Glutathione Depletion Down-Regulates Tumor Necrosis Factor (TNF) α-Induced NF-κB Activity via IkB Kinase (IKK)-dependent and –Independent Mechanisms*

Huan Lou and Neil Kaplowitz

From Research Center for Liver Diseases, Keck School of Medicine, University of Southern California, Los Angeles California 90033

Running title: GSH depletion represses TNFα-induced NF-κB activity

Address correspondence to Neil Kaplowitz, Keck School of Medicine, University of Southern California, 2011 Zonal Avenue, HMR101, Los Angeles, CA 90033; Tel 323-442-5576; Fax 323-442-5425; and E-mail kaplowit@usc.edu

Reduced glutathione (GSH) plays a crucial role in hepatocyte function, and GSH depletion by diethyl maleate (DEM) was shown previously to inhibit expression of NF-κB-target genes induced by TNFα and sensitize primary cultured mouse hepatocytes to TNF-mediated apoptotic killing. Here we demonstrate, in the same system, that GSH depletion down-regulates TNF-induced NF-κB transactivation via two mechanisms depending on the extent of the depletion. With moderate GSH depletion (~50%), the down-regulation is IKK-independent and likely acts on NF-κB transcriptional activity, because TNF-induced IKK activation, IkBα phosphorylation and degradation, NF-κB nuclear translocation, NF-κB DNA binding in vitro, and NF-κB subunit RelA(p65) recruitment to κB sites of target gene promoters, all appear unaltered. On the other hand, with profound GSH depletion (~80%), the down-regulation also is IKK-dependent and a timeline is established linking the inhibition of polyubiquitination of RIP1 in TNFR1 complex to partial blockage of IKK activation, IκBα phosphorylation and degradation, and NF-κB nuclear translocation. Of note, pretreatment with antioxidant trolox protects against the inhibitory effect of profound GSH depletion on IKK activation and NF-κB nuclear translocation but fails to restore expression of NF-κB target genes, revealing both IKK-dependent and –independent inhibition. These findings provide new insights into the complex effects of oxidative stress and redox perturbations on the NF-κB pathway.

TNFα plays an important role in liver injury in many animal models and has been implicated in the progression of human alcoholic liver disease and hepatic viral infections (1-4). TNFα exerts diverse biological effects by activation of multiple pathways promoting inflammation and both cell survival and death (5). Like many cell types, normal hepatocytes do not undergo apoptosis in response to TNFα because successful activation of TNF-induced NF-κB pathway blocks TNF-induced apoptotic pathway (5,6).

NF-κB, a family of dimeric transcriptional factors, regulates the expression of a spectrum of genes involved in cell survival and anti-apoptotic functions and inflammation (7). It exists mainly as RelA(p65) and p50 heterodimer, and is retained in cytoplasm by inhibitor proteins (IkBs) in unstimulated cells (7). TNFα activates various pathways through two receptors, TNFR1 and TNFR2 (5). The signaling from TNFα to NF-κB activation, mediated mainly through TNFR1, has been most extensively studied (5,8,9). Upon TNFα binding, TNFR1 recruits TNF receptor-associated death domain protein (TRADD), receptor-interacting protein 1 (RIP1) and TNF receptor-associated factor 2 (TRAF2) (5,10). RIP1 is polyubiquitinated in TNFR1 complex (10,11). This polyubiquitination process is promoted by TRAF2 and reversed by A20 (8). TRAF2 functions as an ubiquitin ligase (E3) for RIP1 generating polyubiquitin chain linked through lysine-63 of ubiquitin (K63-polyUb) that is insensitive to 26S proteasome (12-14), whereas A20 acts sequentially through its two domains to disassemble K63-polyUb and assemble K48-polyUb bound to RIP1, and thereby designates RIP1 for degradation (14). RIP1 recruits IKK complexes (15) through the interaction of the polyUb chains of polyUb-RIP1 with IKKγ/Nemo in the IKK complex (16,17), and leads to eventual activation of IKKβ by phosphorylation of serines 177 and 181 in the activation loop (8,9). Activated IKK complex phosphorylates IκBα at two serine sites (32 and 36), which trigger its ubiquitination and degradation by 26S proteasome (7). Released NF-
κB then enters nucleus where with further modifications (phosphorylation, acetylation etc.) it binds to specific promoter sequences termed κB sites and turns on the transcription of target genes (9,18).

It is reasonable to speculate that in the context of specific liver injury models or disease conditions, hepatocytes become unable to respond normally to the diverse effects of TNFα, and thereby become sensitized to TNF-induced death. GSH, a cysteine containing tripeptide, as the single most abundant antioxidant for detoxifying enzymes and a determinant of the thiol-disulfide state, is crucial to hepatocyte function (19). Previous work in our laboratory demonstrated that GSH depletion by DEM (0.25-0.5 mM) inhibited TNF-induced expression of NF-κB-responsive survival genes and sensitized primary cultured mouse hepatocytes to TNF-induced apoptosis (20,21). The present studies were designed to delineate the inhibitory effect of GSH depletion on signaling events leading to inhibition of TNF-induced NF-κB transactivation. By reporter gene assay we verified that GSH depletion by DEM down-regulates TNF-induced NF-κB activity. By Western blotting and electrophoretic mobility shift assay (EMSA) we confirmed that GSH depletion by DEM at low concentrations (≤0.2 mM) did not inhibit NF-κB nuclear accumulation and DNA binding activity, but did so partially at a higher concentration (0.5 mM). Therefore, we hypothesized that IKK-independent and -dependent mechanisms are involved and set out to prove this by evaluating DEM effect on major signaling events leading to IKK-dependent NF-κB activation and relating them with the timing and degree of GSH depletion. Further, the role of ROS was assessed indirectly by evaluating the possible protective effect of antioxidant pretreatment. Finally, nuclear p65 was analyzed for phosphorylation, acetylation and recruitment to κB sites of NF-κB-target gene promoters as an initial step to define the IKK-independent mechanism.

EXPERIMENTAL PROCEDURES

Cell Culture, Treatments, Preparation of Protein Extracts and Cellular Glutathione Determination—Primary cultured mouse hepatocytes (PMH) were used in all experiments. Hepatocytes were isolated from male C57BL/6 mice, 7-9 week of age, and plated at 1.2x10⁶ cell/60-mm dish as described previously (22). Three hours after plating, hepatocytes were washed and rested in phenol red-free and serum-free DMEM/F12 medium (3-ml/dish) overnight (~15 hours). The incubation and following treatments were carried out in a 37°C cell culture incubator with 5% CO₂. For drug cotreatments, working stock was added to the culture just before TNFα addition except specified otherwise. After indicated time periods, cells were washed with ice-cold D-PBS followed by further processing. The resulting preparations were stored in aliquots at –80°C. For whole cell extracts, cells were lysed in APB buffer (20 mM Tris-HCl pH7.6, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 20 mM β-glycerophosphate, 1.3 mM p-nitrophenol phosphate, 0.5 mM Na₃VO₄, 1 mM PMSF, and protease inhibitor cocktail (Sigma)) supplemented with 1% Triton-100, 2 mM DTT and 300 mM NaCl. Nuclear extracts were prepared as described (23). The protein concentration of the extracts was determined by the method of Lowry. Recombinant mouse TNFα (20 ng/ml; EMD Biosciences) was used in the set of experiments shown in Fig. 1A-B, 2, 3A-C. To study TNFR1-mediated signaling (24), we used recombinant human TNFα (20 ng/ml; R&D Systems) in the rest of experiments. For stock solutions, aliquots of DEM (0.5 M in DMSO; Sigma), trolox (0.5 M in ethanol; EMD Biosciences), buthionine sulfoximine (BSO, 0.1 M in H₂O, filtered; Sigma) and actinomycin D (ActD, 0.4 mg/ml in ethanol; Sigma) were stored at −20°C as recommended by the manufactures. To determine the cellular glutathione (GSH + 2GSSG) content, cell extracts were prepared with 5% trichloroacetic acid (TCA) and subjected to the GSH-recycling assay (25).

Plasmids, Transient Transfection and Reporter Gene Assays—Plasmids pNF-κB-Luc, pTAL-Luc and pAP1-Luc were from EMD Biosciences. Plasmids pTBP-Luc and c-Fos-Luc were kind gifts from Dr. Johnson DL (University of Southern California). Luciferase reporter plasmids were introduced into PMH by transient transfection using Targefect F-1 (Targeting system) as described previously (26). A Renilla luciferase reporter plasmid (pRL-TK; Promega) was included in all transfection experiments as an internal control for transfection efficiency. Six hours post-transfection PMH were treated with
TNFα and/or DEM as indicated for 4 hours, lysed and assayed for luciferase activities using the dual-luciferase reporter assay system (Promega).

