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The Glutaredoxin/Glutathione System Modulates NF-κB Activity by Glutathionylation of p65 in Cinnamaldehyde-Treated Endothelial Cells

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Reversible protein glutathionylation is an important post-translational modification that provides protection against oxidation. In endothelial cells (ECs), cinnamaldehyde is an electrophilic compound that can increase the intracellular glutathione (GSH) levels or reactive oxygen species (ROS) production depending on the treatment duration. ECs treated with GSH and H_2O_2 show increased sulfhydryl modifications of the p65 subunit of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which are responsible for NF-κB inactivation, and also a block in TNF-α–induced p65 nuclear translocation and inter-cellular adhesion molecule-1 (ICAM-1) expression. In our current study, we find that cinnamaldehyde induces p65 glutathionylation and inhibits TNF-α–induced p65 nuclear translocation and ICAM-1 expression within 12 h of treatment. Our analyses also reveal that p65 glutathionylation is suppressed by a GSH synthesis inhibitor, buthionine sulfoximine (BSO), and we further observed that the inhibitory effects of p65 nuclear translocation and ICAM-1 expression are also suppressed by BSO. NF-E2–related factor-2 small interfering RNA (siRNA) molecules not only inhibit glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modifier subunit (GCLM) induction and increases in GSH but also abolish cinnamaldehyde-induced p65 glutathionylation and its inhibitory effects. The gene expression and activity of glutaredoxin-1 (Grx-1), which catalyzes the formation of protein-glutathione mixed disulfides (protein-SSG), were also found to be increased after cinnamaldehyde treatment. A knock down of endogenous Grx-1 by siRNA or pretreatment with an inhibitor of Grx-1 activity, $CdCl_2$, abolishes p65-SSG formation. In addition, Grx-1 siRNA blocks the inhibition of p65 nuclear translocation and ICAM-1 expression, suggesting that this enzyme is involved in the cinnamaldehyde-mediated NF-κB inhibition. Our current results thus indicate that the GSH/Grx-1–dependent glutathionylation of p65 is likely to be responsible for cinnamaldehyde-mediated NF-κB inactivation and for the enhanced inhibitory effects of cinnamaldehyde upon TNF-α–treated ECs.

Key Words: **cinnamaldehyde; NF-κB; GSH; glutaredoxin-1; glutathionylation.**

Glutathionylation is often considered to be a process that protects sensitive cysteinyl residues from irreversible oxidation and may be preceded by spontaneous reaction of glutathione (GSH) with a corresponding oxidized derivative, for example, S-nitrosyl (–SNO), sulfenic acid (–SOH), or a thiyl radical ($-S^{\bullet}$) (Gallogly and Mieyal, 2007). Reversible posttranslational modifications of proteins by glutathionylation are thought to protect against irreversible oxidation (Shelton *et al.*, 2005). In addition to this protective effect, glutathionylation also results in protein-specific functional changes during the regulation of signaling mediators. The glutathionylation of Ras activates this oncoprotein and leads to the downstream phosphorylation of Akt and p38 and increased cell proliferation (Adachi *et al.*, 2004). The catalytic subunit of protein kinase A is inactivated through the glutathionylation of Cys^{199} (Humphries *et al.*, 2002). Glutathionylation also plays a key role in the regulation of the kinase activity of mitogen-activated protein kinase kinase kinase and in the protein kinase C-α pathway in response to oxidative stress (Cross and Templeton, 2004; Ward *et al.*, 2000). Moreover, transcription factors that function in cell growth, differentiation, and apoptosis appear to be regulated by glutathionylation (Dalle-Donne *et al.*, 2009). Through the introduction of a negatively charged GSH within their DNA-binding sites, glutathionylation also inhibits the DNA-binding activity of c-Jun and NF-κB (Pineda-Molina *et al.*, 2001; Qanungo *et al.*, 2007). Collectively, these data suggest that glutathionylation is a physiologically relevant mechanism for controlling the activation of key signaling pathways. Recent studies have now found that electrophiles can give rise to thiyl radicals and, consequently, propagate protein glutathionylation (Foresti *et al.*, 2005; Starke *et al.*, 2003).

The compounds that contain an α,β-unsaturated carbonyl moiety, including cinnamaldehyde, function as potent Michael reaction acceptors. These electrophiles cause GSH depletion and then interact with the sulfhydryl residues of proteins to modulate signal transduction (Cernuda-Morollón

et al., 2001; Heiss *et al*., 2001; Rossi *et al*., 2000). On the other hand, electrophiles have also been reported to activate NF-E2–related factor-2 (Nrf2) (Liao *et al*., 2008; Wu *et al*., 2006), a transcription factor which been shown to control the expression of many thiol-regulating enzymes, including glutathione S-transferase, glutamylcysteine synthetase, and thioredoxin reductase (Nguyen *et al*., 2004). Our recent data have indicated also that cinnamaldehyde increases the intracellular GSH levels via the induction of glutamylcysteine synthetase at the transcriptional level (Liao *et al*., 2008). These two opposing effects of cinnamaldehyde upon the intracellular GSH concentration are dependent on the treatment duration.

Cinnamaldehyde is a bioactive compound isolated from the stem bark of *Cinnamomum cassia* Presl (Lauraceae) which has been widely used as a natural spice and also in traditional Chinese medicine. Our previous study demonstrated that cinnamaldehyde enhances the suppression of TNF-α–induced monocyte/endothelial cell (EC) interactions by downregulating the expression of the adhesion molecules ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) at noncytotoxic concentrations (Liao *et al*., 2008). Furthermore, depending on the pretreatment time, cinnamaldehyde exerts two distinct mechanisms to suppress TNF-α–induced NF-κB activation. Over short-term pretreatments, cinnamaldehyde blocks the degradation of inhibitor of kappa B-α (IκB-α), whereas over long-term pretreatments its inhibitory effects are independent of the degradation of IκB-α but are mediated through the induction of Nrf2-related genes (Liao *et al*., 2008). However, little is yet known regarding the anti-inflammatory mechanisms associated with the induction of Nrf2-related GSH levels during long-term pretreatments. In our present study, we examined whether intracellular GSH facilitates p65 protein glutathionylation and is thereby a potential mechanism of NF-κB inactivation.

Glutaredoxin (Grx) has been identified as a ubiquitous multifunctional thioltransferase (Mieyal *et al*., 2008). This enzyme generally reduces the glutathionylation of proteins because of the low equilibrium constant of its cysteine residue at position 22 in a conserved dithiol catalytic site for the binding of GSH (Fernandes and Holmgren, 2004). However, if glutathionyl radical–generating conditions prevail, Grx then promotes glutathionylation (Qanungo *et al*., 2007; Starke *et al*., 2003). Grx plays an important role in the shear stress-activated Akt signaling pathway and in NO synthase activation in a Grx-dependent manner in ECs (Wang *et al*., 2007). Grx has also been found to be expressed in atherosclerotic lesions and to play a protective role in human coronary arteries (Okuda *et al*., 2001). This has led to speculation that Grx-mediated glutathionylation is a protective mechanism in vascular diseases. In our present study, we describe the phenomenon of cinnamaldehyde-induced Grx-1 expression, which we further show to be involved in increased p65 glutathionylation by this compound.

MATERIALS AND METHODS

Materials. Bacterially derived TNF-α was purchased from Calbiochem (San Diego, CA). Antibodies against Grx-1 and GSH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). P65 antibodies were obtained from Stressgen Biotechnologies (San Diego, CA). Enhanced chemiluminescence (ECL) reagents were purchased from Pierce (Rockford, IL). Luciferase assay kits were purchased from Promega (Madison, WI). Peroxidase-conjugated anti-rabbit and anti-mouse antibodies were obtained from Amersham (Arlington Heights, IL) and nitrocellulose was obtained from Schleicher & Schuell (Dassel, Germany). Grx-1 siRNA and nonsense siRNA were purchased from Ambion (Austin, TX). All other reagents, including cinnamaldehyde (purity, 98%), were purchased from Sigma (St Louis, MO).

Grx expression construct. To construct the His-tagged Grx-1 expression vector pcDNA-His-GRX, a 320-bp DNA fragment encoding the human *Grx-1* gene was amplified from the complementary DNA (cDNA) library by PCR. The primers used were a 5' oligonucleotide (5'-TTAAGAATTCGCTCAA-GAGTTTGTGAACTGC), containing an *Eco*RI site, and a 3'-oligonucleotide (5'-GAGCTCTAGATTACTGCAGAGCTCCAATCTG), containing an *Xba*I site. The resultant amplified product was digested with *Eco*RI and *Xba*I and subcloned into the pcDNA4-His vector.

