High Concentration of Antioxidants N-Acetylcyesteine and Mitoquinone-Q Induces Intercellular Adhesion Molecule 1 and Oxidative Stress by Increasing Intracellular Glutathione

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High Concentration of Antioxidants N-Acetylcyesteine and Mitoquinone-Q Induces Intercellular Adhesion Molecule 1 and Oxidative Stress by Increasing Intracellular Glutathione

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In endothelial cells, the intracellular level of glutathione is depleted during offering protection against proinflammatory cytokine TNF-α-induced oxidative stress. Administration of anti-inflammatory drugs, i.e., N-acetylcyesteine (NAC) or mitoquinone-Q (mito-Q) in low concentrations in the human pulmonary aortic endothelial cells offered protection against depletion of reduced glutathione and oxidative stress mediated by TNF-α. However, this study addressed that administration of NAC or mito-Q in high concentrations resulted in a biphasic response by initiating an enhanced generation of both reduced glutathione and oxidized glutathione and enhanced production of reactive oxygen species, along with carboxylation and glutathionylation of the cellular proteins. This study further addressed that 1αB kinase, a phosphorylation-dependent regulator of NF-κB, plays an important regulatory role in the TNF-α-mediated induction of the inflammatory cell surface molecule ICAM-1. Of the two catalytic subunits of IKK (IKKα and IKKβ), low concentrations of NAC and mito-Q activated IKKα activity, thereby inhibiting the downstream NF-κB and ICAM-1 induction by TNF-α. High concentrations of NAC and mito-Q instead caused glutathionylation of IKKα, thereby inhibiting its activity that in turn enhanced the downstream NF-κB activation and ICAM-1 expression by TNF-α. Thus, establishing IKKα as an anti-inflammatory molecule in endothelial cells is another focus of this study. This is the first report that describes a stressful situation in the endothelial cells created by excess of antioxidative and anti-inflammatory agents NAC and mito-Q, resulting in the generation of reactive oxygen species, carboxylation and glutathionylation of cellular proteins, inhibition of IKKα activity, and up-regulation of ICAM-1 expression. The Journal of Immunology, 2007, 178: 1835-1844.

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The present investigation examines how TNF-α-dependent ICAM-1 expression in endothelial cells varies with changing levels of GSH/GSSG generated by NAC or mito-Q. The current study focuses on the effects of the excess GSH/GSSG-dependent intracellular stress on the TNF-α-dependent expression of proinflammatory protein ICAM-1.

Materials and Methods

Materials

TNF-α, NAC, DL-buthionine-[-S]-sulfoximine (BSO), and glutaraldehyde were obtained from Sigma-Aldrich. Anti-rabbit ICAM-1 Ab and mouse
normal IgG were purchased from Santa Cruz Biotechnology. Anti-GAPDH, anti-rabbit IgG (total), and NF-κB-p65 (total) Abs were obtained from Chemicon International. Anti-rabbit glutathione peroxidase (GPx) 1, glutathione peroxidase 4 (GPx4), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase Abs (G6PD) were purchased from Abcam. Anti-rabbit γ-glutamylcysteine synthetase (γ-GCS) heavy subunit Ab was obtained from Lab Vision. Monoclonal GSH Ab was purchased from Virogen. Monoclonal IκB kinase (IκK) α and IκKB Abs were purchased from Upstate Biotechnology. Monoclonal phospho-IκB-α was obtained from BD Pharmingen and anti-rabbit phospho-NF-κB-p65 Ab was purchased from Cell Signaling Technology. The monoclonal and polyclonal HRP-conjugated secondary Abs were obtained from Chemicon International. The ECL detection system was purchased from Amersham Biosciences. The sources of all other chemicals are described in their specific and relevant methods sections.

Methods

Cell treatment, viability, and cellular toxicity assessment

Human pulmonary arterial endothelial cells (HPAECs; Cambrex) were grown in complete EGM-2 medium as described by the supplier (Clonetech). Confluent HPAECs were washed and subsequently incubated overnight with EBM-2 basal medium (Cambrex) containing 2% charcoal-dextran-treated FBS (HyClone) and antibiotic-antimycotics. Over night incubation, the medium was replaced with fresh EBM-2 medium and cells were treated with NAC or mito-Q. Mito-Q, a potential new class of antioxidant, is generated by covalently linking phosphonionium ion with coenzyme-Q (a gift from M. Murphy, Medical Research Council, Dunn Human Nutrition Unit, Cambridge, U.K.). After an 18-h treatment with NAC or mito-Q, 10 ng/ml TNF-α was added to the cell culture medium and the combined treatment was continued for another 6 h. Cellular viability was measured by trypan blue exclusion (Invitrogen Life Technologies) and cellular toxicity was assessed by measuring the release of lactate dehydrogenase into the medium (Roche).