**EMSA**—The experiments were performed as described previously (27). NF-κB consensus oligonucleotides (κB; Promega) were labeled with [γ-32P]ATP by T4 polynucleotide kinase (Promega), purified by 8% PAGE, and used as a probe. Nuclear extracts (2 μg) were incubated with the labeled probe (40000-cpm/10 fmol) at 30°C for 20 min in a binding reaction (10 μl) containing 10 mM Tris (pH 7.5), 100 mM KCl, 40 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1 μg poly(dI-dC), and 5 μg BSA. The protein-DNA complexes were fractionated on a native 4% poly(dI-dC), and 5 γ BSA. The protein-DNA complexes were fractionated on a native 4% PAGE with recirculation of TAE buffer (6.7 mM Tris pH 7.5, 3.3 mM sodium acetate and 1 mM EDTA). Labeled Oct-1 oligo (Promega) was used for loading control in parallel experiments. For competition or super shift experiments, nuclear extracts were incubated with unlabeled oligonucleotides (κB or AP-1; Promega), or antibodies as specified at 4°C for 1 hour prior to the addition of the labeled probe. Antibodies against RelA(p65) (100-4165; Rockland) and p50 (sc114; Santa Cruz Biotechnology) were used in super shift assay.

**Western Blotting (WB)**—Whole cell extracts (250 μg) were pre-cleared by normal rabbit IgG plus protein A-agarose followed by immunoprecipitation (IP) with IKKα antibody (5 μl, sc7190; Santa Cruz Biotechnology) and protein A-agarose (20 μl). Precipitates of IKK complex were washed 3 times in APB buffer supplemented with 0.25% Triton X-100, 2 mM DTT and 150 mM NaCl, and once in kinase buffer containing 20 mM Hepes pH 7.2, 20 mM MgCl2, 2 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na3VO4 and 10 μg/ml aprotinin. IKK activities of the precipitates were determined in an in vitro kinase assay (28) in the kinase buffer (20 μl) supplemented with 1 μg of GST-IκBα and 0.3 mM ATP. After 30 min incubation at 30°C, the reaction was stopped and analyzed by WB for the extent of phosphorylation of IκBα with an antibody specific for phospho-IκBα (Ser32/Ser36) (#9246; Cell Signaling Technology). To determine whether DEM is a direct inhibitor of IKK activity, DEM (0.5 mM) was added to the washed immunoprecipitates in kinase buffer (10 μl) in the presence of DTT. After 10 min incubation at 30°C, the precipitates were diluted with KA cocktail (30 μl) and kinase activities were determined. GST-IκBα was prepared from an E. coli strain (JM109) containing plasmid pGST-IκBα using glutathione-Sepharose 4B. This plasmid encodes an amino-terminal GST tag and was overexpressed in E. coli. The GST-IκBα plasmid was used to express GST-IκBα in E. coli JM109 cells. The GST-IκBα was purified by affinity chromatography and activated by succimer (Sigma). The activated GST-IκBα was then used to inhibit the IKK complex. GST-IκBα was added to the IKK complex at a molar ratio of 1:1. The IKK complex was then incubated for 20 min at 30°C, and the phosphorylation of IκBα was determined by WB. The kinase activity of the IKK complex was determined by the addition of [γ-32P]ATP and T4 polynucleotide kinase. The phosphorylation of IκBα was determined by WB with an antibody specific for phospho-IκBα (Ser32/Ser36) (#9246; Cell Signaling Technology).

**Preparation of TNFRI Signaling Complex**—Experiments were performed as described (11) with some modification mainly due to the relative large size and small surface area of PMH. In brief, 5x10^5 PMH were left alone or stimulated with FLAG-tagged human TNFα (0.4 μg/ml; Apotech) systems (Pierce), with West Pico chemiluminescent substrate routinely used, and an enhanced chemiluminescent substrate, West Femto was added (1:10 dilution in West Pico) to increase the sensitivity of detection for low abundance proteins. The probed membranes were restored after treatment with stripping buffer (Pierce), and reprobed with different antibodies sequentially. For protein semi-quantification, the related WB images were first scanned and then analyzed by ImageQuant software.
in the presence or absence of DEM (0.5 mM) for indicated time. In these experiments, DEM was added 2 min prior to TNFα addition to ensure a starting point of 50% GSH depletion in all TNFα/DEM cotreatments. PMH were then washed and lysed in APB buffer (1 ml) supplemented with 0.2% NP-40, 150 mM NaCl, 5 mM NEM, and 1 mM DTT. Whole cell extracts (12 mg) were pre-cleared and subjected to IP overnight with monoclonal anti-FLAG antibody (2 µg, F3165; Sigma) and protein G-Sepharose (30 µl; GE Healthcare). Precipitates of TNFR1 signaling complex were washed and then boiled in 2x sample buffer (pH 8.0, 30 µl) before processing for WB with antibodies specific for TNFR1 (AF-425- PB; R&D Systems), RIP1 (610459; BD Biosciences), TRADD or TRAF2 (sc7868 and sc876; Santa Cruz Biotechnology).

Isolation and Quantitative Analysis of RNA Transcripts—Total RNA was extracted following a previously described protocol (29) from PMH either left untreated, or treated with TNFα plus or minus DEM. Two microgram of total RNA was reverse-transcribed using Omniscript Reverse Transcription Kit (Qiagen) supplemented with 10 µM of random primer (Applied Biosystems). The resulting cDNA (equivalent to 6 ng of total RNA) was subjected to quantitative real-time PCR analysis with TaqMan Universal Master Mix (Applied Biosystems) and ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Primer pairs and TaqMan probes (Biosearch Technologies Inc.) used in quantification of gene expressions are summarized in Table 1 except of the set for IκBα, which was reported previously (30). Each set of these oligonucleotides was designed to encompass an exon-exon junction so that genomic DNA would not be amplified or detected. We used the standard-curve method provided by the system software (SDS2.1) for the relative quantification following manufacture’s instruction (Applied Biosystems). The standard used in construction of relative standard curves was composed of two cDNA stocks (50% of each) derived from total RNAs of PMH stimulated with TNFα for 30 and 180 min, respectively, as in our preliminary experiments this mixture showed high level of the transcripts for the six genes listed in table 1. All data were normalized by 18S ribosomal RNA, and the ratios were shown directly to reflect relative abundances of the mRNA examined under different experimental conditions (Fig. 1D and 9C) or expressed as percentages of the relevant maximal level induced by TNFα (set as 100, Fig. 5D and 8B).

Chromatin Immunoprecipitation (ChIP) and Quantification—The recruitment of RelA(p65) NF-κB subunit to κB sites of certain NF-κB target gene promoters in intact hepatocytes was assessed by ChIP assays following a previously described protocol (31) with minor modifications. PMH were switched to phenol red-free and serum-free DMEM/F-12 medium for 15 hours, then either left untreated, or treated with TNFα, TNFα/DEM, or DEM for indicated time. After cross-linking, cells were washed and lysed on ice in APB buffer supplemented with 150 mM NaCl, 0.5% NP-40 and 0.25% Triton X-100. Nuclei from 8x10^6 hepatocytes were collected, lysed in 0.4 ml of nuclear lysis buffer, sonicated and centrifuged. The supernatant chromatin was precleared, diluted, and divided. Two aliquots, one with 7-µl of RelA(p65) antibody (Rockland), the other without the antibody as negative control, were immunoprecipitated overnight. A third portion, equivalent to only 1% of the IP input, was kept frozen at –80ºC as input chromatin. The immune complex was collected with protein G Sepharose pre-blocked with salmon sperm DNA, washed and eluted. Cross-links on immunoprecipitated and input chromatin were then reversed, and DNAs were purified with the QiaQuick PCR Purification Kit (Qiagen). Immunoprecipitated DNAs (undiluted) and input DNAs (diluted 1:10) were subjected to quantitative real-time PCR using standard curve method as described above. Primer pairs and TaqMan probes (Biosearch Technologies Inc.) used in quantification of gene specific promoter regions are summarized in Table 2. Each set of these oligonucleotides was designed to amplify and detect a specified promoter region encompassing κB sites. The standard used in construction of relative standard curves for the quantification was from an input DNA stock derived from the chromatin sample of untreated PMH. All data for recruitment of RelA(p65) to specified promoters were expressed as percentages of their relevant input DNAs.

Apoptosis and Necrosis Assays—Apoptosis and necrosis were measured by Hoechst 33258 and
Sytox green staining as described previously (20) with Sytox green concentration decreased to 50 nM to accommodate computerized fluorescence image acquiring and analysis with MetaMorph software.

RESULTS

GSH Depletion by DEM Down-Regulates TNF-Induced NF-κB Activity, but Perturbation of Nuclear Accumulation of NF-κB Is Not a Requisite—Previous work in our laboratory demonstrated that GSH depletion by DEM (0.25 and 0.5 mM) inhibits TNF-induced increase of mRNA of endogenous NF-κB-responsive genes, such as IκBα, iNOS and cIAP1, sensitizing PMH to TNF-induced apoptosis (20,21). To explore the underlying mechanism, we proceeded to determine whether κB site is sufficient to confer the down-regulation using reporter gene assays. pNF-κB-Luc, a plasmid with a luciferase reporter gene under the control of a κB site-containing minimal promoter was introduced into PMH by transient transfection. Transfected PMH were either untreated or treated for 4 hours with DEM, TNFα, or TNFα plus DEM before being assayed for luciferase activity.

As expected, stimulation with TNFα increased NF-κB-reporter activity, while GSH depletion by DEM (0.1–0.25 mM) repressed the basal as well as TNF-induced activities (Fig 1A, Left). Importantly, such an inhibitory effect was not seen with pTAL-Luc, a control plasmid lacking the κB sites (Fig 1A, Right). Further experiments revealed that the inhibitory effect was dose-dependent and a ~50% inhibition was observed at a DEM concentration of 0.05 mM (Fig 1B). As additional controls, we examined the effect of GSH depletion on expression of reporter genes driven by TBP-promoter, c-Fos-promoter, or AP-1 site containing promoter, respectively (Fig 1C). In these cases, GSH depletion alone, or in combination with TNFα, either rendered an increase in reporter activity, albeit to different extent, or showed no obvious effect, ruling out a non-specific effect on the assay.