Promoter constructs. Using genomic DNA as a template, a DNA fragment of the 5'-flanking region of the *Grx-1* gene (–1993 to +22 nucleotides) was amplified by PCR and designated Grx (–1993). The primers used were sense, 5'-GGACTGAGTGAGAGGCAGACAATAGTCTCC-3', and antisense, 5'-CGGGAAGAATCCTCAGTTGCAGGTATTGCTTGG-3'. An NF-κB/Luc fragment containing tandem repeats of double-stranded oligonucleotides spanning the NF-κB–binding site of *ICAM-1* (5'-TGGAAATTCC-3') (van de Stolpe *et al*., 1994) was amplified with the primers sense: 5'-CCCGGGTGGAAATTCCTG-GAAATTCCTGGAAATTCCGGAGTCTAGA-3' and antisense: 5'-TCTA-GACTCCGGAATTTCCAGGAATTTCCAGGAATTTCCACCCGGG-3' and then introduced into the pGL3 promoter plasmid (Promega).

EC cultures. The human EC line EA.hy926 (American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere of air and 5% CO_2. Cells were grown in petri dishes for 3 days and allowed to reach confluence. The culture medium was then replaced with serum-free DMEM, and the cells were incubated for a further 12 h prior to experimental treatment.

Cell viability assay. Cell viability was measured using an Alamar blue assay (Serotec, Oxford, UK) according to the manufacturer's instructions. The assay is based on the detection of metabolic activity in living cells using a redox indicator that changes from an oxidized (blue) to a reduced (red) form. The intensity of the red color is proportional to the viability of the cells and is calculated as the difference in the absorbance values at 570 and at 600 nm and expressed as a percentage of the control.

Detection of protein glutathionylation using BioGEE. Biotin-labeled glutathione ester (BioGEE, G36000; Invitrogen) is a cell permeable biotinylated GSH that detects proteins that form adducts to reactive thiols by avidin-agarose pull down. These biotinylated proteins can be observed by SDS-polyacrylamide gel electrophoresis (PAGE) (Brennan *et al*., 2006). The BioGEE mixture is then added to the cell culture medium at a final concentration of 100μM. At designated time points, cell lysates are prepared, precleared with agarose beads, and then incubated with streptavidin-conjugated agarose beads (100 μl/mg of protein) for 30 min at 4°C to specifically bind protein-BioGEE complexes. After centrifugation and washing, the beads are incubated for 30 min with 10mM dithiothreitol (DTT) in PBS/EDTA/SDS to elute proteins. Total glutathionylated proteins were detected by SDS-PAGE followed by silver staining.

Measurement of intracellular ROS. Cells were cultured at 37°C in the presence or absence of the reagents indicated in the figures, washed with PBS,

and then incubated with 20μM of the peroxide-sensitive fluorescent probe 5-(and-6)-carboxy-2,7,dichlorodihydro fluorescein diacetate (carboxy-H_2DCFDA; Molecular Probes, Eugene, OR) for an additional 30 min at 37°C. After two further washes with PBS, the cells were solubilized with 1% SDS and 5mM Tris-HCl (pH 7.4). Fluorescence was measured by spectrofluorophotometry (Shimadzu, Rf-5301PC) with excitation and emission wavelengths of 450 and 520 nm, respectively. Samples were assayed in triplicate.

GSH assay. GSH levels were determined using the method originally described by Kamencic *et al.* (2000). Briefly, Cells were cultured at 37°C in the presence or absence of specific treatment reagents, as indicated in the figures, washed twice with PBS, and then incubated with monochlorobimane (2mM) in the dark for 20 min at 37°C. After two further washes with PBS, the cells were solubilized with 1% SDS and 5mM Tris-HCl (pH 7.4). Fluorescence was measured by spectrofluorophotometry (Shimadzu, Rf-5301PC) with excitation and emission wavelengths of 380 and 470 nm, respectively. Samples were assayed in triplicate. The assay for detecting GSH levels *in vitro* was performed identically but without cell lysates. The levels of intracellular GSH were quantified using a GSH solution as a standard. Samples were assayed in triplicate.

Determination of Grx activity. To determine the Grx activity levels, the reaction mixtures consisted of 0.5mM GSH, 12 units of GSH reductase, 2.5mM S-sulfocysteine, 0.35mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.137M sodium phosphate buffer, pH 7.5, and the enzyme to be assayed. Grx activity was analyzed in EC lysates by monitoring the decrease in absorbance of NADPH at 340 nm using spectrofluorophotometry (Shimadzu, Rf-5301PC) (Mieyal *et al.*, 1991).

Reverse transcription-PCR. Total cellular RNA was extracted from the cells using the phenol-guanidinium isothiocyanate method (Wung *et al.*, 1997). Equal amounts (1 μg) of RNA from cells undergoing different treatments were reverse transcribed for 60 min at 42°C using 50 units of superScript II (Invitrogen). Amplification of cDNA was performed in 25 μl of PCR buffer (10mM Tris-HCl, 50mM KCl, 5mM $MgCl_2$, and 0.1% Triton X-100, pH 9.0) containing 0.6 units of *Taq* DNA polymerase (Promega) and 30 pmol of synthetic specific primers for human *Grx-1*: sense 5′-GGC ATG GCT CAA GAG TTT GTG AA-3′ and antisense 5′-CAT ATG ATT TCT CCA ATT GGG TCC T-3′ or *GAPDH*: sense 5′-TAT CGT GGA AGG ACT CAT GAC C-3′ and antisense 5′-TAC ATG GCA ACT GRG AGG GG -3′. The specific primers used for the amplification of *GCLM* and GCLC were as follows: GCLM, sense 5'-CAG CGA GGA GCT TCA TGA TTG-3' and antisense 5'-TGA TCA CAG AAT CCA GCT GTG C-3'; *GCLC*, sense 5'-GTT CTT GAA ACT CTG CAA GAG AAG-3' and antisense 5'-ATG GAG ATG GTG TAT TCT TGT CC-3' (Neurohr *et al.*, 2003). The first-strand cDNA syntheses were performed in accordance with the manufacturer's instructions. PCR amplifications were performed after a 10-min denaturation at 94°C, followed by repeat cycles of 94°C, 55°C, and 72°C, each for 40 s. The cycle number was specific to each primer set. PCR products were electrophoresed in a 1% agarose gel containing ethidium bromide, and the relative densities of the mRNA levels were determined by scanning densitometry analysis using Uni-photo band tool (EZ lab, Taiwan, ROC). The given values represent the mean with a numerical round off, normalized to the control values of at least three independent experiments from which similar results were obtained.

Transfections and luciferase assays. All transfection experiments were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. For luciferase assays, the cell lysate was first mixed with luciferase substrate solution (Promega), and the resulting activity was measured using a luminometer. For each experiment, luciferase activity was determined in triplicate and normalized using β-galactosidase activity.

Transient transfection with siRNA targeting Nrf2 and Grx-1. The siRNA sequences used in this study were as follows: human Nrf2, 5′-UCCCG-UUUGUAGAUGACAA-3 (Singh *et al.*, 2008), and GRX-1, 5′-CCAA-GAGUGUAUCUGUGAAtt-3 (Wang *et al.*, 2007). A control siRNA, 5-GCAAGCUGACCCUGAAGUUCAU-3, was purchased from Ambion. ECs were seeded onto 60-mm dishes 24 h prior to transfection and then transiently transfected with 100 nmol/l siRNA per dish at 90% confluence with Lipofectamine 2000 reagent. After 24 h of recovery in 10% serum medium, the cells were then cultured in medium without serum for another 12 h prior to treatment.

Preparation of cytosolic and nuclear lysates. To separate cytosol from nuclear proteins, ECs were collected by scraping in cold PBS. The cell pellet was then lysed in 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5mM PMSF, and 0.3% Nonidet P-40 (NP-40). After 5 min of centrifugation at 1500 × g at 4°C, the supernatant was collected and designated the cytosolic fraction. Nuclear proteins were then extracted using a buffer containing 25% glycerol, 20mM HEPES, 0.6M KCl, 1.5mM $MgCl_2$, and 0.2mM EDTA. Protein concentrations were determined using a protein assay DC system (Bio-Rad, Richmond, CA).

