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Regulation of Nuclear Factor-κB, Activator Protein-1, and Glutathione Levels by Tumor Necrosis Factor-α and Dexamethasone in Alveolar Epithelial Cells

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ABSTRACT. The development of an oxidant/antioxidant imbalance in lung inflammation may activate redox-sensitive transcription factors such as nuclear factor-*kappa*B (NF-κB) and activator protein-1 (AP-1), which regulate the genes for proinflammatory mediators and protective antioxidant genes. GSH, a ubiquitous tripeptide thiol, is a vital intra- and extracellular protective antioxidant against oxidative stress, which plays a key role in the control of proinflammatory processes in the lungs. The rate-limiting enzyme in GSH synthesis is γ-glutamylcysteine synthetase (γ-GCS), which consists of a catalytic heavy and a regulatory light subunit. The promoter regions of the human γ-GCS subunits contain AP-1, NF-κB, and antioxidant response elements and are regulated by oxidants, growth factors, inflammatory cytokine tumor necrosis factor-α (TNF-α), and anti-inflammatory agent (dexamethasone) in lung cells. TNF-α depletes intracellular GSH, concomitant with an increase in oxidised glutathione levels in alveolar epithelial cells. TNF-α also induces the activation of NF-κB and AP-1 and the subsequent increase in γ-GCS heavy subunit transcription in these cells. Dexamethasone depleted both basal and TNF-α-stimulated GSH levels by down-regulating the γ-GCS-heavy subunit transcription via a mechanism involving AP-1 (c-Jun). The existence of this fine tuning between the redox GSH levels and the activation of transcription factors may determine the balance of transcription for proinflammatory and antioxidant γ-GCS genes in inflammation. More studies are required to understand the signalling mechanism of the redox regulation of NF-κB and AP-1 and gene transcription in inflammation. This could lead to the development of therapeutic strategies based on the pharmacological manipulation of the production of this important antioxidant in inflammation. BIOCHEM PHARMACOL **60**;8:1041–1049, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. glutathione; TNF-α; dexamethasone; AP-1, NF-κB; alveolar epithelial cells

Reactive oxygen species such as $O_2^{\cdot-}$, liberated by phagocytes recruited to sites of inflammation, are proposed to be a major cause of the cell and tissue damage associated with many chronic inflammatory diseases [1, 2]. Lung cells, in particular alveolar epithelial type II cells, are susceptible to the injurious effects of oxidants. Reduced glutathione is a ubiquitous, essential tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) containing a side chain sulfhydryl (-SH) residue that enables it to protect cells against oxidants (hydroperoxides) and electrophilic compounds [3]. The GSH redox (thiol–disulfide) status is critical for various biological events that include modulation of redox-regulated signal transduction, transcriptional activation of specific genes, storage and transport of cysteine, regulation of cell proliferation, apoptosis, immune modulation, and inflammation [4–7].

Redox-sensitive transcription factors such as NF-κB† and AP-1 (c-Fos/c-Jun) are known to play a key role in proinflammatory processes such as the transcription of cytokine genes and in up-regulating protective antioxidant genes [8–12]. It has been shown that oxidants, antioxidants, inflammatory TNF-α, and anti-inflammatory (glucocorticoids, e.g. dexamethasone) agents modulate the activation of redox-sensitive AP-1 and NF-κB [13–15]. These transcription factor binding sites and AP-1-like ARE are present in the promoter region of γ-GCS, the rate-limiting enzyme in *de novo* GSH synthesis [16, 17]. γ-GCS

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† *Abbreviations:* A549 cells, human alveolar epithelial type II cell line; AP-1, activator protein-1; ARE, antioxidant response element; BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; ELF, epithelial lining fluid; γ-GCS, γ-glutamylcysteine synthetase; γ-GCS-HS, γ-glutamylcysteine synthetase-heavy subunit; γ-GCS-LS, γ-glutamylcysteine synthetase-light subunit; IκB, inhibitory binding protein κB; JNK, c-Jun N-terminal protein kinase; NF-κB, nuclear factor-*kappa*B; ROS, reactive oxygen species; TRE, tetradecanoylphorbol-13-acetate (TPA)-responsive element; and TNF-α, tumor necrosis factor-alpha.

TABLE 1. Inflammatory lung condition associated with altered glutathione levels

Condition	GSH Level	Reference(s)
Smoking		18
Acute	↓	
Chronic	↑	
Idiopathic pulmonary fibrosis	↓	19, 20
Acute respiratory distress syndrome	↓	21
Cystic fibrosis	↓	22
Lung allograft	↓	23
HIV-seropositive	↓	24
Asthma	↑	25

consists of a catalytic γ-GCS-HS and a regulatory γ-GCS-LS. It is possible that the differences in cellular glutathione levels under the influence of oxidative stress and inflammation might play a direct role in the activation of redox-sensitive transcription factors and thereby the regulation of glutathione synthesis by γ-GCS. This review describes the redox control of NF-κB and AP-1 transcription factors and the regulation of glutathione levels and the way in which these transcription factors influence γ-glutamylcysteine synthetase gene expression in response to proinflammatory cytokine TNF-α and the anti-inflammatory agent dexamethasone in lung epithelial cells.

LUNG FLUID GLUTATHIONE LEVELS IN VARIOUS INFLAMMATORY LUNG DISEASES

The antioxidant GSH has been shown to be critical to the lungs' antioxidant defences, particularly in protecting airspace epithelium from oxidant injury and inflammation [2–4, 18]. Alterations in the levels of reduced GSH in the lung lining fluid have been shown under various inflammatory conditions (Table 1). Glutathione is present in increased concentrations in the ELF of chronic smokers, whereas this is not the case in the ELF of acute smokers [18]. GSH is decreased in the ELF in idiopathic pulmonary fibrosis [19, 20], acute respiratory distress syndrome [21], cystic fibrosis [22], lung allograft [23], and HIV-positive patients [24]. In contrast, total concentrations of GSH, including its oxidised form GSSG, are higher in the bronchial and alveolar fluid in patients with mild asthma [25]. A low GSH concentration in the ELF may contribute to an imbalance between oxidants and antioxidants in the lungs and may amplify inflammatory responses (activation of NF-κB and AP-1) and potentiate lung damage [3].