Determination of the levels of GSH and GSGG

The total glutathione (GSH plus GSGG) concentration was measured spectrophotometrically using the 5,5’dithio-bis(2-nitrobenzoic acid)-GSH disulfide reductase recycling assay by recording the increase in absorbance for 3 min at 412 nm. GSSG was selectively measured after assaying samples in which GSH is masked by pretreatment with 2-vinylpyridine. The difference between the two values gave the GSH levels in cells. Calculations were made by using a standard curve for GSSG.

Measurement of the activity of γ-GCS, glutathione peroxidase (cystolic GPx), and GR

γ-GCS activity was assessed using the coupled assay with pyruvate kinase and lactate dehydrogenase (10). The rate of decrease in absorbance was recorded at 340 nm and 37℃. Enzyme specificity was defined as micromoles of NADH oxidized per minute per milligram of protein, which is equal to 1 IU. BSO (50 μM) was used to test the specificity of the reaction. Cystolic GPx and GR activities were spectrophotometrically measured according to the manufacturer’s protocol (Oxis Research).

Measurement of H₂O₂ generation

Extracellular H₂O₂ was measured using an homovanillic acid (HVA) assay (11). Briefly, following treatment with specific pharmacologic agents, confluent cells in a 6-well plate were washed twice and incubated with serum and antibiotic-free 2 ml of EBM-2 basal medium containing 100 μM HVA and 8 U of HRP for 2 h at 37℃. Following incubation, 1 ml of medium was removed and mixed with 75 μl of HVA stop buffer (0.1 M glycine/0.1 M NaOH (pH 12) and 25 mM EDTA in PBS). Fluorescence was read at 312 nm excitation and 412 nm emissions and normalized to cell protein (Bradford reagent; Bio-Rad).

Protein carbonylation assay

Protein carbonylation was measured as described previously (12). Briefly, cell pellets were suspended with lyso buffer containing protease inhibitors and 0.1% Tween 20 and lysed with repeated freezing and thawing for 45 min. For the detection of carbonylation, 800 μl (10 μg) of diluted protein extracts was reacted with 200 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH; prepared in 2.5 M HI) at room temperature for 1 h. Proteins were precipitated with 10% (w/v) ice-cold TCA and washed three times with ether-anel(ethyl acetate (1:1 v/v) to remove excess DNPH, boiled with Laemmli sample buffer containing 2-ME, and loaded on SDS-PAGE, and the membrane was probed with anti-DNPH IgG at 1/2000 dilution (Sigma-Aldrich).

Western blotting of total and phosphoproteins

Western blotting was performed as described previously (14). Briefly, 40 μg of protein was transferred onto a polyvinylidene difluoride membrane (Bio-Rad) that was blocked overnight with 5% nonfat dry milk. The membrane was treated with the following Abs for 2–3 h at room temperature in the presence of 0.1% BSA: anti-RAGE (1/500), anti-ICAM-1 (1/250), anti-GCS (1/4000), anti-GPx1 (1/500), anti-GPx4 (1/2000), anti-GR (1/1000), anti-IκKα (1/1000), anti-IκKB (1/2000), anti-phospho-IκBα (1/1000), anti-NF-κB-p65 (1/2000), anti-phospho-NF-κB-p65 (1/1000), or anti-GAPDH (1/10000) (an internal control). HRP-conjugated secondary Abs were used at a dilution of 1/5000–1/20,000 in the presence of 0.1% BSA for 1 h at room temperature. Signals were developed using the ECL detection system. For detection of phosphoproteins, lysates were treated with a phosphatase inhibitor mixture (Calbiochem). Then, 200 μg of each sample protein was enriched using a phosphoprotein enrichment kit (BD Biosciences and loaded onto gels and subjected to Western blotting.

IKK assay

The IKK assay was performed using the method described previously (15). Precleared lysates were incubated overnight with agargose conjugate-coupled anti-IKKα Ab, washed in lysis buffer, and resuspended in kinase buffer (20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl, and 20 mM β-glycerophosphate) containing 2 μg of GST-IκBα as substrate (US Biological). The mixture was incubated for 1 h at 30℃. The binding reaction was started by addition of radioactive ATP and stopped by the addition of Laemmli buffer. Radiolabeled proteins were resolved on SDS-PAGE, and the activity was measured by phosphor imaging.

Transfection of IΚKα and IΚkB small interfering RNA (siRNA)

Since HPAECs had minimum transfection efficiency, human microvascular endothelial cell 1 (a gift from J. Candal, Centers for Disease Control and Prevention, Atlanta, GA) were used for transfection with 20 – 60 nM small interfering RNA (siRNA) or sense RNA of IKKβ (smart pool siRNA; Chemicon International) for 24 h before carrying out the procedure as described previously (16).