Western blotting. Whole lysates of ECs were prepared as previously described (Wung *et al.*, 1999). A total of 1×10^6 cells were lysed on ice in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture), and whole-cell extracts were boiled for 5 min prior to separation on 10% SDS-PAGE, in which the protein samples were evenly loaded. The proteins were then transferred to a nitrocellulose filter (Millipore, Bedford, MA) in Tris-glycine buffer at 10 V for 1.5 h. The membranes were then blocked with PBS containing 5% nonfat milk and incubated with antibodies for 2 h at 4°C, with gentle shaking. The results were visualized by chemiluminescence using ECL according to the manufacturer's instructions. Relative protein concentrations were determined by scanning densitometry analysis using the Uni-photo band tool (EZ lab). The given values represent the mean with a numerical round off, normalized to control values, of three independent experiments from which similar results were obtained.

Immunoprecipitation. Protein cell lysates (300 μg) were cleared of abundant proteins by preincubation for 1 h with Protein G plus-agarose. The supernatant was then collected and incubated with anti-p65 or IκB monoclonal antibodies for 1 h at 4°C. Protein G plus-agarose was then added overnight at 4°C, and the mixture was then washed three times with PBS. The immunoprecipitated proteins were eluted, and Western blotting for the detection of p65, IκB, or GSH was performed using the corresponding antibodies.

Statistical analysis. Values are expressed as the means ± SD of at least three experiments. Statistics were performed using ANOVA with Tukey's *post hoc* test (SPSS 12.0 software package, Chicago, IL). A confidence limit of $p < 0.05$ was considered to be significant.

RESULTS

The Possible Role of Cinnamaldehyde-Increased GSH and H_2O_2 in p65 Glutathionylation

GSH is a well-studied tripeptide with numerous reported roles in protecting cells from oxidants and maintaining the cellular thiol redox status (Dickinson and Forman, 2002). The cell redox state is particularly important in protein glutathionylation and for the thiol-mediated signal transduction pathways (Dalle-Donne *et al.*, 2009; Sen, 2000). We found in our previous study that cinnamaldehyde increases the cellular GSH levels in ECs after 9 h of exposure (Liao *et al.*, 2008). Although electrophiles can increase the GSH levels via Nrf2-mediated glutamate-cysteine ligase, they also exert transient GSH depletion over short-term treatments (Kelleher *et al.*, 2007). We therefore tested the GSH and ROS levels over identical cinnamaldehyde pretreatment time. As shown in Figure 1A, the GSH levels increase after 9 h of cinnamaldehyde pretreatment

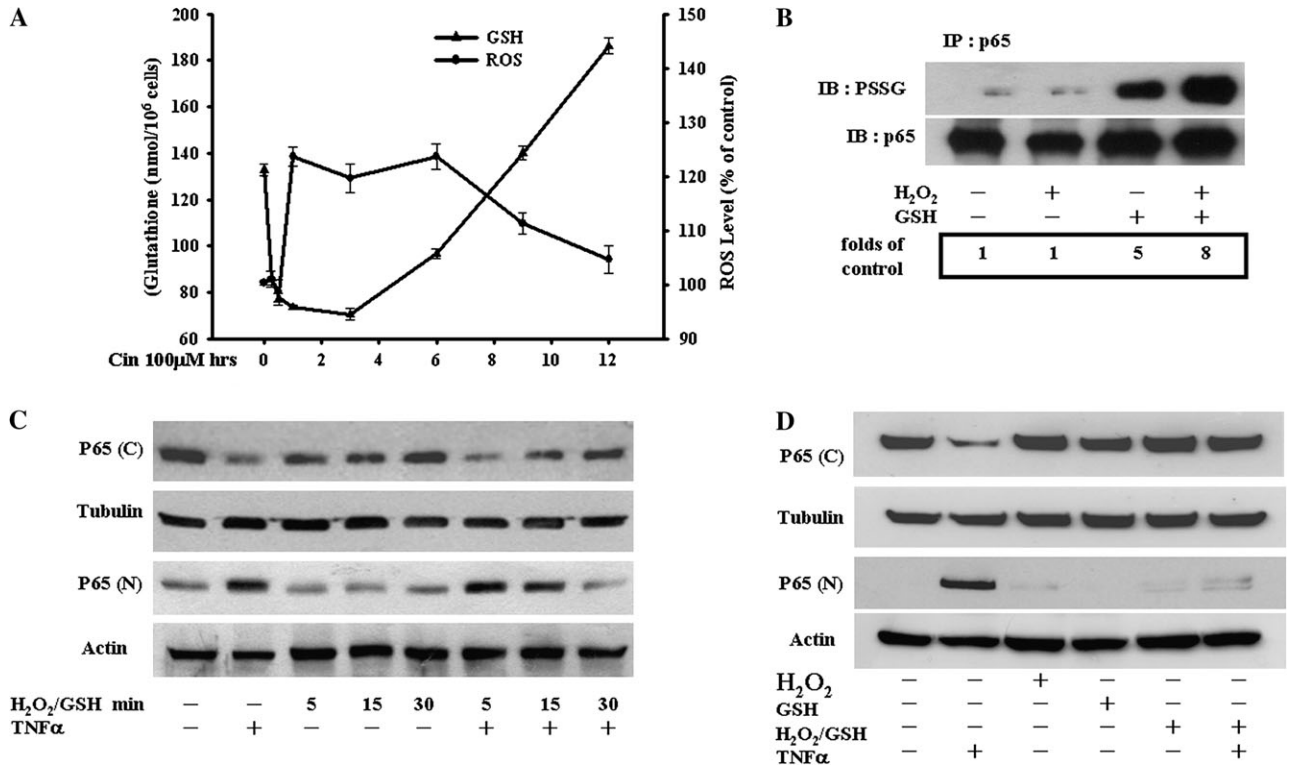

FIG. 1. The possible role of cinnamaldehyde-*increased* GSH and H_2O_2 in p65 glutathionylation. (A) Intracellular GSH levels in ECs incubated with 100μM cinnamaldehyde for 15 and 30 min and for 1, 3, 6, 9, and 12 h. The GSH content of these samples was determined using standard curves generated with known amounts of GSH. Data values are expressed in nmol/10^6 cells. ECs were subjected to similar treatments prior to the measurement of intracellular ROS levels. The results are shown as the mean ± SEM. (B) ECs were exposed to 100 μg/ml H_2O_2 and 20mM GSH for 30 min. S-glutathionylated proteins were immunoprecipitated using an antibody against p65, followed by detection of GSH by Western blotting. The p65 band intensities are used as an internal control. (C) ECs subjected to 100 U/ml TNF-α for 1 h were pretreated with 100 μg/ml H_2O_2 and 20mM GSH for indicated times. Nuclear (N) and cytosolic (C) extracts were then prepared and subjected to Western blot analysis with p65 antibodies. The actin and tubulin band intensities indicate equal loading of each well. (D) ECs subjected to 100 U/ml TNF-α for 1 h were pretreated with or without 100 μg/ml H_2O_2, 20mM GSH, or a combination of H_2O_2 with GSH for 30 min. Nuclear (N) and cytosolic (C) extracts were subjected to Western blot analysis as above.

and persist for over 12 h. In contrast, we could detect an increase in the ROS levels at 1 h following cinnamaldehyde treatment. The biochemical mechanisms by which proteins are S-glutathionylated involve the intermediate formation of protein sulfenic acid moieties that subsequently react with GSH, which then reacts with susceptible protein Cys residues through a thiol exchange mechanism. In a previous study, Reynaert *et al.* (2006) reported that the use of GSH with H_2O_2 induced protein glutathionylation. Other previous studies have also demonstrated that thiyl radicals can interact with the sulfhydryl residues of NF-κB and modulate its activity (Cernuda-Morollón *et al.*, 2001; Heiss *et al.*, 2001; Rossi *et al.*, 2000). Given our finding that the GSH and H_2O_2 levels are increased over the same cinnamaldehyde pretreatment time (Fig. 1A), we further assessed the possible role of GSH and H_2O_2 in p65 glutathionylation and their inhibitory effects. As shown in Figure 1B, the levels of p65 glutathionylation increased after treatment with GSH alone and with GSH and H_2O_2. However, treatment with GSH and H_2O_2 induced higher levels of p65 glutathionylation than GSH alone. To further examine whether GSH with H_2O_2 regulates p65 translocation to the nucleus in TNF-α–treated ECs, we pretreated these cells with these agents over a half-hour time course. After GSH with H_2O_2 pretreatment for 30 min, TNF-α–induced p65 nuclear translocation decreased (Fig. 1C) and there was no detectable induction of this process in H_2O_2- or GSH-treated ECs (Fig. 1D). To clarify the mechanisms underlying the block in TNF-α–induced NF-κB activation, the degradation of IκB-α was then examined over a pretreatment time course. The incubation of ECs for 1 h in the presence of TNF-α alone induced significant degradation of IκB-α. Under these same conditions, GSH with H_2O_2 does not block IκB-α degradation (Figs. 2A and 2B). Thus, the inhibitory effects of GSH with H_2O_2 may be mediated through the inhibition of NF-κB translocation and not through a blockade of IκB-α degradation. Furthermore, pretreatment of ECs with GSH and H_2O_2 for 30 min significantly inhibited TNF-α–induced ICAM-1 expression (Fig. 2C). These data suggest that cinnamaldehyde-*increased* GSH and H_2O_2 *play* a role in p65 glutathionylation and its subsequent functional modifications.

