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

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

Tapan K. Mukherjee, Anurag K. Mishra, Srirupa Mukhopadhyay, and John R. Hoidal

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-acetylcysteine (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 carbonylation and glutathionylation of the cellular proteins. This study further addressed that IκB kinase (IKK), 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, carbonylation and glutathionylation of cellular proteins, inhibition of IKKα activity, and up-regulation of ICAM-1 expression. The Journal of Immunology, 2007, 178: 1835–1844.

The intracellular milieu is usually maintained reduced as a result of the abundant presence of low-molecular-mass-reduced thiol, namely, reduced glutathione (GSH), which neutralizes excess reactive oxygen species (ROS), the normal by-product of aerobic respiration or naturally occurring free radical-generating compounds in the cell (1). However, under inflammatory conditions, high-level exposure to ROS can alter the redox balance of the cell toward oxidative stress (2). The anti-inflammatory drugs are used therefore to inhibit the generation/accumulation of intracellular ROS.

Numerous studies have highlighted a central role of the vascular endothelium in initiating inflammatory responses (3–5). A well-recognized characteristic of endothelial cells is that exposure to the proinflammatory cytokines like TNF-α depletes the GSH level in the cells. This, along with a consequential induction of oxidized glutathione (GSSG) level in the cells, shifts the redox balance toward oxidative stress (6, 7). The penultimate effect of the oxidative stress is manifested by the enhanced expression of a number of proinflammatory cell surface molecules, including ICAM-1. Although ICAM-1 expression may be modulated by the depletion of GSH levels (8, 9), the role of the antioxidant drugs, i.e., N-acetylcysteine (NAC), a cytosol specific antioxidant and precursor of GSH) and mitoquinone-Q (mito-Q, a mitochondrion-specific antioxidant), in ICAM-1 expression has yet to be examined. More importantly, far less attention has been paid to the effects of low vs high levels of such antioxidants on the expression and activity of different proinflammatory proteins, including ICAM-1. This has both mechanistic and clinical importance, since in several therapeutic trials the GSH precursor NAC was used in large excess doses without any benefit and, in some instances, with potential adverse effects. Similarly, it has yet to be determined the effects of various doses of mito-Q on glutathione (GSH/GSSG) synthesis and recycling and the subsequent effects of the mito-Q-dependent GSH/GSSG level on ICAM-1 expression.

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, n-buthionine-[S,R]-sulfoximine (BSO), and glutaraldehyde were obtained from Sigma-Aldrich. Anti-rabbit ICAM-1 Ab and mouse

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normal IgG were purchased from Santa Cruz Biotechnology. Anti-GAPDH, anti-rabbit IxBα (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 IxB kinase (IKK) α and IKKβ Abs were purchased from Upstate Biotechnology. Monoclonal phospho-IxBα 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 (Clonetics). Confluent HPAECs were washed and subsequently incubated overnight with EBM-2 basal medium (Cambrex) containing 2% charcoal-dextran-treated FBS (HyClone) and antibiotic-antimycotics. After overnight 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 phosphonium ion with coenzyme-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 GSSG

The total glutathione (GSH plus GSSG) 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°C. 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 (Oxiris 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°C. 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 420 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 lysis 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 extract was reacted with 200 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH; prepared in 2.5 M HCl) at room temperature for 1 h. Proteins were precipitated with 10% (v/v) ice-cold TCA and washed three times with ethyl alcohol/ethyacetate (1:1 v/v) to remove excess DNPH, boiled with Laemml 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 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 (1/1,500), anti-ICAM-1 (1/250), anti-GCS (1/4,000), anti-GPX1 (1/3,000), anti-GPS4 (1/2,000), anti-GR (1/1,000), anti-IKKα (1/1,000), anti-IKKβ (1/2,000), anti-phospho-IxBα (1/1,000), anti-NF-κB-p65 (1/200), anti-phospho-NF-κB-p65 (1/500), or anti-GAPDH (1/10,000) (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 agarose 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-IxBα as substrate (US Biological), 10 mM ATP, and 10 μM γ-[32P]ATP (Valeant Pharmaceuticals). The mixture was incubated for 1 h at 30°C. 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 IKKα and IKKβ 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 siRNA or sense RNA of IKKα or IKKβ (smart pool siRNA; Chemicon International) for 24 h before carrying out the procedure as described previously (16).

