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Regulation of iNOS mRNA Levels in Endothelial Cells by Glutathione, A Double-Edged Sword

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Both inducible nitric oxide synthase (iNOS) and glutathione are important mediators in various physiological and pathological conditions in humans. In human endothelial cells the intracellular glutathione levels were modulated by N-acetyl-L-cysteine (NAC), a precursor of glutathione and 1,3-bis(chloroethyl)-1-nitrosouresea (BCNU), an inhibitor of glutathione reductase. BCNU significantly decreased reduced glutathione (GSH) but increased oxidized glutathione (GSSG) whereas NAC markedly elevated GSH with a relatively small increase in GSSG. Appropriate concentrations of GSH and GSSG increase the expression of iNOS gene. However, either GSH or GSSG at a too high concentration inhibits its expression, indicating that iNOS gene is fine tuned by the metabolites of glutathione cycle. The changes of iNOS mRNA steady state levels by the glutathione metabolites were associated with a similar alteration in its gene transcription and NF-κB activity. BCNU at high concentrations also shortens the half-life of iNOS mRNA, suggesting a role of GSSG in the stability of the iNOS gene. Thus, the change of glutathione levels *in vitro* can regulate iNOS mRNA steady state levels in a bi-phasic manner in human endothelial cells.

Keywords: Nitric oxide synthase, glutathione, gene expression, transcription factors

Abbreviations: BCNU, 1,3-bis(chloroethyl)-1-nitrosouresea; C-RT-PCR, competitive reverse-transcription polymerase chain reaction; EMSA, electrophoretic mobility shift assay; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; GSSG, oxidized glutathione; IL-1β, interleukin-1beta; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NAC, N-acetyl-L-cysteine; NF-κB, nuclear factor-kappa B; NO, nitric oxide; rGSH, reduced glutathione; TNF-α, tumour necrosis factor alpha

The physiological messenger nitric oxide (NO), which is synthesized from L-arginine by the enzyme NO synthase (NOS), has been implicated in the biology of most cells of the body. Its functions are wide ranging – from vasodilatation to neurotransmission, immune modulation and mediation of tissue damage. Inducible NOS (iNOS) was first cloned from activated mouse macrophage cells.[1,2] Its synthesis is markedly stimulated by various inflammatory agents

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including a number of cytokines in a wide variety of cells. Although endothelial cells are the predominant site of localization of constitutive endothelial NOS, it has now been recognized that they can also express iNOS.

Changes in iNOS expression play a role in the pathogenesis of cardiovascular diseases such as atherosclerosis and hypertension.[3,4] As dysfunction of vascular endothelial cells is a vital step in the development of atherosclerosis, hypertension and many other cardiovascular diseases, it is important to know how iNOS production is regulated within the endothelium.

The synthesis of iNOS and its activity can be regulated in a number of ways, one of which is by antioxidants and reactive oxygen species. Superoxide and hydrogen peroxide in the presence of catalase stimulate the activity of NOS in human epithelial cells and rat cerebellum respectively,[5,6] whereas superoxide dismutase and ascorbic acid inhibit the activity of NOS in human endothelial cells.[7] However, in contrast to other antioxidants, reduction of glutathione levels in rat hepatocytes or murine macrophages abolishes or reduces the induction of NOS by cytokines.[8–10]

Both iNOS and glutathione are important mediators in various physiological and pathological conditions in human. Oxidant/antioxidant systems include a wide range of different agents which function in many different ways. Although a number of studies have addressed the effects of oxidants/antioxidants on NO production or iNOS expression,[5–10] information related to human endothelial cells is still limited whereas none explains their mechanisms of action. In particular, the relationship between the changes in the levels of glutathione and iNOS activity in human endothelial cells has not yet been investigated at the level of mRNA. Here we have investigated how alterations in glutathione levels and oxidation state affect iNOS mRNA transcription, expression and stability in the human endothelial cell line, EA.hy 926 cells.

MATERIALS AND METHODS

Reagents

Human recombinant TNF-α and human recombinant IL-1β were purchased from R&D Systems; Kodak BioMax MS-1 film, Hyperfilm ECL were from Amersham International plc. FCS (fetal calf serum), RPMI 1640 medium, γ-^{32}P-ATP, ^{32}P-UTP and L-glutamine were from ICN Biomedicals Ltd. (Oxfordshire, England); TRI REAGENT (RNA isolation reagent) was from Molecular Research Center (Cincinnati, OH); electrophoretic mobility shift assay (EMSA) reagents were from Promega Co. (Southampton, England); poly (dI-dC) and dNTPs were from Pharmacia Biotech Ltd. (Herts, England); random hexanucleotide primers and M-MLV reverse transcriptase were from Life Technologies Ltd. (Paisley, Scotland); DNA polymerase and other PCR reagents were from Bioline Ltd. (London, England); Molecular Imager and Imaging Densitometer was from Bio-Rad Laboratories (Hercules, CA). Other reagents were from Sigma.

Cell Culture

The human endothelial cell line EA.hy 926 is a hybrid resulting from fusion of primary human umbilical vein endothelial cells with cells selected from a human lung carcinoma[11] and which has been well characterized.[12,13] To induce iNOS expression, cytokines (TNF-α 1000 units/ml + IL-1β 100 units/ml) were added to culture medium for 1–24 h.