ELISA and monocye adhesion assay

Cell surface ELISA for ICAM-1 was performed as described previously (17). A monocyte adhesion assay was performed as described previously (18). Briefly, after the predetermined treatment, cells were washed twice with warm medium and freshly grown human peripheral blood monocytes (U937 cell line from American Type Culture Collection) were added to the endothelial cell monolayer and incubated for 1 h at 37℃ to allow binding. The unattached monocytes were washed by vigorous rinsing. Following two washings with PBS, cells were fixed with 1% glutaraldehyde in PBS. The monocytes bound to the endothelial cells were counted under an inverted microscope with an eyepiece grid. For each well, five fields were selected randomly and the average was calculated.

Northern blotting

Northern blots were performed as described previously (17). Briefly, ICAM-1 cDNA (a gift from T. Springer, Harvard Medical School, Boston, MA) was labeled with [α-32P]dCTP (Valeant Pharmaceuticals) using Ready to Go DNA labeling beads (Amersham Biosciences). The radiolabeled cDNA was purified using a Nick Column (Amersham Biosciences) and added to the prehybridized (4 h) RNA-containing membranes for overnight hybridization. GAPDH cDNA was used as an internal control.

Statistics

Values are expressed as mean ± SD and were obtained from three separate experiments in each group. Differences between the groups were assessed by one-way ANOVA and Newman-Keuls multiple comparison tests. Results having p < 0.05 are considered as significant.
Results
Rescue of TNF-α-dependent glutathione depletion by NAC and mito-Q: role of glutathione synthesis/recycling system

One of the mechanisms whereby TNF-α exerts its proinflammatory effects is through depletion of GSH and subsequent enhancement of oxidative stress (6, 7). NAC has been widely used as an antioxidant and anti-inflammatory agent that induces GSH synthesis and predominantly enhances the cytosolic GSH level (19). Similarly, mito-Q, a mitochondrion-targeted pharmacologic agent, may protect the cells from BSO-induced oxidative stress, although its potential role on the GSH cycle has yet to be determined (20). Moreover, little attention has been given to the effects of high levels of glutathione (GSH/GSSG) that may be generated by NAC or mito-Q on the activity and expression of different components of the glutathione cycle and its subsequent effects on the biosynthesis of proinflammatory molecules.

To begin to investigate the relationship between the GSH level and ICAM-1 expression, HPAECs were incubated with varying concentrations of NAC or mito-Q followed by measurement of GSH and GSSG levels. A comparative analysis of the effects of various doses of antioxidants on the GSH/GSSG ratio indicated that cells had a large net gain of GSH (Fig. 1, A and C) with low to moderate concentrations of NAC or mito-Q, shifting the redox balance toward a reducing environment. However, although low to moderate concentrations of NAC or mito-Q depleted the TNF-α-generated GSSG levels (Fig. 1, B and D, lanes 3–6), a high-level generation of GSSG at higher concentrations of NAC or mito-Q was particularly noticed (Fig. 1, B and D, lane 7). Since high-level GSSG is an indication of oxidative stress, this observation suggested that the cells had been exposed to oxidative stress at higher concentrations of NAC or mito-Q. In this experiment, triphenylmethylphosphonium that was considered as a mito-Q control did not show any statistically significant effect (data not shown).

To determine the effects of NAC or mito-Q on the glutathione cycle, expression, and activity of γ-GCS, the rate-limiting enzyme of GSH synthesis, GPx, the enzyme involved in conversion of GSH to GSSG, and GR, the enzymes involved in recycling of GSSG back to GSH, were assessed. Neither TNF-α, NAC, nor mito-Q in combination or alone influenced the expression of GCS, GPx, and GR (data not shown), indicating that there was no control existing at the level of expression of these enzymes. Upon testing the activities, TNF-α alone showed no significant effect on GCS, GPx, and GR activities (Fig. 2, A–C, lane 2).

A comparative activity analysis showed that mito-Q maximally induced both GPxs (Fig. 2B, lanes 5 and 6) and GR (Fig. 2C, lanes 5 and 6) activities without effecting GCS activity (Fig. 2A, lanes 5 and 6), indicating that mito-Q is predominantly involved in recycling, but not synthesis of GSH. A similar study of NAC-treated cells demonstrated that although NAC significantly induced GSH synthesis by enhanced GCS activity (Fig. 2A, lanes 3 and 4), GR activity remained unchanged by low concentrations of NAC (Fig. 2C, lane 3). There was no significant inhibition of GR activity at high concentrations of NAC (Fig. 2C, lane 4). NAC also moderately utilized GSH by activating GPx activity (Fig. 2B, lanes 3 and 4). However, the results showed net accumulation of GSH by NAC treatment. This indicates that the rate of GSH synthesis by increased GCS activity is much higher than the rate of GSH utilization by GPx activity. These results, along with no effect of NAC on GR activity, suggest that GSH accumulation by NAC is mainly due to induced GSH synthesis, without affecting the recycling of GSSG. These results further indicate that for the moderate to high level induction of GSH/GSSG generation either by glutathione synthesis (NAC) or glutathione recycling (mito-Q), cells utilized at least part of the glutathione pool by inducing GPx activity.

