Notopterygium forbesii Boiss Extract and Its Active Constituent Phenethyl Ferulate Attenuate Pro-Inflammatory Responses to Lipopolysaccharide in RAW 264.7 Macrophages. A "Protective" Role for Oxidative Stress?

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Received May 11, 2009

Oxidative stress and oxidative modification of biomolecules are involved in several physiological and pathophysiological processes. We have previously reported that *Notopterygium forbesii* Boiss (NF), a traditional Chinese medicine, and its active constituents, including phenethyl ferulate (PF), bergaptol, and isoimperatorin, induced oxidative stress with increased levels of reactive species and heme oxygenase-1 in human fetal hepatocytes. The current study determined the effects of NF and PF on the inflammatory effects of lipopolysaccharide (LPS). Exposure of RAW 264.7 macrophages to LPS increased the expression of inducible nitric oxide synthase and cyclooxygenase 2 and stimulated the formation of reactive nitrogen species. In a coculture system, the LPS-activated macrophages also induced expression of cell adhesion molecules (including E-selectin, intercellular cell adhesion molecule 1, and vascular cell adhesion molecule 1) in human umbilical vein endothelial cells (HUVEC) and vascular smooth muscle cells (VSMC). Preincubation of macrophages with NF or PF attenuated the effects of LPS on macrophages as well as their effects on HUVEC and VSMC. These inhibitory effects of NF and PF were decreased in the presence of *N*-acetyl-L-cysteine (NAC). At the same time, NAC also reduced NF- or PF-induced increases in reactive oxygen species (ROS) and Hsp32 protein levels and the formation of protein carbonyls in the macrophages. These results suggest that NF- or PF-induced ROS generation and oxidative modifications of intracellular proteins may be responsible for the inhibitory actions of NF and PF on LPS-induced inflammatory responses. These data add to the growing literature that ROS may sometimes be anti-inflammatory.

Introduction

Oxidative stress is defined as an imbalance between production of reactive species (RS)[1] and antioxidant defense systems (*1*). RS, including oxygen, nitrogen, and chlorine species, depending on their reactivity, can react with cellular components and damage them. Proteins are a major target of oxidative damage (*2*). Damaged proteins, if not repaired or removed promptly, can interfere with normal cell function and viability. One way to remove oxidized proteins is by the ubiquitin proteasome system (*3, 4*).

Cell adhesion molecules (CAMs) such as E-selectin, intercellular cell adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) are expressed in endothelial cells, and VCAM-1 is also expressed in vascular smooth muscle cells (VSMC) (*5*). CAMs are responsible for cell−cell interaction including the "rolling" of the leukocytes along the endothelium as well as their adherence and extravasation. The expression levels of CAMs increase in cells at sites of inflammation and are under the regulation of transcription factors such as nuclear factor κ B (NF-κB), activator protein 1, and peroxisome proliferators-activated receptor (*6*).

Notopterygium forbesii Boiss (NF), a plant belonging to the Umbelliferae family, is known as *Qianghuo* in Chinese. It has been studied as part of our ongoing search for biologically active agents from medicinal plants. The rhizomes and roots of NF have been used in traditional Chinese medicine as crude drugs or in at least 13 prescriptions for the treatment of common cold, rheumatism, and headache (*7*). Moreover, this plant has been reported to possess anti-inflammatory (*8*), diaphoretic, and analgesic (*9*) properties.

We have previously reported that NF and its active constituents, including phenethyl ferulate (PF), bergaptol, and isoimperatorin, induced oxidative stress and rises in heme oxygenase-1 (HO-1/Hsp32) proteins in human fetal hepatocytes (*10*). The current study was undertaken to determine the effects of NF and PF on the pro-inflammatory effects of lipopolysaccharide (LPS), to see if this might help explain some of the beneficial pharmacological effects of NF and PF. For this, we utilized LPS-sensitive RAW 264.7 murine macrophages in a coculture system with human umbilical vein endothelial cells (HUVEC) and VSMC.

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[1] RS, reactive species; CAMs, cell adhesion molecules; ICAM-1, intercellular cell adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; VSMC, vascular smooth muscle cells; NF, *Notopterygium forbesii* Boiss; PF, phenethyl ferulate; LPS, lipopolysaccharide; HUVEC, human umbilical vein endothelial cells; DAF-FM DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DHE, dihydroethidium; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; FBS, fetal bovine serum; PSA, penicillin, streptomycin, amphotericin; RNS, reactive nitrogen species; EBSS, Earle's balanced salt solution; ROS, reactive oxygen species; DNPH, 2,4-dinitrophenylhydrazine; SDS, sodium lauryl sulfate; DTT, dithiothreitol; IGEPAL, polyoxyethylene nonylphenol; Hsp, heat shock protein; NAC, *N*-acetyl-L-cysteine.

10.1021/tx900163h CCC: $40.75
Published on Web 07/31/2009

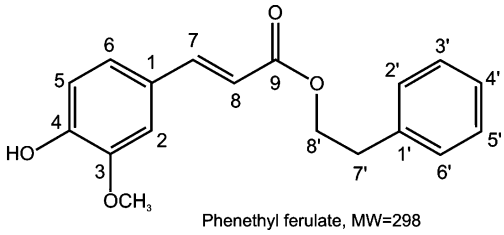

Figure 1. Chemical structure of PF.

Experimental Procedures

Materials. All reagents used including LPSs from *Escherichia coli* 055: B5 (Catalog No. L2880) were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. 4-Amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA), dihydroethidium (DHE), and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent dyes were purchased from Molecular Probes (Eugene, OR). Antibodies against E-selectin, ICAM-1, VCAM-1, Hsp32, Hsp70, and Hsp90 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), as well as monoclonal antibodies used for immunoprecipitation assay, NF-κB p50 (E10), and NF-κB p52 (C-5). Anti-iNOS (inducible nitric oxide synthase) and anti-COX-2 (cyclooxygenase 2) antibodies were purchased from BD Biosciences (San Jose, CA). Antibodies against p100/p52, p105/p50, p65, and RelB were obtained from Cell Signaling Technology (Danvers, MA). Anti-DNP was purchased from Bethyl Laboratories (Montgomery, TX). Fetal bovine serum (FBS) was from PAA Laboratories GmbH (PAA-Strasse 1, Pasching). HUVEC were obtained from Cell Applications, Inc. (San Diego, CA). VSMC (T/G HA) and RAW 264.7 macrophages were obtained from American Type Culture Collection (ATCC, Rockville, MD). Dynabeads Protein G and Trizol reagent were purchased from Invitrogen Corp. (Carlsbad, CA). K-LISA IKKβ Inhibitor Screening Kit (Catalog No. CBA044) was purchased from Calbiochem (San Diego, CA). A superoxide dismutase assay kit (Catalog No. 706002) was purchased from Cayman Chemical (Ann Arbor, MI).

Extraction and Validation of NF. NF (rhizomes) purchased from a local medicinal shop (Tiang Soon Tong Medical Hall, Dover, Singapore) were validated and extracted as described in ref *10*. The concentrated extract was kept at −20 °C and was dissolved in dimethyl sulfoxide (DMSO) before use. The final concentration of DMSO in all assays was kept below 0.05% (unless otherwise stated) and had no effect on the assays.

Purification and Identification of PF from NF. PF was purified and identified as described in ref *10*. The fractions were tested on the macrophages for their ability to inhibit LPS-induced up-regulation of iNOS and COX-2 proteins. The yield of NF was estimated to be about 320 mg/g of dried raw material according to our laboratory extraction protocol (*10*). The NF extract contains approximately 0.92% (w/w) PF. Two other compounds purified and identified from NF, bergaptol and isoimperatorin, did not significantly inhibit the effect of LPS on RAW 264.7 macrophages (data not shown). Therefore, only PF was investigated in this study. The chemical structure of PF is shown in Figure 1.