Competitive RT-PCR (C-RT-PCR)

Cell monolayers were washed with PBS and then RNA was isolated by using TRI REAGENT. C-RT-PCR was performed as described previously.[14] Briefly, a 248 bp of cDNA fragment with the same primer binding sites as target fragment (305 bp) was constructed and the competitor RNA was then synthezised by SP6 RNA polymerase.

Total endogenous RNA with or without synthetic RNA competitor was reverse-transcribed at 37°C for 1 h. PCR was performed using an Omni-Gene™ thermocycler. Initial denaturation was done at 94°C for 2 min followed by 24–40 cycles of amplification. Each cycle consisted of 35 s of denaturation at 94°C, 35 s of annealing at 58°C, and 45 s for enzymatic primer extension at 72°C. PCR products were then size-fractionated through a 2% agarose gel and the bands visualized using ethidium bromide. The amplified endogenous fragment (target) was 305 bp and the synthetic competitor fragment was 248 bp. The densities of the two bands corresponding to the endogenous target and the synthetic competitor amplification products were determined with the Molecular Imager and Imaging Densitometer from Bio-Rad Laboratories (Hercules, CA). The data were then analyzed by Microsoft Excel and InStat computer softwares.

Northern Blot Analysis

A nonisotopic method was used to perform Northern analysis.[15] Briefly, 5 μg of total RNA was fractionated in an 1.2% agarose–formaldehyde gel. Samples from the gel were transferred to nylon membranes. The membranes were prehybridized for 1 h and then hybridized to a biotinylated iNOS probe or G3PDH probe. The signals on the membranes were detected by adding chemiluminescent substrate (Lumi-Phos 530) and the image is captured on X-ray film.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared basically as described by Schreiber *et al.*[16] with slight modifications. Briefly, after washing with PBS, cells were resuspended in 500 μl buffer A (10 mM Hepes, pH 7.9, 4.5 mM KCl, 7 mM Na_2HPO_4, 0.5 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride) and incubated in ice for 15 min. Afterwards, 30 μl of 10% Nonidet P-40 was added to the mixture, and the samples were mixed on a vortex machine for 10 s. Nuclei were collected following a 30-s spin and resuspended in 50 μl of cold buffer C (5 mM Hepes, pH 7.9, 26% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride). The samples were vigorously rocked for 15 min at 4°C, and the supernatants were frozen at −70°C.

EMSA was performed essentially as described earlier with some modification.[17,18] Binding reaction mixtures (10 μl) containing 2 μg protein of nuclear extract, 1 μg poly (dI-dC), ^{32}P-labeled probe, 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl and 10 mM Tris-HCl (pH 7.5) were incubated for 30 min at 25°C. For specificity controls a 100 fold excess of unlabeled probe was applied and in some experiments, 100 fold noncompetitor probe was also used as an additional control. Proteins were separated by electrophoresis through a native 4% polyacrylamide gel at 4°C in a running buffer of 12.5 mM Tris borate, 0.25 mM EDTA (pH 8.0), followed by autoradiography. The sequences of the NF-κB specific probe (binding site is underlined) were 5′-GAT CCA GAG GGG ACT TTC C GA GTA C-3′. The oligonucleotide was labeled with ^{32}P-ATP by T4 polynucleotide kinase and the labeled probe was purified by passing it through a G-25 column.

mRNA Stability Analysis

Cells were stimulated with cytokine mixture for 12 h and either BCNU or NAC was present for only the last 3 h. Thereafter, actinomycin (10 μg/ml) was added. Total RNA was prepared at the indicated time points and used for C-RT-PCR or Northern hybridization as described above.

Nuclear Run-on Analysis

The nuclear run-on transcription assay was performed according to the method described

previously.[19] The amount of sample hybridizing to G3PDH was used for normalization.

Glutathione Measurement

Total glutathione (GSH + GSSG) was determined by the Tietze's enzymatic recycling procedure as described by Griffith.[20] For GSSG measurement, the cell lysates were treated with 2-vinylpyridine (2VP, 2 µl/100 µl sample for 60 min) and expressed as GSSG equivalents. GSH levels were calculated from the difference between total glutathione and GSSG. The total protein content of each sample was determined using the Bradford assay.[21]

Statistical Analysis

All values are expressed as mean ± standard error. Statistical comparisons were performed using one-way analysis of variance (ANOVA) and the Dunnett's test. A p-value of less than 0.05 was taken as statistically significant.