As GSH depletion by 0.1 mM DEM down-regulated NF-κB-driven reporter gene expression, and the depletion was only moderate (see Fig. 3 and detail in later sections), we proceeded to verify the effect on endogenous gene expression. Total RNAs were isolated from PMH after treatments, reverse-transcribed and analyzed by quantitative real-time PCR for transcript levels of IκBα, A20, cIAP1 and iNOS (Fig. 1D and data not shown). Indeed, TNFα stimulated transcription of these NF-κB-responsive genes with the maximal induction level reached at 1 hour for IκBα and A20, and 4 hour for cIAP1 and iNOS. GSH depletion by 0.1 mM DEM cotreatment inhibited the observed induction by TNFα for all four genes at the time points examined, though less severe inhibition for the initial induction was observed for IκBα and A20 compared to the later-response genes (cIAP1 and iNOS), indicating the response is likely to be gene-specific. Increasing DEM to 0.5 mM further enhanced the inhibition.

Having demonstrated that GSH depletion down-regulates TNF-induced NF-κB activity, we next determined whether the down-regulation requires corresponding alteration in TNF-induced NF-κB DNA binding activity or NF-κB nuclear accumulation. Nuclear extracts were prepared from PMH after 3-hour treatments, analyzed by EMSA with 32P-labeled κB-oligonucleotide probes for NF-κB DNA binding activity, and by WB with specific antibody for NF-κB subunit RelA(p65) or p50.

TNF-stimulation, as expected, resulted in an increase in NF-κB DNA binding activity as compared with the untreated control (Fig. 2A), which was κB site specific and composed mainly of p50/p50 homodimer and p50/p65 heterodimer as confirmed by competition and super shift experiments (Fig. 2B, odd lanes). GSH depletion by 0.1 mM DEM did not alter TNF-induced NF-κB DNA binding activity (Fig. 2A). Furthermore, GSH depletion by 0.1 mM DEM exhibited no obvious effect on either the specificity or the dimer pattern of NF-κB DNA binding with the above EMSA results. Nuclear levels of RelA(p65) and p50 were clearly elevated 3-hours after the start of TNFα stimulation. GSH depletion with DEM at 0.025-0.2 mM exerted no effect on the nuclear accumulation of NF-κB, but dose-dependent inhibition occurred with DEM at 0.25-0.5 mM. From this correlation we concluded that the observed decrease in NF-κB DNA binding
activity at 0.5 mM DEM was largely due to decreased nuclear accumulation of NF-κB.

Taken together, our data demonstrated that GSH depletion by DEM (0.1 mM) down-regulated TNF-induced NF-κB activity without interfering with NF-κB accumulation in the nucleus, or NF-κB DNA binding in vitro, suggesting an IKK-IκB pathway independent mechanism. Alternatively, with DEM at 0.5 mM, the down-regulation occurred with concomitant decreases in both NF-κB nuclear accumulation and NF-κB DNA binding activity in vitro, indicating involvement of an additional mechanism, either at the level of or more upstream of IKK, which may have masked the coexistence of nuclear inhibition of NF-κB activity.

GSH Depletion by DEM Is Dose-Dependent, Rapid and Persistent During Initial TNFα Signaling—At first, we examined the relationship between GSH depletion and inhibition of initial TNFα signaling, which is known to be very rapid, to see how the rate of GSH depletion and subsequent levels correlate with inhibition of TNFα signaling. DEM (32,33) is a commonly used agent for acute GSH depletion because it diffuses freely into cells due to its hydrophobicity and forms glutathione conjugates in reactions catalyzed by glutathione S-transferase (Fig. 3A). We therefore performed time course experiments in PMH to examine the kinetics of acute GSH depletion by DEM in detail. As shown in Fig. 3B, the cellular GSH content was not affected initially by TNFα treatment, but dropped immediately after cotreatment of DEM; 50% GSH depletion was observed in 2 min with 0.5 mM DEM, and 40% depletion in 5 min with 0.1 mM DEM. The time required for maximal depletion depended on the initial DEM concentration: DEM at 0.5 mM led to a profound depletion of cellular GSH (~80%) in 5 min, whereas 0.1 mM led to a moderate depletion (~50%) in 10 min. After the initial phase, cellular GSH levels remained relatively steady in the 45-min time course. Thus, DEM-induced GSH depletion is dose-dependent, and with 0.5 mM DEM the depletion is very rapid and marked.

Profound GSH Depletion Interferes with TNF-Induced Activation of IKK Leading to Decreased Nuclear Translocation of NF-κB—Next, we performed a detailed time course study in PMH to examine the possible effect of GSH depletion on TNF-induced activation of IKK-IκB pathway. IKK activity was determined by immunocomplex kinase assay, phosphorylation and degradation of cellular IκBα were examined by WB analysis of whole cell extracts, and nuclear translocation of RelA(p65) and p50 was examined by WB analysis of nuclear extracts.

After TNFα stimulation, IKK activity strongly increased at 5 min and reached the maximal at 10 min (Fig. 4A, top panel, left); consistently the level of phospho-IκBα (Ser32/36) in cells increased sharply at 5 min (third panel, left) followed by a rapid decline of IκBα between 10 to 30 min (fourth panel left) and a concurrent increase of nuclear RelA(p65) and p50 (Fig. 4B, left). In comparison, moderate GSH depletion by cotreatment with 0.1 mM of DEM exerted no effects on the following: (1) the initial activation of IKK at 5 min, (2) phosphorylation and degradation of IκBα, and (3) nuclear translocation of RelA(p65) and p50 (Fig 4A-B, middle panels). In contrast, profound GSH depletion by 0.5 mM DEM resulted in a marked reduction in IKK activity as early as 5 min post TNFα stimulation, followed by decreases in IκBα degradation and corresponding nuclear translocation of both RelA(p65) and p50 (Fig 4A-B, right panels). Although under the latter condition a corresponding decrease in phospho-IκBα at early time points was not observed directly (Fig 4A, third panel), its existence was unmasked after degradation of IκBα was blocked by pretreatment of PMH with M-132, an inhibitor of 26S proteasome (Fig. 4C). The observed inhibition by 0.5 mM DEM on initial IKK activation is not likely due to a direct effect of DEM on IKK activity, as incubation of immunoprecipitates of active IKK complex with DEM (0.5 mM) did not cause reduction of IKK activity in the subsequent KA assay (Fig. 4D, compare lane 4-5 with lane 3). On the other hand, N-ethylmaleimide (NEM, 2 mM), a commonly used agent for blocking protein thiol groups, strongly inhibited IKK activity (compare lane 6 with lane 3).

Thus, we have established that down-regulation of TNF-induced NF-κB activity is IKK-independent when GSH is only moderately depleted (~50%), but becomes also IKK-dependent when GSH is severely depleted (~80%).
Antioxidant Protects Against the Inhibitory Effect of GSH Depletion on TNF-Induced IKK Activation and NF-κB Nuclear Translocation, but Does not Restore NF-κB-Responsive Gene Expression—Because the high DEM dose (0.5 mM) used to produce profound cellular GSH depletion (~80%) also significantly depletes mitochondrial GSH (>55%) (21) and severe mitochondrial GSH depletion is associated with increased generation of reactive oxygen species (ROS) (34), it is possible that ROS may play a role in the inhibitory effect of profound GSH depletion on TNF-induced IKK activation. We therefore examined whether antioxidant pretreatment protects against the inhibitory effect. Trolox (0.5 mM), a potent watersoluble vitamin E analog and a chain-breaking antioxidant (35), was used in the experiments.

As shown in Fig. 5A, GSH depletion by DEM at 0.5 mM inhibited TNF-induced IKK activity; however, with trolox pretreatment, the inhibition was no longer observed, indicating ROS play a major role in the inhibition process. As additional controls, we examined cellular glutathione levels and found that treatment with trolox neither increased the cellular glutathione level nor prevented the depletion of GSH by DEM (Fig. 5B). Furthermore, the inhibition of TNF-induced NF-κB nuclear translocation was prevented by trolox pretreatment (Fig. 5C, lane 6-7), but the protection was not sufficient to prevent inhibition of TNF-induced expression of NF-κB target genes, such as A20 and iNOS, at mRNA levels (Fig. 5D), unmasking the coexistence of nuclear inhibition of NF-κB activity. Thus, the further inhibition of TNF-induced expression of IκBα and A20 by increasing DEM concentration to 0.5 mM (Fig. 1D) is likely the result of IKK-dependent inhibition of NF-κB nuclear translocation.