INTRACELLULAR GLUTATHIONE REDOX STATUS AND THE ACTIVATION OF REDOX-SENSITIVE NF-KB AND AP-1 TRANSCRIPTION FACTORS IN INFLAMMATION

There has been considerable interest in the recent past in the ability of oxidative stresses or changes in the intracellular GSH redox status to trigger signal transduction via the activation of redox-sensitive transcription factors and hence the transcription of specific genes. However, the mechanism and the critical level/balance by which oxidants and GSH redox status modulate the transcription of genes in specific lung cells are not well studied.

Oxidants, either inhaled or produced by inflammatory cells, are directly implicated in the inflammatory responses in lung cells via signalling mechanisms. Redox-sensitive transcription factors such as NF-κB and AP-1 (their activation is affected by the redox GSH balance) [6, 13, 14] have been shown to be activated in epithelial and inflammatory cells during oxidative stress/inflammation, leading to the upregulation of a number of proinflammatory genes [8–10, 13, 26]. Maintenance of a high intracellular (GSH)/(GSSG) ratio (>90%) minimises the accumulation of disulfides and provides a reducing environment within the cell. However, if oxidative stress alters this ratio, this shift in the GSH/GSSG redox buffer influences a variety of cellular signalling processes, such as activation and phosphorylation of stress kinases (JNK, p38, phosphatidylinositol 3-kinase) via sensitive cysteine-rich domains, hydrolysis of sphingomyelinase to ceramide, activation of the transcription factors AP-1 and NF-κB, and nuclear import of active JNK, leading to increased gene transcription [6, 26, 27].

NF-κB

NF-κB exists as a heterodimeric complex consisting usually of p50 and p65/RelA subunits. In unstimulated cells, NF-κB is found in the cytoplasm as an inactive non-DNA-binding form, associated with an inhibitor protein called IκB that masks the nuclear translocation signal and so prevents NF-κB from entering the nucleus. Upon cell stimulation with many NF-κB inducers, IκB-α is rapidly phosphorylated on two serine residues, thus targeting the inhibitor protein for ubiquitination by the E3 ubiquitin ligases, $E3RS^{I\kappa B}$, and subsequent degradation by the 26S proteasome [13–15]. The released NF-κB dimer can then be translocated into the nucleus and activate target genes by binding with high affinity to κB elements in their promoters (Fig. 1). Thus, activation of NF-κB would play a pivotal role in the regulation of many genes involved in inflammation whose products mediate inflammatory responses in the lungs [13, 15].

Oxidative stress, including lipid peroxidation products [28] or depletion of GSH and subsequent increases in cytosolic GSSG in response to oxidative stress, causes rapid ubiquitination and phosphorylation of the IκB complex, leading to the activation of NF-κB [13, 14, 29, 30]. The signalling pathway for the activation of NF-κB may be different in response to oxidative stress as compared to TNF-α, since ROS activate NF-κB without the degradation of IκB-α in lung epithelial cells and human umbilical vein endothelial cells [31, 32]. Under reducing conditions, such as an increase in intracellular GSH following treatment with the cysteine donor *N*-acetyl-L-cysteine, serine