RESULTS

Intracellular Levels of GSH and GSSG After Treatment with BCNU and NAC

BCNU, an inhibitor of glutathione γ reductase, prevents the conversion of oxidized glutathione to GSH. Cells incubated with cytokines and BCNU at concentrations of 20 µM or higher for 12 h significantly decreased GSH levels but elevated the accumulation of GSSG (Figure 1). NAC can increase the intracellular level of GSH by supplying L-cysteine, a substrate for GSH synthesis. Cytokine-pretreated cells incubated with NAC displayed a marked elevation in GSH levels but a relatively small increase in GSSG levels (Figure 2). Cytokines such as TNF-α and IL-1β produce a prooxidant status in a variety of cell types.[22,23] In an agreement with this, cells treated with cytokines in the present experiment showed a decrease in the levels of GSH (15.9 ± 2.0 nmol/mg protein *vs* 23.1 ± 2.2 nmol/mg protein, $p < 0.01$) but an increase in GSSG levels (0.96 ± 0.021 nmol/mg protein *vs* 1.19 ± 0.019

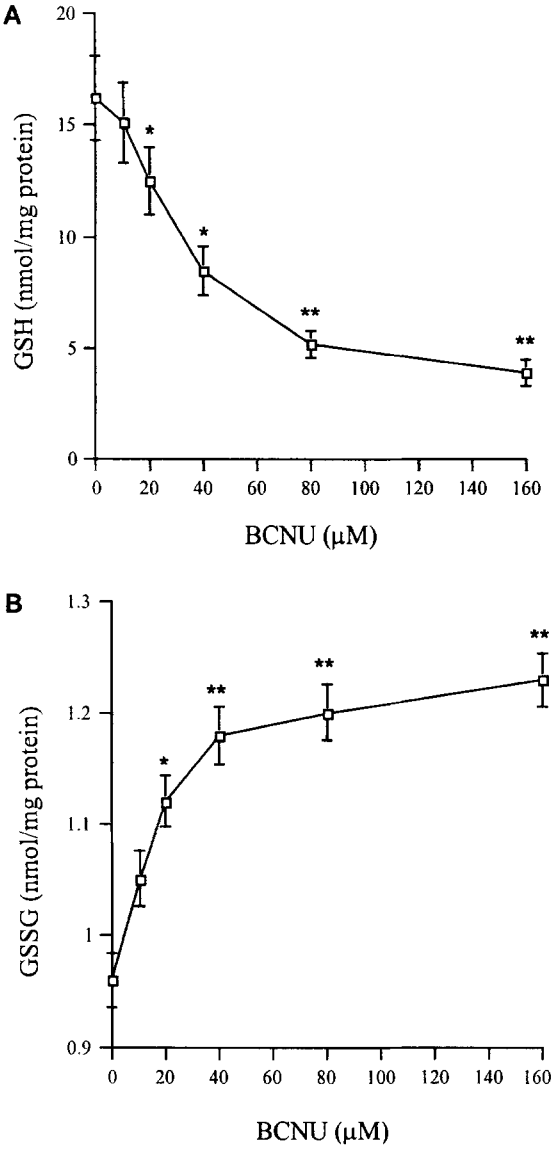

FIGURE 1 Effect of BCNU on glutathione levels. Cells were treated with cytokines for 12 h in the presence of various concentrations of BCNU. Cell lysates were obtained after incubation. Total glutathione and GSSG were determined by an enzymatic method. GSH levels were calculated from the difference between total glutathione and GSSG. The total protein content of each sample was determined using the Bradford assay. Data are expressed as mean ± standard error ($n = 10$).

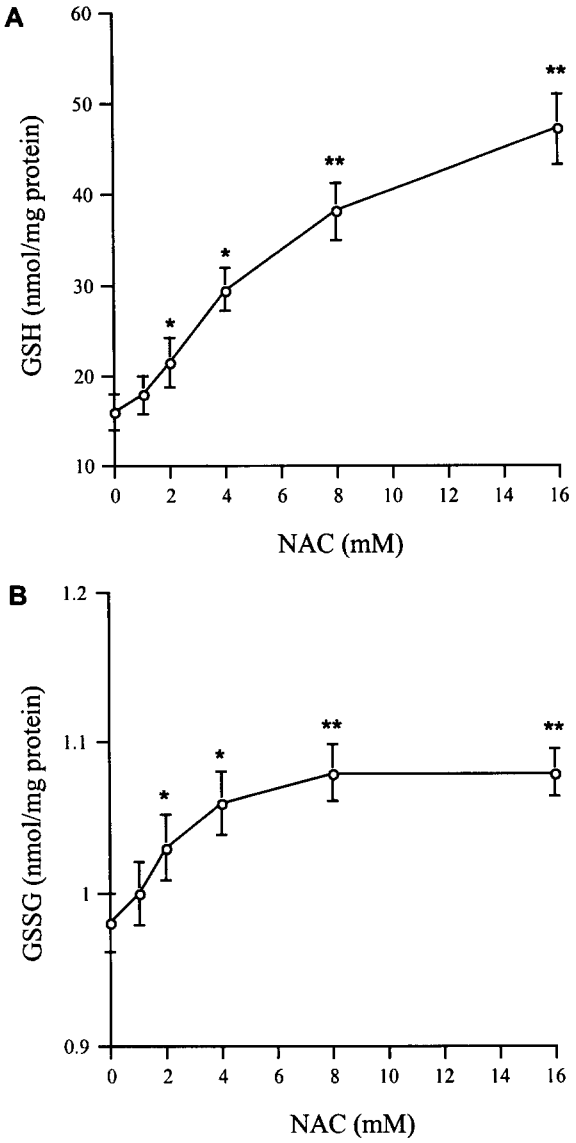

FIGURE 2 Effect of NAC on glutathione levels. Cells were treated with cytokines for 12 h in the presence of various concentrations of NAC. Cell lysates were obtained after incubation. Total glutathione and GSSG were determined by an enzymatic method. GSH levels were calculated from the difference between total glutathione and GSSG. The total protein content of each sample was determined using the Bradford assay. Data are expressed as mean ± standard error ($n = 10$).

nmol/mg protein, $p < 0.05$). BCNU and NAC did not significantly affect glutathione levels in the cells without cytokine treatment (data not shown). Cell viability as determined by a MTT assay was unaffected by any of the above reagents or reagent combinations (data not shown).