To further examine how mito-Q affected the GSH/GSSG cycle, we determined the expression and activity of G6PD, a rate-limiting enzyme of the pentose phosphate pathway that generates cytosolic NADPH, a cofactor utilized by GR. A moderate level induction of G6PD expression and activity was observed by TNF-α (Fig. 3, lane 2) or TNF-α-treated cells pretreated with mito-Q (Fig. 3, lanes 3 and 6). At low concentrations, while mito-Q significantly induced G6PD expression it did not reflect in increased G6PD activity (Fig. 3, lane 5). At high concentrations, mito-Q induced both expression and activity of G6PD (Fig. 3, lane 6). Enhancement of G6PD expression and activity by mito-Q, a mitochondria-specific antioxidant, indicates that mito-Q activity is not limited to mitochondria but also influences the cytosol. Of note, mito-Q also increased isocitrate dehydrogenase activity, an enzyme responsible for mitochondrial NADPH production (data not shown). In contrast to mito-Q, although 3 mM NAC-pretreated cells showed no significant alteration of G6PD expression and activity, 30 mM...
NAC significantly attenuated TNF-α-dependent G6PD expression and activity (Fig. 3, lanes 3 and 4). Increased expression and activity of NADPH-generating enzymes is reflected in increased generation of NADPH (data not shown).

Reversal of TNF-α-dependent ICAM-1 expression by low vs high concentrations of mito-Q or NAC

TNF-α, a pleotropic proinflammatory cytokine, has been implicated in the process of inflammation and sepsis in endothelial cells (4, 5) and is responsible for the induction of TNF-α-dependent cell surface ICAM-1 expression. The foregoing results indicating that NAC or mito-Q dose-dependently increased cellular GSH levels led us to investigate whether NAC or mito-Q similarly attenuate TNF-α-dependent ICAM-1 expression. The results showed that low concentrations of NAC attenuated TNF-α-dependent ICAM-1 mRNA (Fig. 4, A1 and A2, lanes 3–5) and protein (Fig. 4, B1 and B2, lanes 3–5) expression. In contrast, high concentrations of NAC showed no significant effect in attenuating TNF-α-dependent ICAM-1 expression (Fig. 4, B1 and B2, lanes 6 and 7). However, comparison of ICAM-1 expression by low (3 mM) vs high (10–30 mM) concentrations of NAC-pretreated cells indicates a significantly high-level expression of ICAM-1 by high concentrations of NAC.

Similarly, mito-Q at its low concentrations attenuated TNF-α-dependent ICAM-1 mRNA (Fig. 5, A2, lanes 3 and 4) and protein (Fig. 5, B1 and B2, lanes 3 and 4) expression. However, a 3- to 4-fold induction of ICAM-1 expression was particularly noticed at high concentrations of mito-Q (Fig. 5, B1 and B2, lanes 5 and 6) when compared to the mito-Q effect with TNF-α-only-treated cells (Fig. 5, B1 and B2, lane 2). ICAM-1 expression by high concentrations of mito-Q was attenuated by cyclohexamide (data not shown), indicating that mito-Q induced ICAM-1 expression by affecting translation. Of note, a similar biphasic response was also observed when HPAECs were treated by various concentrations of NAC and mito-Q in the absence of TNF-α (data not shown).

Implications of high concentrations of NAC or mito-Q on H₂O₂ generation and protein carbonylation

To determine whether the enhanced level of apparent oxidative stress associated with high concentrations of NAC or mito-Q was responsible for the activation of ICAM-1 expression, we measured the accumulation of H₂O₂ in the cell and protein carbonylation as the parameters of oxidative stress under various treatment conditions. The results showed that the low concentrations of NAC or mito-Q protected the cells from TNF-α-induced oxidative stress by attenuating H₂O₂ accumulation (Fig. 6A, lanes 3 and 5). In contrast, both NAC and mito-Q showed no significant effect in attenuating TNF-α-induced H₂O₂ accumulation at their high concentrations (Fig. 6A, lanes 4 and 6).

Another important characteristic of oxidative stress is the carbonylation of the cellular proteins (21). Increased carbonylation of the proteins was observed by TNF-α and high concentrations of NAC or mito-Q (Fig. 6B, lanes 2, 4, and 6), indicating that at their high concentrations NAC or mito-Q failed to attenuate TNF-α-induced protein carbonylation. In contrast, low concentrations of...
NAC or mito-Q prevented the accumulation of the TNF-α-dependent carboxylated proteins (Fig. 6B, lanes 3 and 5). In these experiments, the internal control GAPDH did not show any change. These results further confirmed that NAC or mito-Q at high concentrations failed to attenuate TNF-α-induced oxidative stress in the cells.