Cell Culture. HUVEC were cultured in Ham's F12K medium supplemented with 0.1 mg/mL heparin, 0.03 mg/mL endothelial cell growth supplement (ECGS), and 10% (v/v) FBS. VSMC were cultured in Ham's F12K medium supplemented with 0.05 mg/mL ascorbic acid, 0.01 mg/mL insulin, 0.01 mg/mL transferrin, 10 ng/mL sodium selenite, 0.03 mg/mL ECGS, 10% (v/v) FBS, 10 mM HEPES, and 10 mM TES. Our group has previously reported that some phenolic compounds were less reactive with Ham's F12 medium (*11*). RAW 264.7 cells, a murine line of monocyte-macrophages, were cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12K medium in the ratio of 1:1 supplemented with 1% (v/v) penicillin, streptomycin, and amphotericin (PSA) and 10% (v/v) FBS. For further confirmation of lack of oxidation of NF and PF, we also measured H_2O_2 in the combined medium by Fox 2 assay (*12*). All cells were grown in a humidified incubator with 5% CO_2 at 37 °C. All cells were maintained in the logarithmic growth phase by routine passage every 3−4 (RAW 264.7 macrophages) or 7−10 days (HUVEC and VSMC).

Coculture Experiment with LPS-Activated RAW 264.7 Macrophages. RAW 264.7 macrophages in medium were seeded overnight at a density of 2.5×10^5 cells per well in 0.4 μm pore size six well cell culture inserts (ThinCert, Greiner, Monroe, NC). Cells at 80% confluency were exposed to LPS (1 μg/mL) for 1 h after pretreatment with NF (5 and 10 μg/mL) or PF (2.5 and 5 μg/mL) for 12 h. Media containing LPS were removed, and cells were washed twice with phosphate-buffered saline (PBS) to remove the remaining LPS. The macrophages in the inserts were transferred onto the top of HUVEC or VSMC wells in direct contact with Ham's F12K medium and then cocultured for 20 h at 37 °C. Because no pharmacokinetic data regarding NF and PF in humans are available in the literature, the concentrations of NF and PF used in this study were based on a dose−response effect in RAW 264.7 macrophages.

Measurement of Intracellular Reactive Nitrogen Species (RNS) with DAF-FM DA Fluorescent Probe. RNS were determined with the DAF-FM DA fluorescent dye using an Infinite 200 microplate reader (TECAN, Durham, NC) (*13*). Macrophages in medium were seeded overnight at a density of 2.0×10^5 cells per well in 24 well plates for attachment. Cells at 80% confluency were treated with NF (5 and 10 μg/mL) or PF (2.5 and 5 μg/mL) for 12 h, followed by LPS (1 μg/mL) exposure for 20 h. After they were washed with PBS, the macrophages were incubated with DAF-FM DA fluorescent dye dissolved in Earle's balanced salt solution (EBSS; final concentration, 5 μM, 0.5 mL) for 30 min at 37 °C. Excess DAF-FM DA solution was subsequently removed by washing twice with PBS. Fluorescence at Ex/Em 485/515 nm was measured every 20 min for 1 h at 37 °C.

Kinetic Study of Rise in Reactive Oxygen Species (ROS) with DHE. ROS were determined with DHE fluorescent dye using the Infinite 200 microplate reader (*14*). Macrophages in medium were seeded overnight at a density of 2.0×10^5 cells per well in 24 well plates for attachment. Cells at 80% confluency were washed twice with PBS and preincubated with DHE fluorescent dye dissolved in EBSS (final concentration, 2 μM, 0.5 mL) for 30 min at 37 °C. Excess DHE solution was subsequently removed by washing twice with PBS. For treatment, 5 and 10 μg/mL of NF or 2.5 and 5 μg/mL of DHE dissolved in EBSS were added to cells, and fluorescence was measured immediately at Ex/Em 510/610 nm every 10 min for 30 min at 37 °C.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis. For RT-PCR analysis of mRNA levels, total RNA of macrophages was isolated after treatment using Trizol reagent. RNA (1 μg) samples were converted to single-stranded cDNA by the ImProm-IITM Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instruction, and the resulting cDNA was amplified by PCR using the GoTagGreen Master Mix (Promega). PCR conditions comprised an initial denaturation step at 94 °C for 1.5 min, followed by 30 cycles of denaturation (94 °C for 1.5 min), annealing (55− 65 °C for 1 min), and extension (72 °C for 1.30 min) steps. The final extension step was set at 72 °C for 2 min. The primers used for amplifying iNOS were 5′-AAT GGC AAC ATC AGG TCG GCC ATC ACT-3′ (For) and 5′-GCT GTG TGT CAC AGA AGT CTC GAA CTC-3′ (Rev); for COX-2 were 5′-GGA GAG ACT ATC AAG ATA GT-3′ (For) and 5′-ATG GTC AGT AGA CTT TTA CA-3′ (Rev); and for β-actin were 5′-TCA TGA AGT GTG ACG TTG ACA TCC-3′ (For) and 5′-CCT AGA AGC ATT TGC GGT GCA CGA TG-3′ (Rev). β-Actin was used as an internal control. At the completion of PCR, 5−10 μL of PCR products was electrophoresed in 1% (w/v) agarose gel in the presence of 0.5 μg/mL ethidium bromide. The amplified DNA fragments were visualized using a Kodak Image Station 2000R (Kodak, Rochester, NY).

Determination of Protein Carbonyl Content. Immunoblot detection of carbonyl groups was performed as described in ref *15*. Briefly, 2.5 μL of total cell lysates (20 μg) was incubated for 15 min at room temperature with 2,4-dinitrophenylhydrazine [DNPH; 20 mM in 10% trifluoroacetic acid (TFA), 5 μL] to form the

dinitrophenylhydrazone carbonyl derivate in the presence of 6% (final concentration, w/v) sodium lauryl sulfate (SDS). DNP-derivatized proteins were resolved by a 7.5−10% SDS-PAGE and detected by Western blotting using anti-DNP antibody.

Preparation of Cytosolic and Nuclear Extracts for NF-κB Immunoblot Analysis. Cytosolic and nuclear extracts of the macrophages were prepared according to the supplier's protocol (Panomics Inc., Redwood City, CA). Briefly, the macrophages in T75 cm^2 flasks at 80% confluency were pretreated with NF (10 μg/mL) or PF (2.5 μg/mL) NF for 12 h prior to LPS (1 μg/ mL) challenge for 1 h. The cells were washed with PBS to remove excess LPS and incubated for another 20 h. For nuclear separation, the cells were washed twice with PBS and incubated with 1 mL of buffer A mix, containing buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, and 10 mM EDTA, 1 mL), 100 mM dithiothreitol (DTT, 10 μL), protease inhibitor cocktail (10 μL), and 10% polyoxyethylene nonylphenol (IGEPAL, 40 μL) on a shaker at 200 rpm for 10 min at 4 °C. Cells were scraped with a sterile scraper, and the cytosolic fraction was collected by centrifugation at 15000*g* for 3 min at 4 °C. The remaining pellet was resuspended in 150 μL of buffer B mix containing buffer B (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, and 5% glycerol, 1 mL), protease inhibitor cocktail (1.5 μL), and 100 mM DTT (1.5 μL) followed by vigorous vortexing for 10 s. The tubes were then shaken horizontally on a shaker at 200 rpm for 2 h at 4 °C. The nuclear extract was collected after centrifugation at 15000*g* for 20 min at 4 °C. Both cytosolic and nuclear extracts were kept at −80 °C until analyzed. Western blotting of the cytosolic and nuclear fractions against histone 1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, respectively, showed satisfactory separation of the nuclei from the cytoplasm of the cells.

Immunoprecipitation. To assess whether transcription factors such as p105/p50 and p100/p52 were oxidatively modified, cell lysates treated with NF or PF were immunoprecipitated with NF-κB p50 and NF-κB p52 monoclonal antibodies and immunoblotted with antibody against DNP for protein carbonyls. Briefly, Dynabeads Protein G-Ig complex were formed according to the manufacturer's procedures. Two hundred micrograms of whole cell lysates was then added into the Dynabeads Protein G-Ig complex and incubated for 1 h at 4 °C with gentle rotation. The beads were washed with PBS (3×), and the supernatant was discarded. The beads were resuspended in RIPA buffer for Western blotting as described.