Effects of BCNU and NAC on Formation of iNOS mRNA Steady State Levels

C-RT-PCR was performed to assess whether the BCNU or NAC affected iNOS mRNA steady state levels. Northern blots were also carried out in some experiments to confirm the results (data not shown). Cells were stimulated with cytokines for 12 h in the presence of various concentrations of BCNU or NAC. BCNU exerted a bi-phasic effect. It stimulated iNOS mRNA steady state levels at a relatively low concentration (20 μM), but inhibited them at higher concentrations (80–160 μM) (Figure 3). NAC at a low concentration (2 mM) increased iNOS mRNA expression, however, at concentrations greater than 4 mM diminished or abolished iNOS mRNA expression induced by cytokines (Figure 4). As shown above, BCNU and NAC had different effects on cellular glutathione contents. BCNU increased the GSSG level whereas NAC mainly elevated the amount of GSH. Surprisingly, both BCNU and NAC at low concentrations increased iNOS mRNA expression induced by cytokines and at higher concentrations had suppressive effects. Since BCNU and NAC change the glutathione content in opposite directions, it would be expected that they neutralize each others' actions when simultaneously applied to the cells. Indeed, here we found that in the presence of a high concentration of BCNU (160 μM), cells were less sensitive to NAC. Thus NAC at concentration of 4 mM caused an increase in iNOS mRNA, while 16 mM NAC still had an inhibitory effect (Figure 5).

Effects of BCNU and NAC on iNOS Gene Transcription

Nuclear run-on experiments were performed to evaluate whether changes in iNOS gene transcription would contribute to the change of iNOS

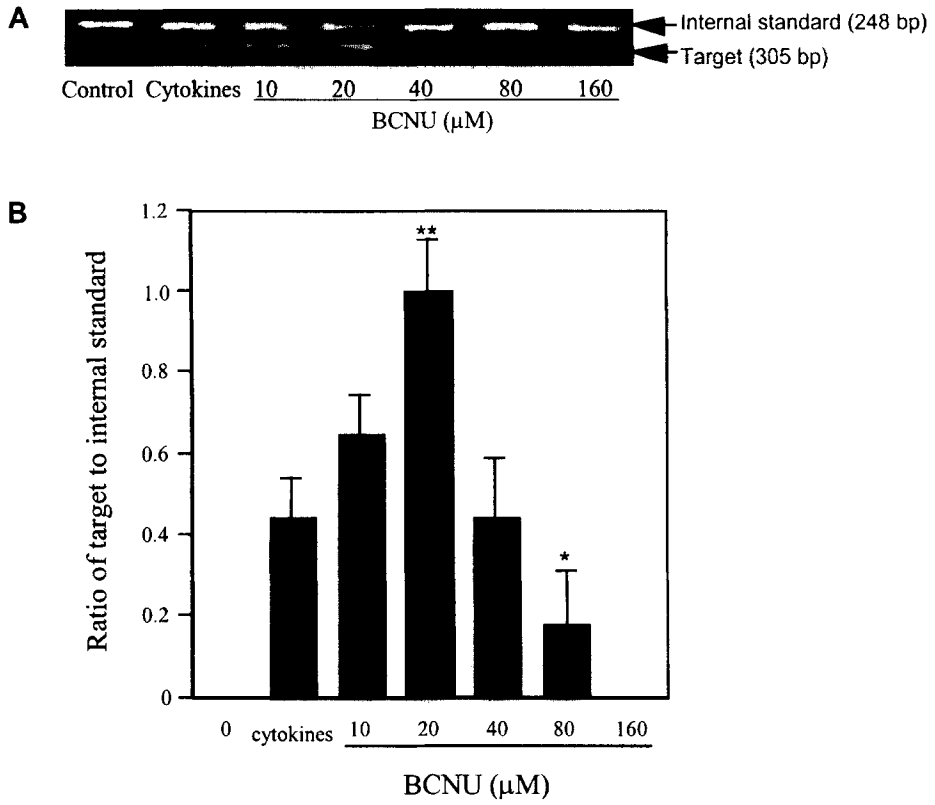

FIGURE 3 Effect of BCNU on iNOS mRNA steady state. Cells were treated with cytokines for 12 h in the presence of various concentrations of BCNU. Total RNA was isolated and competitive RT-PCR were performed to assess iNOS mRNA levels. The bands were analyzed for densities and the ratio of target iNOS mRNA to internal standard was calculated. The internal standard is a synthetic RNA/cDNA which has the same iNOS primer binding sites as the target samples. The experiment was performed six times and each study yielded similar results. Data (B) are expressed as mean ± standard error.

mRNA steady state levels caused by BCNU and NAC treatment. In unstimulated cells iNOS transcriptional activity was virtually undetectable (Figure 6). Incubation of the cells with cytokines for 12 h increases the transcription rate of the iNOS gene. Addition of BCNU or NAC into cell cultures caused similar changes to the transcriptional activity of iNOS gene as found for the steady state of iNOS mRNA expression.

