Thiol regulation of endotoxin-induced release of tumour necrosis factor α from isolated rat Kupffer cells

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Proinflammatory cytokines released by hepatic macrophages (Kupffer cells) have a central role in the pathogenesis of liver injury and the cardiovascular abnormalities of sepsis. Because cytokine release is controlled primarily at the level of gene expression, intracellular signalling mechanisms that control the transcription of cytokine genes are critical links to organ injury. Oxidant stress up-regulates and antioxidants down-regulate the pleiotropic transcription factor NF-κB, a DNA-binding protein that induces the expression of cytokines and vascular adhesion molecules. Thiol-bearing molecules are also important inhibitors of NF-κB activation, but whether this inhibition represents an antioxidant effect is unknown. This study was undertaken to determine whether important endogenous and pharmacological thiols modulate the activation of NF-κB and the release of tumour necrosis factor α (TNF-α) from Kupffer cells and to ascertain whether these effects are mediated through glutathione. Exposure of rat Kupffer cells to a physiologically relevant concentration of lipopolysaccharide (10 ng/ml) activated NF-κB within 1 h and induced the release of TNF-α over 5 h. Cellular glutathione content remained unchanged after lipopolysaccharide exposure, but both glutathione monoethyl ester and *N*-acetyl-L-cysteine increased cellular glutathione levels, blocked NF-κB activation and inhibited the release of TNF-α. Inhibition of glutathione synthesis prevented the NAC-induced increase in Kupffer cell glutathione, yet it did not prevent the inhibition of TNF-α release by NAC. Thus the inhibition of NF-κB activation by pharmacological thiols such as NAC might reflect a more general role of the intracellular thiol redox status in NF-κB regulation rather than the antioxidant properties of these agents.

INTRODUCTION

Kupffer cells, the resident macrophages of the liver, are largely responsible for clearing the post-mesenteric blood of gut-derived bacteria and potent bacterial toxins such as lipopolysaccharide (LPS) [1]. However, activation of Kupffer cells can also contribute to shock and end-organ injury, consequences that might be largely due to the release of macrophage-derived cytokines such as tumour necrosis factor α (TNF-α) [2–4]. Understanding the intracellular events that follow Kupffer cell activation by LPS is therefore important for predicting the circumstances under which this normal host defence mechanism might contribute to injury and for identifying interventions that might prove therapeutically beneficial.

The activation and nuclear translocation of nuclear factor kappa-B (NF-κB) is a necessary step in the stimulation of macrophages to synthesize proinflammatory cytokines [5,6]. NF-κB is a heterodimer of two proteins, p50 and p65, that binds with high affinity to upstream κB regulatory DNA sequences and promotes the transcription of specific immunomodulatory genes such as TNF-α. Unlike most transcription factors, a pool of NF-κB is sequestered in the cytoplasm and only during the process of activation is it mobilized to the nucleus, where it promotes transcription. Preventing the nuclear migration of the heterodimer in the absence of an activating stimulus is its cytoplasmic association with members of the IκB family of proteins [7,8]. In response to specific stimuli, IκB is phosphorylated, which targets this protein for ubiquitination followed by proteolysis [8]. This liberates NF-κB, which then is translocated to the nucleus.

Because of the pivotal role of NF-κB in inflammation and injury, its activation is regulated at multiple levels. The synthesis of IκB and the many processes controlling its degradation are major mechanisms, and maintaining a critical thiol within the DNA-binding cleft of NF-κB in a reduced state and interactions of NF-κB with other proteins at the binding site are others [5–9]. Although maintaining thiol reduction is necessary for NF-κB binding to DNA, the phosphorylation of IκB leading to its degradation and release from NF-κB paradoxically might require the oxidation of critical cytoplasmic thiols, the identities of which remain unknown [9,10].