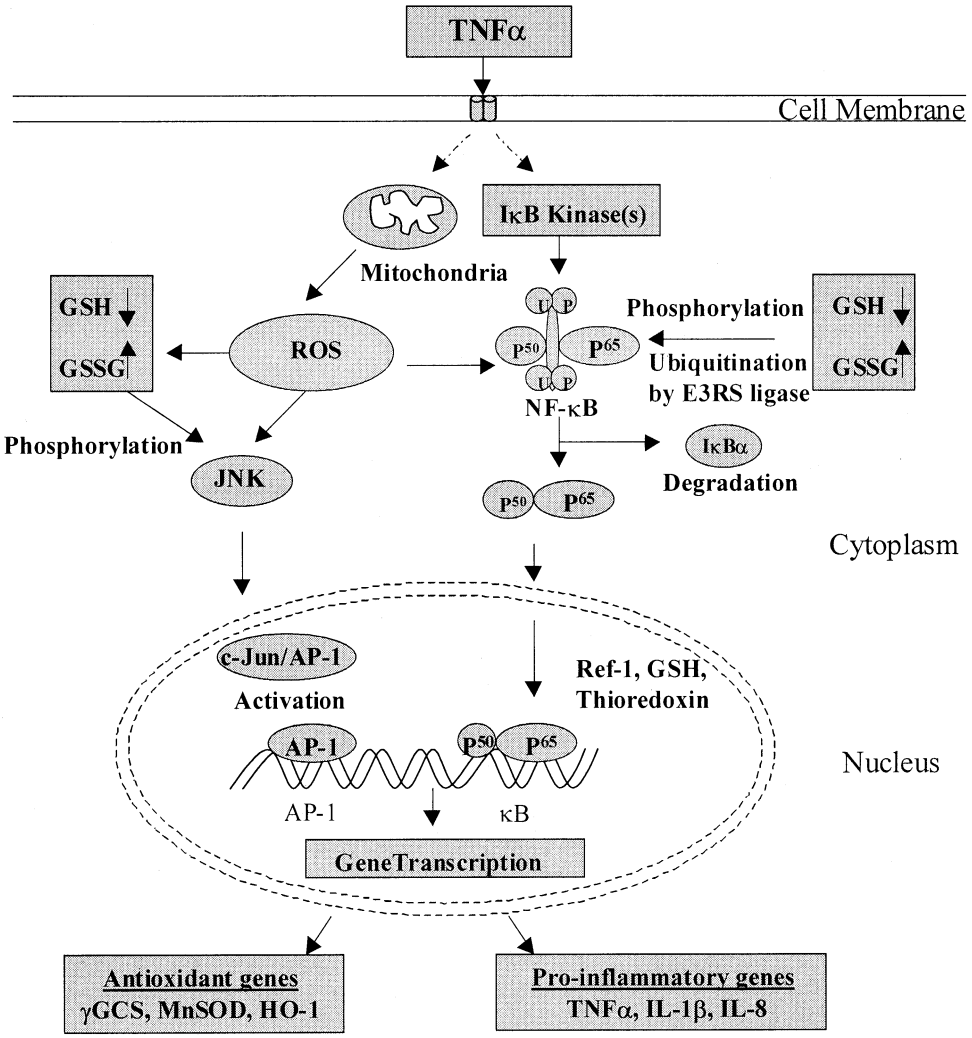

FIG. 1. Model for the mechanism of NF-κB and AP-1 activation leading to gene transcription. TNF-α acts via its receptors on IκB kinase(s) and/or mitochondria to generate ROS, which are involved in the activation of NF-κB. Activation of NF-κB involves the phosphorylation, ubiquitination, and subsequent proteolytic degradation of the inhibitory protein IκB. Free NF-κB then translocates into the nucleus and binds with its consensus sites. The intracellular redox ratio of GSH/GSSG levels and the intranuclear presence of Ref-1, GSH, and thioredoxin can modulate AP-1 and NF-κB activation. Similarly, AP-1 (either c-Jun/c-Jun [homodimer] or c-Fos/c-Jun [heterodimer]) is activated by TNF-α-mediated phorphorylation of the JNK pathway leading to the activation of AP-1, which binds with its TRE consensus region. Activation of NF-κB/AP-1 leads to the co-ordinate expression of protective antioxidant and proinflammatory genes.

phosphorylation on IκB-α following TNF-α treatment is inhibited, leading to the down-regulation of NF-κB in endothelial cells [33]. Similarly, by employing the technique of electron spin reasonable spin trapping with 5-(diethioxyphosphoyl)-5-methyl-1-pyrroline *N*-oxide (DEMPMO), it has been shown that TNF-α-induced NF-κB activation is indeed mediated by an oxidant-sensitive pathway and that this activation was blocked by *N*-acetyl-L-cysteine in macrophages and monocyte-like U937 cells [34, 35]. These data implicate the presence of a redox sensor in the activation of NF-κB in these cells.

A recent study by Manna *et al.* [36] showed that the overexpression of γ-GCS and GSH levels in rat hepatic cells inhibited TNF-α-induced IκB-α degradation and completely inhibited TNF-α-mediated NF-κB activation, NF-κB-dependent gene transcription, stress-activated protein kinase/JNK, and apoptosis. They suggested that inhibition of TNF-α-induced IκB-α degradation by γ-GCS (elevation in GSH levels) is due to the inactivation of ubiquitin-conjugating enzymes, which are shown to be redox-sensitive [37]. Rahman and co-workers have demonstrated that TNF-α exposure produced a marked depletion of intracellular GSH, concomitant with an increase in GSSG levels associated with the activation of NF-κB in lung epithelial cells [38]. This suggests that the intracellular redox state (GSH/GSSG levels) may play a central role in the regulation of NF-κB and the potentiation of the inflammatory responses in lung cells. However, it is not yet known whether oxidative stress and/or an imbalance in GSH redox status may directly stimulate the activity of IκB-α kinase or whether elevated GSH levels inhibit IκB-α kinase activity in lung cells. However, indirect evidence

shows that oxidative stress induces IκB kinase and that *N*-acetyl-L-cysteine inhibits phosphorylation and degradation of IκB-α, suggesting that IκB kinase may act as a primary redox sensor in the activation of NF-κB [39, 40]. It is also possible that changes in intracellular GSH redox status during oxidative stress may affect the proteosome enzymatic activity that leads to the activation of NF-κB [41]. Thus, oxidative stress favours the activation and translocation of NF-κB to the nucleus, and nuclear GSH (reducing environment) facilitates the binding of NF-κB to DNA.

The mechanism of activation of NF-κB under oxidative stress and in altered redox GSH status may be cell-specific and distinct between physiological activators such as TNF-α and interleukin-1β, since diamide, which oxidises GSH to GSSG, and hydrogen peroxide are unable to activate NF-κB in most cell types [42, 43]. Hence, it remains to be elucidated in certain cell types exactly how ROS or alterations in intracellular GSH redox status influence the activation of NF-κB (phosphorylation/ubiquitination/degradation and/or IκB kinase activity). It may be that there are distinct mechanisms at multiple points of activation in different cell types [44].

AP-1

AP-1 is mainly composed of the *Jun* and *Fos* gene products, which form homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexes. This family of transcription factors, which includes heterodimers and homodimers of c-Jun, c-Fos, v-Jun, v-Fos, Fos, Fra 1, Fra 2, Jun D, Jun B, and activating transcription factors (ATF), has been shown to be important in regulating early response genes. AP-1 binds to the TRE present in the promoter region of a wide variety of genes implicated in cell proliferation and tumor promotion. Interaction between Jun and Fos via a "leucine zipper" domain is required for binding to TRE.

DNA binding of the Fos–Jun heterodimer is increased by the reduction of a single conserved cysteine in the DNA-binding domain of each of the proteins [45]. Thiol compounds such as *N*-acetyl-L-cysteine increase unstimulated and phorbol myristate acetate-stimulated AP-1 DNA binding and transactivation in HeLa cells [46, 47]. This may be due to conservation of the redox-sensitive cysteine (sulfhydryl) residue required for the nuclear AP-1 DNA binding through electrostatic interactions with a zinc atom to form a "zinc finger" that binds the DNA. Most recently, Klatt and co-workers [48] have clearly demonstrated that the redox regulation of c-Jun DNA binding is dependent on the ratio of reduced to oxidised glutathione and by the mechanisms that include both protein disulfide formation and *S*-glutathiolation. This observation was supported by the finding that the DNA binding of AP-1 can be enhanced by thioredoxin as well as the nuclear redox protein, Ref-1, and is inhibited by GSSG in many cell types, supporting suggestions that the formation of the disulfide bond of cysteine residues inhibits AP-1 DNA binding [49, 50]. By contrast, oxidative stress imposed by hydrogen peroxide treatment, UV irradiation, TNF-α, depletion of intracellular GSH using DL-buthionine-(SR)-sulfoximine, or alterations in the ratio GSH/GSSG by diamide may also stimulate AP-1 binding [38, 47]. This observation is supported by Wilhelm and co-workers, who demonstrated that perturbation of cellular thiol redox status provides a signal for AP-1 activation, particularly by the induction of stress-activated signal transduction pathways by JNK and p38 kinase [51]. AP-1 is also activated by certain phenolic antioxidant compounds such as butylated hydroxytoluene, butylated hydroxyanisole, and pyrrolidine dithiocarbamate [52, 53]. Thus, both oxidants and antioxidants stimulate AP-1 activation, but differences in biological responses to these agents may be distinct and cell-specific.