Western Blot Analysis. Cells (0.5×10^6 cells per well in six well plates) treated with various concentrations of NF or PF were harvested and lysed with cell lysis buffer (Cell Signaling Technology). Cells were centrifuged at 20000*g* at 4 °C for 15 min using a desktop centrifuge (Centrifuge 5417C, Eppendorf) to remove unbroken cells, nuclei, mitochondria, and other organelles after incubation on ice for 15 min. The protein concentration of the supernatant was determined with the DC Protein Assay Kit (Bio-Rad, Hercules, CA), and 20−50 μg of protein in loading dye was heated at 95 °C for 5 min before loading into a 7.5−10% SDS-PAGE gel for immunoblotting. The separated proteins were transferred to nitrocellulose membranes (Bio-Rad, 0.2 μm) and probed with antibodies after membrane blocking with nonfat milk (5% in Tris buffer containing 0.05% Tween 20, 1 h) against E-selectin (1:500), ICAM-1 (1:500), VCAM-1 (1:500), Hsp32 (1:1000), Hsp70 (1:2000), Hsp90 (1:2000), iNOS (1:5000), COX-2 (1:400), p100/52 (1:1000), p105/p50 (1:1000), p65 (1:1000), RelB (1:1000), DNP (1:500), histone 1 (1:1000), GAPDH (1:1000), α-tubulin (1:5000), and β-actin (1:2000), followed by the appropriate HRP-conjugated secondary antibodies (1:5000). Detection was performed by enhanced chemiluminescence (Pierce, Rockford, IL) and visualized using the Kodak Image Station 2000R.

Statistical Analysis. Differences between means were evaluated using a one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for multigroup analysis ($^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs LPS, and $^{\$\$}p < 0.01$ vs NF or PF alone considered as significant).

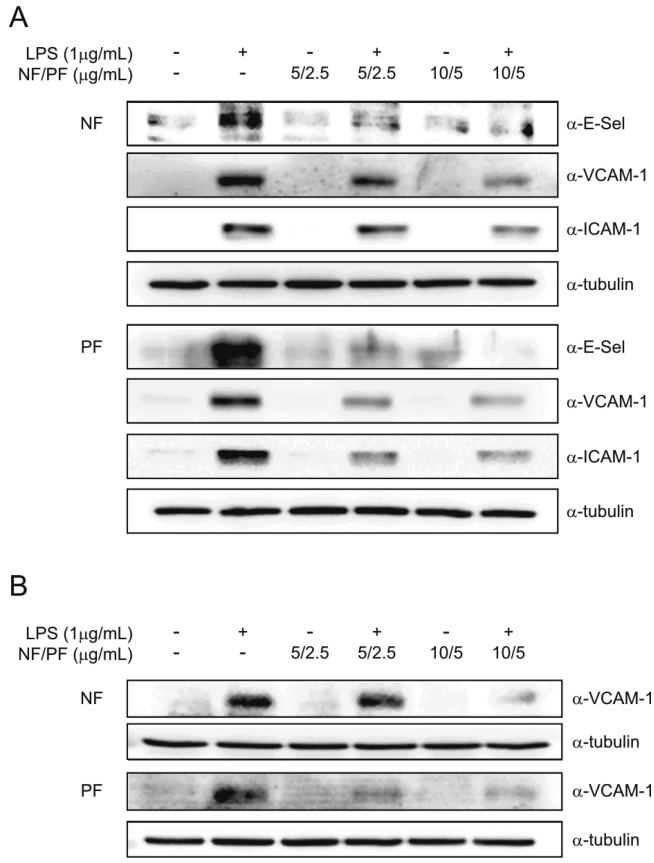

Figure 2. NF and PF attenuate up-regulation of E-selectin, ICAM-1, and VCAM-1 protein levels in HUVEC (A) and VCAM-1 in VSMC (B) induced by LPS-activated macrophages. Macrophages were pre-incubated with NF (5 and 10 μg/mL) or PF (2.5 and 5 μg/mL) for 12 h prior to LPS (1 μg/mL) challenge for 1 h. The macrophages in cell culture inserts following LPS challenge were transferred on to HUVEC and VSMC and cocultured for another 20 h. Total cellular proteins were collected from HUVEC and VSMC for Western blot analyses for E-selectin, ICAM-1, and VCAM-1. α-Tubulin was used to control protein loading. Results are representative of three or more independent experiments.

Results

LPS-Activated Macrophages Induce CAM Proteins in HUVEC and VSMC in a Coculture System: Attenuation by NF and PF. The inflammatory leukocyte-endothelial adhesion process consists of several steps and involves several CAMs such as E-selectin, ICAM-1, and VCAM-1. The expression levels of these CAMs can be induced by cytokines. We used a coculture system to study the interaction of LPS-activated macrophages with HUVEC and VSMC. As shown in Figure 2, macrophages stimulated with LPS (1 μg/mL) for 1 h and washed and transferred on to HUVEC and VSMC for another 20 h were able to induce the expression of E-selectin, ICAM-1, and VCAM-1 proteins in HUVEC (Figure 2A) and VCAM-1 protein in VSMC (Figure 2B). Preincubation of macrophages with NF (5 and 10 μg/mL) or PF (2.5 and 5 μg/mL) for 12 h prior to LPS challenge attenuated the protein levels of E-selectin, ICAM-1, and VCAM-1 induced in HUVEC and VSMC by the activated macrophages. NF showed a dose-dependent attenuation effect on the expression of ICAM-1 and VCAM-1 proteins in HUVEC and VCAM-1 in VSMC. However, both concentrations of PF tested were equally effective in inhibiting the expression of E-selectin, ICAM-1, and VCAM-1 in HUVEC and VCAM-1 in VSMC.

To verify that the effects of NF and PF were not consequences of their potential cytotoxicity, crystal violet assays were

performed to assess cell proliferation of macrophages. NF and PF did not significantly reduce cell proliferation of macrophages ($n = 4$, data not shown) at the concentrations that inhibited the expression of CAMs in HUVEC and VSMC as well as iNOS and COX-2 in the macrophages (see below). Moreover, the protein concentrations, as determined with DC protein assay kit, of HUVEC and VSMC after various treatments did not show significant differences, consistent with no significant effects on cell viabilities.

Effect of NF and PF on LPS-Induced Expression of iNOS and COX-2 mRNA and Protein Levels in the Activated Macrophages. LPS is known to induce the expression of iNOS and COX-2 in macrophages. Once activated, macrophages can produce a significant amount of nitric oxide ($NO^{\bullet}$) over a long period of time. Western blots and RT-PCR analyses were performed to determine whether the inhibitory effects of NF and PF on LPS-mediated responses could be related to the modulation of the expression of iNOS and COX-2 (Figure 3A,B). In resting macrophages, mRNA levels of iNOS and COX-2 were low. Upon stimulation with LPS, both the mRNA and the protein levels of iNOS and COX-2 were markedly increased. Pretreatment with NF (Figure 3A) or PF (Figure 3B) inhibited the increase in iNOS and COX-2 mRNA and protein levels in a concentration-dependent manner. These data suggest that the reduced levels of iNOS and COX-2 proteins observed are due to, at least in part, decreased gene transcription.

Effect of NF and PF on LPS-Induced Increase in RNS in the Activated Macrophages as Determined with DAF-FM DA Fluorescent Dye. To confirm the activity of iNOS, we determined intracellular RNS with DAF-FM DA fluorescent dye. As shown in Figure 3C,D, LPS significantly increased the production of RNS as compared with the control ($p < 0.01$). Pretreatment of cells with NF or PF significantly decreased the levels of RNS as compared with LPS treated cells ($p < 0.01$). NF at 5 and 10 μg/mL were equally effective, but PF showed a dose-dependent decrease in RNS levels. The concentrations of NF and PF used had no quenching effect on DAF-FM DA fluorescent dye (data not shown). Also, NF and PF did not significantly inhibit peroxynitrite-mediated formation of 3-nitrotyrosine as determined by a high-performance liquid chromatography (results not shown), suggesting that NF and PF do not scavenge $NO^{\bullet}$-derived RNS and are acting by decreasing RNS formation.