High Concentration of BCNU Reduces iNOS mRNA Stability

Cells were stimulated with either cytokines or a combination of cytokines plus BCNU or NAC for 12 h after which cells were treated with actinomycin D to inhibit further transcription. Total RNA was isolated and examined by C-RT-PCR at various time points. The decay of iNOS mRNA after stimulation of the cells with cytokines corresponded to a half-life of 10.8 ± 1.2 h (Figure 7). Co-stimulation with NAC and lower concentrations of BCNU (10–80 μM) did not significantly change the mRNA half-life (data not shown). Co-stimulation with a high concentration of BCNU (160 μM) decreased the iNOS mRNA half-life to 7.7 ± 0.9 h (Figure 7). This decrease was significant ($p < 0.05$) when compared with the half-life of 10.8 ± 1.2 h obtained in the cells treated with cytokines.

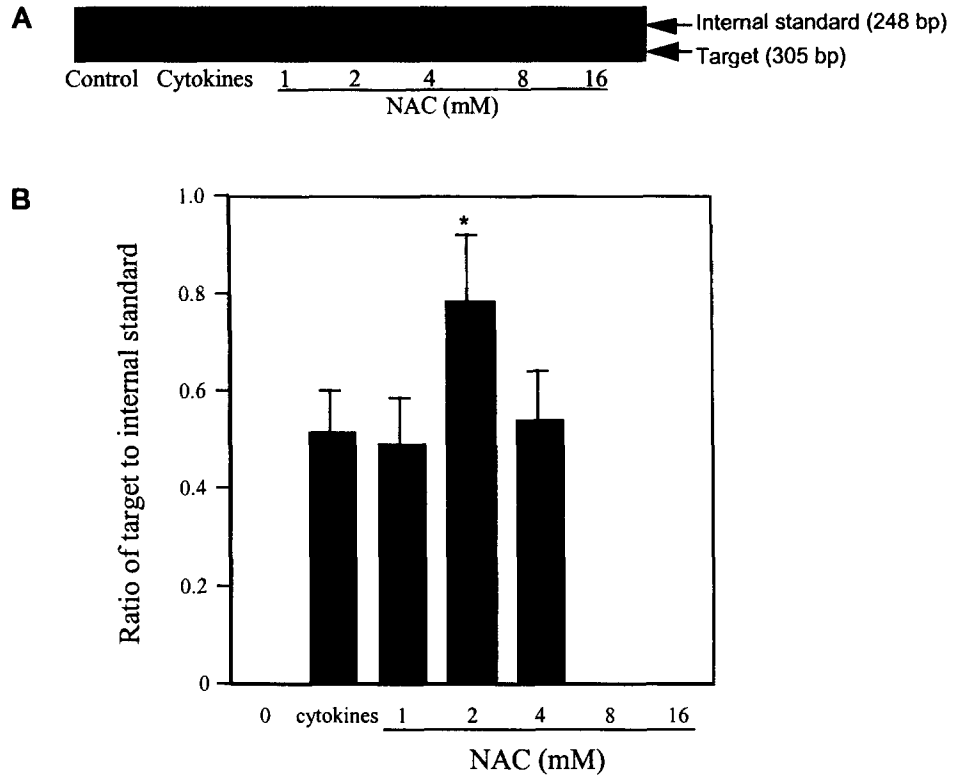

FIGURE 4 Effect of NAC on iNOS mRNA steady state. Cells were treated with cytokines for 12 h in the presence of various concentrations of NAC. Other conditions were as described in Figure 3.

Change of Glutathione Levels Regulates NF-κB Activity

Activation of NF-κB transcription factor has been reported to play a crucial role in induction of iNOS gene in a variety of cell types[24–26] and it is also well known that reactive oxygen species are a powerful stimulator of NF-κB activity.[27] EMSA was carried out to examine the effects of BCNU and NAC on the activation of NF-κB. BCNU had a dual effect on NF-κB activity (Figure 8A). At concentrations of 10–20 μM it increased NF-κB activity. However, at higher concentrations (80–160 μM) BCNU inhibited the activation of NF-κB. NAC had a positive effect on NF-κB activity at the concentration of 2 mM, but at the concentrations higher than 2 mM it completely blocked NF-κB activity (Figure 8B).

DISCUSSION

Here we demonstrate that the changes in intracellular glutathione levels affect iNOS mRNA steady state levels in a bi-phasic fashion in human endothelial cells. Intracellular glutathione levels were readily adjusted by application of BCNU and NAC. BCNU and NAC function change the levels of intracellular glutathione at opposing directions. As an inhibitor of glutathione reductase, BCNU increases GSSG and decreases GSH levels, producing a pro-oxidant state. Conversely, NAC as a precursor of glutathione up-regulates synthesis of GSH. An increase in GSH levels will naturally shift the balance between GSH and GSSG in favor of the former. We also found that NAC caused an increase in GSSG levels, though this elevation was relatively small. This suggests