REGULATION OF GLUTATHIONE LEVELS AND γ-GLUTAMYLCYSTEINE SYNTHETASE

The majority of intracellular glutathione occurs as reduced GSH (1 to 10 mM), depending on the cell type [3]. The synthesis of glutathione requires the presence of two enzymes and the amino acids glycine, cysteine, and glutamate, with cysteine being the rate-limiting substrate. The tripeptide GSH is formed by the consecutive actions of γ-GCS and glutathione synthetase [3]. In general, the activity of γ-GCS determines the rate of glutathione synthesis. The reaction, catalysed by γ-GCS, is feedback-inhibited by GSH [3]. The mammalian γ-GCS holoenzyme is a heterodimer consisting of a γ-GCS-HS 73 kDa and γ-GCS-LS 30 kDa [54]. Although the heavy subunit contains all of the catalytic activity, γ-GCS activity can be modulated by the association of the heavy subunit with the regulatory light subunit [54]. The regulatory properties of γ-GCS-LS are thought to be mediated by a disulfide bridge between the subunits that would allow conformational changes in the active site depending on the oxidative state of the cell [54].

The agents that regulate the levels of intracellular redox GSH might have a direct role in the activation of NF-κB and AP-1 transcription factors and glutathione synthesis. Therefore, attempts were made to understand the molecular regulation of glutathione synthesis in response to various stimuli [3, 55]. This is bearing in mind that an identification and characterisation of the types of diverse stimuli that act as potent inducers of the glutathione synthesis should aid in the development of effective pharmacological strategies for antioxidant treatment involving GSH regulation in inflammation. Our group and other investigators have reported that the promoter (5′-flanking) regions of human γ-*GCS-HS* and γ-*GCS-LS* genes are regulated by a putative c-Jun homodimeric complex–AP-1 sequence [17, 56, 57], a distal ARE containing an embedded phorbol myristate acetate-responsive element, TRE/AP-1, and an electrophile responsive element, EpRE or its functional equivalent ARE, in response to diverse stimuli [16, 58]. A role for NF-κB in the modulation of γ-*GCS-HS* gene expression has also been suggested [59]. However, mutation and

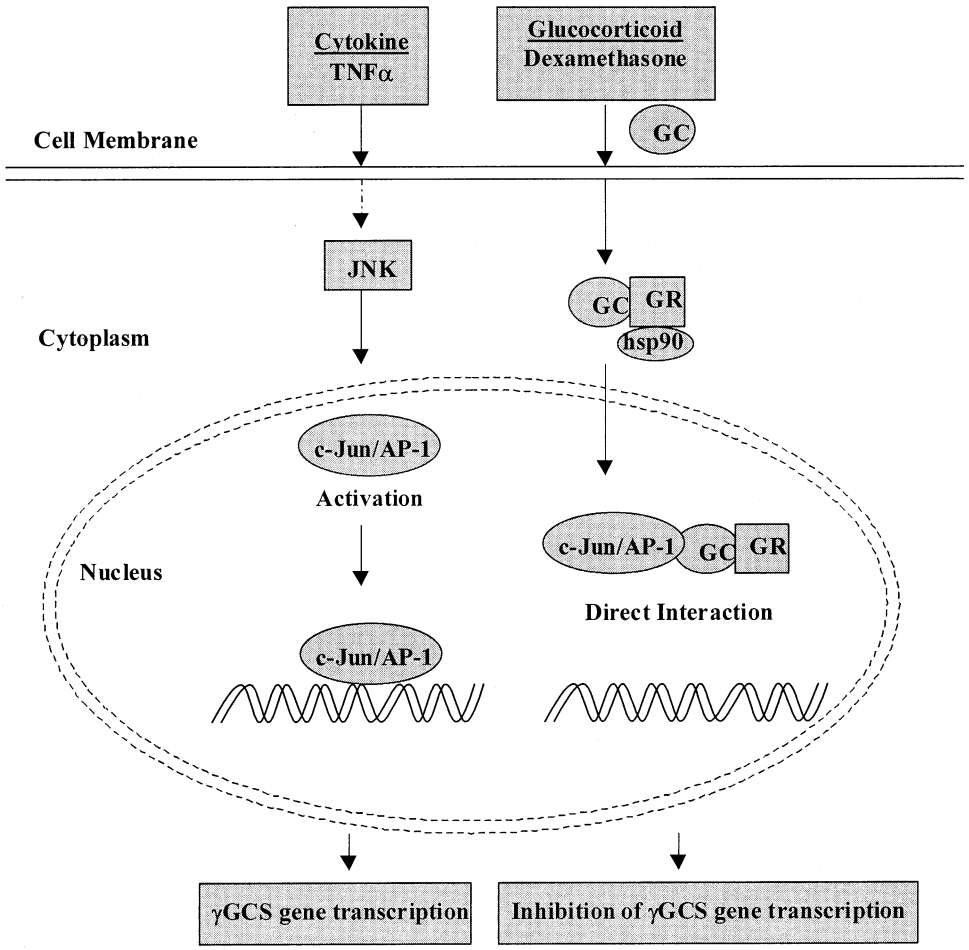

FIG. 2. Model showing the possible mechanism of γ-GCS-HS expression by TNF-α and its repression by corticosteroids (GCs). AP-1 may be activated by a variety of signals including TNF-α and oxidative stress via the activation of JNK, leading to the binding of AP-1 to its TRE consensus regions. AP-1 binding results in the expression of the γ-GCS-*HS* gene, which provides cellular protection/tolerance against inflammatory mediators. Direct interaction between AP-1 and the glucocorticoid receptor (GR) may result in repression of the expression of the γ-GCS-*HS* gene. In this way, steroids may not only inhibit chronic inflammatory effects of cytokines that activate AP-1, but also suppress antioxidant protective gene expression, rendering cells susceptible to various stimuli.

deletion techniques applied to the γ-GCS-HS promoter region have ruled out the possible involvement of NF-κB in the transcriptional up-regulation of the γ-GCS-*HS* gene in alveolar epithelial cells and other cell lines in response to TNF-α and oxidative stress [16, 17, 38]. Thus, it is likely that the expression of the γ-GCS subunit genes is regulated by the different regulatory signals in response to diverse stimuli in specific cells.