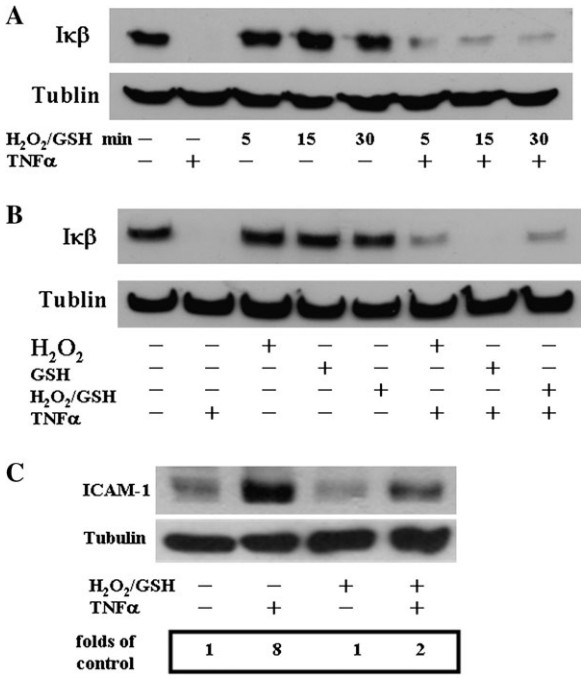

FIG. 2. Effects of GSH and H_2O_2 on the TNF-α–induced degradation of IκB-α and ICAM-1 expression. (A) ECs were induced by 100 U/ml TNF-α for 30 min and a portion of these cells was then pretreated with 100 μg/ml H_2O_2 and 20mM GSH for the indicated times. Cell lysates were then prepared and subjected to Western blot analysis with antibodies against IκB-α and tubulin as indicated. (B) ECs were induced by 100 U/ml TNF-α for 30 min and a portion of these cells was then pretreated with 100 μg/ml H_2O_2, 20mM GSH, or H_2O_2 with GSH for 30 min. Cell lysates were then prepared and subjected to Western blot analysis. (C) ECs were pretreated with 100 μg/ml H_2O_2 and 20mM GSH for 30 min and then incubated with or without 100 U/ml TNF-α for 4 h. Cell lysates were prepared and subjected to Western blot analysis with antibodies against ICAM-1 or tubulin as indicated. The tubulin band intensities indicate equal loading of each well.

Cinnamaldehyde Induces p65 Glutathionylation

As shown in Figure 3A, cells that had been preloaded with biotinylated GSH ethyl ester (BioGEE) prior to cinnamaldehyde exposure showed increased glutathionylation of multiple cellular proteins after this treatment. We further found that the glutathionylation of p65 increased after 6 h of cinnamaldehyde treatment, persisted for over 12 h, and that this induction was higher than that following GSH and N-Acetyl-Cysteine (NAC) cotreatment (Fig. 3B). We next demonstrated that cinnamaldehyde-induced p65 glutathionylation could be reversed by incubation with a reducing agent, DTT (Fig. 3C). We could not detect increased glutathionylation of IκB-α (Fig. 3D). In addition, an examination of cytotoxicity in cinnamaldehyde-pretreated cells using an Alamar blue assay indicated no adverse effects up to a 150μM dose (Fig. 3E). Moreover, TNF-α treatment of cinnamaldehyde-pretreated cells has no adverse effects upon cell viability (Fig. 3F).

Cinnamaldehyde Inhibits TNF-α–Induced NF-κB Activation and ICAM-1 Expression

In our previous study, we showed that after a short-term pretreatment, cinnamaldehyde exerts its anti-inflammatory effects by blocking the degradation of the inhibitory protein IκB-α (Liao *et al.*, 2008). After prolonged cinnamaldehyde treatments, however, the characteristics of the precise inhibitory mechanism remained unclear. We examined this further in our current study by assaying p65 translocation to the nucleus in TNF-α–treated ECs that had been pretreated with cinnamaldehyde at a concentration range of 1–100μM. As shown in Figures 4A and 4B, cinnamaldehyde pretreatment of TNF-α–treated ECs for 12 h over this concentration range indeed inhibits P65 translocation, and the 100μM dose also inhibits ICAM-1 expression (Fig. 4C). We further tested whether cinnamaldehyde inhibits TNF-α–induced p65 activation at the transcriptional level and found that following 100μM cinnamaldehyde pretreatment, the inhibition of TNF-α–induced NF-κB activation in ECs was detectable in a luciferase assay (Fig. 4D). These data together indicate that a 12-h pretreatment with cinnamaldehyde suppresses NF-κB activation in ECs.

The Effects of the Intracellular GSH Levels in Cinnamaldehyde-Treated ECs

We further investigated whether cinnamaldehyde-increased GSH is required for cinnamaldehyde-induced p65 glutathionylation and functional modification. We found that cinnamaldehyde-treated ECs that had been pretreated with 0.5 and 1mM buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamyl cysteine synthetase, showed reduced intracellular GSH levels (Fig. 5A). The lower GSH levels in cells treated with both BSO and cinnamaldehyde compared with BSO alone was found to be because of a depletion of cinnamaldehyde during the first 3 h of exposure and the lack of recovery following BSO inhibition (Fig. 1A). To further demonstrate that this glutathionylation event is mediated by GSH, ECs were pretreated with 0.5 and 1mM BSO to reduce the intracellular GSH levels. This successfully abolished cinnamaldehyde-induced P65 glutathionylation (Fig. 5B). Pretreatment of ECs with BSO at concentrations lower than 0.5mM abolished the suppressive effects of cinnamaldehyde upon p65 translocation (Fig. 5C). In a similar experiment, BSO was also found to suppress the inhibitory effects of cinnamaldehyde upon ICAM-1 expression (Fig. 5D). These results indicate that the inhibitory effects of cinnamaldehyde require an increase in the intracellular GSH levels.

The Effects of Nrf2 upon the Cinnamaldehyde-Induced GSH Levels

Nrf2 is a transcription factor that serves as a sensor for oxidative stress and coordinates the expression of GCL (Ishii *et al.*, 2000) and other antioxidative stress genes in response to