It is perhaps because of the necessity for cytosolic thiol oxidation to effect IκB degradation that the overproduction of reactive oxygen intermediates (ROI) resulting in oxidant stress might be a major, if not central, mechanism of NF-κB activation [11]. Both exogenously applied oxidant stresses and increased endogenous ROI generated by the mitochondrial electron transport chain cause NF-κB activation [12–14]. Conversely, antioxidants exert a profound inhibitory effect on NF-κB activation. Whether thiol antioxidants prevent NF-κB activation by eliminating ROI or by directly participating in the reduction of key regulatory thiols has not been established.

Under normal conditions, the intracellular redox status of thiols is highly reductive. Glutathione is responsible for maintaining this reductive state, and the overwhelmingly reductive state of glutathione is maintained by the high affinity of glutathione reductase for oxidized glutathione [15], coupled with the efficient efflux of oxidized glutathione [16]. Although increases in oxidized glutathione might be required to activate NF-κB [9,17], whether reduced glutathione can prevent NF-κB activation has not been well studied. Indirect evidence is provided by the

Abbreviations used: BSO, L-buthionine sulphoximine; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GSHEE, glutathione monoethyl ester; LPS, lipopolysaccharide; NAC, *N*-acetyl-L-cysteine; ROI, reactive oxygen intermediates; TNF-α, tumour necrosis factor α.

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observation that augmenting cellular glutathione inhibits HIV replication in a chronically infected monocytic cell line [18]. Other thiols have been better studied, including the cysteine pro-drug *N*-acetyl-L-cysteine (NAC), which has a protective effect in hepatic ischaemia-reperfusion injury, perhaps by inhibiting Kupffer cell activation [19]. NAC inhibits NF-κB activation under a variety of conditions, an effect that has been attributed to its ability to prevent glutathione depletion [20]. Although the proposition that NAC inhibits NF-κB by augmenting glutathione synthesis suggests that stimuli that activate NF-κB also deplete glutathione, such alterations in cellular total glutathione homeostasis have been observed only in injury models in whole animals [21].

The present studies were designed to establish how thiols such as NAC and glutathione regulate the activation of NF-κB and subsequent cytokine release from Kupffer cells. Specifically, these studies addressed whether the thiols NAC and glutathione inhibit NF-κB activation and TNF-α release in Kupffer cells, and if so whether the effect of NAC is mediated through augmented intracellular glutathione.

MATERIALS AND METHODS

Materials

Reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise specified. Glutathione monoethyl ester (GSHEE) was synthesized as the hemisulphate salt and converted into the bicarbonate salt as previously described [22].

Animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN, U.S.A.) weighing 250–300 g were allowed unrestricted access to food and water. Animals were used in accordance with procedures approved by the institutional Animal Care Committee and within the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were quarantined for at least 2 days before Kupffer cell isolation.

Isolation and culture of Kupffer cells

Kupffer cells were isolated from rat liver by collagenase–pronase perfusion and purified by centrifugal elutriation [23]. All buffers for this procedure were formulated in pyrogen-free water (Abbott Labs., Chicago, IL, U.S.A.). Both viability, determined by Trypan Blue exclusion, and purity, determined by endogenous peroxidase staining, exceeded 90 %. Kupffer cells were cultured overnight in Dulbecco's modified essential medium containing 4.5 g/l glucose, 25 mM Hepes (BioWhittaker, Walkersville, MD, U.S.A.) and 10 % (v/v) heat-inactivated fetal calf serum (BioWhittaker).

Treatment of Kupffer cells with LPS, GSHEE, NAC and L-buthionine sulphoximine (BSO)

After overnight culture, cells were stimulated with 10 ng/ml LPS (*Escherichia coli* serotype 0111:B4) in serum-free Dulbecco's modified essential medium at 37 °C. GSHEE was prepared as a 0.4 M stock solution, pH 6.5, and NAC was prepared as a 0.4 M stock solution in 1 mM HCl; both were filter-sterilized and added to cell culture media 2 h before LPS treatment. BSO was prepared as a 0.1 M filter-sterilized solution in water and was added to cell culture media to achieve a 1 mM final concentration 1 h before the addition of NAC.