TUMOR NECROSIS FACTOR-α

TNF-α is a ubiquitous proinflammatory cytokine recognised as an important mediator of inflammatory events in the lungs. It induces chronic inflammatory changes associated with an increase in a variety of defence mechanisms including antioxidants [11]. TNF-α is an important inflammatory mediator in inflammatory lung diseases such as COPD and acute respiratory distress syndrome and is present in increased amounts in the BALF and sputum in COPD patients [60]. Recently we and other investigators [38, 61] have shown rapid depletion of intracellular GSH by TNF-α exposure in lung epithelial cells and hepatocytes *in vitro*, associated with the activation of NF-κB and AP-1. This is followed by a rebound increase in GSH in epithelial and endothelial cells as an adaptive response to oxidant stress, occurring as a result of up-regulation of γ-GCS-HS and activation of AP-1 [38, 61, 62] (Fig. 2). However, TNF-α-mediated regulation of glutathione levels has not been studied in other cellular systems *in vitro* or *in vivo*. Thus, it may be surmised in alveolar epithelial cells that oxidative stress that is imposed by inflammatory mediators may acutely deplete GSH, activate redox-sensitive transcription factors, and render lung epithelial cells susceptible to the amplification of inflammatory responses.

The signalling mechanism whereby TNF-α exerts its effect is currently not known. TNF-α has been shown to generate ROS in certain cell types, particularly the superoxide anion and hydrogen peroxide, by leakage from the electron transport chain in mitochondria [34, 61, 63]. However, this has been questioned by Liochev and Fridovich [64], who

suggested that TNF-α actually increases the production of arachidonate by activation of phospholipase A_2. Arachidonate will then be converted to the corresponding alkyl hydroperoxide by lipoxygenase. Superoxide anion increases "free" iron by oxidising prosthetic 4Fe–4S clusters of dehydratases, such as aconitase [64]. Ferrous iron in turn reacts with alkyl hydroperoxides, in an analogue of the Fenton reaction, to produce alkoxyl radicals, which can initiate the oxidation of polyunsaturated lipids by a free radical chain reaction [64]. This mechanism could trigger transcriptional up-regulation of glutathione synthesis, possibly by JNK/stress-activated protein kinase [38, 65]. With regard to this, it has been shown that exposure of alveolar epithelial cells *in vitro* to oxidants such as hydrogen peroxide, to redox recycling compounds such as menadione, and to lipid peroxidation products (4-hydroxy-2-nonenal) causes an initial depletion of GSH, associated with increased formation of GSSG, leading to the activation of NF-κB and AP-1 [66–68]. However, chronic oxidative stress leads to a rebound increase in GSH, concomitant with increased expression of mRNA for the γ-*GCS* gene in alveolar epithelial cells [17] other cells *in vitro* [58, 69]. Thus, the short-term effects of inflammatory mediator and various oxidant/oxidant-generating systems appear to up-regulate the gene for glutathione synthesis, providing an adaptive mechanism to subsequent proinflammatory effect/oxidative stress [69, 70]. This outlines the importance of glutathione and γ-GCS against cytotoxic effects of various agents and the fact that some of the actions of TNF-α are regulated by the glutathione-controlled redox status of the cell.

DEXAMETHASONE

Glucocorticoids, such as dexamethasone, are widely used as anti-inflammatory agents in various inflammatory lung diseases. Corticosteroids act to reduce inflammation and cellular damage by two different mechanisms: by direct binding of glucocorticoids to their consensus glucocorticoid response element site, which activates transcription processes, and by an indirect mechanism involving nuclear protein interactions [71]. These latter effects may be mediated by an interaction between the glucocorticoid–receptor complex and transcription factors such as NF-κB and AP-1 [13, 15].

Airway epithelium is one of the most important targets for inhaled glucocorticoids in lung diseases [15]. Exposure of alveolar epithelial cells (A549) to dexamethasone decreases both basal and stimulated GSH levels (TNF-α-treated), without any change in GSSG [38, 72]. However, the ratio of GSH/GSSG levels was decreased by dexamethasone, suggesting that dexamethasone may impose oxidative stress in alveolar epithelial cells [38]. Dexamethasone alone or in combination with TNF-α also decreases γ-*GCS-HS* gene expression in alveolar epithelial cells *in vitro* by a transcriptional mechanism involving inhibition of AP-1 transcription factor [38]. However, dexamethasone did not inhibit TNF-α-mediated activation of NF-κB, ruling out the possible involvement of NF-κB in the regulation of γ-GCS-HS by TNF-α. This observation has been confirmed by mutation and deletion studies showing that the promoter proximal sequence of the γ-*GCS-HS* gene, containing a putative AP-1-binding site, plays an important role in the transcriptional up-regulation of the γ-*GCS-HS* gene in TNF-α-treated alveolar epithelial cells [38]. Furthermore, it has been shown that the modulation of γ-*GCS-HS* gene expression by TNF-α and dexamethasone occurs by a mechanism involving c-Jun homodimer [38, 56, 61]. Depletion of liver GSH in mice and inhibition of GSH synthesis by dexamethasone have been observed in rat hepatic cells [73, 74]. Inhaled corticosteroid such as beclomethasone dipropionate decreased erythrocyte GSH levels in patients with asthma [75]. This suggests that corticosteroids may deplete the intracellular pool of GSH systemically in circulating cells, which renders them susceptible to oxidative stress. Thus, it may be implied that the use of dexamethasone in patients with inflammatory lung diseases may prevent synthesis of the protective antioxidant GSH.

CONCLUSIONS

Glutathione is an important protective antioxidant in the lungs which is altered in the ELF of several inflammatory lung diseases. GSH redox status also plays an important role in the signalling pathways that activate redox-sensitive transcription factors such as NF-κB and AP-1. The regulation of intracellular glutathione levels and the activation of NF-κB and AP-1 may determine the balance between the transcription of proinflammatory mediators and antioxidant genes. Hence, study of the mechanism of GSH regulation and critical redox GSH levels that mediate the amplification of signal transduction and gene transcription in inflammation is an important area of further research.