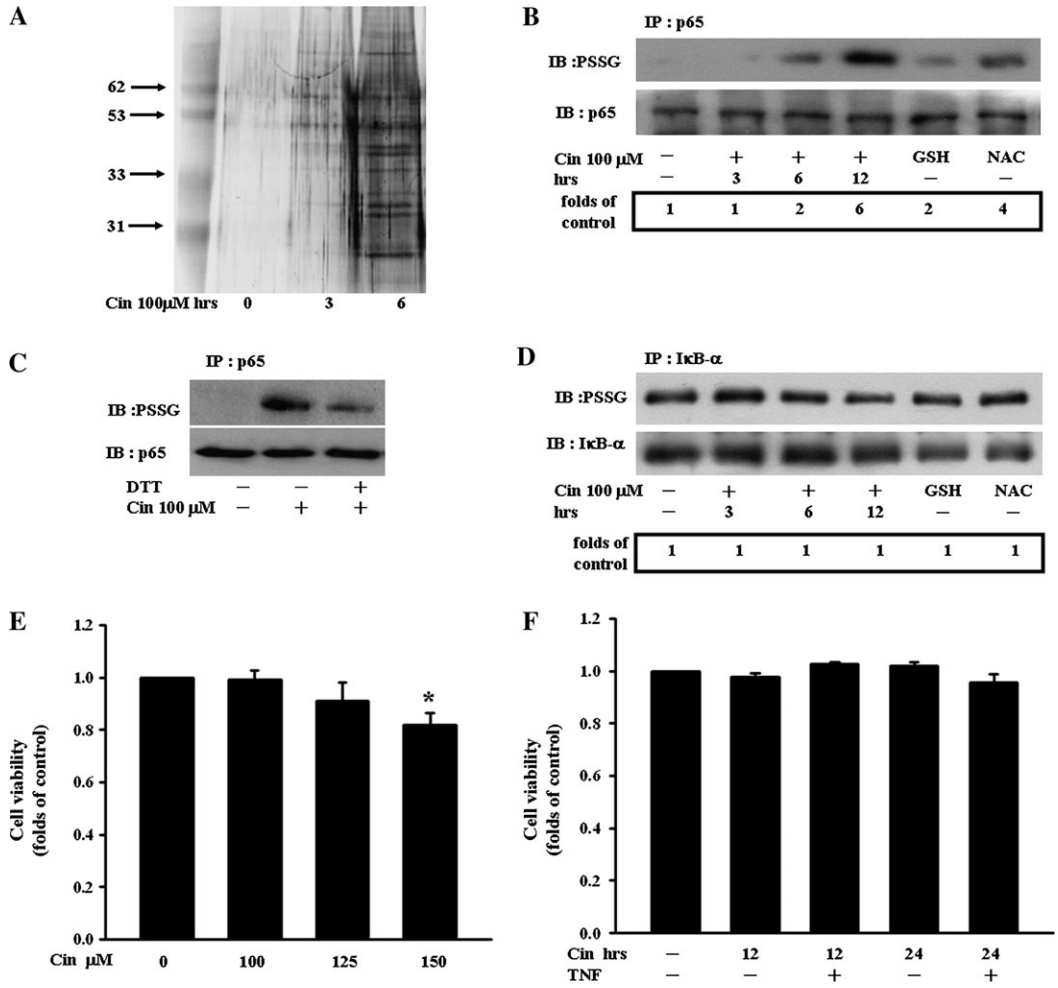

FIG. 3. The cinnamaldehyde induces p65 glutathionylation. (A) ECs were treated with 100μM BioGEE for 1 h and loaded with 100μM cinnamaldehyde for the indicated times to induce protein glutathionylation. (B) ECs were exposed to 100μM cinnamaldehyde for the indicated periods. Some experiments involved treatment with GSH (20mM) or NAC (20mM) for 12 h. S-glutathionylated P65 was immunoprecipitated and detected using an antibody against protein-SSG. (C) ECs were exposed to 100μM cinnamaldehyde for 12 h. Some samples were further incubated with the DTT reducing agent (10mM) for 1 h at 37°C. (D) ECs were exposed to 100μM cinnamaldehyde for the indicated times. Some experiments involved treatment with GSH (20mM) or NAC (20mM) for 12 h. S-glutathionylated proteins were immunoprecipitated using an antibody against IκB, followed by detection of GSH by Western blotting. (E) ECs were incubated with cinnamaldehyde with indicated concentration for 24 h, and cell viability was measured spectrophotometrically using an Alamar blue assay, according to the manufacturer's instructions. Data are expressed as the mean ± SEM of three independent experiments. (F) ECs were incubated with 100μM cinnamaldehyde without or with TNF-α (100 U/ml) for 12 or 24 h, and cell viability was measured spectrophotometrically using an Alamar blue assay. Data are expressed as the mean ± SEM of three independent experiments.

oxidative stimulations. In a previous study from our laboratory, we reported that cinnamaldehyde activates Nrf2 and also Nrf2-related genes including heme oxygenase-1 and glutamate-cysteine ligase (Liao *et al.*, 2008). To further assess the mediating role of Nrf2 in the effects of cinnamaldehyde, a more targeted inhibition of Nrf2 using siRNA was undertaken. ECs were transfected with Nrf2 siRNA to reduce the Nrf2 protein level (Fig. 6A) and this successfully abolished the induction of both the GCLC and GCLM expression following 12 h of cinnamaldehyde pretreatment (Fig. 6B). These data suggest that cinnamaldehyde promotes GCLC and GCLM expression through the activation of the Nrf2 pathway.

The Inhibitory Effects of Cinnamaldehyde Are Dependent upon Nrf2 Activation

To further delineate the role of Nrf2 in cinnamaldehyde-induced p65 glutathionylation and the inhibitory effects of this modification, we directly targeted Nrf2 in pretreated ECs using siRNA. As shown in Figure 7A, Nrf2 siRNA abolished cinnamaldehyde-induced P65 glutathionylation. We next demonstrated that cinnamaldehyde-induced p65 nuclear translocation could be reversed by Nrf2 siRNA (Fig. 7B). As shown in Figure 7C, Nrf2 siRNA prevents the inhibition of ICAM-1 expression in cinnamaldehyde-treated Ecs, indicating that Nrf2

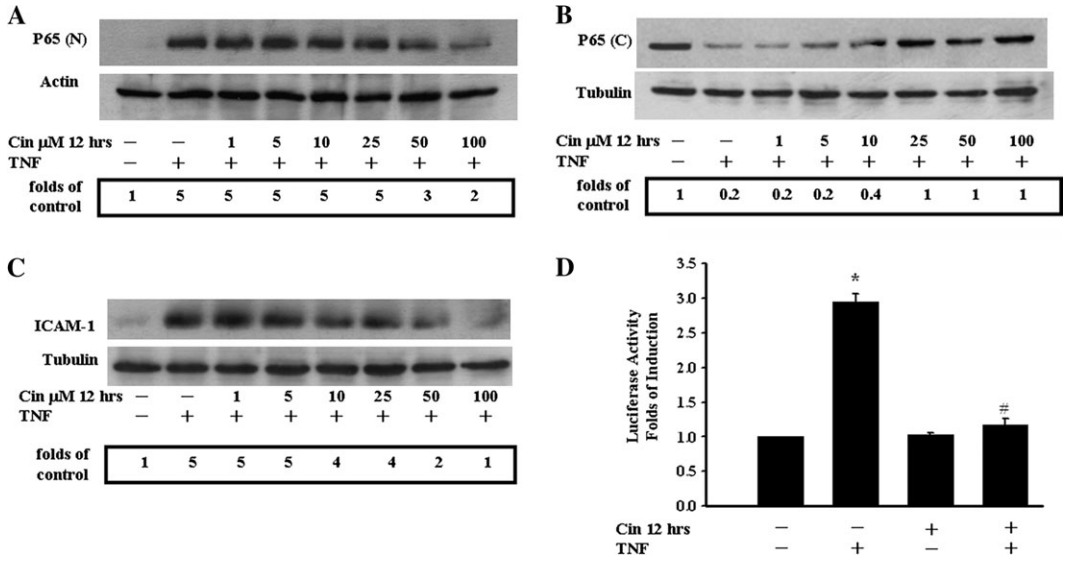

FIG. 4. Cinnamaldehyde inhibits TNF-α–induced NF-κB activation and ICAM-1 expression. (A) ECs were pretreated with cinnamaldehyde for 12 h at the indicated concentrations and then incubated with or without 100 U/ml TNF-α for 1 h. Nuclear extracts (N) were then prepared and subjected to Western blot analysis. (B) ECs were pretreated with cinnamaldehyde for 12 h at the indicated concentrations and then incubated with or without 100 U/ml TNF-α for 1 h. Cytosolic fractions (C) were then prepared and subjected to Western blot analysis. (C) ECs were pretreated with cinnamaldehyde for 12 h at the indicated concentrations and then incubated with or without 100 U/ml TNF-α for 4 h. Cell lysates were prepared and subjected to Western blot analysis. (D) ECs were cotransfected with the NF-κB luciferase reporter construct and β-galactosidase for 16 h. Cells were then exposed to 100μM cinnamaldehyde for 12 h and with or without 100 U/ml TNF-α for another 4 h. Luciferase activity was normalized against β-galactosidase activity, and the untreated value was taken as 1. $^{*}p < 0.05$ compared with untreated ECs; $^{\#}p < 0.05$ compared with TNF-α alone (mean ± SEM).

mediates these effects. These results together suggest that Nrf2 is indeed involved in cinnamaldehyde-induced p65 glutathionylation and NF-κB inhibition.

Induction of Grx-1 Expression by Cinnamaldehyde

Grx has been found to promote the glutathionylation of proteins during glutathionyl radical generation (Starke *et al.*, 2003; Qanungo *et al.*, 2007). To investigate whether cinnamaldehyde increases Grx-1 in ECs, we investigated the transcription levels and activity of this enzyme. As shown in Figure 8A, treatment of the cells with 100μM cinnamaldehyde significantly induces Grx-1 expression which increases over the incubation time course (Fig. 8B). We therefore next analyzed whether cinnamaldehyde also induces Grx-1 promoter activity and as shown in Figure 8C found that the promoter activity of Grx-1 was also activated by 100μM cinnamaldehyde treatment. However, the protein levels of Grx-1 were only marginally increased when ECs were treated with cinnamaldehyde over the incubation time course (Fig. 8D). In parallel experiments, cinnamaldehyde (100μM, 12 h) increased the Grx activity in ECs when compared with untreated cells (Fig. 8E).