Effect of NF and PF on LPS-Induced Nuclear Translocation of NF-κB Subunits. NF-κB is a transcription factor that plays an important role in LPS-mediated responses (*16*). We studied translocation of NF-κB subunits after cytosolic and nuclear separation of treated macrophages. NF-κB activation in RAW 264.7 macrophages was observed 60 min after LPS exposure (1 μg/mL, data not shown). However, we incubated HUVEC and VSMC with LPS-activated macrophages in the coculture system for 20 h for the expression of E-selectin, ICAM-1, and VCAM-1 proteins. As shown in Figure 4, LPS induced translocation of p50 and p65 from the cytosol into the nucleus. Preincubation with NF or PF prior to LPS challenge reduced nuclear translocation of p50 but not p65 proteins. However, LPS exposure increased p52 and RelB protein levels in both the cytosolic and the nuclear fractions of the macrophages. Likewise, preincubation with NF and PF attenuated LPS-induced increase in p52 and RelB protein levels.

IκB kinases (IKK) play a major role in the activation and regulation of the transcription factor, NF-κB (*17*). Using a K-LISA IKKβ inhibitor screening kit (Calbiochem, Catalog No. CBA044), both NF (5, 10, 20, and 40 μg/mL) and PF (2.5, 5,

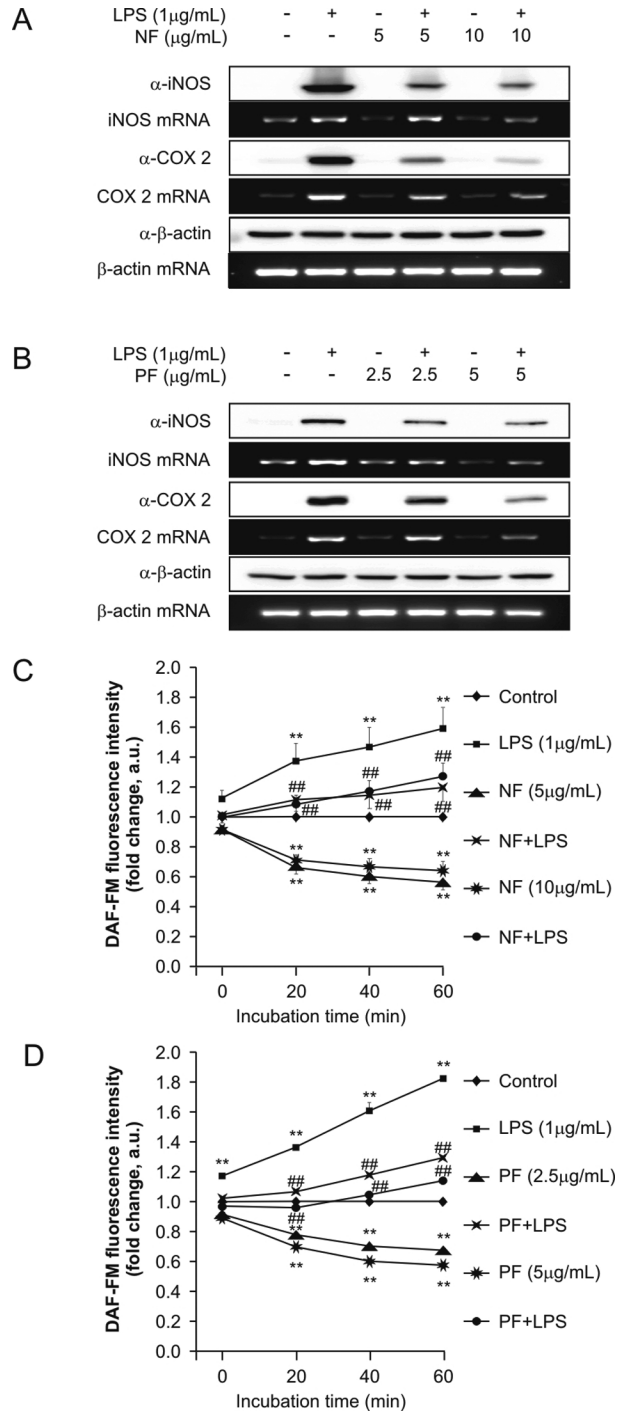

Figure 3. NF and PF attenuate LPS-induced up-regulation of iNOS and COX-2 mRNA and protein levels in macrophages (A and B), as well as $NO^{\bullet}$-derived formation of RNS (C and D). Macrophages were preincubated with NF (5 and 10 μg/mL) or PF (2.5 and 5 μg/mL) for 12 h prior to LPS (1 μg/mL) challenge for 1 h. The macrophages in cell culture inserts following LPS challenge were transferred on to HUVEC and VSMC for another 20 h. Total RNA from the macrophages was extracted with Trizol, and semiquantitative RT-PCR, as described in the Experimental Procedures, was performed to determine iNOS and COX-2 gene expression. Total cellular proteins were collected from the macrophages in a separate experiment for Western blot analyses for iNOS and COX-2. β-Actin was used to control protein loading in every blot performed. Results are representative of at least three independent experiments. (C and D) Macrophages after treatment as described in the Experimental Procedures were incubated with 5 μM DAF-FM DA for 30 min, and fluorescence at Ex/Em 488/515 nm was measured every 20 min for 1 h at 37 °C after excess DAF-FM DA was removed. Data are means ± SDs of three separate experiments performed in four replicates. $^{**}p < 0.01$ vs control, and $^{\#\#}p < 0.01$ vs LPS.

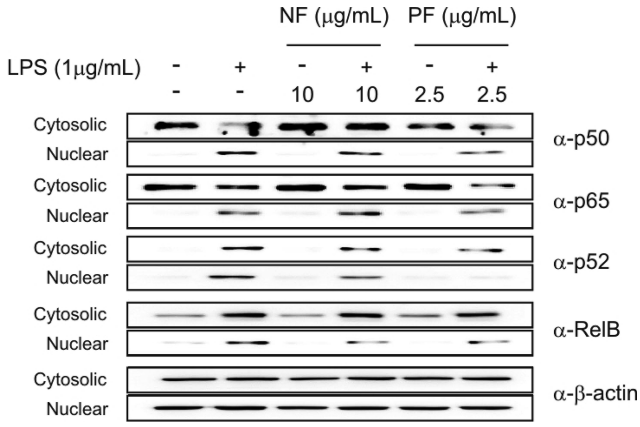

Figure 4. NF and PF attenuate LPS-induced nuclear translocation of NF-κB subunits. Macrophages preincubated with NF (10 μg/mL) or PF (2.5 μg/mL) for 12 h prior to LPS challenge (1 μg/mL for 1 h, washed, and incubated for another 20 h) were collected for nuclear and cytosolic separation as described in the Experimental Procedures. NF-κB subunits including p50, p65, p52, and RelB proteins were analyzed by Western blotting. β-Actin was used to control protein loading. Results are representative of two or more independent experiments.

10, and 20 μg/mL) at the concentrations tested did not significantly inhibit IKKβ activity, suggesting that the inhibitory effects of NF and PF on LPS-induced NF-κB activation in the macrophages were unlikely due to an effect on IKKβ inhibition (data not shown).