The promoter regions of γ-*GCS* genes (γ-*GCS-HS* and γ-*GCS-LS*) contain AP-1, ARE, and NF-κB response elements, and are regulated by oxidants, phenolic antioxidants, proinflammatory mediators, and anti-inflammatory agents in lung cells.

Although the molecular mechanism of γ-GCS is well characterised, its regulation in response to specific stimuli via the activation of specific transcription factor has not been studied in detail. Proinflammatory cytokines such as TNF-α produced a transient depletion of GSH concomitant with an increase in GSSG levels associated with the activation of both AP-1 and NF-κB in lung epithelial cells. This suggests that TNF-α exposure produced changes in thiol–disulfide equilibrium in lung epithelial cells and redox-mediated intracellular signalling. Corticosteroids, such as dexamethasone, deplete intracellular GSH by down-regulating the transcription of the γ-*GCS-HS* gene by a mechanism involving AP-1 (c-Jun homodimer). Thus, TNF-α and dexamethasone modulate GSH levels and γ-*GCS-HS* gene expression specifically by their effects on AP-1 in lung epithelial cells. These findings have implications for the proinflammatory responses in the regulation of

redox GSH levels and the activation of NF-κB and AP-1 in inflammation. However, more studies are required to elucidate the involvement of various proinflammatory and anti-inflammatory agents on the redox regulation of NF-κB and AP-1 and their control on glutathione levels and the regulation of glutathione synthesis. Understanding of such cellular and molecular redox regulating mechanisms in inflammation may provide antioxidant therapeutic strategies for the treatment of various inflammatory conditions.

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References

1. Morcillo EJ, Estera J and Cortijo J, Oxidative stress and pulmonary inflammation: Pharmacological intervention with antioxidants. *Pharmacol Res* **40:** 393–404, 1999.
2. Rahman I and MacNee W, Role of oxidants/antioxidants in smoking-induced lung diseases. *Free Radic Biol Med* **21:** 669–681, 1996.
3. Rahman I and MacNee W. Lung glutathione and oxidative stress: Implications in cigarette smoke-induced airways disease. *Am J Physiol* **277:** L1067–L1088, 1999.
4. Brown LA, Glutathione protects signal transduction in type II cells under oxidant stress. *Am J Physiol* **266:** L172–L177, 1994.
5. Droge W, Schulze-Osthoff K, Mihm S, Galter D, Schenk H, Eck H, Roth S and Gmunder H, Functions of glutathione and glutathione disulfide in immunology and immunopathology. *FASEB J* **8:** 1131–1138, 1994.
6. Arrigo A-P, Gene expression and the thiol redox state. *Free Radic Biol Med* **27:** 936–944, 1999.
7. Hall AG, The role of glutathione in the regulation of apoptosis. *Eur J Clin Invest* **29:** 238–245, 1999.
8. Lakshminarayanan V, Drab-Weiss EA and Roebuck KA, Hydrogen peroxide and tumor necrosis factor-alpha induce differential binding of the redox-responsive transcription factors AP-1 and NF-κB to the interleukin-8 promoter in endothelial and epithelial cells. *J Biol Chem* **273:** 32670–32678, 1996.
9. Saccani A, Saccani S, Orlando S, Sironi M, Bernasconi S, Ghezzi P, Mantovani A and Sica A, Redox regulation of chemokine receptor expression. *Proc Natl Acad Sci USA* **97:** 2761–2766, 2000.
10. Desai A, Huang X and Warren JS, Intracellular glutathione redox status modulates MCP-1 expression in pulmonary granulomatous vasculitis. *Lab Invest* **79:** 837–847, 1999.
11. Das KC, Lewis-Molock Y and White CW, Thiol modulation of TNF alpha and IL-1 induced MnSOD gene expression and activation of NF-kappa B. *Mol Cell Biochem* **148:** 45–57, 1995.
12. Yamada N, Yamaya M, Okinaga S, Lie R, Suzuki T, Nakayama K, Takeda A, Yamaguchi T, Itoyama Y, Sekizawa K and Sasaki H, Protective effects of heme oxygenase-1 against oxidant-induced injury in the cultured human tracheal epithelium. *Am J Respir Cell Mol Biol* **21:** 428–435, 1999.
13. Rahman I and MacNee W, Role of transcription factors in inflammatory lung diseases. *Thorax* **53:** 601–612, 1998.
14. Mercurio F and Manning AM, NF-κB as a primary regulator of the stress response. *Oncogene* **18:** 6163–6171, 1999.
15. Barnes PJ and Adcock IM, Transcription factors and asthma. *Eur Respir J* **12:** 221–234, 1998.
16. Mulcahy RT, Wartman MA, Bailey HH and Gipp JJ, Constitutive and β-naphthoflavonone-induced expression of the human γ-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *J Biol Chem* **272:** 7445–7454, 1997.
17. Rahman I, Smith CA, Antonicelli F and MacNee W, Characterisation of γ-glutamylcysteine-heavy subunit gene promoter: Critical role for AP-1. *FEBS Lett* **427:** 129–133, 1998.
18. Morrison D, Rahman I, Lannan S and MacNee W, Epithelial permeability, inflammation and oxidant status in the airspaces of chronic smokers. *Am J Respir Crit Care Med* **159:** 473–479, 1999.
19. Cantin AM, Hubbard RC and Crystal RG, Glutathione deficiency in the epithelial lining fluid of the lower respiratory tract in idiopathic pulmonary fibrosis. *Am Rev Respir Dis* **139:** 370–372, 1989.
20. Rahman I, Skwarska E, Henry M, Davis M, O'Connor CM, FitzGerald MX, Greening A and MacNee W, Systemic and pulmonary oxidative stress in idiopathic pulmonary fibrosis. *Free Radic Biol* **27:** 60–68, 1999.
21. Bunnel E and Pacht ER, Oxidised glutathione is increased in the alveolar fluid of patients with the adult respiratory distress syndrome. *Am Rev Respir Dis* **148:** 1174–1178, 1993.
22. Roum JH, Behl R, McElvancy NG, Borok Z and Crystal RG. Systemic deficiency of glutathione in cystic fibrosis. *J Appl Physiol* **75:** 2419–2424, 1993.
23. Baz MA, Tapson VF, Roggli VL, Van Tright PV and Piantodosi CA, Glutathione depletion in epithelial lining fluid of lung allograft patients. *Am J Respir Crit Care Med* **153:** 742–746, 1996.
24. Buhl R, Holroyd KJ, Mastrangeli A, Cantin AM, Jaffe HA, Wells FB, Saltini C and Crystal RG, Systemic glutathione deficiency in symptom-free HIV seropositive individuals. *Lancet* **2:** 1294–1298, 1989.
25. Smith LJ, Houston M and Anderson J, Increased levels of glutathione in bronchoalveolar lavage fluid from patients with asthma. *Am Rev Respir Dis* **147:** 1461–1464, 1993.
26. Hutter D and Greene JJ, Influence of the cellular redox state on NF-*kappa*B-regulated gene expression. *J Cell Physiol* **183:** 45–52, 2000.
27. Singh I, Pahan K, Khan M and Singh AK, Cytokine-mediated induction of ceramide production is redox-sensitive. *J Biol Chem* **273:** 20354–20362, 1998.
28. Bowie AG, Moynagh PN and O'Neill LA, Lipid peroxidation is involved in the activation of NF-κB by tumour necrosis factor but not interleukin-1 in the human endothelial cell line ECV304. *J Biol Chem* **272:** 25941–25950, 1997.
29. Ginn-Pease ME and Whisler RL, Optimal NF-κB mediated transcriptional responses in Jurkat T cells exposed to oxidative stress are dependent on intracellular glutathione and costimulatory signals. *Biochem Biophys Res Commun* **226:** 695–702, 1996.
30. Mihm S, Galter D and Droge W, Modulation of transcription factor NF kappa B activity by intracellular glutathione levels and by variations of the extracellular cysteine supply. *FASEB J* **9:** 246–252, 1995.
31. Janssen-Heininger YM, Macara I and Mossman BT, Cooperativity between oxidants and tumor necrosis factor in the activation of nuclear factor-kappa B. *Am J Respir Cell Mol Biol* **20:** 942–952, 1999.
32. Canty TG Jr, Boyle EM Jr, Farr A, Morgan EN, Verrier ED and Pohlman TH, Oxidative stress induces NF-kappa B nuclear translocation without degradation of IkappaBalpha. *Circulation* **100**(Suppl 19): II361–364, 1999.
33. Cho S, Urata Y, Iida T, Goto S, Yamaguchi M, Sumikawa K and Kondo T, Glutathione downregulates the phosphorylation of IκB: Autoloop regulation of the NF-κB-mediated expression of NF-κB subunits by TNF-α in mouse vascular