The Effects of Cinnamaldehyde-Induced Grx-1 on P65 Nuclear Translocation and ICAM-1 Expression

To further demonstrate the role of Grx-1 in cinnamaldehyde-induced p65 glutathionylation, Grx-1 siRNA and an inhibitor of Grx activity, cadmium chloride ($CdCl_2$), were used in cinnamaldehyde-treated cells. As shown in Figure 9A, Grx-1 siRNA transfection completely inhibits Grx-1 expression in cinnamaldehyde-treated ECs. As shown in Figures 9B and C, however, p65 glutathionylation was increased after 12 h of cinnamaldehyde treatment but was abolished by the inhibition of Grx-1 via either $CdCl_2$ or Grx-1 siRNA. As shown in Figure 9D, Grx-1 siRNA also abolishes the suppressive effects of cinnamaldehyde upon p65 translocation. In a similar experiment, Grx-1 siRNA was further found to inhibit the suppressive effects of cinnamaldehyde upon ICAM-1 expression (Fig. 9E). These results indicate that Grx-1 indeed mediates the inhibitory effects of cinnamaldehyde in TNF-α–treated ECs.

DISCUSSION

In our previous study, we demonstrated that cinnamaldehyde enhances the suppression of TNF-α–induced monocyte/EC interactions by downregulating the expression of the adhesion molecules ICAM-1 and VCAM-1 at noncytotoxic concentrations (Liao *et al.*, 2008). In our present study, we propose the model shown in Figure 10 to illustrate the inhibitory effects by long-term pretreatment. Briefly, the cinnamaldehyde-induced cellular dynamic redox state can be divided into two stages that are dependent upon the treatment duration. In the first stage, GSH depletion and the H_2O_2 levels increase after 1 h of cinnamaldehyde treatment, which may enhance the hydroxy radical levels. These radicals in turn could react with GSH to form thiyl

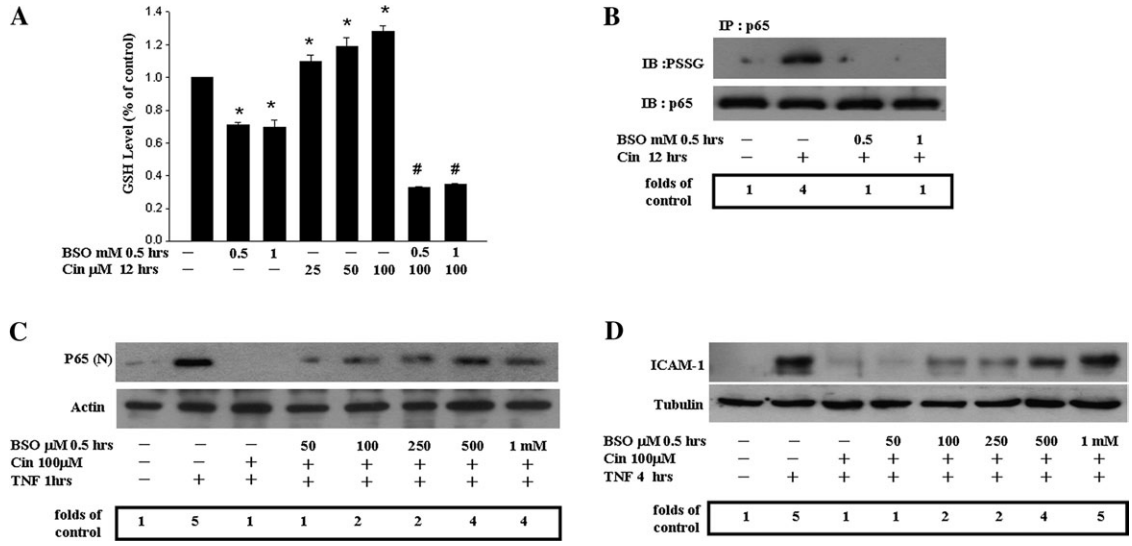

FIG. 5. The effects of intracellular GSH level in cinnamaldehyde-treated ECs. (A) Intracellular GSH levels of ECs incubated with 25, 50, or 100μM cinnamaldehyde for 12 h. ECs were pretreated with BSO (0.5 or 1mM) for 30 min and then cultured without further treatment or exposed to 100μM cinnamaldehyde for 12 h. Data values are expressed as a percentage of the untreated control, which was set at 100%. Results are presented as the mean ± SEM ($n = 3$). *$p < 0.05$ compared with untreated ECs. (B) ECs were pretreated with BSO at the indicated concentrations for 30 min and then cultured without further treatment or exposed to 100μM cinnamaldehyde for 12 h. S-glutathionylated P65 was then immunoprecipitated and detected using antibodies against protein-SSG. (C) ECs subjected to 100 U/ml TNF-α for 1 h were pretreated with BSO at the indicated concentration for 30 min and then cultured without further treatment or exposed to 100μM cinnamaldehyde for 12 h. Nuclear extracts (N) were then prepared and subjected to Western blotting. (D) ECs subjected to 100 U/ml TNF-α for 4 h were pretreated with BSO at the indicated concentration for 30 min and then cultured without further treatment or exposed to 100μM cinnamaldehyde for 12 h.

radicals. In the second stage, after 9 h of cinnamaldehyde treatment, the GSH levels recover to basal levels. The increased number of GSH molecules may react with hydroxy radicals and further enhance thiyl radical formation. The possible increase in thiol radical levels may in turn induce p65 glutathionylation under Grx-1 catalysis. Hence, these inhibitory effects are mediated through two distinct mechanisms that are dependent on the dynamic redox state. In short-term pretreatments, the inhibitory effects of cinnamaldehyde are the result of a decrease of GSH and increase of ROS. However, over a long-term pretreatment, cinnamaldehyde exerts anti-inflammatory effects which appear to be mediated through p65 glutathionylation and involve increased GSH, ROS, and Grx-1 interactions.

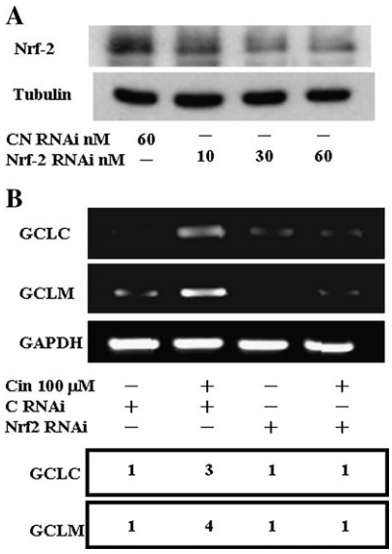

FIG. 6. The effects of Nrf2 on cinnamaldehyde-induced GSH levels. (A) ECs were transfected with control or Nrf2 siRNA for 36 h and the intracellular protein levels of Nrf2 were determined by Western blotting. (B) ECs were transfected with control or Nrf2 siRNA for 36 h and then exposed to 100μM cinnamaldehyde for 12 h. The mRNA levels of GCLM and GCLC genes were determined by RT-PCR.

In our previous study, we demonstrated that during short-term pretreatments, cinnamaldehyde blocks the degradation of IκB-α which results in the inhibition of NF-κB activation (Liao *et al.*, 2008). Lou and Kaplowitz1 (2007) have also indicated in their previous report that GSH depletion downregulates TNF-α–induced IκB kinase activity. Thus, one possible mechanism is that GSH depletion by cinnamaldehyde provides an inappropriate redox state for IκB kinase activity to thereby cause a block in the degradation of IκB-α. However, another possibility may be that during conditions of low intracellular GSH, cinnamaldehyde directly reacts with IκB-α upstream signals via a Michael reaction to block these pathways. The details of these mechanisms remain to be further identified.