NF and PF Induce Rapid Rises in Intracellular Superoxide Anion Radicals. The generation of ROS such as superoxide anion radicals ($O_2^{\bullet-}$) was monitored by the fluorescence emission of DHE. DHE can be oxidized by $O_2^{\bullet-}$ to a fluorescent product, 2-hydroxyethidium, which can intercalate into nuclear DNA and fluoresces strongly at around 610 nm when excited at 510 nm (*14*). As shown in Figure 5A, NF and PF induced rapid generation of $O_2^{\bullet-}$ in the macrophages as determined by the increase in DHE fluorescence. The increase in fluorescence was time-dependent but was the same at both levels of NF or of PF tested. Fluorescence induced by NF and PF was significantly higher 10 min after exposure as compared to the control at both concentrations tested ($p < 0.01$), and the initial increase was more rapid with PF. Both NF and PF had generated similar fluorescence intensities 30 min after exposure. We also used a different probe for ROS, namely, H_2DCFDA. However, NF and PF did not significantly increase DCF fluorescence intensity (data not shown). DCF is known not to respond well to $O_2^{\bullet-}$ or H_2O_2 (*18*).

Superoxide was also measured after LPS treatments. As shown in Figure 5B, macrophages treated with NF or PF alone showed significantly higher DHE fluorescence as compared to control. However, LPS treatment alone showed less DHE fluorescence ($p < 0.05$) than control. In the presence of NF or PF, LPS treatment also significantly reduced the DHE fluorescence as compared to NF or PF alone ($p < 0.01$). This could possibly be explained by an increase in total SOD activity after LPS challenge. Exposure of RAW 264.7 macrophages to NF (5 and 10 μg/mL) or PF (2.5 and 5 μg/mL) for 1, 3, 6, and 12 h did not significantly affect total SOD activity as measured with a superoxide dismutase assay kit (Cayman Chemical, Catalog No. 706002). However, total SOD activity increased significantly after LPS treatment alone (1 μg/mL, 20 h) and in the presence of NF or PF (12 h pretreatment) (data not shown). Similarly, SOD2 but not SOD1 protein levels were also up-regulated by treatment with LPS and remained high in the presence of NF or PF (data not shown).

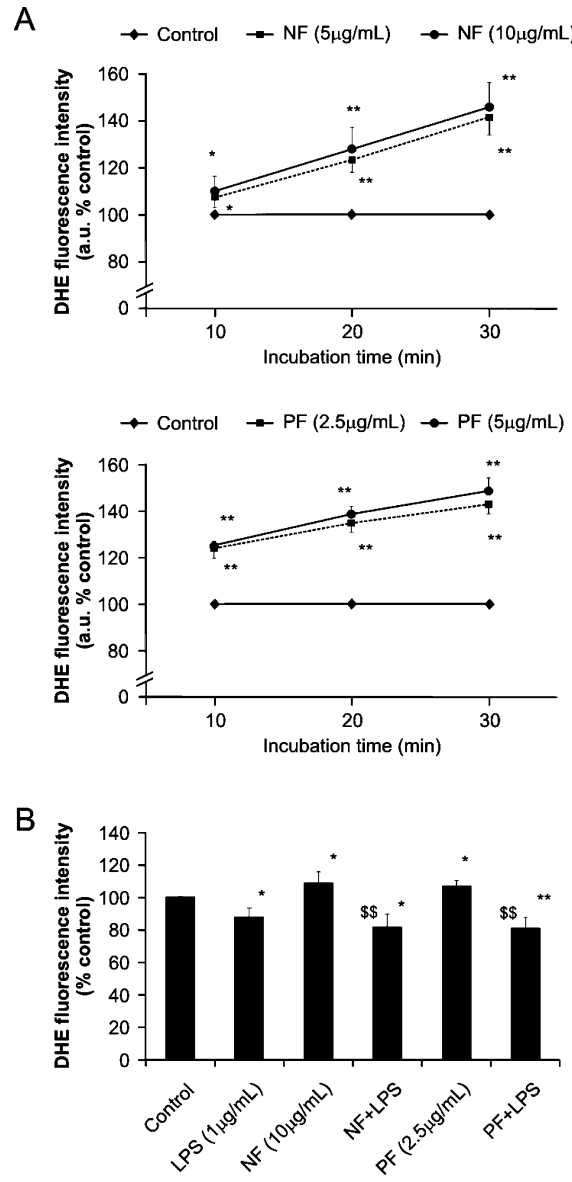

Figure 5. NF- and PF-induced rises in intracellular ROS as determined by DHE. (A) Macrophages were preincubated with 2 μM DHE for 30 min, and measurement at Ex/Em 510/610 nm every 10 min for a total of 30 min at 37 °C was started immediately with 0, 5, and 10 μg/mL of NF or 0, 2.5, and 5 μg/mL of PF after excess DHE was removed. (B) LPS treatment decreased the rise in DHE fluorescence. Macrophages were preincubated with NF (10 μg/mL) or PF (2.5 μg/mL) for 12 h prior to LPS (1 μg/mL) challenge for 1 h, washed, and incubated for another 20 h. Macrophages after challenge were incubated with 2 μM DHE for 30 min, and end point measurement at Ex/Em 510/610 nm was started after excess DHE was removed by washing with PBS. Data are means ± SDs of three separate experiments performed in four replicates. $^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control, and $^{\$\$}p < 0.01$ vs NF or PF alone.

To confirm that NF and PF had not reacted with the cell culture ingredients to generate H_2O_2 [an artifact that can affect many cell culture studies with natural products (*19*)], we measured H_2O_2 in the combined medium by Fox 2 assay (*12*). Incubating NF (20 μg/mL) or PF (5 μg/ mL) in the full culture media (DMEM/Ham's F12 + 1% PSA + 10% FBS) for 30 min at 37 °C did not generate a detectable amount of H_2O_2 in the media (data not shown). Moreover, preincubation of macrophages with catalase (EC 1.11.1.6. 1000 U/mL) for 30 min prior to the stimulation with NF or PF did not affect the increases in DHE fluorescences (results not shown). Hence, the effects of NF and PF in this system were not an artifact of pro-

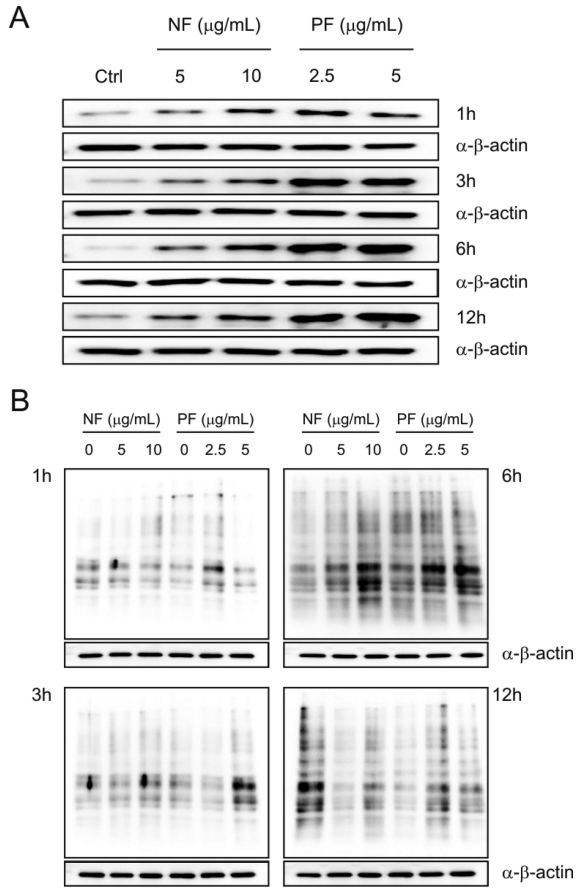

Figure 6. NF- and PF-induced up-regulation of Hsp32 protein levels (A) and accumulation of protein carbonyls (B). Total cellular proteins of macrophages incubated with NF (5 and 10 μg/mL) or PF (2.5 and 5 μg/mL) for 1, 3, 6, and 12 h were collected for Western blot analyses using specific antibody for Hsp32 and for derivatization with DNPH as describe in the Experimental Procedures. Protein carbonyl derivatives in cell lysates were determined by Western blot analyses for DNP. β-Actin was used to control protein loading. Results are representative of three or more independent experiments.