Kupffer cell glutathione and DNA assays

Cells were scraped in 0.5 ml ice-cold distilled water and sonicated at 0 °C. Aliquots of the sonicate were immediately acidified with 0.4 M trichloroacetic acid containing 0.1 mM EDTA (final concentrations) and centrifuged at 16000 ***g*** for 3 min at 4 °C. Total glutathione (GSH + GSSX; X = glutathione or other acid-soluble thiol) was measured in the supernatant by reduction with dithiothreitol (DTT), derivatization with orthophthalaldehyde and separation by HPLC as previously described [24], with modifications [25]. The DNA content of the cell sonicate was measured by fluorescence with 2 μg/ml bis-benzimide (Hoechst 33258) and calf thymus DNA as a standard.

Kupffer cell viability

Potential drug toxicity was determined by both Trypan Blue and propidium iodide exclusion after 7 h of treatment with NAC and GSHEE.

TNF-α assay

Antigenic TNF-α concentrations in Kupffer cell culture media were measured by sandwich ELISA as previously described [26].

RNA isolation and Northern blot analysis

Kupffer cell RNA was isolated by using the RNASTAT 60 reagent (Tel-Test B, Friendswood, TX, U.S.A.) in accordance with the manufacturer's instructions. Total RNA from 5×10^6 cells was separated by electrophoresis in a 1 % (w/v) agarose/formaldehyde gel and transferred to a nylon membrane by capillary transfer. Total RNA in each lane was determined by ethidium bromide staining. Detection of mRNA for γ-actin (ATCC, Rockville, MD, U.S.A.) [27] and TNF-α (Chiron Corporation, Emeryville, CA, U.S.A.) [28] was performed by random priming of the specific cDNA followed by hybridization by standard techniques ([29], vol. 2, pp. 7.37–7.58). Northern blot analysis of γ-actin mRNA reflected loading variability observed with ethidium bromide staining for total RNA.

Preparation of nuclear extracts

The medium overlying the cells was removed and replaced with ice-cold PBS. Kupffer cells were harvested by scraping followed by centrifugation (1000 ***g*** for 10 min at 4 °C). Nuclear extracts were prepared by a modified procedure of Schreiber et al. [30]. Cell pellets were suspended in buffer containing 20 mM Tris/HCl, pH 7.8, 5 mM $MgCl_2$, 0.5 mM DTT, 0.3 M sucrose, 1 mM PMSF, 0.2 mM EGTA, 5 mM β-glycerol phosphate and a protease inhibitor cocktail (final concentrations 1 μg/ml antipain, 5 μg/ml aprotinin, 0.3 μg/ml leupeptin and 0.3 μg/ml benzamidine). The plasma membrane was lysed by the addition of Nonidet P40 (final concentration 0.5 %, v/v), and nuclei were harvested by centrifugation at 2000 ***g*** for 5 min at 4 °C. The nuclear envelope was lysed in hypertonic buffer (10 mM Tris/HCl, pH 7.8, containing 5 mM $MgCl_2$, 350 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EGTA, 10 mM β-glycerol phosphate, 25 % glycerol and the protease inhibitor cocktail). After centrifugation at 12000 ***g*** for 15 min at 4 °C, the supernatant (nuclear extract) was collected. The protein concentration of the nuclear extracts was determined with the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Electrophoretic mobility shift assay (EMSA) for NF-κB

Binding reactions were established in 25 μl of binding buffer as described previously [31], using 3 μg of nuclear extract protein per reaction. In the binding reaction, nuclear extracts were incubated for 15 min at 20 °C with 0.1 ng (10000 c.p.m.) of ^{32}P-end-labelled double-stranded NF-κB consensus oligonucleotide (Promega, Madison, WI, U.S.A.). The binding buffer contained 0.75 mM DTT as a reducing agent to allow optimal binding of NF-κB to the labelled oligonucleotide *in vitro*. For competition studies, a 50-fold excess of unlabelled NF-κB oligonucleotide was incubated with nuclear extracts for 5 min before the addition of labelled NF-κB oligonucleotide. For studies verifying NF-κB binding activity by supershift analysis, 2 μl of anti-p50 antibody (Transcruz Gel Supershift reagent; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was added after completion of the binding reaction and the incubation was continued for 2 h at 20 °C. Nuclear extracts were also analysed for the presence of Oct-1 binding proteins by using ^{32}P-end-labelled double-stranded Oct-1 consensus oligonucleotide (Boehringer-Mannhein, Indianapolis, IN, U.S.A.). All samples were subjected to electrophoresis through a 4 % (w/v) polyacrylamide gel at a constant 155 V; the gels were dried before autoradiography.