Profound GSH Depletion Interferes with TNF-Induced Polyubiquitination of RIP1 in TNFR1 Complex and Subsequent Activating Phosphorylation of IKK—Since the initial IKK activation at 5 min, not the maximal activation at 10 min, was most severely affected by profound GSH depletion (Fig. 4A), and the inhibition could be prevented by antioxidant pretreatments (Fig. 5A), we speculated that the targeted signaling step(s) is likely upstream of IKK. Thus, we examined the effect of profound GSH depletion on TNFR1 complex during initial TNFα signaling following a previously published protocol (11). PMH were either unstimulated or stimulated with FLAG-tagged human TNFα in the presence or absence of 0.5 mM DEM, and cell extracts were prepared 2 and 5 min post stimulation. TNFα-engaged TNFR1 was separated by IP with a FLAG-specific antibody and further analyzed by WB for levels of TNFR1, TRADD, RIP1, and TRAF2 in the complex.

As expected, TNFα stimulation led to engagement of TNFR1 and the recruitment of TRADD, RIP1 and TRAF2 to the signaling complex as early as 2 min post-stimulation (Fig. 6A). Note that TNFα induced an ongoing process of polyubiquitination of RIP1 in TNFR1 complex: polyubiquitinated RIP1 (polyUb-RIP1) appeared at 2 min as a ladder of slow migrating bands (80-200 kDa) with non-ubiquitinated RIP1 (~75 kDa, pointed by arrowhead) being the predominant form; by 5 min, the relative amount of polyUb-RIP1 (mainly 150-250 kDa) predominated over non-ubiquitinated RIP1 (Fig. 6A, third panel, and 6C). These changes in RIP1 were not observed in cell extracts (Fig. 6B, third panel). Unlike RIP1, TNFR1-associated polyubiquitination of TRAF2 was not detectable within 5 min of stimulation, but a TRAF2 immuno-reactive band (~65 kDa) that migrated slightly slower than the unmodified form of TRAF2 (56 kDa) was readily visible (Fig. 6A, fourth panel).

In comparison, profound GSH depletion showed no inhibitory effect at 2 min on either receptor engagement or the recruitment of TRADD, RIP1 and TRAF2, but the level of TNF-induced polyUb-RIP1 was reduced, especially at 5 min (6A, third panel and 6C). Interestingly, the decrease in polyUb-RIP1 was associated with a relatively higher level of non-ubiquitinated RIP1 at 2 min, but was no longer so at 5 min, suggesting the involvement of either decreased polyubiquitination of RIP1 or increased deubiquitination of polyUb-RIP1 at both 2 and 5 min, and increased degradation of RIP1 at 5 min. The larger TRAF2 form noticed above was also decreased by profound GSH depletion (Fig. 6A, bottom panel), though the significance is unknown. As additional controls, we examined cell extracts directly by WB analysis and found that, with or without profound GSH depletion, TNFα stimulation did not affect total protein levels.
of TNFR1, TRADD, RIP1 and TRAF2 in PMH (Fig. 6B). Thus the observed decrease in polyUb-RIP1 level in TNFR1 complex was likely due to changes in polyubiquitination or deubiquitination process, rather than the recruitment of RIP1.

RIP1 is an essential mediator for TNF-induced IKK-NF-κB activation and recent studies have shown increasing evidence suggesting a crucial role of polyUb-RIP1 in the process. We reasoned that if the role of polyUb-RIP1 in TNFα signaling holds true in our model, a decrease in TNFR1 associated polyUb-RIP1 should result in decreased signaling downstream, such as activating phosphorylation of IKK. TNFα induces IKK activity by phosphorylation of serines 177 and 181 in the activation loop of IKKβ (S176/S180 in IKKα), which can be detected by WB analysis using a specific phospho-IKK antibody. As shown in Fig. 6D, TNFα induced a transient activation of IKK by phosphorylation on these sites, which appeared at 5 min, peaked at 10 min and started to decline at 15 min. This activating phosphorylation was indeed decreased at both 5 and 10 min under the condition of profound GSH depletion, which correlated well with the decreased IKK activity at these time points (Fig. 4A, top panel), whereas total IKKβ levels remained stable.

Since in TNFα-TNFR1 mediated signaling both p38 and JNK pathways are downstream of RIP1, we examined their activation by WB with antibodies specific for phospho-p38 and phospho-JNK (Fig. 6E). In response to TNFα, phosphorylation of p38 and JNK peaked at 5 and 15 min, respectively. However, peak phosphorylation of both was inhibited with profound GSH depletion. In addition, TNF-induced phosphorylation of c-Jun by JNK was decreased (Fig. 6E, third panel).

So far our data established a time line linking profound GSH depletion by 0.5 mM DEM to the decreased polyubiquitination of RIP1 in TNFα-TNFR1 signaling complex, and further to the downstream events in IKK-IκB pathway. In supporting this view, TNF-induced transient activation of p38 and JNK signaling pathways were found to be inhibited by profound GSH depletion, suggesting the targeting step is most likely shared by the three and located upstream.

**Moderate GSH Depletion by 0.1 mM DEM Does not Reduce TNF-Induced Nuclear Levels of Phospho-p65 (S536) and Acetyl-p65 (K310), nor Interfere with TNF-Induced Recruitment of p65 to Promoters of NF-κB Target Genes—**As post-translational modification of p65 affects its transcriptional activities, we next performed time course studies to determine TNF-induced phosphorylation of p65 at S276, S468 and S536, as well as acetylation of p65 at K310 by WB using specific phospho-p65 antibodies and a specific acetyl-p65 (K310) antibody.

We observed that in PMH, p65 was phosphorylated at S536 in response to TNF, but not at S276 or S468 (Fig. 7A and data not shown). The phosphorylation peaked 5 min after TNF exposure, but its level in nuclei quickly declined afterwards (Fig. 7A). Cotreatment with 0.1 mM DEM showed no clear effect on the process (Fig. 7A). The inhibitory effect of cotreatment with 0.5 mM DEM is likely due to decreased nuclear translocation of p65, as both reductions were prevented by trolox pretreatment (Fig. 7A, top and middle panels).

On the other hand, we observed that nuclear level of acetyl-p65 (K310) was clearly elevated 1-hour after TNF stimulation (Fig. 7B, top panel, compare lane 4 with lane 1). Cotreatment with 0.1 mM DEM did not inhibit the acetylation (compare lane 5 with lane 4), nor did cotreatment with 0.5 mM DEM (data not shown).

We then determined NF-κB DNA binding activity in intact cells by ChIP assay. PMH were treated, fixed, and used for nuclei isolation. Chromatins were then released from nuclei, fragmented and immunoprecipitated with an antibody raised against carboxyl-terminal of p65 protein. The recruitment of p65 to κB sites of IκBα, A20 and cIAP1 promoters was analyzed by quantitative real-time PCR. As expected, stimulation with TNFα resulted in increased recruitment of p65 to these promoters in a three-hour time course study (Fig. 7C, left and middle panels). For the immediate-early response genes IκBα and A20, the first peak of p65 recruitment between 30 and 60 min correlated well with the sharp increase of their mRNA levels during the same period (Fig. 1D, left two panels), whereas, the recruitment for late response gene cIAP1, (Fig. 7C, right panel) occurred much earlier than the significant increase of mRNA (Fig. 1D, top right panel). Cotreatment with 0.1 mM DEM did not inhibit TNF-induced recruitment of p65 for all...
three genes (Fig. 7C), suggesting that transcriptional activity of p65, not DNA binding activity of p65, is inhibited by moderate GSH depletion.

Taken together, our data indicated that moderate GSH depletion does not disrupt TNF-induced steady state nuclear phospho-p65 (S536) and acetyl-p65 (K310), nor TNF-induced binding of p65 to κB sites of NF-κB target gene promoters. To further delineate the inhibition of NF-κB transcriptional activity in our model, detailed analysis is required to elucidate the state of phosphorylation and acetylation of promoter-recruited p65 and its association and dissociation with coactivator and corepressor complexes but is beyond scope of this paper.

Moderate GSH Depletion Sensitizes PMH to TNF-Induced Apoptosis—An alternative approach for GSH depletion is pretreatment of PMH with BSO, an inhibitor of GSH synthesis. A moderate GSH depletion (30%) was achieved by BSO pretreatment (33 μM) for 16 hours without interfering with cell viability. BSO pretreatment showed no effect on TNF-induced IKK activation as well as phosphorylation and degradation of IxBα (Fig. 8A), but inhibited TNF-induced expression of NF-κB-responsive genes, such as IxBα and A20, at mRNA level (Fig. 8B). Of note, TNFα causes further decrease of GSH level (50% after 2-hour exposure) in PMH pretreated with BSO (25 μM). Thus, we have verified that moderate GSH depletion in PMH, either acutely or slowly, inhibits TNF-induced NF-κB activity downstream of IKK-IxB pathway.

Profound GSH depletion by 0.5 mM DEM causes necrosis, and in the presence of TNF the death of PMH is a combination of apoptosis and necrosis with sustained JNK activation (20). In contrast, moderate GSH depletion by 0.1 mM DEM caused neither JNK activation (data not shown), nor increase in necrotic death as compared with the control (0.6±0.6% vs. 0.4±0.6% by 24 hours). However, it sensitized PMH to TNF-induced apoptosis (Fig. 8C) with preceding cleavage of caspase-8 and caspase-3 (Fig. 8D); so did pretreatment with BSO (25 μM; Fig. 8C and data not shown). These are expected, as moderate GSH depletion inhibits TNF-induced NF-κB transactivation and therefore the expression of its targeting anti-apoptotic and survival genes, such as A20, c-IAP1, iNOS and cFLIPL (Fig. 1D and 9C). In addition, the JNK activation caused by 0.1 mM DEM cotreatment was greater and lasted longer than that by TNFα alone, (Fig. 9A). Consistently, cellular level of cFLIPL protein, an endogenous inhibitor of caspase 8, dropped considerably (Fig. 9B), as JNK activation promotes cFLIPL degradation (36).