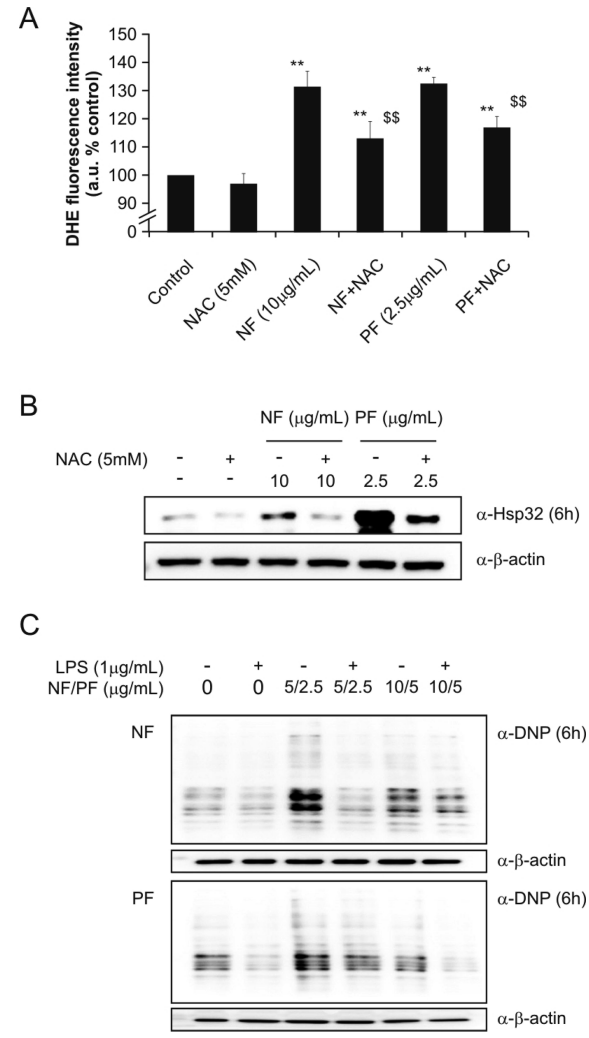

Figure 7. NAC suppresses NF- and PF-induced rises in intracellular ROS, up-regulation of Hsp32 proteins, and accumulation of protein carbonyls in macrophages. (A) Macrophages were preincubated with 5 mM NAC for 3 h prior to loading of DHE (2 μM) for 30 min. Excess DHE was removed by washing macrophages with PBS. Measurement at Ex/Em 510/610 nm was started immediately after stimulation of macrophages with 10 μg/mL of NF or 2.5 μg/mL of PF. Differences in DHE fluorescence intensity between 0 and 30 min were used to compare the effect of NAC. Data are means ± SDs of three separate experiments performed in four replicates. $^{**}p < 0.01$ vs control, and $^{\$\$}p < 0.01$ vs NF or PF alone. (B and C) Total cellular proteins of macrophages pretreated with NAC (5 mM) prior to NF (10 μg/mL) or PF (2.5 μg/mL) challenge for 6 h were collected for Western blot analyses using specific antibody for Hsp32 and derivatization with DNPH for protein carbonyl determination. β-Actin was used to control protein loading. Western blot results are representative of at least two independent experiments.

oxidant events occurring in cell culture media, and the ROS detected with DHE were unlikely to be of H_2O_2 origin.

NF and PF Increase Hsp32 Protein Levels. Many cells respond to moderate oxidative stress by increasing their defense system, such as the synthesis of heat shock proteins (Hsp). Because NF and PF induced rapid rises in intracellular ROS, we were interested to know whether Hsp were affected in the macrophages. Western blot analyses show that Hsp32 protein levels increased in a dose- and time-dependent manner (Figure 6A). Hsp32 proteins were induced as early as 1 h after exposure to NF and PF at both concentrations, and their levels remained elevated up to 12 h. Moreover, PF, a constituent of NF, showed a stronger effect on Hsp32 induction as compared to NF. However, the protein levels of Hsp70 and 90 remained relatively constant within the time frame of these experiments (data not shown). DMSO, the vehicle in which NF and PF were solubilized (0.025%, final concentration), did not produce significant changes in Hsp32 protein expression.

NF and PF Increase Formation of Protein Carbonyl Derivatives. To further explore whether ROS generated are capable of causing protein modifications, protein carbonyl derivatives (*15*) were determined in macrophages incubated with NF and PF. As shown in Figure 6B, immunoblotting of protein carbonyl groups with DNP antibody 1 and 3 h after NF and PF exposure at both concentrations showed very little increases in carbonyl formation. However, protein carbonyls increased markedly by 6 h after exposure, and the accumulation of carbonyl derivatives decreased by 12 h, suggesting their removal.

Effects of *N*-Acetyl-L-cysteine (NAC) on NF- and PF-Induced Increase in RS, Hsp32 Protein Levels, and Protein Carbonyls. Many antioxidants have been used in various studies to counteract the damage caused by RS. Preincubation of macrophages with 5 mM NAC, the thiol antioxidant and redox modulator, for 3 h prior to the addition of NF (10 μg/mL) and PF (2.5 μg/mL) decreased the rises in $O_2^{\bullet -}$ as determined by the decrease in DHE fluorescence (Figure 7A). Because NAC is a precursor of glutathione (GSH), we determined intracellular GSH levels of macrophages after NF and PF stimulation. Cells treated with NF (5 and 10 μg/mL) and PF (2.5 and 5 μg/mL) for 1, 3, and 6 h had unchanged intracellular GSH levels, but

at 12 h, intracellular GSH levels increased significantly by 20−60% as compared to control ($p < 0.01$) (data not shown). Hsp32 protein levels induced by NF and PF were also markedly attenuated in the presence of NAC (Figure 7B) by 6 h. Because GSH only rises at a later time, the effects of NF and PF on ROS and Hsp32 protein induction may involve mechanisms other than changing GSH levels.

To further confirm the role of ROS induced by NF and PF in the macrophages, we studied the effect of NAC on the formation of protein carbonyls. Pretreatment with NAC (5 mM) for 30 min prior to the addition of NF (5 and 10 μg/mL) or PF (2.5 and 5 μg/ mL) for 6 h attenuated the accumulation of protein carbonyls in a dose-dependent manner (Figure 7C). These results suggest that NF- and PF-induced ROS and oxidative stress can be attenuated by NAC, which probably acts as a free radical scavenger.

NAC Attenuates Effects of NF and PF on LPS-Induced Up-regulation of iNOS and COX-2 Protein Levels and RNS in Macrophages. We next determined the effect of NAC on LPS-inducible proteins such as iNOS and COX-2 in macrophages treated with NF and PF. Figure 8A confirms the attenuating effect of NF and PF on LPS-induced up-regulation of iNOS and COX-2 protein levels. In the presence of NAC, the effect of NF and PF was reduced markedly, and iNOS and COX-2 levels rose. Likewise, NF (10 μg/mL) and PF (2.5 μg/mL) significantly inhibited LPS-induced increases in intracellular RNS derived from NO$^{\bullet}$ (Figure 8B, $p < 0.01$). Preincubation with NAC diminished the inhibitory effects of NF and PF on LPS-induced formation of RNS (Figure 8C). Furthermore, the LPS-induced increase in RNS remained significant ($p < 0.05$) in the presence of NAC.

NAC Attenuates the Effects of NF and PF on CAMs Protein Expression in HUVEC and VSMC and NF-κB Subunits in LPS-Activated Macrophages. We also studied the effect of NAC on HUVEC and VSMC cocultured with LPS-activated macrophages. As shown in Figure 9A,B, NF and PF attenuated the up-regulation of E-selectin, ICAM-1, and VCAM-1 proteins in HUVEC and VCAM-1 proteins in VSMC induced by LPS-activated macrophages. The inhibitory effects of NF and PF decreased in the presence of NAC except in one case; the effect of NF on decreasing VCAM-1 protein in HUVEC remained.

The attenuation of nuclear translocation of p50 and the increase in p52 and RelB protein levels in the nuclear fractions by NF or PF (Figure 4) were also abolished in the presence of NAC (Figure 10). However, nuclear p65 proteins were not affected, and the changes of other NF-κB subunits in the cytosolic fractions were complex and difficult to comprehend.