Statistical analysis

Statistically significant differences among groups were identified by one-way analysis of variance (ANOVA) and the Student–Newman–Keuls method of multiple pairwise comparisons (SigmaStat; Jandel Scientific Software, San Rafael, CA, U.S.A.).

RESULTS

GSHEE treatment inhibits TNF-α release

To determine whether glutathione could prevent LPS-induced Kupffer cell activation, Kupffer cells were treated with GSHEE, a cell-permeant glutathione pro-drug capable of directly increasing intracellular glutathione levels [32]. After 2 h of exposure to GSHEE, cells were stimulated with LPS (10 ng/ml) and the release of TNF-α was measured by ELISA 6 h after the addition of LPS. Whereas 1 mM GSHEE had no significant effect on LPS-induced TNF-α release (Figure 1), 7.5 mM GSHEE inhibited release by 80 % and treatment with 15 mM GSHEE led to unmeasurable TNF-α levels in the medium (less than 0.03 ng/μg of DNA). TNF-α release from cells treated with GSHEE alone was undetectable (results not shown). Treating Kupffer cells with 30 mM GSHEE for 7 h did not diminish viability as indicated by the absence of increased uptake of either Trypan Blue or the membrane-impermeant nuclear dye propidium iodide (results not shown).

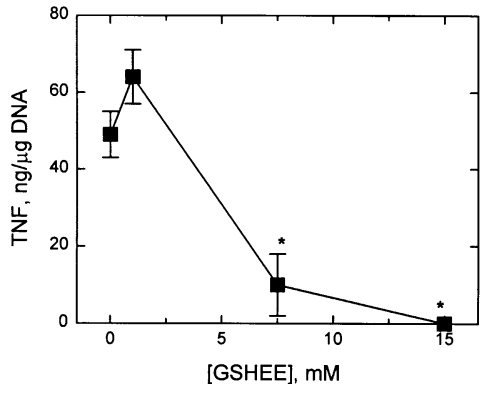

Figure 1 GSHEE inhibits TNF-α release from LPS-stimulated Kupffer cells

TNF-α was released from rat Kupffer cells treated with LPS (10 ng/ml) for 6 h. Pretreating cells with 7.5 or 15 mM GSHEE beginning 2 h before LPS treatment significantly inhibited TNF-α release compared with controls, whereas 1 mM GSHEE had no significant effect. Error bars denote S.E.M. $^*P < 0.05$; $n = 4$ independent experiments.

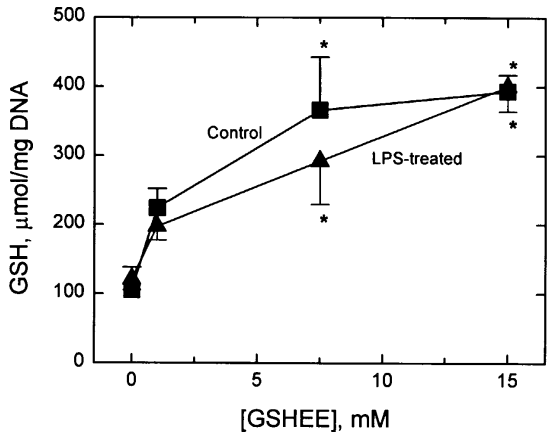

Figure 2 Kupffer cell glutathione content after treatment with LPS and GSHEE

LPS treatment (10 ng/ml) did not significantly alter Kupffer cell glutathione levels (total glutathione, GSH + GSSX; see the Materials and methods section) after 1 h. Treating cells with 7.5 or 15 mM GSHEE, beginning 2 h before LPS, significantly increased cellular glutathione ($^*P < 0.05$ compared with cells not treated with GSHEE or LPS; $n = 4$ independent experiments). Error bars denote S.E.M. The glutathione content of GSHEE-untreated and treated Kupffer cells was not significantly altered by exposure to LPS.