**IKKα as a mediator of glutathione-dependent ICAM-1 expression: identification of IKKα-glutathione complex**

To investigate how glutathione-dependent oxidative stress affects ICAM-1 expression, IKK activity was monitored. Although it is widely recognized that of the two catalytic subunits of the IKK complex, IKKβ activates NF-κB (22–24) and therefore plays a pivotal role in ICAM-1-mediated inflammatory reactions (25), the exact role of IKKα in ICAM-1 expression is not known. However, recently IKKα was established as a negative regulator of mouse macrophage activation and inflammation by its suppression of NF-κB activity, indicating that IKKα may have a varied regulatory role in NF-κB activation (15). These observations led us to speculate that IKKα might play a pivotal role in controlling the inflammation by suppressing NF-κB activity in vascular endothelium. Our results indicated that low concentrations of NAC and mito-Q showed no effect on TNF-α-dependent glutathionylation of IKKα (Fig. 7A, lanes 4 and 6). The extent of complex formation was profoundly increased by NAC or mito-Q at their high concentrations (Fig. 7A, lanes 5 and 7), indicating that a high level of glutathionylation of the IKK complex.

**Implication of glutathionylation of IKKα on NF-κB activity**

To investigate how glutathionylation could influence the activity of IKKα or IKKβ, kinase activity assays were performed. Using GST-IkBα as a substrate, NAC or mito-Q inhibited TNF-α-dependent IkBα activity at low concentrations, but failed to do so at high concentrations (Fig. 7B). Using siRNA against IKKα and IKKβ, it was observed that the inhibitory properties of 10 nM mito-Q were retained in the presence of IKKβ siRNA but were lost in the presence of IKKα siRNA (Fig. 7B). Of note, no significant effect of sense RNA was observed in this experiment (data not shown). These results indicated that IKKα might be involved in transmitting an inhibitory signal in the presence of low concentration of mito-Q, which might be lost at high concentrations.

To further elucidate how IKK might affect the ICAM-1 expression, we determined the phosphorylation of IkBα, translocation of NF-κB-p65 from cytoplasm to nucleus, and phosphorylation of NF-κB-p65. Our results indicated that at low concentrations, NAC or mito-Q inhibited TNF-α-dependent phosphorylation of IkBα (Fig. 8A, lanes 3 and 5) and translocation of NF-κB-p65 (Fig. 8, B1,

![FIGURE 4](image-url) Northern and Western blots showing the effects of preincubation of NAC on the TNF-α-dependent ICAM-1 mRNA and protein expression in HPAECs. Cells were treated exactly as discussed in Fig. 1. A1 and A2, ICAM-1 mRNA and its quantitative densitometric units, respectively. B1 and B2, ICAM-1 protein and its quantitative densitometric units, respectively. *, Significant alteration than the TNF-α-treated cells only (A and B, lane 2). **, Significant alteration than low concentration of NAC and TNF-α-treated cells only (A and B, lane 5). This figure is a representation of four similar experiments.

![FIGURE 5](image-url) Northern and Western blots showing the effects of preincubation of mito-Q on the TNF-α-dependent ICAM-1 mRNA and protein expression in HPAECs. Cells were treated exactly as discussed in Fig. 1. A1 and A2, ICAM-1 mRNA and its quantitative densitometric units, respectively. B1 and B2, ICAM-1 protein and its quantitative densitometric units, respectively. *, Significant alteration than the TNF-α-only-treated cells (A1, A2, B1, and B2, lane 2). This figure is a representation of four similar experiments.
but showed a significant difference from the low concentration of NAC (NF-κB, lanes 3 and 5). High concentrations of NAC or mito-Q (A and B, lanes 4 and 6) showed no significant difference in the ROS generation and carbonylated proteins in comparison to TNF-α-only-treated cells (lane 2) but showed a significant difference from the low concentration of NAC (A and B, lane 3) or mito-Q (A and B, lane 5). The bar graph in B is not shown. * and **, Significant alteration than the TNF-α-only-treated cells and low concentration of NAC or mito-Q plus TNF-α-treated cells, respectively. This figure is a representation of five similar experiments.

Evidences that glutathionylation of preproteins or proteins alters their functional form: implications of ICAM-1 glutathionylation

ICAM-1 is a cell surface adhesion molecule belonging to the Ig superfamily, which interacts with the counterreceptors on monocytes/macrophages and initiates inflammatory and oxidative reactions (26). To determine whether differential expression of cell surface ICAM-1 mediated by different concentrations of NAC or mito-Q (Figs. 4 and 5) also alters its monocyte-binding capacity, a monocyte-binding assay was performed. The results suggested that NAC or mito-Q attenuated TNF-α-dependent increased monocyte adhesion at low concentrations (Fig. 9A, lanes 3 and 5). In contrast, high concentrations of NAC or mito-Q showed no significant difference in monocyte adhesion (Fig. 9A, lanes 4 and 6) when compared with its effect with TNF-α-only-treated cells (Fig. 9A, lane 2). Similarly, results of the ELISA showed that while at low concentrations NAC or mito-Q attenuated TNF-α-dependent cell surface expression of ICAM-1 (Fig. 9B, lanes 3 and 5); high concentrations of NAC or mito-Q showed no significant difference in cell surface ICAM-1 expression (Fig. 9B, lanes 4 and 6) in comparison to TNF-α-only-treated cells (Fig. 9B, lane 2). In comparison to low concentrations, high concentrations of mito-Q and NAC showed significantly higher levels of monocyte adhesion and cell surface ICAM-1 expression. Three- to 4-fold higher levels of ICAM-1 protein expression by high concentrations of NAC or, particularly, by mito-Q (Fig. 5, B1 and B2, lanes 5 and 6) in comparison to TNF-α-only-treated cells but similar levels of cell surface ICAM-1 expression by TNF-α-only-treated cells and high concentration of NAC or mito-Q (Fig. 9B, lanes 4 and 6) indicate that ICAM-1 could not be efficiently translocated from endoplasmic reticulum (ER) to the cell surface in the presence of high concentrations of NAC or mito-Q.