Proteins Immunoprecipitated with NF-κB p52 Transcription Factor Might Be Oxidatively Modified. Because the LPS-induced up-regulation of proteins such as iNOS, COX-2, and CAMs under the regulation of NF-κB was attenuated in the presence of NF and PF, we asked the question whether p105/p50 and p100/p52 proteins were affected. As shown in Figure 11A, incubation with NF (10 μg/ mL) or PF (2.5 μg/mL) for 12 h prior to LPS challenge attenuated LPS-induced up-regulation of p100/p52 protein levels. Interestingly, the basal protein levels of p100 were also reduced by treating the macrophages with NF or PF, but mRNA levels were not affected (data not shown). This suggests that the reduction of p100 protein levels was probably due to post-translational modification and degradation. p105/p50 proteins were not affected by NF or PF stimulation (data not shown). Next, samples from macrophages treated with NF (10 μg/mL) or PF (2.5 μg/mL) for 6 h were immunoprecipitated with monoclonal antibody against NF-κB p50 and NF-κB p52. When proteins immunoprecipitated with the latter antibody were derivatized with DNPH and subjected to SDS/PAGE, NF- and PF-treated cell lysates showed increased carbonyl derivatives formation with anti-DNP antibody (Figure 11B). These results suggest that proteins immunoprecipitated with NF-κB p52 antibody might be oxidatively modified.

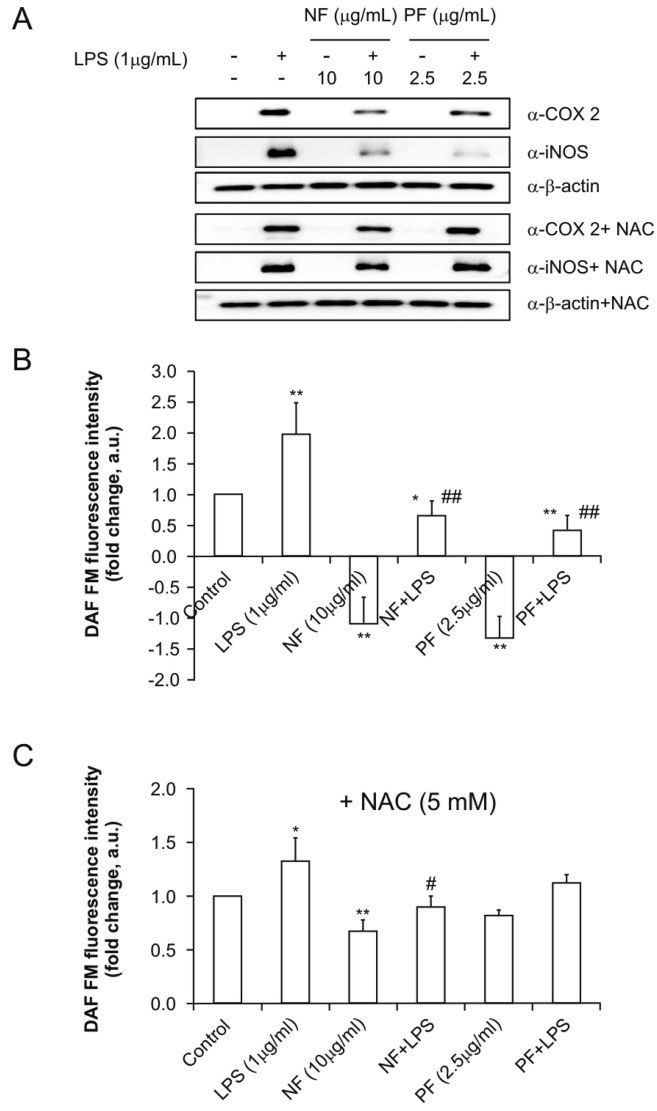

Figure 8. NAC ameliorates NF- and PF-induced up-regulation of iNOS and COX-2 protein levels and NO$^{\bullet}$-derived RNS in LPS-activated macrophages. (A) Macrophages were exposed to NF (10 μg/mL) or PF (2.5 μg/mL) for 12 h after preincubation with 5 mM NAC for 30 min. The macrophages were then challenged with LPS (1 μg/mL) for 1 h and kept in the incubator for another 20 h. Total cellular proteins of macrophages were collected for Western blot analyses using antibodies for iNOS and COX-2. β-Actin was used to control protein loading. Results are representative of three independent experiments. (B and C) For RNS estimation, the macrophages were pre-loaded with DAF-FM DA (5 μM) for 30 min, and measurement at Ex/Em 510/610 nm commenced immediately after excess DAF-FM DA was removed. Differences in DAF-FM fluorescence intensity between 0 and 30 min were used to compare the effect of NAC on RNS formation. Data are means ± SDs of three separate experiments performed in four replicates. $^{*}p < 0.01$ vs control, $^{**}p < 0.01$ vs control, and $^{\#\#}p < 0.01$ vs LPS alone.

Discussion

Oxidative stress and the oxidative modification of cellular components are involved in a number of physiological and

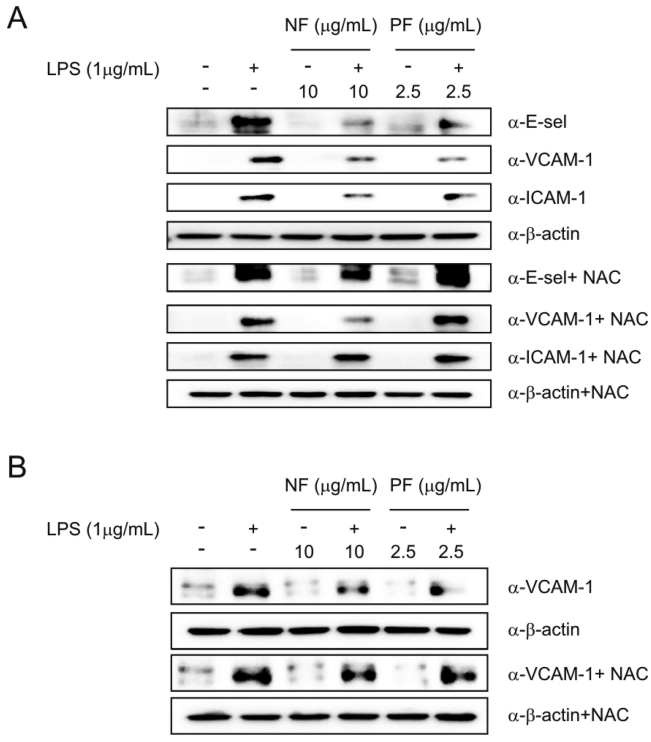

Figure 9. NAC suppresses the effects of NF and PF on CAMs in HUVEC and VSMC. Macrophages in cell culture inserts were preincubated with or without NAC (5 mM) for 30 min prior to exposure to NF (10 μg/mL) or PF (2.5 μg/mL) for 12 h. The macrophages were then challenged with LPS (1 μg/mL) for 1 h, washed with PBS, and cocultured for 20 h with HUVEC (A) and VSMC (B). Total cellular proteins from HUVEC and VSMC were collected for Western blot analyses using antibodies for E-selectin, ICAM-1, and VCAM-1. β-Actin was used to control protein loading. Results are representative of two or more independent experiments.