GSHEE treatment increases Kupffer cell glutathione levels

The finding that GSHEE prevented LPS-induced TNF-α release from Kupffer cells suggested that LPS activation might be mediated through glutathione depletion. However, the glutathione content of Kupffer cells 1 h after LPS treatment was identical in normal cells and LPS-treated cells (Figure 2; 0 mM GSHEE). Additionally, the glutathione content of Kupffer cells remained constant 15 and 30 min after LPS treatment, suggesting that a transient fluctuation of glutathione that might trigger TNF-α release did not occur (glutathione at 0, 15, 30 and 60 min: 99 ± 20, 97 ± 14, 98 ± 10, 111 ± 15 μmol/mg DNA, means $\pm$ S.E.M.; $P > 0.9$). Treating Kupffer cells with 7.5 or 15 mM GSHEE, the concentrations that prevented TNF-α release, increased glutathione levels 3.5-fold compared with those in untreated cells, confirming the efficacy of this agent in Kupffer cells (Figure 2) and demonstrating that subsequent LPS treatment did not alter the glutathione content of cells treated with GSHEE.

GSHEE and NAC inhibit NF-κB activation

Thiol compounds can modulate the activation of NF-κB, an essential process that precedes LPS-mediated TNF-α transcription and translation. To determine whether the inhibitory effect of GSHEE on Kupffer cell TNF-α release might be mediated through the inhibition of NF-κB activation, the nuclear levels of this transcription factor were measured by EMSA with nuclear extracts obtained from Kupffer cells exposed to LPS after pretreatment with GSHEE or NAC. Baseline NF-κB in untreated cells varied from undetectable to a faint band, whereas LPS

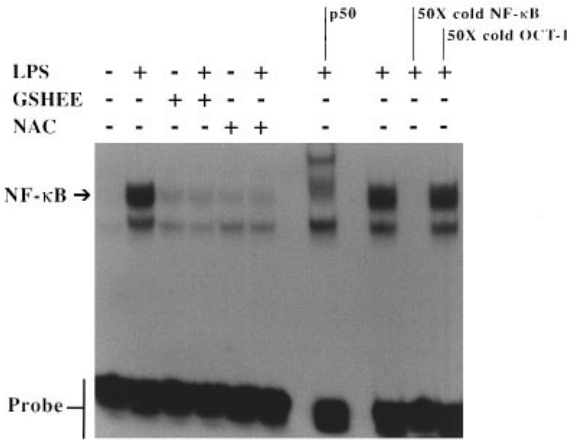

Figure 3 Kupffer cell NF-κB activation by LPS and its prevention by thiols

EMSA of Kupffer cell nuclear proteins demonstrates minimal constitutive activation in cells cultured for 24 h in endotoxin-free medium (left lane). Treating cells with LPS (10 ng/ml) for 1 h caused robust NF-κB activation (second lane) that was markedly inhibited by pretreating the cells with 15 mM GSHEE or 15 mM NAC for 2 h (fourth and sixth lanes). Treatment with either GSHEE or NAC in the absence of LPS resulted in faint bands of NF-κB activation (third and fifth lanes). The identity of the putative NF-κB band was established by co-incubation of the radiolabelled probe and nuclear protein extract from LPS-treated cells with an antibody to the p50 subunit of NF-κB, resulting in an upward shift (supershift) of the band (seventh lane). The specificity of the binding activity found in the nuclear extracts for the probe was confirmed by the loss of detectable binding with unlabelled ('cold') competition with the NF-κB but not Oct-1 consensus oligonucleotides (ninth and tenth lanes, the eighth lane is identical with lane 2). An additional binding activity to the NF-κB probe is also induced in these cells by LPS and is represented by the shifted band below the identified NF-κB band. The identity of this binding activity has not been established. The results shown are from a single experiment and are representative of those seen in three independent experiments.