Notice that the apoptotic death was slow and moderate (for 0.1 mM DEM cotreatment, 10.3±2.6% by 24 hours and 18.1±5.2% by 36 hours; for 25 μM BSO pretreatment, 8.3±3.3% by 36 hours) as compared with TNFα/ActD (an inhibitor of new RNA formation), which caused 40% apoptosis (no necrosis) after 6-hour exposure. The activation of caspase-8 (2-4 hours) was not different between the two (Fig. 8D, top panels), but the latter showed strong amplification in the subsequent caspase-3 activation (middle panels). At present the cause of the difference has not been defined, but it is clear that ActD causes more complete inhibition of NF-κB pathway (Fig. 1D and 9C) and stronger sustained JNK activation (Fig. 9A) than seen with 0.1 mM DEM in response to TNFα.

DISCUSSION

Previous work in our laboratory demonstrated that GSH depletion by DEM inhibits TNFα-induced transcription of endogenous NF-κB-responsive genes, sensitizing PMH to TNFα-induced apoptosis (20,21). This phenomenon has broad potential implications in relation to the role of TNFα in liver diseases associated with GSH depletion or oxidative redox perturbations, and represents a unique example of sensitization to the lethal effect of TNFα. Therefore, to fully understand the mechanism for this effect we performed the current studies. Using the same model, we demonstrate systematically that GSH depletion down-regulates TNFα-induced NF-κB transactivation via IKK-independent and -dependent mechanisms.

Our data establish that moderately depleting cell GSH (~50%) by 0.1 mM DEM (Fig. 3B) represses expression of NF-κB reporter gene in a κB site dependent manner (Fig. 1A-C), and inhibits expression of endogenous NF-κB-responsive anti-apoptotic genes (Fig. 1D, and 9C), which contributes to the sensitization of PMH to TNF-induced apoptosis (Fig. 8C-D). Similar
results were obtained when moderate GSH depletion was achieved via an alternative approach, inhibition of GSH synthesis by BSO pretreatment (Fig. 8B-C). The down-regulation is independent of the IKK-\(\text{I}\kappa\text{B}\) pathway, as it does not affect TNF-induced IKK activation, \(\text{I}\kappa\text{B}\) degradation, and \(\text{NF}-\kappa\text{B}\) nuclear translocation (Fig. 4 and Fig. 8A). Furthermore, it does not interfere with \(\text{NF}-\kappa\text{B}\) DNA binding activity in vitro (Fig. 2) or \(\text{p}65\)-DNA binding in vivo (Fig. 7C), indicating repression of \(\text{NF}-\kappa\text{B}\) transcriptional activity.

Transcriptional activity of \(\text{NF}-\kappa\text{B}\) can be regulated by post-translational modifications, such as phosphorylation and acetylation, which, in many cases, facilitate binding of transcriptional coactivators and enhance the transcription of target genes (9,18). In PMH, we observed TNF-induced nuclear \(\text{p}65\) phosphorylation at S536 and acetylation at K310, and the two processes were not reduced by cotreatment with DEM (0.1 mM) (Fig. 7A-B, and data not shown). Further investigation is necessary to evaluate the state of phosphorylation and acetylation of promoter-recruited \(\text{p}65\) and effect of moderate GSH depletion. Moreover, redox-regulation of \(\text{NF}-\kappa\text{B}\) on a conserved RxxRxRxxC motif has been reported. For \(\text{p}50\), redox-regulation involves its C62 and affects primarily its DNA binding affinity (38), whereas for c-Rel, oxidation of C27 prevents its phosphorylation and reduces its transcriptional activity (39), though the affected phosphorylation site and the underlying mechanism remain unknown. C-Rel is a member of Rel family with transcriptional activity, forms a dimer with \(\text{p}50\), \(\text{p}65\) or itself, and is present in PMH. Therefore, it will be interesting to know if moderate GSH depletion interferes with any of these events induced by TNF\(\alpha\).

On the other hand, our data also demonstrate that severely depleting cell GSH (~80%) by 0.5 mM DEM (Fig. 3B) represses expression of \(\text{NF}-\kappa\text{B}\)-responsive genes below basal levels (21), at least partially through blocking TNF-induced nuclear translocation of \(\text{NF}-\kappa\text{B}\) (Fig. 2C and 4B) in an IKK-\(\text{I}\kappa\text{B}\) pathway dependent manner (Fig. 4A and 4C), though DEM at 0.5 mM does not function as a direct inhibitor of IKK activity (Fig. 4D). Interestingly, the targeting step is likely at the level of polyUb-RIP1 in TNF-engaged TNFR1 signaling complex, as a decrease in polyUb-RIP1 (Fig. 6A and 6C) preceded the decrease in activating phosphorylation of IKK (Fig. 6D) and subsequent decrease in IKK activity, \(\text{I}\kappa\text{B}\) phosphorylation and degradation (Fig. 4A and 4C).

It is well known that RIP1 is an essential mediator of TNF\(\alpha\)-induced IKK-\(\text{NF}-\kappa\text{B}\) activation. Oxidants, such as \(\text{H}_{2}\text{O}_{2}\) and diamide (40,41), or agents leading to increased \(\text{H}_{2}\text{O}_{2}\) production, such as L-mimosine (42), have been reported to block TNF\(\alpha\)-induced IKK-\(\text{I}\kappa\text{B}\) pathway. For L-mimosine, it is likely due to decreased recruitment of RIP1 to TNFR1 complex, as levels of RIP1 in both receptor complex and cell are reduced by its pretreatment (42). Similarly, \(\text{H}_{2}\text{O}_{2}\) pretreatment destabilizes cellular RIP1 and is able to completely block TNF\(\alpha\)-induced IKK activation (40). Unlike the case of L-mimosine, profound GSH depletion in our model system does not affect recruitment and cellular level of RIP1 during initial TNF\(\alpha\) signaling, indicating differences between their underlying mechanisms. Considering the critical roles of polyUb-RIP1 in recruitment of IKK complex to TNFR1 signaling complex, the decrease in polyUb-RIP1 in the receptor complex in our studies is likely to contribute to the inhibition of TNF\(\alpha\) signaling down the IKK-\(\text{I}\kappa\text{B}\) pathway. This view is supported by our observation that TNF-induced activation of p38 and JNK pathways was simultaneously inhibited by profound GSH depletion (Fig. 6E), both of which are downstream of RIP1 in TNFR1-mediated TNF\(\alpha\) signaling. Because TRAF2 is the ubiquitin ligase for TNF\(\alpha\)-induced polyubiquitination of RIP1 and A20 is the deubiquitinase that reverses the reaction, effects of GSH depletion on the activity of TRAF2 and the recruitment and activity of A20 need to be explored in future work.

Additionally, we have provided evidence suggesting that ROS play a major role in the inhibition of initial TNF\(\alpha\) signaling in our model, as pretreatment with antioxidant trolox afforded protection against the effects of profound GSH depletion by DEM on TNF-induced IKK activation (Fig. 5A) without preserving GSH (Fig. 5B). The source and critical ROS species remain to be explored. Importantly, the protective effect of trolox, which restores \(\text{NF}-\kappa\text{B}\) nuclear translocation as well (Fig. 5C), is still not sufficient to prevent the inhibition of expression of \(\text{NF}-\kappa\text{B}\) target genes (Fig. 5D), revealing that
profound GSH depletion not only interferes with NF-κB nuclear translocation but also inhibits nuclear NF-κB activity. Thus, GSH depletion can inhibit TNF-induced NF-κB transactivation via IKK-dependent and –independent mechanisms: with profound GSH depletion both mechanisms are in operation, whereas for moderate GSH depletion the inhibition occurs independent of IKK pathway.

It is of interest that a major distinction between high and low concentration of DEM is depletion of mitochondrial GSH (21), suggesting that this may be the source of oxidative inhibition of IKK activation. Since mild GSH depletion, which spares mitochondrial GSH (21), is not expected to promote oxidative stress, our findings suggest thiol-disulfide redox balance itself influences the assembly and activity of the NF-κB transcriptional apparatus. Even if moderate GSH depletion is unaccompanied by increased oxidized glutathione (GSSG), the Nernst equation predicts that the redox potential is related to [GSH]²/[GSSG]. So decreased GSH without a change in GSH/GSSG can exert significant effects on redox susceptible thiols on proteins.

Finally, the strongly sustained JNK activation seen in ActD cotreatment was not observed in PMH treated with TNFα plus 0.1 mM DEM (Fig. 9A). This is likely due to incomplete inhibition of NF-κB activity by 0.1 mM DEM as compared to ActD (Fig. 1D and 9C), and probably accounts for the less striking sensitization to TNF-induced apoptosis compared with ActD.

In summary, we demonstrate in PMH that GSH depletion down-regulates TNF-induced NF-κB transactivation via IKK-dependent and –independent mechanisms. Although more work is required to assess the source and species of ROS and mechanism of decreased polyUb-RIP1 with profound GSH depletion and mechanism of inhibition of transcription by moderate GSH depletion, the findings underscore the potential for oxidative stress and redox perturbations to impair cell survival pathways in response to TNFα.