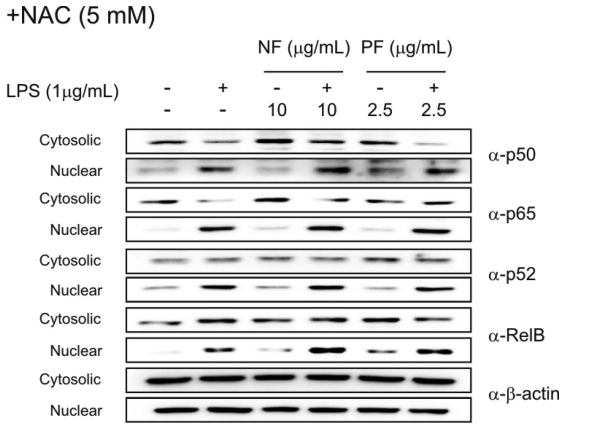

Figure 10. NAC blocks the effects of NF and PF on LPS-induced nuclear translocation of NF-κB subunits. Macrophages were exposed to NF (10 μg/mL) or PF (2.5 μg/mL) for 12 h after preincubation with 5 mM NAC for 30 min. The macrophages were then challenged with LPS (1 μg/mL) for 1 h and incubated for another 20 h. Macrophages after treatment were collected for nuclear and cytosolic separation as descried in the Experimental Procedures. Cytosolic and nuclear p50, p65, p52, and RelB proteins were analyzed by Western blotting. β-Actin was used to control protein loading.

pathophysiological processes, such as aging, inflammation, atherosclerosis, carcinogenesis, and neurodegenerative diseases (*1*). ROS, particularly H_2O_2, have generally been considered to contribute to various diseases by activating transcription factors, such as NF-κB, with resultant increased production of proinflammatory mediators (*20, 21*). However, depending on cell type and NF-κB activators (such as LPS, TNF, and phorbol dibutyrate), H_2O_2 has been shown to inhibit inflammatory responses (*22*−*26*). For example, Malmberg and colleagues (*23*) have shown that low concentrations of H_2O_2 decreased cytokines production in stimulated T cells and higher concentrations of H_2O_2 induced apoptosis. Moreover, neutrophils from acatalasemic mice or neutrophils treated with aminotriazole were less sensitive to LPS challenge as compared to neutrophils from control mice, suggesting a role for H_2O_2 in modulating the inflammatory effects of LPS (*26*).

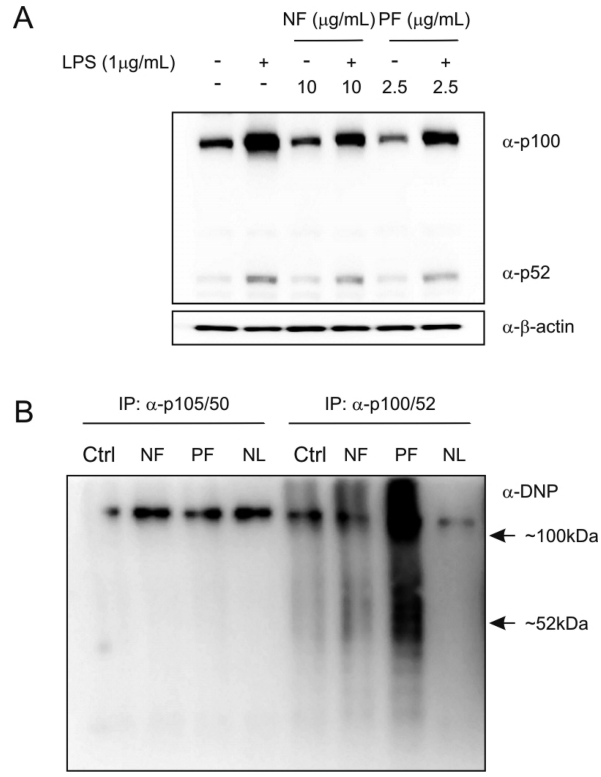

Figure 11. NF- and PF-treated protein samples from macrophages immunoprecipitated with NF-κB p52 antibody might be oxidatively modified. (A) Macrophages were pretreated with NF (10 μg/mL) or PF (2.5 μg/mL) for 12 h prior to the addition of LPS (1 μg/mL) for 1 h and kept for another 6 h after LPS was removed. Total cellular proteins were collected for Western blot analyses using antibodies against p100/p52. β-Actin was used to control protein loading. Results are representative of three independent experiments. (B) Macrophages treated with NF (10 μg/mL) or PF (2.5 μg/mL) for 6 h were harvested for immunoprecipitation assay with NF-κB p50 and NF-κB p52 antibodies. The immunoprecipitates were subjected to SDS-PAGE after derivatization with DNPH and immunoblotted with anti-DNP antibody against protein carbonyls. NL, no lysate control.

RS such as hydroxyl radicals ($OH^{\bullet}$), peroxyl radicals ($RO_2^{\bullet}$), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$) can oxidize amino acid residues in proteins to form products with carbonyl groups (aldehydes and ketones), which can be measured after derivatization with DNPH (*15, 27*). Our protein carbonyl data and the preliminary immunoprecipitation experiments with NF-κB p52 suggest that NF- and PF-induced ROS might be responsible for causing oxidative modification of intracellular proteins, including proteins related to the transcriptional activity of NF-κB p52. This is also supported by the inhibitory effects of NF and PF on LPS-induced activation of NF-κB and expression of CAM proteins in HUVEC and VSMC as well as iNOS, COX-2, and RNS in the activated macrophages. A coculture system was utilized to demonstrate that macrophage stimulation with LPS, such as occurs during inflammation, induced the expression of CAM proteins such as E-selectin, ICAM-1, and VCAM-1 in HUVEC and VCAM-1 in VSMC. In the activated macrophages, the mRNA and protein

expression levels of iNOS and COX-2 and RNS formation were also up-regulated following LPS challenge. Oxidative modification of IκBα (*28*) and IKK (*29*) by taurine chloramines and H_2O_2, respectively, have been demonstrated to inhibit NF-κB activation.

Schreck and colleague (*30*) have shown that $O_2^{\bullet-}$ generating systems were poor activators of NF-κB. In our current study, NF- and PF-induced ROS as determined by the increases in DHE fluorescence did not result in nuclear translocation of NF-κB subunits (Figure 4). We think the ROS generated are mostly, if not entirely, $O_2^{\bullet-}$ because NF and PF did not increase DCF fluorescence intensity and preincubation with catalase also did not attenuate the increases in DHE fluorescence. Moreover, $O_2^{\bullet-}$ generated could have reacted rapidly with RNS such as $NO^{\bullet}$ produced by LPS-inducible iNOS and, thus, attenuated the responses of LPS (*31*). However, the rapid reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$ also produces RS such as $ONOO^-$ (*32*). $ONOO^-$ readily protonates and breaks down under physiological conditions to give highly reactive oxidizing and nitrating species that are able to cause oxidative modification of biomolecules (*32*−*34*).

NAC is a thiol compound and a precursor of L-cysteine and GSH. Hence, it can be used as an antioxidant to scavenge free radicals by interacting with RS such as $OH^{\bullet}$ and H_2O_2 (*35*). NAC has been demonstrated to suppress cytokines-stimulated expression of VCAM-1, ICAM-1, and E-selectin in endothelial cells through different mechanisms (*36, 37*). NAC also inhibited the induction of HO-1 by TNF-α and IL-1 in endothelial cells, suggesting a role of RS (*38*). Our results with NAC supported our hypothesis that NF- and PF-induced oxidative stress is responsible for the attenuation of pro-inflammatory effects of LPS in macrophages as well as the effects of LPS-activated macrophages on HUVEC and VSMC in coculture.

Many compounds isolated from medicinal plant extracts have been demonstrated to inhibit or suppress cytokine-induced iNOS, COX-2, CAMs mRNA, and protein expression levels in cultured macrophages and endothelial and smooth muscle cells (*39*−*42*). Some compounds were found to inhibit the activation of the transcription factor NF-κB by cytokines. However, the inhibitory effects of "active" compounds are often assumed to be acting by antioxidant mechanisms. Our data suggest that they can sometimes do the opposite. Overall, our data and others suggest that ROS can sometimes be anti-inflammatory in vivo, for example, at sites of chronic or prolonged inflammation (*23, 31, 43*).

Acknowledgment. We thank Alvin Loo Eng Kiat for testing H_2O_2 levels in this study using the FOX 2 assay. We are grateful to the Biomedical Research Council of Singapore for their research support (BMRC 03/1/21/18/213).

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TX900163H