Table 1 Kupffer cell glutathione content and TNF-α release after treatment with LPS, NAC and BSO

NAC (15 mM final concentration) was added 2 h before the addition of LPS. After exposure to LPS (10 ng/ml) for 6 h, cellular glutathione content and media TNF-α were measured as described in the Materials and methods section. NAC caused a 2.1-fold increase in cellular glutathione, and the inhibition of glutathione synthesis with BSO (1 mM) added 1 h before NAC prevented the NAC-induced increase in cellular glutathione. LPS treatment did not accelerate glutathione loss when compensatory synthesis was blocked (NAC + BSO). LPS stimulated the release of TNF-α over 6 h, and pretreatment with NAC decreased LPS-stimulated TNF-α release by 70%; preventing the NAC-induced rise in cellular glutathione with BSO did not impair the ability of NAC to inhibit TNF-α release (NAC + BSO). Values for glutathione and TNF-α determinations represent means ± S.E.M. ($n = 3$–6 independent experiments). $^*P < 0.05$ compared with respective control cells not treated with LPS. All LPS-treated cells released significantly greater amounts of TNF-α than the respective controls.

	GSH (μmol/mg of DNA)	TNF-α (ng/μg of DNA)
No LPS	126 ± 22	2 ± 2
+ NAC	268 ± 58*	0.4 ± 0.3
+ NAC + BSO	85 ± 18	0.7 ± 0.4
LPS-treated	125 ± 40	40 ± 7
+ NAC	279 ± 49*	12 ± 3*
+ NAC + BSO	84 ± 20	11 ± 4*

treatment caused substantial activation of NF-κB (Figure 3). Both GSHEE and NAC treatment inhibited LPS-mediated NF-κB activation. The identity of the shifted band as NF-κB was established by both supershift and unlabelled-competition assay

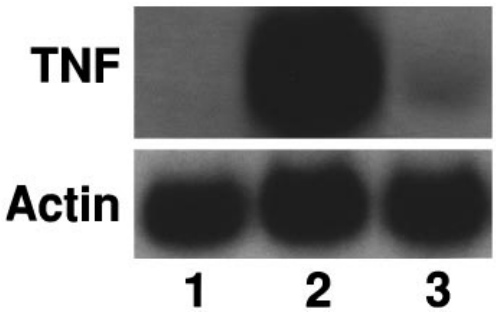

Figure 4 Effects of NAC on steady-state mRNA levels for TNF-α in LPS-treated Kupffer cells

Northern blot analysis of Kupffer cell RNA demonstrates that steady-state TNF-α mRNA was significantly induced after 3 h of stimulation with 10 ng/ml LPS (compare lane 2 with lane 1). NAC (30 mM) added 1 h before stimulation with LPS suppressed the increase in steady-state TNF-α mRNA (lane 3). γ-Actin mRNA was measured as an index of RNA loading. The autoradiogram is representative of five independent studies.

conditions. The binding activity of Oct-1, a constitutively present transcription factor, was constant under the conditions described (results not shown).

NAC increases Kupffer cell glutathione levels

Although glutathione is the most abundant intracellular non-protein thiol, NAC is frequently used experimentally to modulate NF-κB activation. The rationale for using NAC is its availability, its safety as a pharmacological agent and the unproven assumption that its action is mediated through the stimulation of glutathione synthesis. To establish whether NAC and LPS alter cellular glutathione levels, Kupffer cells were pretreated for 2 h with NAC and exposed to LPS. LPS had no effect on Kupffer cell glutathione content, and treatment with NAC caused the cellular glutathione content to increase more than 2-fold (Table 1). This increase was due to synthesis *de novo* as shown by its inhibition with BSO, which specifically inhibits γ-glutamylcysteine synthetase, an essential enzyme in the biosynthetic pathway of glutathione. LPS treatment did not impair the ability of NAC to increase glutathione, nor did it accelerate the loss of glutathione. Homeostatic regulation of intracellular glutathione is a dynamic process; under conditions of increased utilization, glutathione synthesis can increase to maintain normal levels. That increased glutathione consumption did not occur after LPS treatment is shown by the equivalent glutathione levels in LPS-treated cells and in non-LPS-treated cells even when glutathione synthesis was inhibited by BSO (Table 1). NAC treatment did not diminish Kupffer cell viability as measured by either Trypan Blue or propidium iodide exclusion (results not shown).