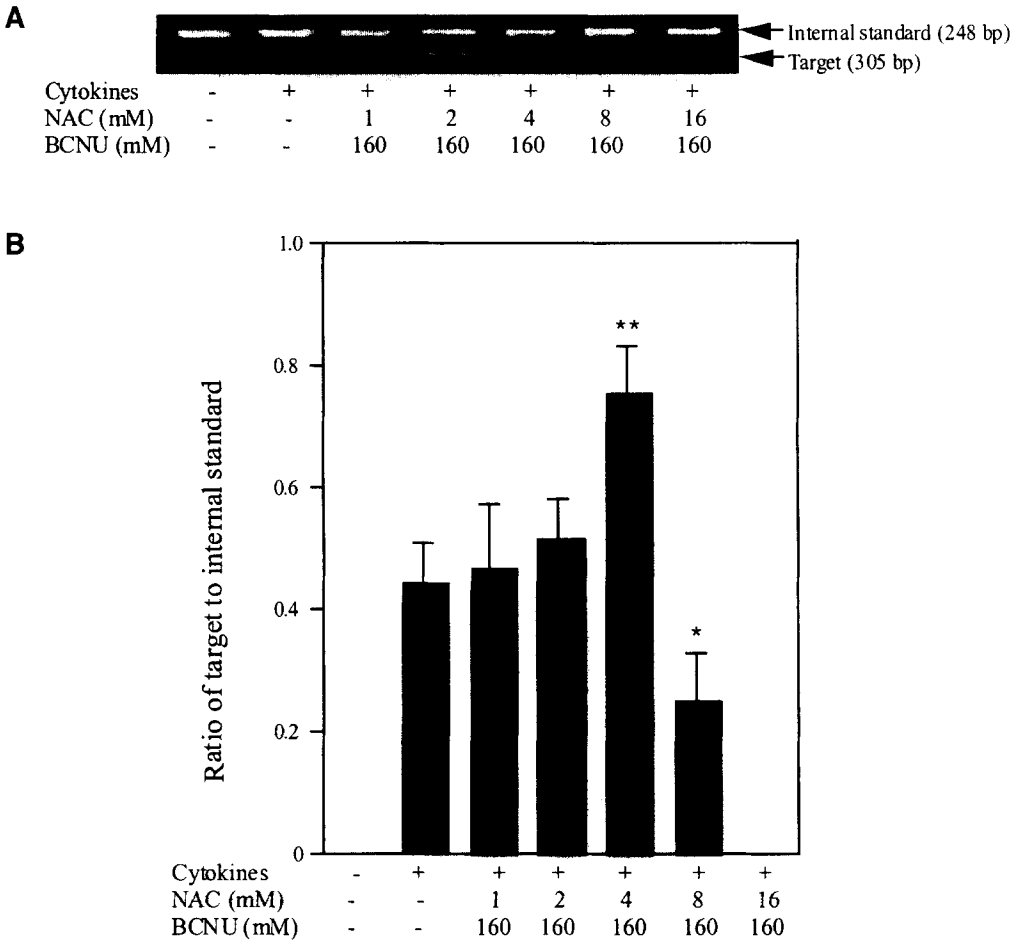

FIGURE 5 Regulation of steady state of iNOS mRNA by NAC in the presence of BCNU. Cells were treated with cytokines for 12 h in the presence of 160 μM BCNU and various concentrations of NAC. Other conditions were as described in Figure 3.

that the cytokine-stimulated cells used in our experiments were in an oxidant condition and so turned extra GSH into GSSG to return the balance between antioxidants and oxidants. It appears somewhat strange that both BCNU and NAC increase iNOS mRNA at low concentrations but inhibit them at high concentrations, as their effects on glutathione are opposite. However, both agents at low concentrations increased GSSG levels whereas at higher concentrations BCNU significantly reduced GSH and increased GSSG while NAC markedly elevated GSH with only a slight increase in GSSG. Thus, it appears that up-regulation and down-regulation of iNOS mRNA are influenced by different metabolites (GSSG and GSH) of the glutathione cycle. Clearly these different metabolites may exert completely different actions depending on their concentrations.

Transcription factor NF-κB is required for the induction of iNOS gene in response to LPS and cytokines including TNF-α and IL-1β[24–26] and the binding site sequence for NF-κB is present in the iNOS gene promoter of several species including human, mouse and rat.[28–31] Interestingly both TNF-α and IL-1β also promote the

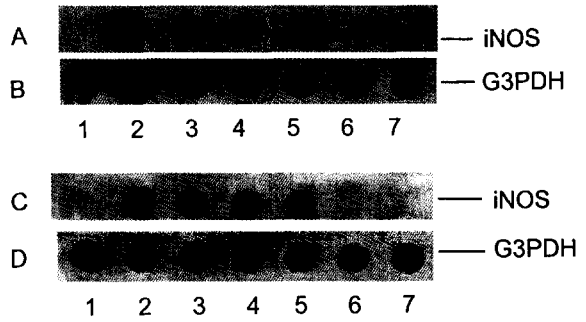

FIGURE 6 Regulation of iNOS gene transcription by BCNU and NAC. Cells were stimulated with either cytokines or a combination of cytokines plus BCNU or NAC for 12 h. Nuclei were isolated and the transcriptional rate of the iNOS and G3PDH genes were determined by hybridizing ^{32}P-labeled nuclear RNA transcripts to iNOS or G3PDH probe immobilized onto the membranes. A and B: cells treated with BCNU; C and D: cells treated with NAC, Lane 1: control; Lane 2: cells treated cytokines alone; Lane 3: cells treated with 10 μM BCNU or 1 mM NAC; Lane 4: cells treated with 20 μM BCNU or 2 mM NAC; Lane 5: cells treated with 40 μM BCNU or 4 mM NAC; Lane 6: cells treated with 80 μM BCNU or 8 mM NAC; Lane 7: cells treated with 160 μM BCNU or 16 mM NAC.

production of reactive oxygen species,[22,23] stimulating cells to generate mitochondrial oxygen radicals and oxidized glutathione.[26] In our experiments stimulation of endothelial cells by TNF-α and IL-1β similarly produced an oxidant stress characterized by an increase in GSSG and a decrease in GSH. In agreement with others, we also found that changes of glutathione levels altered the NF-κB activity. However, instead of linear relationship between NF-κB activity and oxidant levels, we showed NF-κB activity to be regulated along a bell-shaped curve, depending on the levels of GSSG and GSH. A small increase in the GSSG levels caused by treatment with BCNU elevated the activity of NF-κB, whereas a marked increase in GSSG levels together with a profound drop in GSH levels reduced NF-κB activity. This finding indicates that NF-κB activity is blocked by either a significant reduction of GSH levels or a marked increase in GSSG amount. Furthermore, we also found that a significant increase in GSH levels had an inhibitory effect on NF-κB activity as the high concentration of NAC blocked the activation of NF-κB. Therefore, the activity of NF-κB is finely regulated by modulations in glutathione and this modulation plays an important role in regulation of iNOS mRNA expression in endothelial cells. However, whether other factors may still contribute to the pathways of glutathione's regulatory effects on iNOS expression cannot be excluded.[32]

Why glutathione exerts a bi-phasic effect on NF-κB activity is unclear. It is well known that NF-κB is a cytoplasmic complex composed of hetero- and homo-dimers from the Rel/NF-κB family of proteins.[33] These dimers are complexed to a member of the IkB family such as IkB-α or IkB-β which inhibits the nuclear localization signal in the Rel homology domain of the NF-κB complex. IkB-α and IkB-β preferentially bind to c-Rel and RelA (p65) dimers. Upon stimulation, phosphorylation, ubiquination and proteolytic degradation of IkB-α or IkB-β occur and this process will allow the NF-κB complex to rapidly translocate to the nucleus where it binds to its consensus DNA sequence. It is possible that within a relatively narrow concentration range, GSSG promote the release of NF-κB dimers from IkB family and initiates nuclear translocation whereas excessive GSSG inhibits the DNA binding of NF-κB dimers.[34] A specific concentration of GSH may also be required for the DNA binding site to accept NF-κB dimers. High levels of GSH may either shut the binding site or stabilize the NF-κB-IkB-α/IkB-β complex. Others have also found that the depletion of GSH by either BCNU or DL-buthionine-[S,R]-sulfoximine inhibited the activity of NF-κB in T cells.[35,36] And this activity of NF-κB was blocked by a high concentration of GSH elevated by NAC or cysteine treatment.[35,37]

Our experiments also indicate that the bi-phasic effect of glutathione on iNOS mRNA formation is primarily due to its regulation of the transcriptional activity of the iNOS gene. A decrease in the stability of iNOS mRNA might also partly account for the decrease in mRNA levels since BCNU at high concentrations shortened the half life of

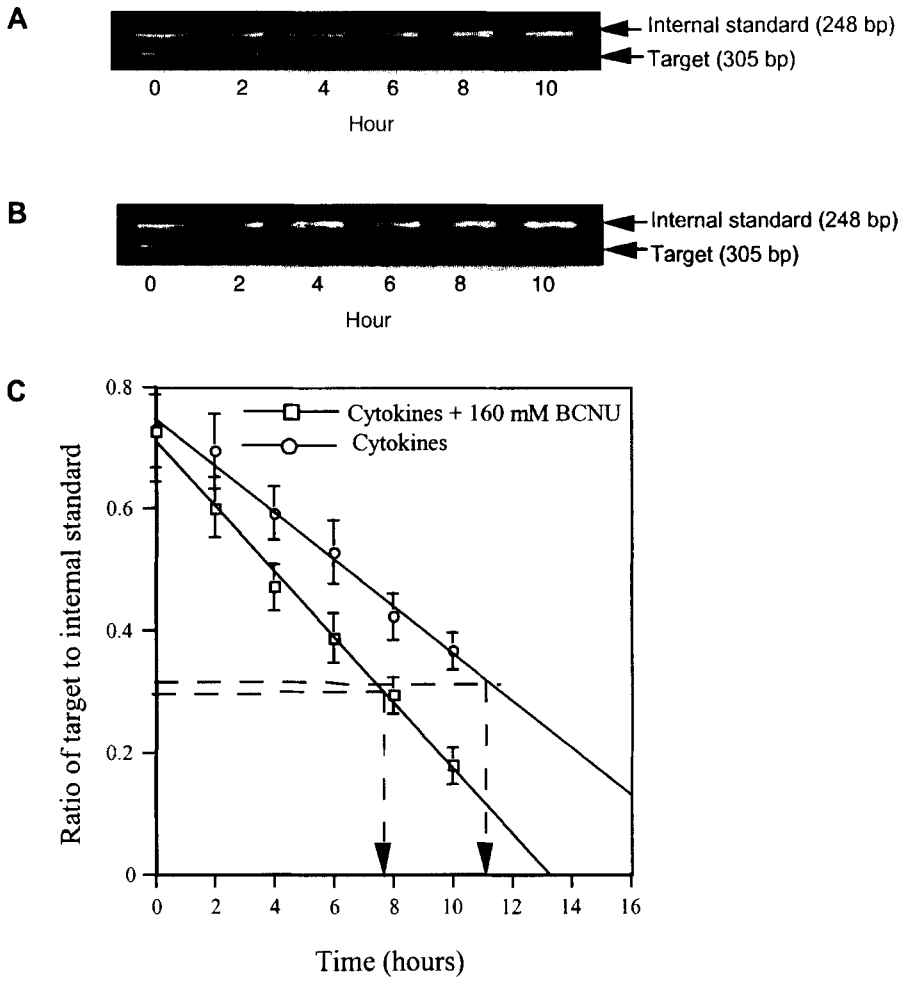

FIGURE 7 Reduction of iNOS mRNA stability by BCNU. Cells were stimulated with cytokines (A) for 12 h and BCNU (B) was presented for only the last 3 h. The cells were then treated with actinomycin D to inhibit further transcription. Total RNA was isolated and examined by C-RT-PCR at various time points. The bands were analyzed for densities and the ratio of target iNOS mRNA to internal standard was calculated. The half-life of iNOS mRNA was determined according to the curves (C).

iNOS mRNA. A high concentration of BCNU changed glutathione metabolism in two ways, increasing GSSG levels and decreasing GSH levels. It is most likely that the increased GSSG results in a shorter half life of iNOS mRNA and it is known that oxidant stress can damage the nucleotide.[38]

From the results presented in this paper it is clear that the induction of iNOS mRNA in human endothelial cells is regulated by glutathione in a bi-phasic manner. Specific concentrations of both GSSG and GSH are needed for optimal stimulation of iNOS mRNA. High concentrations of GSSG and/or GSH exhibit a suppressive effect. The effects of glutathione on the steady state of iNOS mRNA are executed at its gene transcriptional level and are closely related to transcription factor, NF-κB. Further studies are needed to define the precise mechanisms by which GSSG and GSH regulate iNOS mRNA.

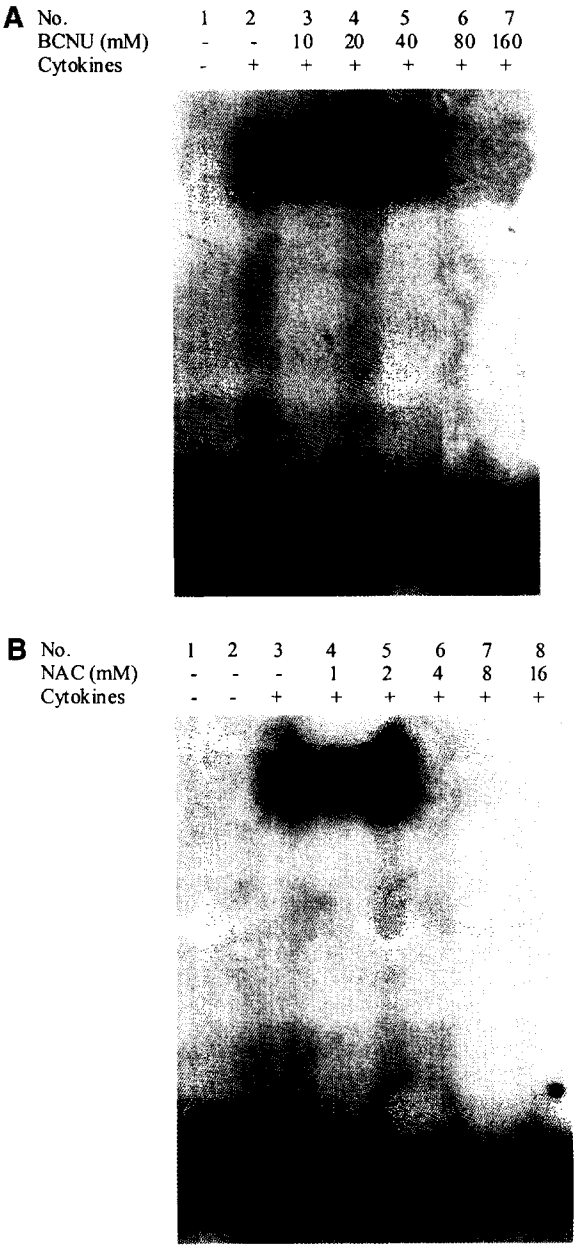

FIGURE 8 Regulation of NF-κB activity by BCNU and NAC. Cells were stimulated with either cytokines or a combination of cytokines plus BCNU or NAC for 12 h. The nuclear extracts were isolated and EMSA was performed by using a NF-κB specific probe. Lane 1 in (B) was a specificity control using a 100 fold excess of unlabeled probe.

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