ELISA and monocyte 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 medium and freshly grown human peripheral blood mononuclear cells (U937 cell line from American Type Culture Collection) were added to the endothelial cell monolayer and incubated for 1 h at 37°C 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.

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 5 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, A1 and 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 cycloheximide (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 carbonylated 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-IκBα as a substrate, NAC or mito-Q inhibited TNF-α-dependent IκBα 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 IκBα, 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 IκBα (Fig. 8A, lanes 3 and 5) and translocation of NF-κB-p65 (Fig. 8, B1, B2).
and B2, lanes 3 and 5). High concentrations of NAC or mito-Q showed no significant effect on TNF-α-dependent phosphorylation of IkBα (Fig. 8, A and B, lanes 4 and 6) but showed significant changes when compared with the results with low concentrations of NAC or mito-Q (Fig. 8, B1 and B2, lanes 3 and 5). Additional results indicate that siRNA against IKKα (Fig. 8C, lanes 5-7) but not against IKKβ (data not shown), induced the phosphorylation of NF-κB-p65, again confirming that IKKα is involved in inhibiting the activities of NF-κB.

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 (20). 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 with anti-GSH Ab, followed by Western blot with IKKα Ab. Jurkat cell lysate was used as a positive control for IKKα (lane 1). For IkBα kinase assay, TNF-α treatment was given for 20 min instead of 6 h and siRNA was transfected 24 h before NAC or mito-Q treatment. This figure is representative of five similar experiments.

FIGURE 6. Immunochemical detection of H2O2 and carbonylated proteins in HPAECs. Cells were treated exactly as discussed in Figs. 1A, H2O2 production and B, protein carbonylation. TNF-α alone (A and B, lane 2) enhanced the ROS generation and carbonylated protein level of the cells. Low concentrations of NAC or mito-Q significantly attenuated TNF-α-dependent ROS generation (A, lanes 3 and 5) and protein carbonylation (B, lanes 3 and 5). High concentrations of NAC and mito-Q (A and B, lane 4 and 6) showed no significant difference in the ROS generation and carbonylation of 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.

FIGURE 7. Western blot and kinase assay showing detection of glutathionylation of IKKα and its effect on IkBα activity in HPAECs. A. For detection of glutathionylated IKKα Cells were treated exactly as discussed in Fig. 1, immunoprecipitated using anti-GSH Ab, and followed by Western blot with IKKα Ab. Jurkat cell lysate was used as a positive control for IKKα (lane 1). B. For IkBα kinase assay, TNF-α treatment was given for 20 min instead of 6 h and siRNA was transfected 24 h before NAC or mito-Q treatment. This figure is representative of five similar experiments.
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.
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 pharmacologic 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 enhanced 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 (≥C = 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 pKa 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 IKKβ, but not IKKα is critical for cytokine-induced iκBα degradation (22, 24). Although the exact role of IKKα in cellular inflammation has yet to be documented, one recent observation indicates that IKKα inhibits NF-κB activity in mouse macrophages (15).

It was not known how human IKKα and IKKβ behave in the presence of the high concentration of GSH/GSSG. In the present investigation, a high-level binding of both IKKα (Fig. 7A) and IKKβ (data not shown) to GSH/GSSG at high concentrations of NAC or mito-Q was observed. However, by using GST-iκBα as substrate, IKK assay demonstrated a decrease in kinase activity by low concentrations of mito-Q or the siRNA of IKKβ (Fig. 7B). In contrast, an increased kinase activity was observed when IKKα was inhibited by siRNA. Increased phosphorylation of iκBα, translocation of NF-κB-p65 from cytoplasm to nucleus, and increased phosphorylation of NF-κB-p65 by siRNA of IKKα (Fig. 8), but not by IKKβ siRNA (data not shown) indicate that under a moderate level of GSH/GSSG that quenches excess H2O2 generated by TNF-α, IKKα 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 IKKα activity that inhibited the IKKβ activity. This inhibitory signal may be altered particularly at high concentrations of mito-Q due to the enhanced oxidative stress that induces IKKβ activity. Since IKKβ 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 IKKβ that was generated by the prereactive stress conditions gets consumed. This may explain how IKKβ continues to phosphorylate iκBα 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 to 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 concentration 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 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

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