The NAC-mediated inhibition of TNF-α release is independent of glutathione

The ability to augment Kupffer cell glutathione with NAC along with the ability to prevent glutathione synthesis *de novo* provided the opportunity to establish whether the inhibitory effect of NAC on TNF-α release was mediated by the induction of glutathione synthesis. Treating cells with NAC substantially blocked LPS-induced increases in TNF-α mRNA (Figure 4) and TNF-α release from Kupffer cells (Table 1). More importantly, the effect of NAC on TNF-α release was unaltered by concomitant inhibition of glutathione synthesis by BSO. The inability of BSO

to prevent inhibition by NAC demonstrated that the inhibitory effect of NAC is independent of its role as a glutathione precursor. Thus these results show that NAC must act either directly on components of the LPS-induced signal transduction pathway or through an alternative metabolic pathway. A toxic effect of NAC leading to diminished viability or functional capacity, which could cause decreased TNF-α release, was excluded by the findings of unimpaired dye exclusion and unimpaired TNF-α release when NAC was removed and the cells were allowed to recover overnight before LPS treatment (results not shown).

DISCUSSION

The synthesis and release of TNF-α by Kupffer cells is induced by exposure to LPS and is regulated at the level of gene transcription [33]. Because TNF-α is responsible for many of the cardiovascular and end-organ derangements characteristic of septic shock, the regulation of its transcription is a critical step that links the initial stimulus to these adverse sequelae.

The heterodimeric protein NF-κB is a major *cis*-acting transcriptional regulator of many proinflammatory adhesion molecules and cytokines including TNF-α. The presence of NF-κB has been demonstrated in Kupffer cells [34], and the activation of NF-κB in Kupffer cells by stimuli such as LPS is associated with the release of TNF-α [35,36]. Analogously to findings in other cell types, antioxidants such as pyrrolidine dithiocarbamate and α-tocopherol inhibit LPS-induced NF-κB activation and TNF-α release from cultured Kupffer cells [35,36].

The mechanism underlying the regulatory role of antioxidants on NF-κB activation has remained elusive. One interpretation is that ROI serve as a common mechanism for all stimuli of NF-κB activation [6,11,37–41] and are therefore central to the intracellular signalling events triggered by LPS [6]. However, not all experimental evidence is explained by this hypothesis [10]. An alternative explanation is that ROI constitute but one mechanism of altering the intracellular ratio of oxidized to reduced thiols, and that it is the latter redox ratio that regulates NF-κB activation [9,17,20]. For example, preventing the reduction of oxidized glutathione by inhibiting glutathione reductase with 1,3-bis-(2-chloroethyl)-1-nitrosourea elevates oxidized glutathione levels and activates NF-κB in monocytic cells [9], and the activation of T-cell NF-κB by hydrogen peroxide requires glutathione, implicating oxidized glutathione rather than the peroxide itself as the necessary intermediary [17].

These studies were undertaken to establish the general regulatory role of endogenous and pharmacological thiol compounds on NF-κB activation and TNF-α release. After stimulation of Kupffer cells with a physiologically relevant concentration of LPS (10 ng/ml), activation of NF-κB was evident within 1 h (Figure 3). Unlike freshly isolated Kupffer cells, which were found to have a substantial level of basal NF-κB activation [34], unstimulated cells prepared with meticulous avoidance of spurious endotoxin exposure and cultured overnight showed very little, if any, baseline NF-κB activation. The significance of the Kupffer cell NF-κB activation detected by EMSA on Kupffer cell activation was confirmed by the markedly increased TNF-α levels detected in the media of LPS-treated cells (Figure 1 and Table 1).

These findings were used to explore the role of thiols in modulating NF-κB activation. The activation of NF-κB in Kupffer cells and the subsequent increase in TNF-α mRNA levels and release of TNF-α were inhibited by the cysteine pro-drug NAC. NAC provides the necessary cysteine for glutathione synthesis and its ability to feed into this pathway has been proposed to be the basis of its inhibitory effect on NF-κB activation in other cell types. However, these experiments demonstrate that, at least in Kupffer cells, serving as a glutathione precursor is not the mechanism by which NAC inhibits NF-κB activation. Blocking the incorporation of cysteine into glutathione with BSO, a specific inhibitor of γ-glutamylcysteine synthetase, fully prevented the capacity of NAC to increase cellular glutathione. Despite this metabolic block, the ability of NAC to inhibit LPS-induced TNF-α release remained intact, demonstrating unequivocally that the effect of NAC is not mediated by augmented glutathione synthesis. This observation is also consistent with the beneficial effect that NAC has against ischaemia-reperfusion injury or LPS-induced mortality even when glutathione synthesis is similarly blocked with BSO [19,21].

The cell-permeant glutathione pro-drug GSHEE also proved to be an effective agent for preventing LPS-induced NF-κB activation and TNF-α release. GSHEE is rapidly de-esterified intracellularly by esterases and serves as an effective delivery agent for glutathione, a peptide that is unable to cross cell membranes in its native form. Although a distinction cannot be made between a biological effect of GSHEE and an effect of glutathione in these experiments, the rapid intracellular conversion of GSHEE into glutathione suggests that the effects of GSHEE are likely to be mediated through glutathione. Glutathione formed endogenously or provided by GSHEE serves as a specific substrate for several well-defined intracellular processes. These include the elimination of peroxides such as hydrogen peroxide and lipid hydroperoxides, covalent conjugation with reactive electrophiles, and the reduction of protein thiols. Whether one of these important biological functions of glutathione underlies the mechanisms by which GSHEE inhibits NF-κB activation and TNF-α release was not established by these experiments. However, the simplest explanation for the findings with both NAC and GSHEE is that any cell-permeant reduced thiol, when provided in sufficient quantity, can alter the intracellular thiol redox equilibrium, leading to the inhibition of IκB phosphorylation/proteolysis and the suppression of NF-κB activation [9,17]. Alternatively, the augmented cysteine provided by NAC could feed into a metabolic pathway leading to products other than glutathione that are capable of modulating NF-κB activation, and the augmented glutathione provided by GSHEE could serve as a specific substrate for a glutathione-dependent process.

What is evident from these experiments is that the function of glutathione supplementation is not to counterbalance an increased utilization of glutathione caused by LPS-induced oxidant stress in Kupffer cells, a hypothesized general model for NF-κB activation [20]. Two findings from the current study suggest that LPS treatment does not generate sufficient oxidant stress to alter glutathione homeostasis in Kupffer cells. First, Kupffer cell glutathione was not depleted by LPS treatment as might be expected if there were an increased production of ROI. Secondly, the turnover of glutathione in Kupffer cells was not accelerated by LPS. Although glutathione synthesis could potentially compensate for a small net loss induced by LPS, cells treated with LPS had the same glutathione levels as untreated cells even when synthesis of glutathione *de novo* was blocked with BSO, suggesting that LPS did not stimulate glutathione turnover.

These experiments demonstrate that LPS-induced depletion of glutathione is not a necessary event for NF-κB-mediated Kupffer cell activation by LPS. However, thiols clearly have an important regulatory role in this proinflammatory signalling pathway, as was shown by the inhibition of NF-κB activation by using pharmacological levels of thiol agents, including glutathione. The sensitivity of the NF-κB signalling pathway to generalized inhibition by thiols suggests that it is the overall redox status of

cellular thiols, rather than the presence of oxidant species, that governs key signalling events needed for the release of IκB from NF-κB and the expression of NF-κB-dependent proinflammatory genes.

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