endothelial cells. *Biochem Biophys Res Commun* **253:** 104–108, 1998.
34. Wang S, Leonard SS, Castranova V, Vallynathan V and Shi X, The role of superoxide radical in TNF-alpha induced NF-kappaB activation. *Ann Clin Lab Sci* **29:** 192–199, 1999.
35. Vlahopoulos S, Boldogh I, Casola A and Brasier AR, Nuclear factor-kappaB-dependent induction of interleukin-8 gene expression by tumor necrosis factor alpha: Evidence for an antioxidant sensitive activating pathway distinct from nuclear translocation. *Blood* **94:** 1878–1889, 1999.
36. Manna SK, Kuo MT and Aggarwal BB, Overexpression of γ-glutamylcysteine synthetase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappa B and activator protein-1. *Oncogene* **18:** 4371–4382, 1999.
37. Obin M, Shang F, Gong X, Handelman G, Blumberg J and Taylor A, Redox regulation of ubiquitin-conjugating enzymes: Mechanism insights using the thiol-specific oxidant diamide. *FASEB J* **12:** 561–569, 1998.
38. Rahman I, Antonicelli F and MacNee W, Molecular mechanism of the regulation of glutathione synthesis by tumour necrosis factor-α and dexamethasone in human alveolar epithelial cells. *J Biol Chem* **274:** 5088–5096, 1999.
39. Spiecker M, Darius H and Liao JK, A functional role of IκB-ε in endothelial cell activation. *J Immunol* **164:** 3316–3322, 2000.
40. Flory E, Kunz M, Scheller C, Jassoy C, Stauber R, Rapp UR and Ludwig S, Influence virus-induced NF-κB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IκB kinase. *J Biol Chem* **275:** 8307–8314, 2000.
41. Ukada K, Wangpoengtrakul C, Osawa T, Toyokuni S, Tanaka K and Uchida K, 4-Hydroxy-2-nonenal-mediated impairment of intracellular proteolysis during oxidative stress. *J Biol Chem* **274:** 23787–23793, 1999.
42. Bonizzi G, Piette J, Merville MP and Bours V, Cell type-specific role for reactive oxygen species in nuclear factor-kappaB activation by interleukin-1. *Biochem Pharmacol* **59:** 7–11, 2000.
43. Bowie A and O'Neill LA, Oxidative stress and nuclear factor-κB activation: A reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* **59:** 13–23, 2000.
44. Li N and Karin M, Is NF-*kappa*B the sensor of oxidative stress? *FASEB J* **13:** 1137–1143, 1999.
45. Abate C, Patel L, Rausher FJ and Curran T, Redox regulation of fos and jun DNA-binding activity *in vitro*. *Science* **249:** 1157–1161, 1990.
46. Bergelson S, Pinkus R and Daniel V, Intracellular glutathione levels regulate Fos/Jun induction and activation of glutathione *S*-transferase gene expression. *Cancer Res* **54:** 36–40, 1994.
47. Meyer M, Schreck R and Baeuerle PA, Hydrogen peroxide and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J* **12:** 2005–2015, 1993.
48. Klatt P, Molina EP, De Lacoba MG, Padilla CA, Martinez-Galisteo E, Barcena JA and Lamas S, Redox regulation of c-Jun DNA binding by reversible *S*-glutathiolation. *FASEB J* **13:** 1481–1490, 1999.
49. Galter D, Mihm S and Droge W, Distinct effects of glutathione disulphide on the nuclear transcription factors kB and the activator protein-1. *Eur J Biochem* **221:** 639–648, 1994.
50. Hirota K, Matsui M, Iwata S, Nishiyama A, Mori K and Yodoi J, AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc Natl Acad Sci USA* **94:** 3633–3638, 1997.
51. Wilhelm D, Bender K, Knebel A and Angel P, The level of intracellular glutathione is a key regulator for the induction of stress-activated signal transduction pathways including Jun N-terminal protein kinases and p38 kinase by alkylating agents. *Mol Cell Biol* **17:** 4792–4800, 1997.
52. Choi HS and Moore DD, Induction of c-*fos* and c-*jun* gene expression by phenolic antioxidants. *Mol Endocrinol* **7:** 1596–1602, 1993.
53. Pinkus R, Weiner LM and Daniel V, Role of oxidants and antioxidants in the induction of AP-1, NF-*k*B and glutathione *S*-transferase gene expression. *J Biol Chem* **271:** 13422–13429, 1996.
54. Griffith OW, Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic Biol Med* **27:** 922–935, 1999.
55. Lu SC, Regulation of hepatic glutathione synthesis: Current concepts and controversies. *FASEB J* **13:** 1169–1183, 1999.
56. Sekhar KR, Meredith MJ, Kerr LD, Soltaninassab SR, Spitz DR, Xu ZQ and Freeman ML, Expression of glutathione and γ-glutamylcysteine synthetase mRNA is Jun dependent. *Biochem Biophys Res Commun* **234:** 488–593, 1997.
57. Tomonari A, Nishio K, Kurokawa H, Arioka H, Ishida T, Fukumoto H, Fukuoka K, Nomoto T, Iwamoto Y, Heike Y, Itakura M and Saijo N, Identification of *cis*-acting DNA elements of the human gamma, glutamylcysteine synthetase heavy subunit gene. *Biochem Biophys Res Commun* **232:** 522–527, 1997.
58. Monova H and Mulcahy RT, An electrophile response element (EpRE) regulates β-naphthoflavone induction of the human γ-glutamylcysteine synthetase regulatory subunit gene: Constitutive expression is mediated by an adjacent AP-1 site. *J Biol Chem* **273:** 14683–14689, 1998.
59. Iwanga M, Mori K, Iida T, Urata Y, Matsuo T, Wasungaga A, Shibata S and Kondo T, Nuclear factor *kappa* B dependent induction of γ-glutamylcysteine synthetase by ionizing radiation in T98G human glioblastoma cells. *Free Radic Biol Med* **24:** 1256–1268, 1998.
60. Keating SV, Collins PD, Scott DM and Barnes PJ, Differences in interleukin-8 and tumour necrosis factor-induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* **153:** 530–534, 1996.
61. Morales A, Garcia-Ruiz C, Miranda M, Mari M, Colell A, Ardite E and Fernandez-Checa JC, Tumour necrosis factor increases hepatocellular glutathione by transcriptional regulation of the heavy subunit chain of γ-glutamylcysteine synthetase. *J Biol Chem* **272:** 30371–30379, 1997.
62. Urata Y, Yamamoto H, Goto S, Tsushima H, Akazawa S, Yamashita S, Nagataki A and Kondo T, Long exposure to high glucose concentration impairs the responsive expression of gamma-glutamylcysteine synthetase by interleukin-1 beta and tumor necrosis factor-alpha in mouse endothelial cells. *J Biol Chem* **271:** 15146–15152, 1996.
63. Goossens V, De Vos K, Vercammen D, Steeman M, Vancompernolle K, Fiers W, Vandenabeele P and Grooten J, Redox regulation of TNF signaling. *Biofactors* **10:** 145–156, 1999.
64. Liochev SI and Fridovich I, How does superoxide dismutase protect against tumor necrosis factor: A hypothesis informed by effect of superoxide on "free" iron. *Free Radic Biol Med* **23:** 668–671, 1997.
65. Rahman I, Bel A, Mulier B, Lawson MF, Harrison DJ, MacNee W and Smith CA, Transcriptional regulation of γ-glutamylcysteine synthetase-heavy subunit by oxidants in human alveolar epithelial cells. *Biochem Biophys Res Commun* **229:** 832–837, 1996.
66. Shi MM, Kugelman A, Iwamoto T, Tian L and Forman HJ, Quinone-induced oxidative stress elevates glutathione and induces γ-glutamylcysteine synthetase activity in rat lung epithelial L2 cells. *J Biol Chem* **269:** 26512–26517, 1994.