REFERENCES

1. Bradham, C. A., Plumpe, J., Manns, M. P., Brenner, D. A., and Trautwein, C. (1998) Am J Physiol 275, G387-392
2. Schwabe, R. F., and Brenner, D. A. (2006) Am J Physiol Gastrointest Liver Physiol 290, G583-589
3. Kaplowitz, N. (2005) Nat Rev Drug Discov 4, 489-499
4. Albano, E. (2006) Proc Nutr Soc 65, 278-290
5. Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003) Cell Death Differ 10, 45-65
6. Papa, S., Zazzeroni, F., Pham, C. G., Bubici, C., and Franzoso, G. (2004) J Cell Sci 117, 5197-5208
7. Ghosh, S., and Karin, M. (2002) Cell 109 Suppl, S81-96
8. Chen, Z. J., Bhoj, V., and Seth, R. B. (2006) Cell Death Differ 13, 687-692
9. Hayden, M. S., and Ghosh, S. (2004) Genes Dev 18, 2195-2224
10. Harper, N., Hughes, M., MacFarlane, M., and Cohen, G. M. (2003) J Biol Chem 278, 25534-25541
11. Micheau, O., and Tschopp, J. (2003) Cell 114, 181-190
12. Lee, T. H., Shank, J., Cusson, N., and Kelliher, M. A. (2004) J Biol Chem 279, 33185-33191
13. Shi, C. S., and Kehrl, J. H. (2003) J Biol Chem 278, 15429-15434
14. Wertz, I. E., O'Rourke, K. M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D. L., Ma, A., Koonin, E. V., and Dixit, V. M. (2004) Nature 430, 694-699
15. Zhang, S. Q., Kovalenko, A., Cantarella, G., and Wallach, D. (2000) Immunity 12, 301-311
16. Wu, C. J., Conze, D. B., Li, T., Srinivasula, S. M., and Ashwell, J. D. (2006) Nat Cell Biol 8, 398-406
17. Ea, C. K., Deng, L., Xia, Z. P., Pineda, G., and Chen, Z. J. (2006) Mol Cell 22, 245-257
18. Chen, L. F., and Greene, W. C. (2004) Nat Rev Mol Cell Biol 5, 392-401
19. Han, D., Hanawa, N., Saberi, B., and Kaplowitz, N. (2006) *Am J Physiol Gastrointest Liver Physiol* **291**, G1-7

20. Nagai, H., Matsumaru, K., Feng, G., and Kaplowitz, N. (2002) *Hepatology* **36**, 55-64

21. Matsumaru, K., Ji, C., and Kaplowitz, N. (2003) *Hepatology* **37**, 1425-1434

22. Feng, G., and Kaplowitz, N. (2002) *Am J Physiol Gastrointest Liver Physiol* **282**, G825-834

23. Alkalay, I., Yaron, A., Hatzubai, A., Jung, S., Avraham, A., Gerlitz, O., Pashut-Lavon, I., and Ben-Neriah, Y. (1995) *Mol Cell Biol* **15**, 1294-1301

24. Tartaglia, L. A., Weber, R. F., Figari, I. S., Reynolds, C., Palladino, M. A., Jr., and Goeddel, D. V. (1991) *Proc Natl Acad Sci U S A* **88**, 9292-9296

25. Tietze, F. (1969) *Anal Biochem* **27**, 502-522

26. Hatano, E., and Brenner, D. A. (2001) *Am J Physiol Gastrointest Liver Physiol* **281**, G1357-1368

27. Sen, R., and Baltimore, D. (1986) *Cell* **46**, 705-716

28. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) *Nature* **388**, 548-554

29. Chomczynski, P., and Sacchi, N. (1987) *Anal Biochem* **162**, 156-159

30. Anest, V., Hanson, J. L., Cogswell, P. C., Steinbrecher, K. A., Strahl, B. D., and Baldwin, A. S. (2003) *Nature* **423**, 659-663

31. Ma, H., Shang, Y., Lee, D. Y., and Stallcup, M. R. (2003) *Methods Enzymol* **364**, 284-296

32. Boyland, E., and Chasseaud, L. F. (1967) *Biochem J* **104**, 95-102

33. Kubal, G., Meyer, D. J., Norman, R. E., and Sadler, P. J. (1995) *Chem Res Toxicol* **8**, 780-791

34. Garcia-Ruiz, C., Colell, A., Morales, A., Kaplowitz, N., and Fernandez-Checa, J. C. (1995) *Mol Pharmacol* **48**, 825-834

35. Hayakawa, M., Miyashita, H., Sakamoto, I., Kitagawa, M., Tanaka, H., Yasuda, H., Karin, M., and Kikugawa, K. (2003) *Embo J* **22**, 3356-3366

36. Chang, L., Kamata, H., Solinas, G., Luo, J. L., Maeda, S., Venuprasad, K., Liu, Y. C., and Karin, M. (2006) *Cell* **124**, 601-613

37. Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005) *Cell* **120**, 649-661

38. Nishi, T., Shimizu, N., Hiramoto, M., Sato, I., Yamaguchi, Y., Hasegawa, M., Aizawa, S., Tanaka, H., Kataoka, K., Watanabe, H., and Handa, H. (2002) *J Biol Chem* **277**, 44548-44556

39. Glineur, C., Davioud-Charvet, E., and Vandenbunder, B. (2000) *Biochem J* **352 Pt 2**, 583-591

40. Pantano, C., Shrivastava, P., McElhinney, B., and Janssen-Heininger, Y. (2003) *J Biol Chem* **278**, 44091-44096

41. Han, D., Hanawa, N., Saberi, B., and Kaplowitz, N. (2006) *Free Radic Biol Med* **41**, 627-639

42. Panopoulos, A., Harraz, M., Engelhardt, J. F., and Zandi, E. (2005) *J Biol Chem* **280**, 2912-2923

FOOTNOTES

*This work was supported by the grant from the National Institutes of Health (DK067215). Hepatocytes were provided by the Cell Culture Core of the USC Research Center for Liver Disease (DK48522).*

*The abbreviations used are: ActD, actinomycin D; BSO, buthionine sulfoximine; ChIP, chromatin immunoprecipitation; DEM, diethyl maleate; EMSA, electrophoretic mobility shift assay; GSH, reduced glutathione; GSSG, oxidized glutathione; IKK, IκB kinase; IP, immunoprecipitation; KA, kinase assay; K48-polyUb, lysine 48-linked polyubiquitin chain; K63-polyUb, lysine 63-linked polyubiquitin chain; PMH, primary cultured mouse hepatocytes; polyUb-RIP1, polyubiquitinated RIP1; RIP1, receptor-interacting protein 1; ROS, reactive oxygen species; TCA, trichloroacetic acid; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; TRADD, TNF receptor-associated death domain protein; TRAF2, TNF receptor-associated factor 2; WCE, whole cell extracts; WB, Western Blotting.*

**FIGURE LEGEND**

**Fig. 1.** Effect of GSH depletion by DEM on TNF-induced NF-κB reporter activity and expression of endogenous NF-κB target genes. A, PMH were transfected with luciferase reporter plasmids with or without κB sites (pNF-κB-Luc and pTAL-Luc). A Renilla luciferase reporter plasmid (pRL-TK) was
cotransfected for normalization of transfection efficiency. Six hours after transfection PMH were either untreated or treated for 4 hours with TNFα (20 ng/ml), DEM (01 and 0.25 mM), or TNFα plus DEM, and then assayed for luciferase activities. Each bar represents the mean ± SD (n = 3). B-C, Experiments were carried out as in A with results expressed as fold-increases relative to the untreated control (set as 1). DEM in a lower concentration range (0.025 ~ 0.1 mM) was used in B to define the lowest effective concentration for the down-regulation. Luciferase reporter plasmids driven by different promoter elements as indicated were utilized in C to evaluate the effect of DEM on the assay. D, Total RNAs were prepared from PMH either left untreated, or stimulated with TNFα (20 ng/ml) plus or minus DEM as indicated for 1, 2 or 4 hours corresponding to time of peak induction of each gene, and reverse-transcribed. The resulting cDNA was analyzed by quantitative real-time PCR for the expression of IkBα, A20, cIAP1 and iNOS, respectively. Cotreatment with ActD (0.5 µg/ml) was used as a positive control for inhibition of new RNA formation. All data were normalized to 18S ribosomal RNA, and the ratios are shown for relative mRNA levels. Each bar represents the mean ± SD (n = 3).

Fig. 2. Effect of GSH depletion by DEM on TNF-induced NF-κB DNA binding activity or NF-κB nuclear accumulation. A, PMH were either untreated or treated for 3 hours with TNFα (20 ng/ml), or TNFα plus DEM. Nuclear extracts were then prepared and analyzed by EMSA with [32P]-labeled oligonucleotide probe, κB (top panel) or Oct-1 (bottom panel). B, EMSA was carried out as in A with some modifications. For competition and supershift experiments, nuclear extracts were incubated, as indicated, with or without either 100-fold molar excess of unlabeled oligonucleotides, or an antibody, prior to addition of the labeled probe. C, Nuclear extracts (NUC) prepared as in A were analyzed by WB with specific antibodies to show nuclear accumulation of p65 and p50 at the 3-hour time point. β-actin levels are also shown for loading controls. Results shown in A to C are representative of at least three independent experiments.

