction for 25 cycles using the following primer sets: MIP-2, 5′-TGGGTGGGATGTAGCTAGTTCC-3′ (sense), 5′-AGTTTGCC TTGACCCTGAAGCC-3′ (anti-sense); mouse GAPDH, 5′-ATGG TGAAGGTCGGTGTGAACG-3′ (sense), 5′-GTTGTCATGGATG ATCTTGGCC-3′ (anti-sense). PCR products were visualized with UV light after resolving on 1.2% agarose gel.

Western blotting

We performed SDS-PAGE and Western blot analysis as described previously (24). After we pretreated the cells with saponin extracts, nano saponin-Pt-complex, or NAC for 1 h and then treated them with DNFB (5 μg/ml) for 2 h, we harvested cells and lysed them in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 1% NP-40, and Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). Immunoreactive proteins against anti-p38, anti-phosphor-p38 (Thr180/ Tyr182), anti-ERK1/2, anti-phosphor-ERK1/2 (Thr202/Tyr204) antibodies were detected by horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology Inc, Beverly, MA, USA) and an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Measurement of intracellular ROS levels

2′,7′-Dihydrorhodamin (Invitrogen, USA) was used as an oxidation-sensitive fluorescent probe for analysis of intracellular ROS levels as described manufacturer's specifications. RAW 264.7 cells were maintained in phenol red-free media for 8 h. To verify the effect of NAC, Saponin extracts and nano saponin-Pt conjugates on the DNFB-induced ROS production, we pretreated the cells with these materials for 1 h and then treated them with DNFB (5 μg/ml) for 10 min. After incubation, the cells were fixed with 4% paraformaldehyde (USB Corp., Cleveland, OH, USA) for 10 min. The cells were subsequently transferred to coverslips and mounted in Fluoromount-G (Southern Biotechnology Associates Inc, Birmingham. AL, USA). The samples were scanned with a Zeiss LSM 510 Meta laser scanning confocal device (Carl Zeiss, Germany).

Apoptosis detection (TUNEL assay)

We performed terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay in accordance with the manufacturer's specifications. RAW 264.7 cells were placed in a complete medium for 24 h before being treated with DNFB (5 μg/ml) for 12 h and/or pretreated with saponin, saponin-pt com-

plex, NAC for 1 h as indicated in the individual experiments. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 5 min at room temperature, and end-labeled with TdT using the DeadEnd™ Fluorometric TUNEL system (Promega, Madison, WI, USA). DNA staining (0.5 μg/ml Hoechst No. 33258; Sigma) was used to identify cell nuclei. The DNase I treatment served as a control of TUNEL-positive cells. After washing with PBS-T, cells were observed with fluorescence microscopy (Carl Zeiss, Germany).

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