In the second stage, after 9 h of cinnamaldehyde treatment, the GSH levels recover to basal levels. The increased number of GSH molecules may react with hydroxy radicals and further enhance the thiyl radical levels. The possible increased thiol

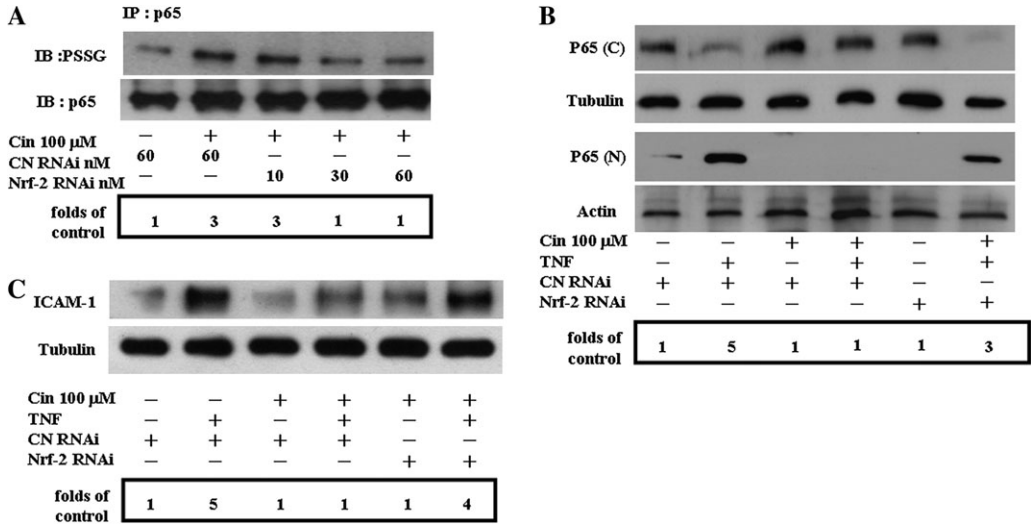

FIG. 7. The inhibitory effects of cinnamaldehyde are dependent upon Nrf2 activation. (A) ECs were transfected with control or Nrf2 siRNA for 36 h and exposed to 100μM cinnamaldehyde for 12 h. S-glutathionylated P65 was detected as above. (B) ECs were transfected with control or Nrf2 siRNA for 36 h, exposed to 100μM cinnamaldehyde for 12 h, and then to 100 U/ml TNF-α for 1 h. Nuclear (N) and cytosolic (C) extracts were then prepared and subjected to Western blot analysis. Relative inductions were actin-normalized nuclear fraction compared with control values. (C) ECs were transfected with control or Nrf2 siRNA for 36 h, exposed to 100μM cinnamaldehyde for 12 h, and then to 100 U/ml TNF-α for 4 h.

radical levels may in turn induce p65 glutathionylation under Grx-1 catalysis. Furthermore, the p65 target of glutathionylation by cinnamaldehyde links the nuclear translocation of this protein with inhibition of NF-κB activity and ICAM-1 expression. On the other hand, the higher levels of GSH may interact with cinnamaldehyde and decrease its inhibitory effects upon IκB-α degradation after 6 h pretreatment (Liao *et al.*, 2008). During this stage, the inhibitory effects of cinnamaldehyde are independent of the degradation of IκB-α but are mediated via the induction of Nrf2-related genes.

In our previous study, we found that the α,β-unsaturated carbonyl moiety of 15d-PGJ_2 is crucial for the inhibition of the interleukin-6–stimulated phosphorylation of tyr705on STAT3 and that this is dependent on its own electrophilic reactivity in ECs (Wung *et al.*, 2006). The ability of chalcone to inhibit STAT3 and NF-κB activation is also defined by an α,β-unsaturated carbonyl structure (Liu *et al.*, 2007). Curcumin, another α,β-unsaturated carbonyl structure containing phytochemicals, has also been found to exert anti-inflammatory effects in various types of cells (Motterlini, 2003). In addition, acrolein, a highly electrophilic α,β-unsaturated aldehyde, exhibits a cytoprotective mechanism in a GSH-dependent manner at sublethal concentrations (Wu *et al.*, 2006). Otherwise, the anti-inflammatory ability of electrophiles is facilitated by the propensity to modulate the intracellular GSH levels (Liu *et al.*, 2008). The electrophilic activity of active compounds is therefore crucial for their cytoprotective properties. In our present study, we demonstrate that BSO and the knock down of Nrf2 by siRNA abolish the glutathionylation of p65 after a long-term incubation with cinnamaldehyde. Thus, an increase in the GSH levels is a major requirement for the glutathionylation of p65. Foresti *et al.* (2005) have also indicated that electrophilic phytochemicals can undergo oxidation and successively give rise to thiyl radicals, which would then propagate oxidative stress reactions intracellularly. Thiyl radicals can interact with the sulfhydryl residues of intracellular targets, including NF-κB (Cernuda-Morollón *et al.*, 2001; Heiss *et al.*, 2001; Rossi *et al.*, 2000). Hence, the increase in intracellular GSH may result in the generation of sulfhydryl radicals, which then causes the glutathionylation of signal transduction proteins. Taken together, the current evidence implicates the glutathionylation of p65 as a general mechanism by which electrophiles may regulate NF-κB activation via the modulation of the intracellular GSH levels.

Remarkably, protein glutathionylation is an important posttranslational modification that induces functional changes in the targeted proteins. Grx contains a dithiol in its conserved catalytic site sequence and has a low equilibrium constant for GSH in its cysteine residue at position 22 (Fernandes and Holmgren, 2004). Recent studies have demonstrated that increased Grx expression and activity may prevent the cytokine-induced glutathionylation of proteins (Reynaert *et al.*, 2006; Shelton *et al.*, 2007). On the other hand, in the event that glutathionyl radical–generating conditions prevail, Grx promotes the glutathionylation of proteins and shows functional changes (Starke *et al.*, 2003). Consistent with the results of this previous study, Grx was found in another report to be involved in NAC-induced glutathionylation of NF-κB in hypoxic pancreatic cancer cells (Qanungo *et al.*, 2007). In our present study, Grx appeared to mediate glutathionylation and the inactivation of NF-κB in ECs treated with cinnamaldehyde, suggesting that enhancing Grx activity

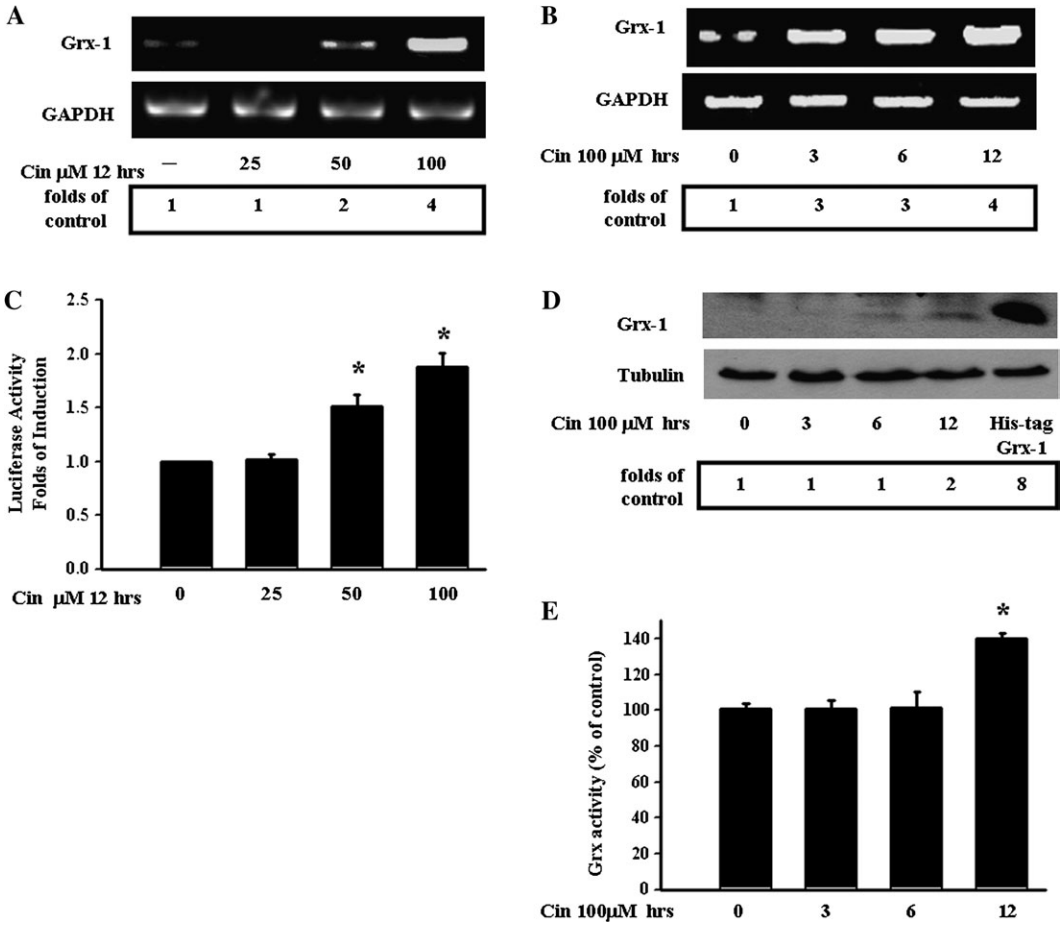

FIG. 8. Induction of Grx-1 expression by cinnamaldehyde. (A) ECs were treated with cinnamaldehyde at the indicated concentrations for 12 h and then subjected to RT-PCR analysis of Grx. (B) ECs were treated with 100μM cinnamaldehyde for the indicated times and then subjected to RT-PCR analysis of Grx. (C) Cells were transfected with the ARE-luciferase construct (Grx-1) and after 12 h were maintained in low-serum medium for a further 6 h and then stimulated with 100μM cinnamaldehyde for an additional 12 h. The cells were then lysed and analyzed for luciferase activity. Induction is shown as an increase in the normalized luciferase activity in the treated ECs, relative to the control. Results are the means ± SEM from at least three separate experiments. *$p < 0.05$ versus treated ECs. (D) EC cultures were incubated with 100μM cinnamaldehyde for the indicated periods. Some experiments involved ECs transfected with the His-tag-Grx for 12 h, which were then lysed and analyzed by Western blotting with antibodies against Grx-1 or tubulin as indicated. (E) EC cultures were incubated with 100μM cinnamaldehyde for the indicated periods and a Grx activity assay was performed ($n = 3$ or 4 cell cultures). *$p < 0.05$, relative to control.