67. Liu RM, Gao L, Choi J and Forman HJ, γ-glutamylcysteine synthetase: mRNA stabilisation and independent subunit transcription by 4-hydroxy-2-nonenal. *Am J Physiol* **275:** L861–869, 1998.
68. Lo YY, Wong JM and Cruz TF, Reactive oxygen species mediate cytokine activation of c-Jun NH2-terminal kinases. *J Biol Chem* **271:** 15703–15707, 1996.
69. Woods JS, Kavanagh TJ, Corral J, Reese AW, Diaz D and Ellis ME, The role of glutathione in chronic adaptation to oxidative stress: Studies in a normal rat kidney epithelial (NRK52E) cell model of sustained upregulation of glutathione biosynthesis. *Toxicol Appl Pharmacol* **160:** 207–216, 1999.
70. Liu RM, Hu H, Robison TW and Forman HJ, Increased γ-glutamylcysteine synthetase and γ-glutamyltranspeptidase activities enhance resistance of rat lung epithelial L2 cells to quinone toxicity. *Am J Respir Cell Mol Biol* **14:** 192–197, 1996.
71. Tsai MJ and O'Malley BW, Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* **63:** 451–486, 1994.
72. Rahman I, Bel A, Mulier B, Donaldson K and MacNee W, Differential effects of oxidants and dexamethasone on γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase in alveolar epithelial cells. *Am J Physiol* **275:** L80–L86, 1998.
73. Madhu C, Maziasz T and Klaassen CD, Effect of pregnenolone-16 alpha-carbonitrile and dexamethasone on acetaminophen-induced hepatotoxicity in mice. *Toxicol Appl Pharmacol* **115:** 191–198, 1992.
74. Lu SC, Kuhlenkamp J, Garcia-Ruiz C and Kaplowitz N, Hormone-mediated down-regulation of hepatic glutathione synthesis in the rat. *J Clin Invest* **88:** 260–269, 1991.
75. Pennings HJ, Borm PJ, Evelo CTA and Wouters EF, Changes in levels of catalase and glutathione in erythrocytes of patients with stable asthma, treated with beclomethasone dipropionate. *Eur Respir J* **13:** 1260–1266, 1999.