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Glutathione Downregulates the Phosphorylation of IκB: Autoloop Regulation of the NF-κB-Mediated Expression of NF-κB Subunits by TNF-α in Mouse Vascular Endothelial Cells

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Nuclear factor-kappa B (NF-κB) regulates gene expression upon immune and inflammatory responses. It has been demonstrated that redox regulation by thiols is involved in the signal-transduction cascade. In this study, we examined the effect of glutathione (GSH) on the NF-κB activity and the expression of NF-κB subunits induced by tumor necrosis factor-α (TNF-α) using mouse vascular endothelial cells. GSH inhibited the serine phosphorylation of IκB-α by TNF-α, leading to the downregulation of NF-κB-DNA binding activity followed by decreased expression of p65/p50 and IκB mRNAs. The regulation of the autoregulatory loop for the NF-κB activation and the expression of NF-κB subunits may be important in endothelial cells in response to cytokines.

Nuclear factor-kappa B (NF-κB) is part of a family of dimeric transcriptional factors (1). NF-κB is present in the cytosol of unstimulated cells in a complex with its inhibitor (IκB) and through the dissociation from IκB (2), transcriptionally activates various cellular genes involved in immune response, inflammation, oxidative stress and embryonic development (3–5). In human disease, activation of NF-κB is involved in immunodeficiency virus gene expression (6) and atherogenesis (7). NF-κB is characterized as a heterodimer with two subunits, p65/p50 (8). In the process of the activation of NF-κB by cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), phosphorylation (9), ubiquitination (10) and proteolysis (11) of IκB-α, an IκB family, (12) are involved. After phosphorylation, IκB is rapidly degraded. These events allow the dissociation of NF-κB from IκB and lead NF-κB to migrate to the nucleus, where it activates its target genes.

Phosphorylation of IκB-α by various kinases has been shown to dissociate NF-κB-IκB-α complexes (9). Phosphorylation of IκB-α on two serine/threonine residues are necessary for the rapid degradation by the proteasome but not for the dissociation of NF-κB-IκB-α complexes (13). Furthermore, the effect of inhibition of IκB-α phosphorylation on the expression of endothelial cell adhesion molecules has been found (14, 15), although the signaling pathway leading to activation of NF-κB is not fully understood.

Glutathione (γ-glutamylcysteinyl glycine, GSH) is thought to be an important factor in cellular function and defense against oxidative stress, such as radiation and drug resistance (16, 17). The role of GSH in Fas-mediated apoptosis has been reported (18, 19).

Another important role of GSH is to maintain the redox potential within cells. Regulation of the NF-κB activation by GSH has been reported in cells treated with TNF-α (20), lipopolysaccharide (21) and oxidative stress (22). We found that treatment of glioblastoma cells with buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine synthetase (γ-GCS), increases the sensitivity of the γ-GCS induction by ionizing radiation mediated by NF-κB (16). Thus, intracellular thiol levels seem to play a key role in regulating the activation process of NF-κB (23).

IκB mRNA is upregulated by TNF-α, IL-1β or lipopolysaccharide, the same agents that activate NF-κB. The upregulation of IκB is antioxidant sensitive (24, 25). Antioxidants, such as pyrrolidine dithiocarbam-

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Abbreviations used: GSH, glutathione (γ-glutamylcysteinyl glycine); NF-κB, nuclear factor-κB; IκB, inhibitory molecule of NF-κB; MHE, mouse hemangioendothelioma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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ate, have been demonstrated specifically to inhibit the activation of NF-κB and pretreatment of cells with pyrrolidine dithiocarbamate prior to stimulation with lipopolysaccharide abolished the induction of IκB mRNA. The mechanism by which the expression of IκB is regulated by TNF-α is caused by the presence of NF-κB binding sites on the promoter region of the IκB gene (26).

The questions to be clarified are whether or not TNF-α regulates the expression of NF-κB subunits and if so, whether or not GSH participates in the regulation of the expression of NF-κB subunits. In this study, we examined the effect of GSH on the phosphorylation on IκB-α and expression of p65/p50 and IκB-α by TNF-α.

MATERIALS AND METHODS

Materials. Glutathione reductase was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Dulbecco's modified Eagle's medium, fetal calf serum and the NF-κB-binding protein detection system were from Life Technologies, Inc (Gaithersburg, MD). [γ-^{32}P]ATP was from DuPont NEN. Recombinant human TNF-α was from Ohtsuka Pharmaceutical Co. (Tokyo, Japan). Polyclonal antibodies against p65/p50 and IκB-α were from Santa Cruz Biotech. Inc. (Santa Cruz, CA). Monoclonal antibody against phosphoserine and isopropyl GSH were from Sigma. The Proto Blot kit (Western blot AP system kit) was from Promega Corp. (Madison, WI).

Cell culture. The mouse hemangioendothelioma (MHE) cell line established from the thyroid, expressing factor VIII and vimetidine (27), was used as the vascular endothelial cells. MHE cells can incorporate acetylated low-density lipoprotein and stain positively for factor VIII antigen (28). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in 5% CO_2 under 100% humidity.

Estimation of glutathione. The concentrations of intracellular GSH were estimated enzymatically as described by Beutler (29).

Northern blots. The complementary oligonucleotide probes for p65/p50 and IκB-α were synthesized as described previously (30–32). These probes were radiolabeled with ^{32}P using a random primer and Northern blotting essentially as described by Sambrook *et al.* (33). The relative radioactivity was expressed as a ratio of PSL corrected by the measured intensity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Electrophoretic mobility shift assay. Nuclei extracts were prepared according to the method described by Abmayr and Workman (34). The electrophoretic mobility shift assay for NF-κB was performed as described by Sen and Baltimore (35) with slight modification. Briefly, nuclear extracts were incubated with a NF-κB-specific ^{32}P-oligonucleotide. The binding reaction proceeded in a 25 μl volume containing 10 μg of nuclear extract, 5 μl of a binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 4% glycerol), 0.08 mg/ml nonspecific competitor DNA and labeled oligonucleotide (3000–6000 cpm). After a 20-min binding reaction at room temperature, the samples were loaded on a 6% nondenaturing polyacrylamide gel and subjected to electrophoresis in 25 mM Tris, 22.5 mM borate and 0.25 mM EDTA, pH 8.0. For the specificity control, a 100-fold excess of unlabeled probe was applied. The sequence of the binding site for the NF-κB probe was 5′-GGGATTTCC-3′. The DNA binding activity of the extracts was quantified by estimating the amount of the ^{32}P-labeled NF-κB. The DNA complex was excised from the dried gels and was expressed as PSL.

Immunoblotting. Cells were lysed by freeze-thawing in phosphate-buffered saline (0.137 M NaCl, 2.68 mM KCl and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) containing the phosphatase inhibitor, 30 mM NaPPi, 1 mM EDTA and 0.5 mM PMSF.

Immunoprecipitation of IκB-α was performed according to the method described by Menon *et al.* (36) with slight modification. IκB-α in the cell lysate (100 μg of total protein) was mixed with anti-IκB-α antibody preconjugated to protein A-agarose beads for 2 h. The immunoprecipitated IκB-α was washed with 1 ml of 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100 and 0.1% SDS followed by washing with 1 M urea in the buffer and then resolved by electrophoresis on 10% SDS–polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. Serine-phosphorylated proteins were detected using anti-phosphotyrosine antibody. Blots were developed by enhanced chemiluminescence (Amersham Corp., Buckinghamshire, UK).

Fifty micrograms of cytosolic protein samples was subjected to 10% SDS–polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes and immunologically stained using rabbit anti-p65/p50 and IκB-α polyclonal IgG. The bound antibodies were made visible with an alkaline phosphatase-coupled second antibody using a Proto Blot kit.

Statistical analysis. The data are given as the means ± SD. Differences were calculated by Student's two-tailed *t* test.

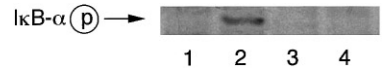

FIG. 1. Serine-phosphorylation of IκB-α. The effect of GSH on the serine-phosphorylation of IκB-α was studied. Cells were treated with 100 units/ml of TNF-α for 15 min. Lysates (100 μg of total protein) were immunoprecipitated with anti-IκB-α antibody and then resolved by electrophoresis on 10% SDS–polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. Serine-phosphorylated proteins were detected using anti-phosphotyrosine antibody. Lane 1, control; lanes 2 and 4, +100 units/ml of TNF-α; lanes 3 and 4, MHE cells previously incubated with 10 mM GSH ester.

RESULTS

GSH inhibits the TNF-α-induced phosphorylation of IκB-α. We studied the effect of GSH on the serine-phosphorylation of IκB-α by TNF-α. Treatment of MHE cells with 100 units/ml of TNF-α for 15 min resulted in stimulation of the phosphorylation rates of IκB-α (Fig. 1, lanes 1 and 2). No stimulation of the phosphorylation rates of IκB-α was observed when the concentration of GSH increased to 2-fold (22.5 ± 1.1 versus 11.7 ± 1.3 nmol/10^6 cells) by the previous treatment of MHE cells with 10 mM GSH ester for 24 h (lanes 3 and 4).

GSH downregulates the TNF-α-induced NF-κB-DNA binding activity. The effect of GSH on the DNA-binding activity of NF-κB by TNF-α was estimated using an electrophoretic mobility shift assay. The NF-κB-DNA binding activity increased 5-fold in MHE cells by the treatment of 100 units/ml TNF-α (Fig. 2, lanes 4 and 5, 860 PSL versus 4350 PSL). Next, the effect of the increase in the concentration of intracellular GSH on the NF-κB-DNA binding activity was studied. The TNF-α-induced NF-κB-DNA binding activity was reduced to 30% by the previous treatment of MHE cells

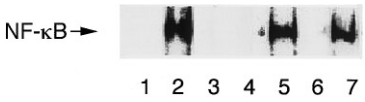

FIG. 2. Electrophoretic mobility shift assay of NF-κB. The DNA-binding activity of NF-κB was estimated using an electrophoretic mobility shift assay. MHE cells were incubated with or without GSH ester for 24 h and then the cells were treated with 100 units/ml of TNF-α for 1 h. Nuclear extracts from each of these cells were incubated with a NF-κB-specific [^{32}P]-oligonucleotide for 30 min and then loaded on to a 6% non-denaturing polyacrylamide gel. For specificity controls, nuclear extracts from Hela cells were used (lanes 2 and 3). Lane 3, a 100-fold excess of unlabeled probe was applied. Lanes 4 and 5, control MHE cells; lanes 6 and 7, MHE cells previously incubated with 10 mM GSH ester; lanes 5 and 7, +TNF-α. Positions of specific DNA-protein complexes are indicated by an arrow.

with 10 mM GSH ester for 24 h (860 PSL versus 3040 PSL) (lanes 6 and 7).

GSH downregulates the expression of p65/p50, and IκB-α by TNF-α. We studied the effect of TNF-α on the expression of p65/p50 and IκB-α on Northern blots. Figure 3 shows the results of the induction of p65/p50 and IκB-α mRNA by TNF-α. The TNF-α induced p65/p50 and IκB-α mRNA with a peak at the 6th hour. TNF-α increased p65 mRNA by 205%, p50 mRNA by 190% and IκB-α mRNA by 165% at the 6th hour, respectively (lanes 1 and 2).

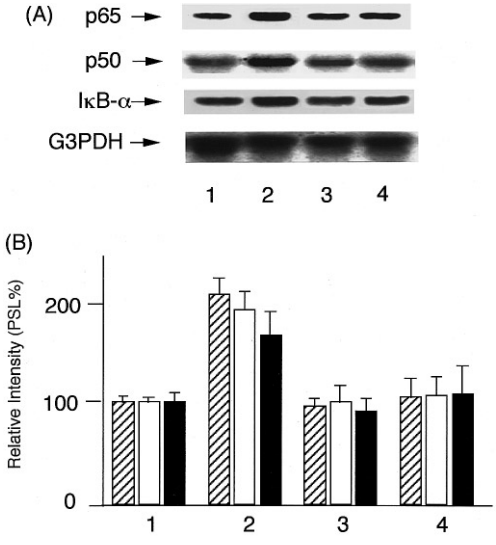

FIG. 3. Northern blots of p65/p50 and IκB-α. After incubating MHE cells with or without TNF-α for 6 h, about 30 μg of each total RNA extracted from the cells was fractionated by electrophoresis through 1% agarose gel, transferred to nylon membranes and hybridized with ^{32}P-labeled nick-translated DNAs (A). Lanes 1 and 2, control MHE cells; lanes 3 and 4, MHE cells previously incubated with 10 mM GSH ester; lanes 2 and 4, +100 units/ml of TNF-α. The relative amounts of radioactivity are expressed as percentage of PSL (B). Dashed bars correspond to the expression of P65 mRNA, open bars that of p50 mRNA and closed bars that of IκB-α mRNA. Each numbered bar corresponds to the lane in A. Values are means ± SD of three independent analyses.

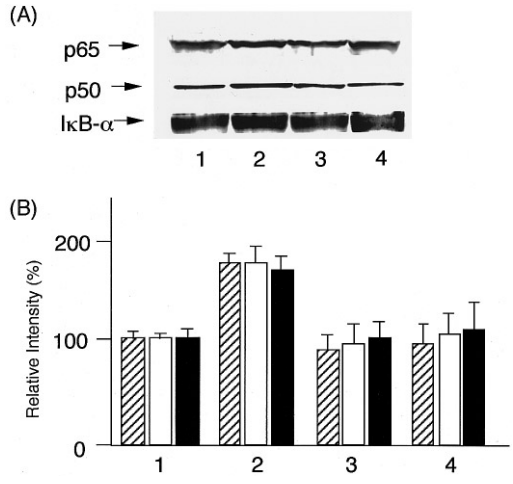

FIG. 4. Western blots of p65/p50 and IκB-α. After incubating MHE cells with or without TNF-α for 12 h, about 50 μg of each total protein extracted from the cells was immunologically stained using rabbit anti-p65/p50 and IκB-α polyclonal IgG (A). Lanes 1 and 2, control MHE cells; lanes 3 and 4, MHE cells previously incubated with 10 mM GSH ester; lanes 2 and 4, +100 units/ml of TNF-α. The relative amounts of the immunoactivity are expressed as percentage of the control (B). Each numbered lane corresponds to the lane in (A). Dashed bars indicate the expression of p65, open bars that of p50 and closed bars that of IκB-α. Values are means ± SD of three independent analyses.

Figure 4 shows the results of the effect of TNF-α on the immunological levels of these components after the 12th h of incubation were approximately 170% for p65, 170% for p50 and 163% for IκB-α, respectively (lanes 1 and 2). The amount of these proteins stimulated by TNF-α paralleled with the degree of each mRNA induced.

Next, the effect of intracellular concentrations of GSH on the expression of p65/p50 and IκB-α by TNF-α was examined. Previous treatment of MHE cells with 10 mM GSH ester for 24 h increased the concentration of GSH by 2 times and downregulated the TNF-α-induced expression of p65/p50 and IκB-α (Fig. 3, lanes 3 and 4). The TNF-α-induction was only 110% of the control cells for p65 mRNA, 112% for p50 mRNA and 115% for IκB mRNA, respectively. Their *de novo* synthesis may not be for NF-κB but for rapid response to extracellular stimuli. IκB function to inhibit the activity of NF-κB through forming inactive NF-κB-IκB complexes in the cytosol.

DISCUSSION

Reversible protein–serine phosphorylation by protein–serine kinases is an important step for a variety of cellular processes, including proliferation and differentiation (37). TNF-α phosphorylates serine-phosphorylation site of IκB-α during activation process of NF-κB (9). Since phosphorylation of IκB-α correlates

with dissociation of NF-κB-IκB complexes (11), the evidence observed in this study that GSH downregulates the phosphorylation of IκB-α may be a cause of redox regulation of NF-κB by GSH. GSH may reduce the induction of phosphorylation by scavenging reactive oxygen species. Transcription initiation represents the major regulatory event controlling specificity of gene expression. This is carried out by the combined action of transcriptional factors (38). Rapid response to external signals is often mediated by inducible activation of transcriptional factors by posttranscriptional mechanisms, as opposed to their *de novo* synthesis. Nelson *et al.* (39) and Wall *et al.* (40) reported that the induction of NF-κB transcription in the pre-B cell line does not require new protein synthesis. Sen and Baltimore (41) demonstrated that the previous treatment of pre-B cells with cycloheximide does not inhibit the activation of NF-κB, suggesting the activation of the NF-κB, comprised of p65/p50 complex, by the DNA alkylating agents does not require the induction of the synthesis of the NF-κB subunits. Tan *et al.* reported the induction of p50 mRNA by TNF-α (42). It is possible that the synthesis of p65/p50 is regulated by the inducible autoregulatory pathway as well as that of IκB. However, no data has been shown on the induction of p65/p50 together with that of IκB.

It has been observed that IκB is rapidly ubiquitinylated and degraded after its phosphorylation at Ser 32 and 36 (43). The induction of NF-κB and IκB creates an autoregulatory loop in which increased synthesis of inhibitor and substrates of active NF-κB serve to restore the uninduced state of the cell as suggested by Brown *et al.* (44). The ability of activated NF-κB (p50/p65) to stimulate the expression of NF-κB-IκB complexes could result in the reaccumulation of these complexes in the cytosol. Such an autoregulatory mechanism may be important to control the cellular localization of the p50/p65 dimer and/or to control the specificity of the NF-κB-mediated response (45).

Endothelial cells play important roles in selective transport, anticoagulation, lipid metabolism, vascular tension, vascularization and immunological regulation. The secretions of same cytokines or growth factors from endothelial cells and their binding to specific receptors mediate these functions (46). GSH plays a ubiquitous role as a basal regulator of cellular redox state, then such a role of GSH may be important to understand the vascular endothelial cell function in response to cytokines for the development of anti-inflammatory therapeutics. Recognition of the pathological significance and the regulatory mechanism of NF-κB in human disease should be a critical determinant of selecting therapy for clinical studies.

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