Recent studies show that under strongly reducing conditions, ER secretes unfolded proteins (27), and these unfolded proteins could be the targets of proteolytic and endosomal degradation. To clarify whether ICAM-1 preproteins could bind to excess GSH/GSSG, NAC- or mito-Q-treated cell extracts were immunoprecipitated with anti-GSH Ab and probed with anti-ICAM-1 Ab on a Western blot. A distinct ICAM-1 band was noticed in the immunoprecipitated extract, indicating that GSH had formed a complex with ICAM-1, thereby possibly interfering with its structure. Moreover, the extent of glutathionylation was increased by TNF-α-only-treated cells and low concentrations, high concentrations of mito-Q and NAC showed significantly higher levels of monocyte adhesion and cell surface ICAM-1 expression. Three- to 4-fold higher levels of ICAM-1 protein expression by high concentrations of NAC or, particularly, by mito-Q (Fig. 5, B1 and B2, lanes 5 and 6) in comparison to TNF-α-only-treated cells but similar levels of cell surface ICAM-1 expression by TNF-α-only-treated cells and high concentration of NAC or mito-Q (Fig. 9B, lanes 4 and 6) indicate that ICAM-1 could not be efficiently translocated from endoplasmic reticulum (ER) to the cell surface in the presence of high concentrations of NAC or mito-Q.
**Discussion**

Oxidative stress is one of the most significant events in the immunobiology of inflammatory responses. Oxidative stress has been extensively studied for many years and its possible clinical ramifications have been explored in considerable depth. Anti-inflammatory drugs have been used to combat oxidative stress and inflammation in cells. However, little is known when these anti-inflammatory agents are used in high concentrations. The present study involving HPAECs implicates that the antioxidants NAC and mito-Q at high concentrations induce the generation of excess GSH/GSSG and this high level of GSH/GSSG is at least partially responsible for enhanced generation of ROS, carbonylation, and glutathionylation of proteins, which results in the enhanced expression of proinflammatory protein ICAM-1.

**FIGURE 8.** Western blots linking the alteration of TNF-α-dependent phosphorylation of IκBα, translocation, and phosphorylation of NF-κBp65 with the pretreatment of NAC or mito-Q and the possible connections with IKKα. Cells were treated exactly as discussed in Fig. 1, except TNF-α treatment was for 25 min instead of 6 h. A1. The comparative levels of phospho-IκBα with total IκBα. A2. The arbitrary densitometric units of pIκBα. Induction of phosphorylation of IκBα was observed in the presence of TNF-α (A1, A2, lane 2) that was significantly attenuated by a low concentration of NAC (A1, A2, lane 3) and mito-Q (A1, A2, lane 5). High concentrations of NAC (A1, A2, lane 4) and mito-Q (A1, A2, lane 6) showed no significant effect in comparison to TNF-α-only-treated cells (A1, A2, lane 2) but showed a significant difference when its results were compared with low concentrations of NAC- or mito-Q-treated cells (A1, A2, lanes 3 and 5). B1 and B2. The biphasic action of low vs high concentrations of NAC and mito-Q on the translocation of NF-κBp65 from cytoplasmic extracts (CE) to nuclear extracts (NE) and its arbitrary densitometric unit, respectively. C. The detection of phospho-NF-κBp65. A high-level phosphorylation of NF-κBp65 in the presence of IKKα siRNA indicates that IKKα may inhibit NF-κBp65 activity (C, lanes 5–7). C. Lane 1 represents the TNF-α-stimulated HeLa cell lysate as positive control. * and **, Significant alteration than the TNF-α-only and low concentration of NAC or mito-Q plus TNF-α-treated cells, respectively This figure is a representation of four similar experiments.