Fig. 3. Dose-dependence of GSH Depletion by DEM. A, Conjugation of GSH with DEM by glutathione S-transferase (GST) generates S-[α,β-diethoxylcarbonyl ethyl]glutathione (DECG). SG, glutathione. B, PMH were either untreated or treated with TNFα (20 ng/ml, ◦), or TNFα plus DEM (0.1 mM, ●; 0.5 mM, ▲) for indicated time. Total cellular GSH levels were then determined by the GSH-recycling assay. Results are presented as percentages of GSH in the untreated control. Each point represents the mean ± SD (n = 3). Total cellular GSH for untreated controls was 66 ± 16-nmol/10⁶ cells. With 0.5 mM DEM, a profound GSH depletion (~80%) was reached in 5 min, while with 0.1 mM DEM, moderate depletion (50%) peaked at 10 min.

Fig 4. Effect of GSH depletion by DEM on TNF-induced IKK-IκB pathway activation. A, WCE were prepared from PMH either untreated or treated, over the indicated time course in a 37°C cell culture incubator, with TNFα (20 ng/ml) or TNFα plus DEM (0.1 and 0.5 mM). IKK complexes were immunoprecipitated with IKKα antibody and assayed for kinase activity (KA) using GST-IκBα and cold ATP as substrates. WB was utilized to visualize the reaction product, phospho-IκBα (Ser32/36) (p-IκBα) with a specific antibody (top panel). In parallel, 20% of the input WCE was subjected to direct WB to show levels of IKKa (loading control), endogenous p-IκBα and IκBα (lower panels). B, Nuclear extracts were prepared from PMH treated as described in A, and subjected to WB to verify changes in nuclear levels of NF-κB subunits p65 and p50. ‡, a non-specific band shown as a loading control. C, First, one set of PMH was pretreated for 1 hour with proteasome inhibitor MG-132 (25 µM) to block TNFα-induced IκBα degradation, while the other set was left alone; then PMH from both sets were either unstimulated or stimulated for 15 min with TNFα (20 ng/ml) or TNFα plus DEM (0.5 mM). WCE were then prepared and analyzed by WB to examine endogenous p-IκBα level. IκBα and β-actin levels are shown as loading controls. D, IKK complex was immunoprecipitated from WCE prepared from untreated or TNF-stimulated PMH and washed. The active IKK complex was then incubated with DEM (0.5 mM) or NEM (2 mM) for 10 min at 30°C followed by KA assay. IKKα levels in precipitates are shown for equal loading. Unlike NEM, DEM did not inhibit IKK activity directly. Results presented in A-D were confirmed at least in three independent experiments.
Fig. 5. Protective effect of antioxidant trolox on GSH depletion-triggered suppression of IKK-NF-κB activation. A, PMH were pretreated with or without trolox (0.5 mM) for 1 hour followed by TNFα stimulation. TNF-treatment was performed in a 37°C water-bath for only 5 and 10 min in the presence or absence of DEM (0.5 mM). IKK-KA was then performed as described in Fig. 4A legend (top panel) with IKKα in precipitates shown for equal loading. Rel. activity, IKK activities were normalized by corresponding IKKα levels, then expressed as percentages relative to the relevant maximal activity induced by TNFα. B, Cellular glutathione levels were examined for PMH exposed to DEM (0.5 mM) for indicated time with or without trolox pretreatment as in A. Results are presented as percentages of glutathione in the untreated control. Each bar represents the mean ± SD (n = 3). C, PMH were treated with or without trolox as described in A, prior to 1-hour TNFα stimulation in the presence and absence of DEM (0.5 mM). Nuclear extracts were then prepared and subjected to WB to verify nuclear translocation of p65 with histone H3 as a loading control. D, PMH were treated with or without trolox as described in A, prior to 1- and 4-hour TNFα stimulation in the presence and absence of DEM (0.5 mM) and total RNAs were prepared. The mRNA levels for A20 at 1-hour and iNOS at 4-hour were determined by quantitative real-time PCR as described in Fig. 1D legend, and expressed as percentages of the maximal level induced by TNFα alone. Each bar represents the mean ± SD (n = 3).

Fig. 6. Effect of profound GSH depletion on polyubiquitination of RIP1 in TNFR1 complex and activating phosphorylation of IKK, p38 and JNK. A-C, PMH were stimulated with FLAG-tagged TNFα (0.4 μg/ml) for indicated time in the presence or absence of DEM (0.5 mM), and collected for cell extracts. Engaged TNFR1 complexes were brought down by IP with anti-FLAG antibody (α-FLAG) and subjected to WB with specific antibodies to detect TNFR1, TRADD, RIP1 and TRAF2 as shown in A. IgG (L), IgG light chain of α-FLAG detected by anti-TNFα1 antibody. PolyUb-RIP1, polyubiquitinated RIP1. Arrowheads point to non-ubiquitinated forms. In parallel, Cell extracts were subjected to direct WB to examine levels of total TNFR1, TRADD, RIP1, TRAF2 and β-actin (loading control) as shown in B. Identical experiments were performed three times and changes of TNFR1-associated RIP1 in both polyubiquitinated (gray stack bar) and non-ubiquitinat ed (open stack bar) forms were summarized in C. All data were expressed relative to the non-ubiquitinated RIP1 recruited at 2 min after TNFα. D, PMH were treated similarly as in A over a 15-min time course, and then cell extracts were analyzed by WB to evaluate TNFα-induced activating phosphorylation of IKK (p-IKK) with an antibody specific for phospho-IKKα/β (Ser176/180). IKKβ is shown as a loading control. Results were verified in at least three independent experiments. E, PMH were treated as in D. WB for phospho-p38 (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), phospho-c-Jun (Ser63) and β-actin in cell extracts are shown.

Fig. 7. Effect of moderate GSH depletion on TNF-induced p65 phosphorylation, acetylation and recruitment to promoters of endogenous NF-κB-responsive genes. A, PMH were pretreated with or without trolox (0.5 mM) for 1 hour followed by incubation with TNF, TNF plus DEM, or DEM alone for the time indicated. Nuclear extracts were then prepared and examined by WB for phospho-p65 at serine 536 (p65-pS536), p65 and Histone H3 (17-kDa) as a loading control. p65 was phosphorylated after TNF stimulation, but level of p65-pS536 quickly declined in nuclei. B, Nuclear extracts were prepared from PMH treated with TNF in the presence or absence of 0.1 mM DEM for the indicated time. WB detected p65, acetyl-p65 at K310 (p65-Ac-K310, pointed by the arrowhead) and HDA3 (50-kDa) as a loading control. A band migrates faster than p65 in a 10% gel but not so in a 10.5-14% gel as in A, though its identity is currently unknown. C, PMH were either left untreated, or stimulated with TNF (20 ng/ml) plus (●) or minus (○) DEM (0.1 mM) over the indicated time course. Chromatin immunoprecipitation (ChIP) assays were performed using an antibody raised against C-terminal of p65 protein. Recruitment of p65 to the promoters of IkBα, A20 and cIAP1 genes was detected by real-time quantitative PCR. All data were expressed as percentage of the relevant DNA input (equivalent to 1% of total IP input). As negative controls, ChIP assays were also performed in the absence of antibody and analyzed similarly; the non-specifically precipitated promoter for each gene analyzed was usually below the detection level (data not shown).
**Fig. 8.** Effect of moderate GSH depletion on the fate of TNF-treated PMH. **A-B**, PMH were treated for 16 hours with or without BSO, an inhibitor of GSH synthesis, followed by TNF stimulation. WCE and total RNA were then prepared. IKK activity and protein levels of p-IκBα and IκBα (A), and mRNA levels of IκBα and A20 (B) were determined. **C**, PMH were incubated with TNF (20 ng/ml), TNF/DEM (0.1 mM), or TNF subsequent to BSO exposure (25 µM, 5 hours) for 36 hours and examined by Hoechst 33258 staining for normal (lightly stained) and apoptotic (brightly stained and shrunken/fragmented) nuclei. PMH, untreated and treated with DEM (0.1 mM) or BSO (25 µM) were included for comparison. **D**, WCE were prepared from PMH treated with TNF (20 ng/ml) or TNF/DEM (0.1 mM) for the time indicated, and subjected to WB detection for active forms of caspase-8 and caspase-3. PMH treated with TNF/ActD (0.5 µg/ml) were used as positive controls for caspase activation.

**Fig. 9.** Effect of moderate GSH depletion on JNK activation in TNF-treated PMH. **A-B**, WCE prepared from PMH treated with TNF, TNF/ActD (0.5 µg/ml) or TNF/DEM (0.1 mM), were examined by WB for phospho-JNK (Thr183/Tyr185) and JNK (A), and FLIP L as well as β-actin as a loading control (B). **C**, Total RNA was prepared from PMH 1 and 2 hours after the same treatments as in **A-B**, and analyzed for the relative level of c-FLIP L mRNA as described in Fig. 1D legend.