promotes anti-inflammation. We further found that the cellular GSH levels are rapidly reduced over short-term treatments with cinnamaldehyde but increased after 9 h of exposure. This biphasic GSH profile may raise the levels of thiyl radicals, which could in turn interact with the sulfhydryl residues of proteins and thereby propagate protein glutathionylation mediated by Grx. Hence, the interaction of biphasic GSH and Grx may be a general phenomenon induced by compounds containing an α,β-unsaturated carbonyl moiety. Our current findings enrich our understanding of the role of such molecules in signal transduction and may potentially aid the identification of new therapeutic targets for a range of diseases.

A growing number of transcription factors have been shown to be modified by glutathionylation, including AP-1, Sp1, and cyclic adenosine monophosphate response element–binding protein and have also been demonstrated to be redox sensitive (Mieyal *et al.*, 2008). These observations implicate glutathionylation as a common mechanism of transcriptional regulation. NF-κB serves as a prototype for transcription factors whose activity is dependent upon the redox status of protein thiols. A previous study has shown that the NO-mediated S-nitrosylation of a cysteine within the RHD of p65 can function to inhibit the DNA-binding activity of this protein, thereby affecting the transcription of a broad range of inflammatory mediators (Kelleher *et al.*, 2007). The *in vitro* glutathionylation of p50 has also been shown to inhibit its DNA-binding activity (Pineda-Molina *et al.*, 2001). Previous studies have also found that NF-κB activation is inhibited by the glutathionylation of upstream mediators of the cytoplasmic NF-κB signaling pathways (Reynaert *et al.*, 2006). However, in our previous study, we found that the inhibitory effects of cinnamaldehyde

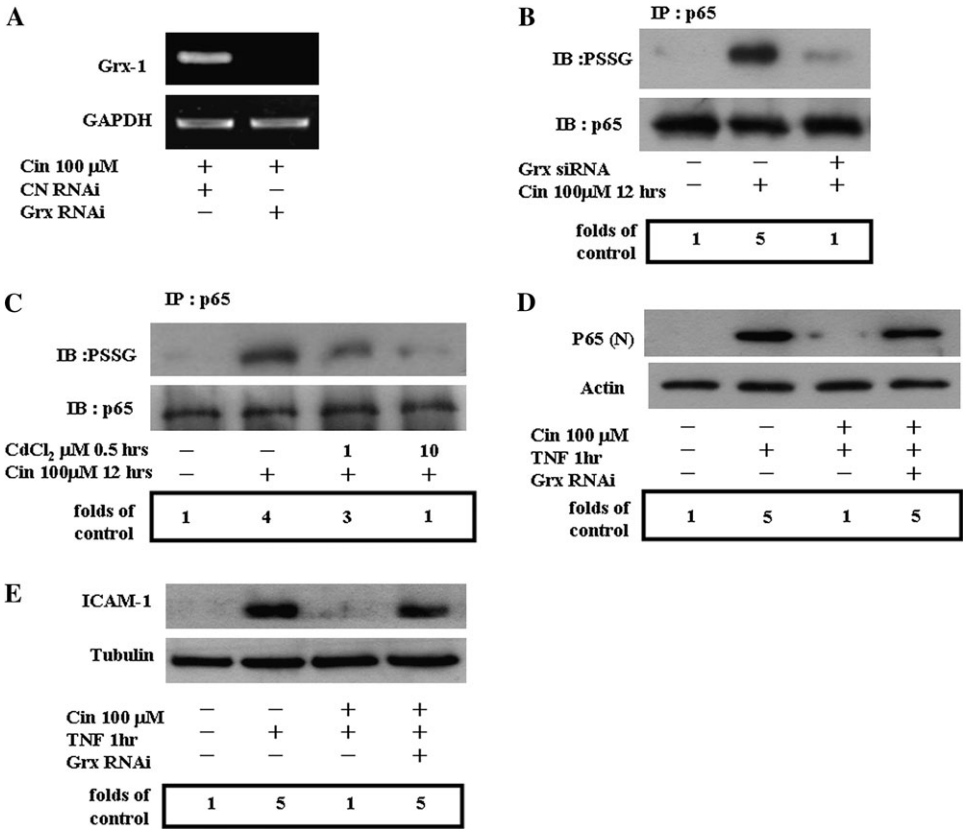

FIG. 9. The effects of cinnamaldehyde-induced Grx-1 on P65 nuclear translocation and ICAM-1 expression. (A) ECs were transfected with control or Grx siRNA for 36 h and exposed to 100μM cinnamaldehyde for 12 h. Gene expression of Grx was determined by RT-PCR. (B) ECs were transfected with control or Grx-1 siRNA for 36 h and then exposed to 100μM cinnamaldehyde for 12 h. S-glutathionylated proteins were detected as above. (C) ECs were pretreated with $CdCl_2$ (1 or 10μM) for 30 min and then exposed to 100μM cinnamaldehyde for 12 h. (D) ECs were transfected with control or Grx siRNA for 36 h and exposed to 100μM cinnamaldehyde for 12 h and then 100 U/ml TNF-α for 1 h. (E) ECs were transfected with control or Grx siRNA for 36 h and exposed to 100μM cinnamaldehyde for 12 h and then 100 U/ml TNF-α for 4 h.

upon NF-κB were not exerted through its binding activity but via its nuclear translocation (Liao *et al.*, 2008). We further demonstrate in our current study that this inhibition of nuclear translocation is mediated by p65 glutathionylation via GSH and Grx-1 activity.

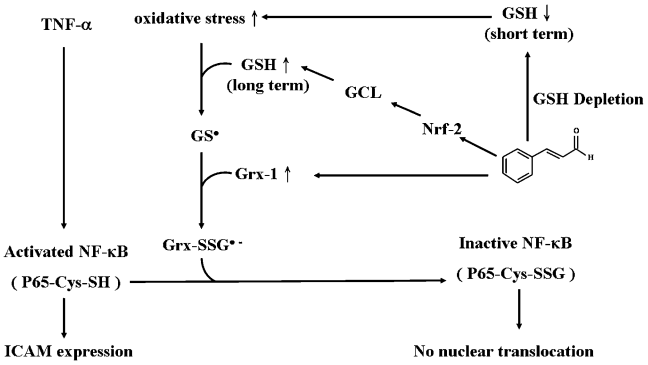

FIG. 10. Proposed model of the inhibitory effects of cinnamaldehyde upon NF-κB activation. Increase in the intracellular levels of GSH and Grx-1 by means of the delayed treatment with cinnamaldehyde promotes p65 glutathionylation, which then inhibits p65 nuclear translocation thought Nrf2 pathway. See the ''Discussion'' section for details.

In our present experiments also, we examined whether the GSH upregulation modulated by cinnamaldehyde facilitates p65 protein glutathionylation and results in a potential mechanism underlying the inhibition of NF-κB. In addition, we demonstrate the role of Grx-1 in the glutathionylation of inflammatory signal proteins in ECs. As a regulatory mechanism, protein glutathionylation enhances our understanding of the role of Grx-1 in signal transduction and may potentially aid in the identification of new therapeutic targets for a variety of diseases. In this regard, cinnamaldehyde-regulated glutathionylation is likely to be an important mechanism by which inflammatory pathways and signaling mediators are modulated and, thus, may also provide new insights into novel anti-inflammation strategies.

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