**FIGURE 9.** Glutathionylation of ICAM-1 and its connection with decreased monocyte-binding and cell surface expression of ICAM-1 in HPAECs. A. Monocyte adhesion assay; B. whole-cell ELISA; C. Detection of glutathionylated ICAM-1 by immunoprecipitation followed by Western blotting; D. arbitrary densitometric units of experiment C. TNF-α induced monocyte adhesion (A, lane 2), cell surface expression of ICAM-1 (B, lane 2), and glutathionylation of ICAM-1 (C and D, lane 2). Low concentrations of NAC (A–D, lane 3) and mito-Q (A–D, lane 5) significantly attenuated TNF-α-dependent enhanced monocyte adhesion (A), cell surface expression of ICAM-1 (B), and glutathionylation (C and D) of ICAM-1. High concentrations of NAC (A–D, lane 4) and mito-Q (A–D, lane 6) failed to attenuate TNF-α-dependent monocyte adhesion, cell surface expression, and glutathionylation of ICAM-1. In comparison to low concentrations (A–D, lanes 3 and 5), high concentrations (A–D, lanes 4 and 6) of mito-Q and NAC showed a significantly higher level of monocyte adhesion and cell surface ICAM-1 expression and glutathionylation of ICAM-1. *, and **, Significant alteration than the TNF-α-only-treated cells or low concentration of NAC- or mito-Q-treated cells, respectively. This figure is a representation of three similar experiments.
Depletion of naturally occurring intracellular antioxidant GSH is one of the initial events in many inflammatory responses (28). Having re-established the fact that GSH depletion increases the ICAM-1 expression (data not shown), we rescued the GSH-depleted cells using two antioxidants, NAC or mito-Q (Fig. 1), predominantly cytosolic and mitochondrial targeted pharmacological agents, respectively (19, 29). Of note, although the role of NAC in the cytosolic GSH synthesis is well established, more studies are needed on its possible role in GSSG recycling. Similarly, mito-Q may protect the cells from BSO-dependent GSH depletion and oxidative stress (20); however, the exact mechanism of action of mito-Q on the GSH/GSSG cycle has yet to be determined. Although the use of cytosolic- and mitochondrial-targeting drugs reveals the respective roles they play in maintaining a reduced environment, the role of the total glutathione (GSH plus GSSG) level in the cells as a whole is more important in sustaining an overall reduced atmosphere, since cytosolic and mitochondrial GSH plus GSSG pools are interdependent and together constitute the total cellular glutathione pool (30).

The present investigation on the effects of antioxidants on the GSH/GSSG cycle show that NAC or mito-Q dose-dependently increase cellular GSH levels (Fig. 1) by GSH synthesis and recycling, respectively (Fig. 2). Moreover, they do so by controlling the activity (Fig. 2), but not the expression of different enzymes involved in this cycle (data not shown). The predominant action of NAC is mediated by enhancing GCS activity that is responsible for increased de novo synthesis of GSH (Fig. 2A). In contrast, the predominant action of mito-Q is mediated by enhancing recycling of GSH by increasing GR activity (Fig. 2C). Increased GR activity by mito-Q is mediated by enhancing the activity of G6PD, a rate-limiting enzyme of the pentose phosphate pathway that is responsible for increased supply of NADPH, a cofactor of GR activity (Fig. 3). Therefore, the results indicate that NAC or mito-Q use different mechanisms to enhance the levels of GSH.

High-level generation of GSH by NAC and mito-Q shows a biphasic action on TNF-α-dependent ICAM-1 expression. Although at low to moderate concentrations NAC and mito-Q attenuates TNF-α-dependent ICAM-1 expression, a high concentration of NAC and mito-Q enhances TNF-α-dependent ICAM-1 expression (Figs. 4 and 5). The exact reason of the biphasic action of NAC and mito-Q is not known. Most possibly, binding of the cysteine molecules of the de novo-generated ICAM-1 (human ICAM-1 has 13 cysteine molecules) with the excess GSH/GSSG leads to misfolding or nonfolding of ICAM-1. This causes proteolytic degradation of ICAM-1 which might stimulate the ER to secrete more and more proteins by a positive feedback mechanism in an effort to generate sufficient functional ones. We very strongly believe that the number of cysteines in the de novo-generated protein is the determining factor of the level of generation of ICAM-1 since the receptor for advanced glycation end-product (RAGE), another cell surface proinflammatory protein, which has only three cysteines, does not show any noticeable level of induction in the presence of high concentrations of NAC or mito-Q (data not shown). This at the same time explains as to why no corresponding change is seen at the RNA levels of this protein. Since ICAM-1 is a proinflammatory molecule and it is well established that an increased level of oxidative stress is involved in the enhanced level of ICAM-1 expression, the different parameters of oxidative stress in the presence of high concentrations of NAC and mito-Q were determined.

To examine the level of oxidative stress of HPAECs in the presence of low vs high concentrations of NAC or mito-Q, the generation of ROS and carbonylation of proteins were determined. Of note, an enhanced generation of ROS or carbonylation of proteins indicates enhanced oxidative stress in cells. Carbonylation is the process of covalent addition of the carbonyl group (\(\text{C} = \text{O}\)) to proteins and phospholipids leading to the proteolysis, loss of membrane integrity, senescence, and death of cells; however, no relationship has presently been drawn between protein carbonylation and high-level usage of anti-inflammatory agents (21). Attenuation of TNF-α-dependent ROS generation and protein carbonylation by low concentrations of NAC or mito-Q but not by their high concentrations indicates that the cells lost the attenuative capacity against oxidative stress (Fig. 6). Similarly, cells treated with a very high concentration of glutathione ester induced carbonylation of proteins, which leads to increased senescence (data not shown), indicating that the enhanced level of GSH/GSSG contributes at least partially to the oxidative stress of cells.

In the present study, high levels of GSH/GSSG not only affected the cellular ROS accumulation and protein carbonylation, but also caused glutathionylation of proteins. Recent studies have shown that protein thiols respond to the varying GSH/GSSG ratios by forming mixed disulfides with GSSG through thiol-disulfide exchange between the thiolate anion and GSSG (protein thiols typically have pK_a values of ~8–9, but these can vary widely depending on the local environment of the cysteine residues) (31). Some exposed, noncatalytic protein thiols may also help buffer the GSH/GSSG ratio by reacting with GSSG to release one or two GSH molecules, leaving a protein-mixed disulfide or a protein disulfide (32). This can maintain the GSH/GSSG pool in a reduced state during transient oxidative stress. Of the different GSH-binding proteins, the role of the IKK complex is particularly important, since it is well established that IKK acts in relaying the inflammatory signals generated by TNF-α to NF-κB and finally to the ICAM-1 promoter (23, 25). Gene disruption studies of the murine IKK genes have shown that IKK_β, but not IKK_α is critical for cytokine-induced iκB degradation (22, 24). Although the exact role of IKK_α in cellular inflammation has yet to be documented, one recent observation indicates that IKK_α inhibits NF-κB activity in mouse macrophages (15).

It was not known how human IKK_α and IKK_β behave in the presence of the high concentration of GSH/GSSG. In the present investigation, a high-level binding of both IKK_α (Fig. 7A) and IKK_β (data not shown) to GSH/GSSG at high concentrations of NAC or mito-Q was observed. However, by using GST-ικB_α as substrate, IKK assay demonstrated a decrease in kinase activity by low concentrations of mito-Q or the siRNA of IKK_β (Fig. 7B). In contrast, an increased kinase activity was observed when IKK_α was inhibited by siRNA. Increased phosphorylation of iκB_α, translocation of NF-κB-p65 from cytoplasm to nucleus, and increased phosphorylation of NF-κB-p65 by siRNA of IKK_α (Fig. 8), but not by IKK_β siRNA (data not shown) indicate that under a moderate level of GSH/GSSG that quenches excess H_2O_2 generated by TNF-α, IKK_α might be able to send an inhibitory inflammatory signal to NF-κB. The present report claims for the first time that an overall moderately reducing environment promoted IKK_α activity that inhibited the IKK_β activity. This inhibitory signal may be altered particularly at high concentrations of mito-Q due to the enhanced oxidative stress that induces IKK_β activity. Since IKK_β takes a dominant role in transferring the inflammatory signal in proinflammatory and prooxidative stress conditions (22, 24), it continues to work without interruption, until all of the IKK_β that was generated by the prereductive stress conditions gets consumed. This may explain how IKK_β continues to phosphorylate iκB_α or NF-κB-p65 under a strong reducing environment.

Lastly, this study examines the effects of high-level expression of ICAM-1 mRNA and proteins (in the presence of high concentration of NAC and mito-Q) on the cell surface ICAM-1 level and...
its binding ability with the counterreceptor on monocytes. A high-level synthesis of mito-Q-dependent ICAM-1 protein without a proportionate increase of monocyte binding indicates that despite high levels of ICAM-1 protein synthesized (Figs. 4 and 5), most of it does not translocate to the cell surface in a fully functional form (Fig. 9, A and B). Recent studies indicate that under a strongly reducing condition mediated by thioredoxin, cells secrete unfolded proteins from ER and these de novo-generated unfolded proteins becomes targets of proteolytic and endosomal degradation (27). In the present investigation, a high-level glutathionylation of ICAM-1 is observed in the presence of TNF-α and high concentrations of NAC and mito-Q (Fig. 9C). We strongly believe that de novo-generated glutathionylated ICAM-1 become unfolded to form an inactive protein-glutathione complex, and this unfolded protein-glutathione complex instead of getting translocated to the cell surface gets translocated to endosomes and lysosomes and gets subjected to the proteolytic degradations. We believe this could be responsible for the decreased monocyte binding under high levels of ICAM-1 expression. Confirmatory results come from cell surface ELISA studies, which show that the endothelial cell surfaces in fact have relatively low levels of ICAM-1 under a strongly glutathionie environment, despite a high-level intracellular expression of ICAM-1 at high concentrations of mito-Q (Fig. 9B). Different situations culminating in oxidative stress are schematically represented in Fig. 10.

This investigation represents a compelling backdrop to re-examine the oxidative damage treatment paradigm based solely on the use of antioxidants in high concentrations.

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References