**TABLES**

**Table 1.** Sets of primer pairs and TaqMan probe for quantification of transcript levels of NFκB-responsive genes by quantitative real-time PCR.

| Transcript | Forward (F) and reverse (R) primers and TaqMan probe (T) |
|------------|----------------------------------------------------------|
| A20        | F 5’-GAACCAGAGATTCATGAAGCA-3’  
   R 5’-CCTGTGATTTGAGGATGT-3’  
   T 5’-FAM-AAGACGGAAGACAGCTGATCAAGCCCA-BHQ-3’ |
| cIAP1      | F 5’-TTGAGACGCTTGTGTTCGACTTCC-3’  
   R 5’-GGCCAAATGCAACACTGT-3’  
   T 5’-FAM-CCCAGGAGAAGAAATGCTGACCTACAGA-BHQ-3’ |
| iNOS       | F 5’-CCAGCTCAAGAGCCAGAAAC-3’  
   R 5’-CCATTATCTTTACTGATCCAGAAG-3’  
   T 5’-FAM-TTGAGGAGCCCAAAGCCACAG-BHQ-3’ |
| c-FLIP L   | F 5’-TCAAGTATAACTCAGAGCTCCAGAAT-3’  
   R 5’-ACGGATGTCCGGAGGTAAGAA-3’  
   T 5’-TAAGAGCCAAGATTTGGAATACCCTGGA-3’ |

**Table 2.** Primer and probe sets for quantitative real-time PCR analysis in ChIP assays.

| Promoter | Forward (F) and reverse (R) primers and TaqMan probe (T) |
|----------|----------------------------------------------------------|
| IκBα     | F 5’-TGAGGCGCTCGAGGAGAAGTAC-3’  
   R 5’-CCTCCACGTGAGAACCTAAACC-3’  
   T 5’-FAM-CGCCAGTGTCATCGCAG-BHQ-3’ |
| A20      | F 5’-CTCCGGCTCCGAGACTACA-3’  
   R 5’-GGGGGACGGCTTTAGAG-3’  
   T 5’-FAM-TTGGCCTTTCTGATGATGCTGCAG-BHQ-3’ |
| cIAP1    | F 5’-AGGACGACGAGGACGAAAGAAT-3’  
   R 5’-GACGGGAAGAAAATGGGAAA-3’  
   T 5’-FAM-AACAGGACCGCTAAATAGGCTGCA-BHQ-3’ |
Figure 1

A. 

|       | pNFκB-Luc | pTAL-Luc |
|-------|-----------|----------|
| TNF   | - + + +   | - + + +  |
| DEM   | 0 0.1 0.25 | 0 0.1 0.25 |

B. 

|       | pNFκB-Luc |
|-------|-----------|
| TNF   | - + + +   |
| DEM   | 0 0.025 0.05 0.1 |

C. 

|       | pTBP-Luc | c-Fos-Luc | pAp1-Luc |
|-------|----------|-----------|----------|
| TNF   | - + + +  | - + + +   | - + + +  |
| DEM   | - + + +  | - + + +   | - + + +  |

D. 

|       | IkBα | cIAP1 |
|-------|------|-------|
| TNF   | - + + + | + + + + |
| DEM (mM) | - 0.1 0.5 - 0.1 0.5 - 0.1 0.5 | - 0.1 0.5 - 0.1 0.5 |
| ActD  | - - - + + | - - - + + |
| Time (hr) | 1 2 | 2 4 |
Figure 2

A.

| TNF | DEM (mM) | 3 hours |
|-----|----------|---------|
|     | 0        | 0       |
|     | 0.1      | 0.5     |

p65/p50

p50/p50

Oct1

1 2 3 4

B.

| Antibody |
|----------|
| p65      |
| p50      |

| 100 x cold |
|------------|
| κB         |
| AP-1       |

TNF + + + + + + + + + + (3 hours)

DEM - + - + + + + + (0.1 mM)

Super shift

p65/p50

p50/p50

C.

| TNF | DEM (mM) |
|-----|----------|
|     | 0        |
|     | 0.05     |
|     | 0.1      |
|     | 0.5      |
|     | 0.5      |

p65

p50

β-actin

1 2 3 4 5 6 7 8
Figure 3

A.  
GSH + EtOOCH=CHCOOEt
\[ \xrightarrow{\text{GST}} \text{EtOOCH}_2\text{CH(COOEt)-SG} \]

B.  
Graph showing the change in total cellular glutathione (% control) over time (min) with different treatments.
Figure 4

A. 

| DEM (mM) | 0 | 0.1 | 0.5 |
|----------|---|-----|-----|
| TNF (min) | 0 | 5 | 10 |

IP: anti-IKKα

B. 

| DEM (mM) | 0 | 0.1 | 0.5 |
|----------|---|-----|-----|
| TNF (min) | 0 | 15 | 50 |

NUC

C.

| MG-132 | − | + |
|--------|---|---|
| DEM (mM) | 0 | 0.5 |
| TNF (min) | 0 | 15 |

WCE

D.

| TNF (min) | 0 | 10 |
|-----------|---|---|

WCE IP: anti-IKKα

| DTT (mM) | 2 | 0 | 0 |
| DEM (mM) | 0 | 0 | 0.5 |
| NEM (mM) | 0 | 0 | 0 |

KA IKKα

β-actin
Figure 5

A.

| Time (min) | None | Trolox (0.5 mM) |
|------------|------|-----------------|
| 0          |      |                 |
| 5          |      |                 |
| 10         |      |                 |

| TNF (mM)  |      |                 |
|-----------|------|-----------------|
| 0.5       |      |                 |

| DEM (mM)  |      |                 |
|-----------|------|-----------------|
| 0.5       |      |                 |

B.

**Cellular glutathione** (vs % control)

- DEM (0.5 mM) 0' 10'
- Trolox (0.5 mM) -- + +

C.

| Trolox     |      |                 |
|------------|------|-----------------|
|            |      |                 |
|            |      |                 |

| TNF (mM)  |      |                 |
|-----------|------|-----------------|
| 0.5       |      |                 |

| DEM (mM)  |      |                 |
|-----------|------|-----------------|
| 0.5       |      |                 |

| Histone H3 | p65 |
|------------|-----|
| 1 2 3 4 5 6 7 8 9 |

D.

**Relative mRNA level**

- A20
- iNOS

| TNF (mM)  |      |                 |
|-----------|------|-----------------|
| 0.5       |      |                 |

| DEM (mM)  |      |                 |
|-----------|------|-----------------|
| 0.5       |      |                 |

| Trolox (0.5 mM) |      |
|-----|------|
|     | + + + + + |

| Trolox (0.5 mM) |      |
|-----|------|
|     | + + + + + |
Figure 6

A. 

| FLAG-TNFα | 0' | 2' | 5' |
|-----------|----|----|----|
| DEM (mM)  | 0  | 0.5| 0  | 0.5|

IP: α-FLAG

| kDa | TNFR1 | IgG (L) | TRADD | PolyUb-RIP1 | RIP1 | TRAF2 |
|-----|-------|---------|-------|------------|------|-------|
| 50  |       |         |       |            |      |       |
| 25  |       |         |       |            |      |       |
| 20  |       |         |       |            |      |       |
| 15  |       |         |       |            |      |       |
| 10  |       |         |       |            |      |       |
| 7.5 |       |         |       |            |      |       |
| 5   |       |         |       |            |      |       |

B. 

TNFR1, TRADD, RIP1, TRAF2, β-actin

C. 

RIP1 in TNFR1 complex

| TNFα | 2' | 2' | 5' | 5' |
|------|----|----|----|----|
| DEM  | -  | +  | -  | +  |

D. 

| FLAG-TNFα | 0' | 2' | 5' | 10' | 15' |
|------------|----|----|----|-----|-----|
| DEM (mM)   | 0  | 0.5| 0  | 0.5 | 0.5 |

p-IKK, IKKβ

E. 

| DEM (mM) | 0' | 0  | 15' | 5' | 0.5 | 15' |
|----------|----|----|-----|----|-----|-----|
| FLAG-TNFα| p-p38 |   | p-JNK |   | p-c-Jun |   |

β-actin
Figure 7

A. 

|        | 0' | 5' | 10' | 20' |
|--------|----|----|-----|-----|
| TNF   | -  | +  | +   | +   |
| DEM   | -  | -  | 0.1 | 0.5 |
| Trolox| +  | -  | -   | -   |

(mM)

B. 

C. 

IkBα promoter

A20 promoter

cIAP1 promoter

% DNA input

Hours
Figure 8

A.

B.

C.

D.
Figure 9

A.

| Time (hr) | 20’ | 2  | 3  | 20’ | 2  | 4  | 20’ | 2  | 3  |
|-----------|-----|----|----|-----|----|----|-----|----|----|
| TNF       | –   | +  | +  | +   | +  | +  | +   | +  | +  |
| ActD      | –   | –  | –  | +   | +  | +  | +   | +  | –  |
| DEM (mM)  | –   | –  | –  | –   | –  | –  | 0.1 | 0.1| 0.1|

B.

C. Relative mRNA level

| Time (hr) | 1 | 2 |
|-----------|---|---|
| TNF       | – | + |
| ActD      | – | + |
| DEM (mM)  | 0.1 | 0.5 |

p-JNK

JNK

c-FLIP

β-actin
Glutathione depletion down-regulates tumor necrosis factor (TNF) α-induced NF-κB activity via IκB Kinase (IKK)-dependent and -independent mechanisms
Huan Lou and Neil Kaplowitz

J. Biol. Chem. published online August 9, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M706145200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts