Pyrrolidine dithiocarbamate up-regulates the expression of the genes encoding the catalytic and regulatory subunits of γ-glutamylcysteine synthetase and increases intracellular glutathione levels

Angela C. WILD and R. Timothy MULCAHY[1]

Department of Human Oncology, University of Wisconsin Medical School, 600 Highland Avenue, K4/316 CSC, Madison, WI 53792, U.S.A.

Time- and dose-dependent increases in the steady-state mRNA levels of the genes encoding the catalytic and regulatory subunits of the enzyme γ-glutamylcysteine synthetase (GCS) were observed in HepG2 human hepatocarcinoma cells after exposure to pyrrolidine dithiocarbamate (PDTC). PDTC was demonstrated to manifest both antioxidant and pro-oxidant properties in HepG2 cells, as assessed by the decreased fluorescence of the redox-sensitive dye Dihydrorhodamine 123 and by the oxidation of glutathione respectively. Attempts to characterize the signalling pathway from PDTC exposure to increases in the expression of the GCS catalytic and regulatory subunit genes demonstrated that induction by PDTC could be partially blocked by treatment with the thiol agent *N*-acetylcysteine and by the copper chelator bathocuproine disulphonic acid. These findings suggested that the up-regulation of the two genes resulted from a PDTC-induced pro-oxidant signal, which was partially copper-dependent. In summary, these studies demonstrate that PDTC exposure elicits a cellular response in HepG2 cells, characterized by the induction of the genes encoding the two subunits of the enzyme GCS and increased *de novo* synthesis of the cellular protectant GSH.

Key words: *N*-acetylcysteine, copper, gene expression, thiuram disulphide.

INTRODUCTION

Glutathione, the most prevalent intracellular non-protein thiol, is critical for preserving the proper cellular redox balance and for its role as a cellular protectant. Glutathione directly scavenges reactive oxygen species (ROS), participates in the GSH peroxidase-catalysed reaction eliminating hydrogen peroxide and other organic peroxides and forms conjugates with potentially harmful electrophilic species in reactions catalysed by glutathione S-transferases [1]. Accordingly, cells must maintain optimal glutathione levels to cope with a variety of stresses and to ensure the maintenance of a reducing environment in the cell. GSH (reduced glutathione), accounting for 90–95% of total glutathione levels in the cell, is maintained primarily by reduction of GSSG (oxidized glutathione) by glutathione reductase and via *de novo* synthesis.

An increase in cellular glutathione levels has been reported following exposure to a number of xenobiotics and, in some cases, is thought to represent an adaptive response to these insults. The increases in glutathione have often been associated with increases in the activity of γ-glutamylcysteine synthetase (GCS) which catalyses the rate-limiting reaction in glutathione synthesis. The dimeric GCS holoenzyme consists of a 28 kDa regulatory subunit (GCS_l) and a 73 kDa catalytic subunit (GCS_h), both of which can be induced by exposure to certain chemical and physical insults. Consequently, there has been much interest in defining the regulation of GCS and the two genes encoding the enzyme's catalytic and regulatory subunits. To date, studies examining the transcriptional regulation of these genes have focused primarily on the GCS_h gene. Increases in GCS_h steady-state mRNA levels have been observed in response to numerous compounds, including β-naphthoflavone (βNF) [2,3], dimethoxy-1,4-naphthoquinone (DMNQ) [4,5], menadione [5,6], dietary 2,3-t-butyl-4-hydroxyanisole (BHA) [7,8], methyl mercury hydroxide [9,10], diethylmaleate [11], t-butylhydroquinone (tBHQ) [12–14], hydrogen peroxide [6], tumour necrosis factor-α [15,16], buthionine sulphoximine (BSO) [12,17] and ionizing radiation [18,19]. Moreover, increases in the RNA levels of the GCS_h gene have frequently been attributed to transcriptional up-regulation [2,4–6,11–13,15,16,18,19]. The regulation of the gene encoding GCS_l has been less well studied, but increases in GCS_l RNA levels have been reported following exposure to βNF [2,20], DMNQ [21], tBHQ [12,14], diethylmaleate [12] and BSO [12]. The studies cited above, as well as those involving other agents such as t-butylhydroperoxide, phenethyl isothiocyanate and chronic exposure to hydrogen peroxide (A. C. Wild, J. J. Gipp, A. M. Erikson and R. T. Mulcahy, unpublished work), suggest that, in the majority of cases, elevations of GCS enzyme activity and glutathione synthesis are the result of transcriptional up-regulation of both the GCS_h and GCS_l genes.

Investigators have speculated about the identity of the regulatory 'signal' required for GCS_h and GCS_l gene induction after exposure to menadione [5], DMNQ [5,21], BHA [7], tBHQ [13] and BSO [17]. The generation of ROS, glutathione depletion, formation of glutathione conjugates and changes in GSSG or the GSH/GSSG ratio have all been postulated to be potential signals contributing to the GCS elevations and increased intracellular glutathione levels induced by exposure to these xenobiotics. Although definitive evidence is lacking, ROS [5,13,21], glutathione depletion [17] and the formation of glutathione conjugates [7] specifically, have been hypothesized to be

Abbreviations used: NAC, *N*-acetyl-L-cysteine; ActD, actinomycin D; BCPS, bathocuproine disulphonic acid; βNF, β-naphthoflavone; BSO, buthionine sulphoximine; DHR, dihydrorhodamine 123; DMNQ, dimethoxy-1,4-naphthoquinone; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GCS, γ-glutamylcysteine synthetase; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; RPA, RNase protection assay; tBHQ, t-butylhydroquinone; BHA, 2,3-t-butyl-4-hydroxyanisole.

[1] To whom correspondence should be addressed (e-mail mulcahy@mail.bascom.wisc.edu).

required for the transcriptional activation of the GCS_h or GCS_l genes. Analysis of the various compounds that up-regulate the two genes also suggests that some form of oxidative stress may be involved in gene regulation. To examine the potential involvement of an altered redox status in the βNF induction of GCS_h and GCS_l, we evaluated the effect of the 'antioxidant' pyrrolidine dithiocarbamate (PDTC) on *GCS* expression in βNF-treated HepG2 cells. These studies revealed that PDTC itself is a potent inducer of both GCS subunit genes.

PDTC is a member of the dithiocarbamate family, well known for its ability to bind free or protein-bound metal [22]. Dithiocarbamates exert both antioxidant and pro-oxidant effects in cells. Their antioxidant behaviour includes eliminating hydrogen peroxide [23] and scavenging the superoxide radical [23], peroxynitrite [24] and the hydroxyl radical [24] and lipid peroxidation products such as the peroxyl radical [25,26]. The reaction of dithiocarbamates with reactive oxygen and nitrogen species generates dithiocarbamate thiyl radicals which ultimately dimerize to form thiuram disulphides [23,24,26], the oxidized form of dithiocarbamates. Thiuram disulphides are responsible for much of the pro-oxidant effects of dithiocarbamates, characterized by their potent oxidation of GSH and protein thiols [22,27,28]. The formation of thiuram disulphides can also be metal-dependent, exemplified by the copper(II)-dependent oxidation of diethyldithiocarbamate and PDTC [29]. In the analysis of dithiocarbamate action, the antioxidant behaviour of these agents has been more often acknowledged, with less appreciation for their pro-oxidant character. For example, although inhibition of nuclear factor κB activation by PDTC has often been attributed to its radical-scavenging properties [30,31], it has recently been demonstrated that PDTC may exert its inhibitory effect on nuclear factor κB via direct oxidation of critical thiols of the transcription factor [32,33]. The pro-oxidant consequences of dithiocarbamate action, including that of PDTC and diethyldithiocarbamate, have recently been highlighted with respect to their influence on apoptosis [22,34,35]. The present study describes the effects of PDTC exposure on GCS_h and GCS_l gene expression and attempts to characterize the conditions generated by PDTC exposure that are required for increases in *GCS* gene expression.

MATERIALS AND METHODS

Cell culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum and 50 μg/ml gentamicin. PDTC was dissolved in doubly distilled water and diluted 1000-fold by addition to fresh medium in order to initiate cell treatment. Based on propidium iodide exclusion assays, approx. 90 % of HepG2 cells were viable after treatment with 100 μM PDTC; viability was comparable to that of untreated cells. For experiments involving pre-incubation of PDTC-treated HepG2 cells with thiol compounds or metal chelators, the pre-incubation was initiated for 30 min–1 h, before PDTC addition, as indicated in the Figure legends. Agents used for pre-treatment were replenished at the time of PDTC addition.

N-Acetyl-L-cysteine (NAC), actinomycin D (ActD), cycloheximide, BSO and bathocuproine disulphonic acid (BCPS) were purchased from Sigma.

RNA isolation and RNase protection

Total cellular RNA was isolated from HepG2 cells using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.) according to the procedure recommended by the manufacturer. Messenger RNA quantification was accomplished using the RPAII (Ambion, Austin, TX, U.S.A.) RNase protection assay (RPA), as described previously [2].

GSH and GSSG

HepG2 cells were plated in duplicate at 10^6 cells/60 mm plate and were harvested 3 days later, after treatment as described. Cells were rinsed with PBS, incubated for 2–3 min in 187.5 μl of Cell-Dissociation Solution (Sigma) and scraped off the plates. Lysates were transferred to fresh microcentrifuge tubes containing 62.5 μl of 20 % sulphosalicyclic acid, to give a final concentration of 5 % sulphosalicyclic acid. The samples were immediately vortexed, incubated on ice for 15 min and centrifuged for 15 min at 4 °C at approx. 16000 ***g***. Supernatants were transferred to new microcentrifuge tubes and kept on ice pending assay or were stored at 4 °C and assayed within 24 h. The pellets were resuspended in 250 μl of 0.1 M NaOH for subsequent determination of protein content using a DC protein assay system (Bio-Rad).

For total glutathione measurements, an assay based on the original Tietze glutathione assay was used [36]. The supernatants were diluted 1:40 with 5 % sulphosalicyclic acid, and 10 μl aliquots of the dilutions were pipetted onto a 96-well plate. A standard curve consisting of 5–50 ng of glutathione was also prepared and duplicate aliquots were pipetted onto the plate. A 100 μl amount of 143 mM sodium phosphate buffer/6.3 mM EDTA, pH 7.5, containing 1.05 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Aldrich) and 0.35 mM NADPH (Sigma), was added to each sample, followed by the addition of 50 μl of 5 units/ml glutathione reductase (Sigma). The plate was read several times over a 5 min period at a wavelength of of 412 nm to measure the formation of 5-thio-2-nitrobenzoic acid by the reaction of glutathione and DTNB. Concentrations were calculated from the standard curve and are expressed as pmol glutathione/μg of protein.

For determination of GSSG, the same DTNB recycling assay was performed. Before transferring the lysates into a 96-well plate, 2-vinylpyridine was added to each lysate to derivatize the GSH, as described by Griffith [37]. Briefly, 2 μl of 2-vinylpyridine (Aldrich) and 6 μl of triethanolamine (Sigma) were simultaneously mixed with 100 μl of the sample lysates (at pH 6–7) and incubated in the dark at room temperature for 1 h. Portions of the derivatized samples (10 μl) and duplicate aliquots of 5–50 ng GSSG standards were transferred onto the 96-well plate, followed by the addition of the solutions containing DTNB and NADPH and glutathione reductase, as described above. The kinetic assay was monitored for 10 min to quantify GSSG levels.

Dihydrorhodamine 123 (DHR) measurements

HepG2 cells were stained as described by Miles et al. [38] and Mancini et al. [39]. Briefly, cells on tissue-culture plates were incubated with 25 μM DHR for 30 min at 37 °C. After incubation, cells were washed twice with PBS, harvested using non-enzymic Cell-Dissociation Solution (Sigma) and resuspended in PBS. The oxidized forms of DHR were detected using excitation/emission wavelengths of 488 and 530 nm respectively. To exclude cellular debris and evaluate cell viability, samples were also stained with propidium iodide (Sigma) in parallel with the DHR staining, according to the manufacturer's recommendations.

Statistical analyses

Results are presented as means ± S.E.M. Where indicated, experiments with a sample size of only two are plotted as the means ± ranges. Statistical comparisons were performed by paired Student's *t*-tests. A value of $P < 0.05$ was considered statistically signficant.

RESULTS

PDTC induces expression of GCS_h and GCS_l subunit genes

PDTC treatment of HepG2 cells resulted in dose- and time-dependent increases in the steady-state GCS_h and GCS_l mRNA levels (Figures 1A and 1B). As observed by RPA, changes in gene expression were statistically significant as early as 3 h after the addition of 100 μM PDTC ($P < 0.01$) (Figure 1C). Maximal levels (8- to 10-fold) were detected 6 h after PDTC addition, and returned to untreated levels by 12 h post-treatment (Figure 1C). To determine whether these PDTC-induced increases were attributable to an increase in the rate of transcription, HepG2 cells

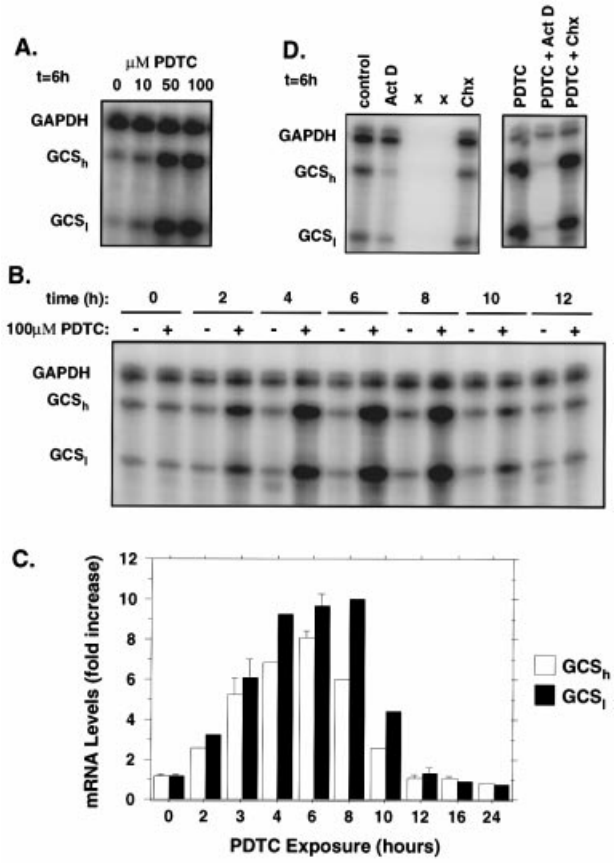


Figure 1 Steady-state mRNA levels of GCS_h and GCS_l subunits of GCS after exposure of HepG2 cells to PDTC

(**A**) RPA of total RNA isolated from HepG2 cells treated with 0, 10, 50 and 100 μM PDTC. HepG2 cells were incubated in complete medium for 48 h, followed by treatment with increasing doses of PDTC for 6 h. Total RNA was isolated using TRI reagent, and 20 μg of total RNA per sample was analysed by RPA. (**B**) A representative RPA analysis of total RNA isolated from HepG2 cells after exposure to either medium alone (−) or 100 μM PDTC (+) for 0, 2, 4, 6, 8, 10 and 12 h. (**C**) Fold increases in the message levels of the *GCS*$_h$ and *GCS*$_l$ genes after exposure to 100 μM PDTC, based on RPA gel quantification. Quantification was determined using a Molecular Dynamics PhosphorImager and ImageQuant software. For the 0, 3, 6 and 12 h time points, $n = 7$–13; $n = 2$ for the 16 and 24 h time points. The histogram bars at 2, 4, 8, and 10 h represent a single determination. (**D**) A representative RPA gel of total RNA isolated from HepG2 cells exposed to 10 μg/ml ActD, 0.5 μg/ml cycloheximide (Chx) and/or 100 μM PDTC for 6 h. The lanes marked with an X were not loaded.

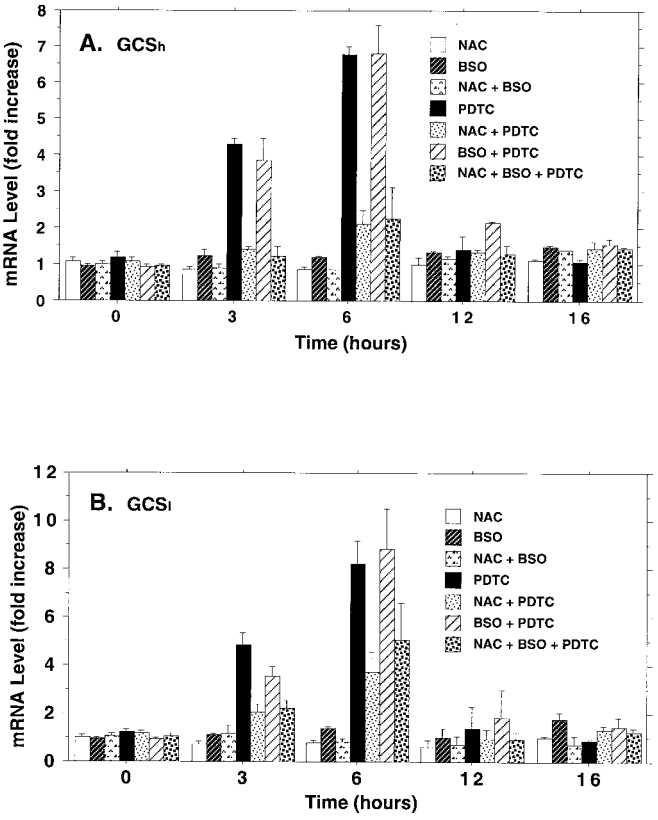


Figure 2 Fold increases in GCS_h and GCS_l mRNA levels after treatment of HepG2 cells with PDTC, NAC and BSO

(**A**) Quantification of GCS_h mRNA levels by RPA. Total RNA was isolated from HepG2 cells treated with 10 mM NAC, 1 mM BSO, 100 μM PDTC or combinations of these compounds at 0, 3, 6, 12 and 16 h. HepG2 cells were pre-treated for 1 h with NAC and/or BSO before the addition of PDTC. For time points 0, 3 and 6 h, $n = 3$–6. For the 12 and 16 h time points, results are means and ranges of only two determinations. (**B**) Quantification of GCS_l mRNA levels, details as for (**A**).

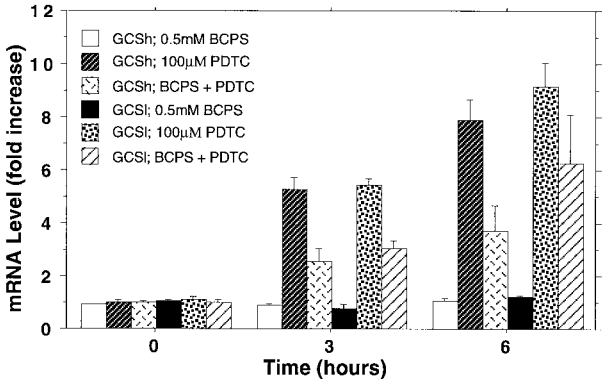


Figure 3 Changes in GCS_h and GCS_l message levels following incubation of HepG2 cells with the copper chelator BCPS and PDTC

Cells were pre-treated with 0.5 mM BCPS for 30 min, before the addition of 100 μM PDTC at 0 h. Total RNA was isolated at 0, 3 and 6 h and was analysed by RPA ($n = 4$).

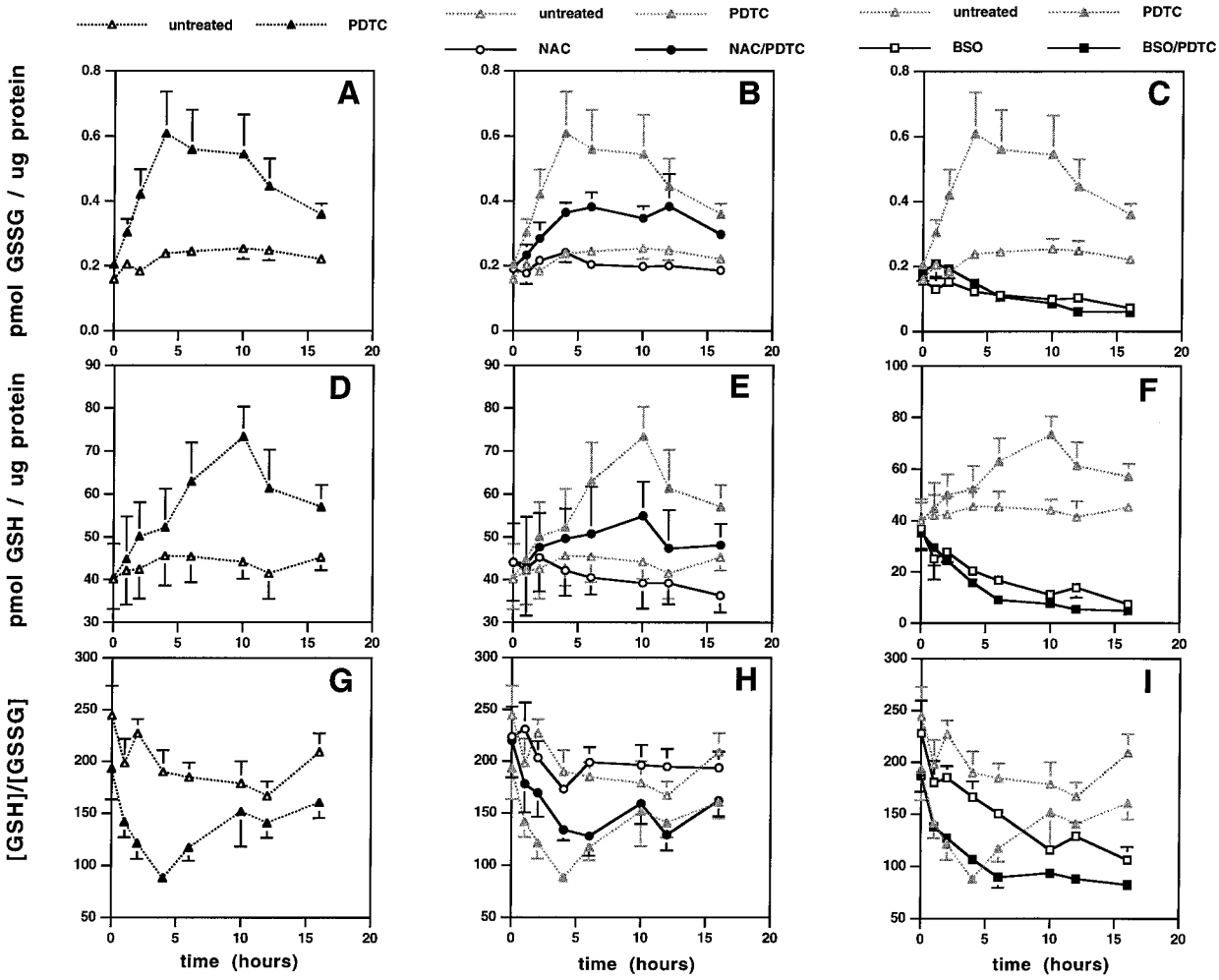


Figure 4 Changes in GSH and GSSG levels after PDTC exposure of HepG2 cells

GSH and GSSG levels were measured after exposure of HepG2 cells to 100 μM PDTC, 10 mM NAC, 1 mM BSO or combinations of these compounds. As indicated previously, cells were treated for 1 h with NAC or BSO before the addition of PDTC. Cells were harvested for GSSG and GSH determination as described in the Materials and methods section at 0, 1, 2, 4, 6, 10, 12 and 16 h. (**A**–**C**) Changes in GSSG. The results plotted in (**A**) are re-plotted as light, broken lines in (**B**) and (**C**) for comparison. (**D**–**F**) Quantification of GSH levels in HepG2 cells after treatment with PDTC, NAC and BSO. The results plotted in (**D**) are re-plotted as light, broken lines in (**E**) and (**F**). (**G**–**I**) The ratio of GSH:GSSG is plotted, based on the equation ([total GSH] − [GSSG])/[GSSG]. The results in (**G**) are re-plotted as light, broken lines in (**H**) and (**I**). All plots display means ± S.E.M. of four independent determinations.

were incubated with PDTC and the transcriptional inhibitor ActD. As shown in Figure 1(D), HepG2 cells exposed to 10 μg/ml ActD exhibited no inducible response to treatment with 100 μM PDTC, implying an increased rate of transcription of the two genes in response to the dithiocarbamate. New protein synthesis was not required for the PDTC response, since cycloheximide treatment did not prevent PDTC-induced changes in the expression of the GCS_h and GCS_l genes (Figure 1D).

The role of redox status in PDTC-induction of GCS subunit genes

To test the hypothesis that PDTC influenced the expression of the genes encoding GCS via a pro-oxidant signal, we attempted to block experimentally any such signal by pre-treating HepG2 cells with the antioxidant NAC. NAC alone (10 mM) had no effect on the steady-state RNA levels of the two genes (Figure 2). However, when combined with PDTC, NAC resulted in a significant diminution of the PDTC-induced changes in GCS_h and GCS_l gene expression, but failed to abolish gene induction completely. PDTC-induced gene expression at 6 h was reduced approx. 4-fold (from 7- to 2-fold) for GCS_h ($P < 0.01$) and 2-fold (from 8- to 4-fold) for GCS_l ($P < 0.01$) in cells treated with NAC.

Modulation of PDTC-induced changes in GCS_h and GCS_l gene expression by the administration of NAC may result from the ability of NAC to directly scavenge ROS, to serve as a precursor for glutathione by providing a source of the limiting substrate cysteine or by its ability to reduce protein disulphides [40]. BSO, a specific inhibitor of the GCS enzyme, was used to evaluate the importance of NAC as a glutathione precursor (Figure 2). Treatment of HepG2 cells with 1 mM BSO alone did not significantly influence GCS_h and GCS_l mRNA levels at the times examined; however, this dose of BSO resulted in significant decreases in glutathione within 1 h of administration (see Figure 4F). BSO also failed to alter PDTC-induced increases in GCS_h and GCS_l mRNA levels. Finally, the effect of BSO on the ability of NAC to mute the PDTC response was evaluated. Even in the presence of BSO, NAC was still capable of blunting PDTC induction of the GCS_h and GCS_l genes, suggesting that the

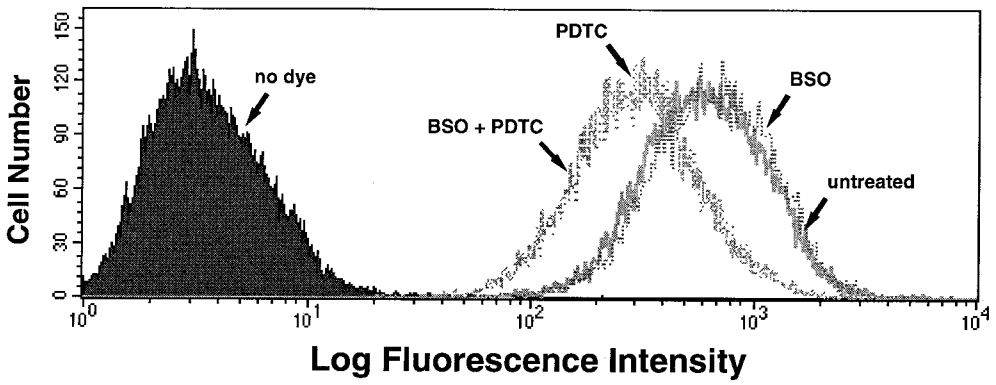


Figure 5 Fluorescence of the redox-sensitive dye DHR after PDTC treatment of HepG2 cells, assessed by flow cytometric analysis

These traces are representative results of 2–3 separate experiments. HepG2 cells were pre-treated with or without 1 mM BSO for 1 h, followed by exposure to 100 μM PDTC for 6 h. After the addition of DHR at 30 min before cell harvest, cells were prepared as described in the Materials and methods section and analysed by flow cytometry. The oxidized forms of DHR were detected using excitation/emission wavelengths of 488/530 nm respectively.

negative effect of NAC on the PDTC induction of the genes was independent of glutathione synthesis.

The exposure of thymocytes to PDTC generates a copper-dependent oxidation of glutathione and protein sulphydryls, contributing to apoptotic cell death [22,28,41]. To evaluate the significance of copper to the PDTC effect on *GCS* gene expression, HepG2 cells were incubated with the non-permeable copper chelator BCPS, 30 min before PDTC exposure. Co-treatment of cells with BCPS and PDTC for 3 h significantly (GCS_h, $P < 0.03$; GCS_l, $P < 0.01$) decreased the mRNA levels of GCS_h and GCS_l compared with levels detected in cells treated with PDTC alone. PDTC-dependent increases in GCS_h and GCS_l continued to be suppressed by BCPS pre-treatment after 6 h (Figure 3). Consequently, part of the PDTC effect on the expression of the *GCS* genes may involve copper-dependent mechanisms.

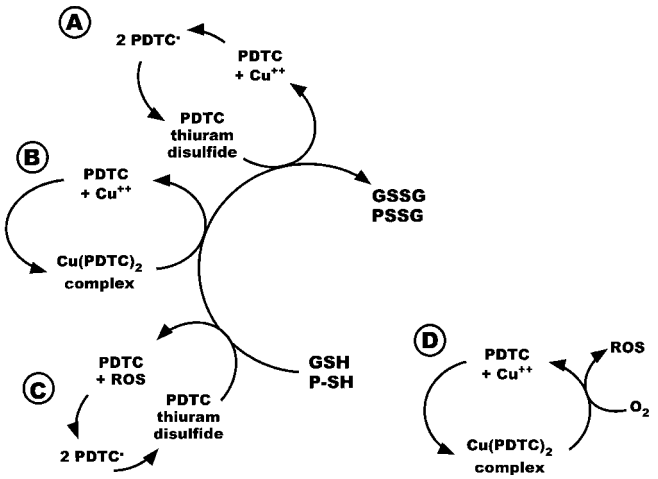
To determine whether PDTC treatment resulted in a shift to a more pro-oxidant intracellular state, the levels of GSH and GSSG in control and PDTC-treated HepG2 cells were compared. Within 1 h of 100 μM PDTC addition, HepG2 cells displayed a significant increase in the level of GSSG (Figure 4A). GSSG levels were increased to 2.5-fold over control within 4 h of PDTC treatment ($P < 0.04$). As was the case for *GCS* gene induction, NAC partially blocked the PDTC-induced increase in intracellular GSSG levels (Figure 4B). Within 1 h of PDTC exposure, the ratio of GSH:GSSG, commonly used as a biochemical indicator of the redox status of cells, was reduced significantly ($P < 0.02$) (Figure 4G). The decrease in the GSH:GSSG ratio reached its lowest point (50% of control) at 4 h after PDTC addition, indicative of a shift to a more oxidizing cellular environment. As compared with untreated cells, PDTC-treated cells exhibited significant increases in GSH within 2 h of treatment; GSH reached peak levels 10 h post-treatment ($P < 0.01$) (Figure 4D). This increase was attributable to *de novo* glutathione synthesis since it could be blocked by BSO treatment (Figure 4F). As levels of GSH increased, the GSH:GSSG ratio progressively increased over the next 10 h (Figure 4G) until it approached the GSH:GSSG ratios in untreated cells.

To further assess the cellular redox status of PDTC-treated cells, changes in the oxidation of the redox-sensitive dye, DHR, were examined in PDTC-treated HepG2 cells. DHR accummulates in the cytosol and is converted into a fluorescent compound after oxidation by a number of pro-oxidants, including hydroxyl radicals, peroxynitrite or peroxyl radicals [42]. Treatment of HepG2 cells with 100 μM PDTC resulted in a reduction in the fluorescence of DHR, when compared with the fluorescence in cells not exposed to PDTC (Figure 5). In constrast, treatment of HepG2 cells with hydrogen peroxide resulted in the increased fluorescence of DHR, as expected (results not shown). Using this measure of cellular oxidative stress, PDTC exerted an antioxidant effect in HepG2 cells, consistent with its previously described radical-scavenging properties. The reduced fluorescence of DHR observed in PDTC-treated cells was not attributable to increased synthesis of glutathione because this shift in the fluorescence was still evident in HepG2 cells co-treated with PDTC and BSO (Figure 5).

DISCUSSION

Our studies revealed that PDTC treatment of HepG2 cells resulted in potent mRNA increases for the genes encoding GCS_h and GCS_l, and that these increases were in large attributable to the transcriptional up-regulation of the two genes. These findings were consistent with a recent report describing elevated glutathione levels following PDTC exposure in bovine aortic endothelial cells [43]. The magnitude of the increases in the GCS_h and GCS_l transcripts after PDTC exposure was greater than the maxima typical of treatment with non-toxic doses of other xenobiotics, such as hydrogen peroxide, βNF and t-BHQ. The model presented here proposes that the induction of the GCS_h and GCS_l genes and the consequential increase in total glutathione represent part of the HepG2 cellular response to the pro-oxidant challenge generated by PDTC exposure.

The current experiments were designed to determine whether a PDTC-induced redox signalling cascade was involved in GCS_h and GCS_l induction by evaluating the cellular consequences of PDTC exposure and the ability of other compounds to modulate the PDTC-induced expression of these genes. The GCS_h and GCS_l genes are responsive to a wide variety of agents, including oxidants [4–6], phenolic antioxidants [7,8,12,13], heavy metals [9,10,44] and radiation [18,19]. Therefore it is likely that changes in the expression of the *GCS* genes result from multiple regulatory signals. Accordingly, efforts to elucidate the regulation of *GSH1*, the gene encoding yeast GCS, demonstrated that menadione and hydrogen peroxide activate *GSH1* via distinct mechanisms [45]. Several mechanisms for the activation of the human *GCS* genes



Scheme 1 Potential mechanisms for the oxidation of glutathione and protein sulphydryls by PDTC

The two species hypothesized to be responsible for the PDTC pro-oxidant effects include the PDTC thiuram disulphide and a $Cu(PDTC)_2$ complex. The PDTC thiuram disulphide can be formed either by the oxidation of PDTC by copper(II) (**A**) or by ROS (**C**), followed by dimerization of the thiyl radical. The putative $Cu(PDTC)_2$ complex results in thiol oxidation (**B**) or the generation of ROS (**D**), both by redox cycling of the copper transported intracellularly by the metal chelator PDTC. It is hypothesized that the oxidation of a protein sulphydryl(s) to a disulphide or to a higher oxidation state ultimately results in the up-regulation of the genes encoding the catalytic and regulatory subunits of GCS.

by certain xenobiotics have recently been proposed. For example, the formation of GSH conjugates was hypothesized to be an important biochemical effect responsible for increased GCS_h expression after BHA treatment [7]. In constrast, evaluation of the quinones DMNQ and menadione suggested that glutathione conjugates were not important regulatory signals for these agents and implicated increases in hydrogen peroxide in *GCS* gene induction [5,21]. Clearly, the cellular signalling pathways leading to the increased expression of the genes encoding GCS are not yet fully defined. The studies presented here predict yet another mechanism operative in the induction of the human GCS_h and GCS_l genes, namely the activation of a critical regulatory protein(s) by thiol oxidation.

Dithiocarbamates, including PDTC, are capable of exerting opposing effects on the cellular redox balance by decreasing single-electron radical species (reduction) and causing a two-electron oxidation of GSH and protein thiols as a consequence of redox cycling [22]. It has been proposed that thiuram disulphides (oxidized dithiocarbamates) or copper–dithiocarbamate complexes represent the chemical species responsible for the pro-oxidant effects of dithiocarbamates [28,29]. Thiuram disulphides can be generated by copper-dependent oxidation of dithiocarbamates (Scheme 1, A) [29] or by copper-independent oxidation of dithiocarbamate compounds by ROS, such as the peroxyl radical or hydrogen peroxide (Scheme 1, C) [23,24,26]. In both cases, oxidation of the dithiocarbamate is followed by dimerization of dithiocarbamate thiyl radicals, generating the thiuram disulphide. Thiuram disulphides (Scheme 1, A and C) or copper–dithiocarbamate complexes (Scheme 1, B) are thought to redox cycle and, through their reduction, result in the oxidation of GSH or protein sulphydryls. Hence, dithiocarbamates, including PDTC, can function as both pro-oxidants and antioxidants. This dual functionality of PDTC may account for the apparent contradictory effects we observed in PDTC-treated HepG2 cells, specifically oxidation of GSH to GSSG and reduction in the fluorescence of the redox-sensitive dye DHR.

The thiol agents NAC (Figure 2), glutathione ethyl ester and WR-1065 (results not shown) were able to inhibit partially the PDTC induction of the GCS_h and GCS_l genes, suggesting that the effect of PDTC on GCS_h and GCS_l gene expression is mediated, at least in part, via a pro-oxidant pathway. According to the model describing the pro-oxidant effects of PDTC, reduction of the PDTC thiuram disulphide or prevention of disulphide formation by NAC, WR-1065 or glutathione ethyl ester could conceivably prevent the oxidation of a redox-labile, cellular regulatory protein(s), or perhaps glutathione itself, thereby inhibiting the GCS induction pathway.

After PDTC treatment, the GSH:GSSG ratio decreased significantly, indicative of oxidation. This decreased ratio was due to a significant increase in GSSG levels. Restoration of the GSH:GSSG ratio occured in PDTC-treated cells after an increase in the *de novo* synthesis of glutathione. Such alterations in GSSG levels and the GSH:GSSG ratio have been implicated in signalling, leading to induction of some redox-responsive genes. However, the current studies failed to demonstrate a consistent correlation between *GCS* gene induction and high GSSG levels, suggesting that increased GSSG levels do not represent a key signalling component in the PDTC-induced expression of the GCS_h and GCS_l subunit genes. For example, BSO and PDTC co-treatment of HepG2 cells resulted in the induction of GCS_h and GCS_l expression with a magnitude and kinetics comparable with that induced in cells exposed to PDTC alone, yet the co-treated cells did not exhibit increases in the absolute levels of GSSG (Figure 4C). We propose that changes in GSSG levels and in the GSH:GSSG ratio are critical indicators for the PDTC-induced changes in the cellular redox status; however, they may not themselves be involved in the up-regulation of *GCS* gene expression by PDTC.

ROS are increasingly recognized as potent activators of signalling cascades, culminating in the up-regulation of stress response genes. It is possible therefore that one or more ROS might be operative in PDTC-induced *GCS* gene expression, particularly after taking into account the role of redox-active copper in the mechanism of action of PDTC. As noted earlier, PDTC induces a pro-oxidant state by chelating copper and transporting it intracellularly [28,29,41] where it potentially influences the cellular redox status by two distinct mechanisms (see Scheme 1). First, copper may either directly oxidize PDTC, resulting in the promotion of PDTC thiuram disulphides (Scheme 1, A) or form $Cu(PDTC)_2$ complexes (Scheme 1, B). Both of these species are hypothesized to be capable of oxidizing GSH and protein sulphydryls. The second mechanism involves ROS generation due to increased levels of intracellular copper (Scheme 1, D) [46]. A partial role for copper-dependent mechanisms in PDTC-induced *GCS* expression was confirmed by the experiments which demonstrated that the copper chelator BCPS partially inhibited the PDTC effect on *GCS* gene expression (Figure 3). The fact that this partial inhibition required high concentrations of BCPS suggests that either PDTC can transport other metals capable of substituting for copper or that PDTC can manipulate gene expression by metal-independent reactions.

Although experiments designed to identify specific ROS involved in PDTC induction of the *GCS* genes were not conducted, the role of certain species can at least be challenged by the data collected in the current experiments. Specifically, PDTC-dependent increases in intracellular copper and subsequent copper redox cycling predict the generation of superoxide, the hydroxyl radical and peroxyl radicals [46], which directly, or indirectly through downstream products, oxidize

DHR to its fluorescent state. PDTC treatment resulted in decreased DHR fluorescence, while increasing *GCS* expression, suggesting that these reactive oxygen intermediates are not directly involved in PDTC induction of the human *GCS* subunit genes. In fact, the potent radical-scavenging properties of dithiocarbamates would probably preclude the involvement of ROS, such as the hydroxyl radical, the peroxyl radical, superoxide and hydrogen peroxide, as effective signalling messengers for PDTC-induced *GCS* expression [23–26]. The role of hydrogen peroxide as a signal transducer for *GCS* gene expression in PDTC-treated cells is also discounted by additional experiments (results not shown) in which cells co-treated with PDTC and catalase displayed no change in the PDTC-inducibility of GCS_h and GCS_l, as compared with PDTC treatment alone. Our hypothesis, that ROS are not involved in the mechanism of PDTC activation of *GCS* gene expression, is also supported by results from Burkitt et al. [29], who proposed that the potent radical scavenging properties of dithiocarbamates eliminated ROS generated by copper redox cycling. Similar to proposals by Burkitt et al. [29], our data support a model in which the copper-dependent (Scheme 1, A, B and D) and -independent (Scheme 1, C) formation of the PDTC thiuram disulphide, rather than increases in specific ROS, is responsible for the PDTC effects on GSH oxidation and *GCS* gene induction.

In conclusion, we have demonstrated that PDTC is a potent inducer of human *GCS* subunit gene expression and that this effect on gene transcription is attributable, in part, to the ability of PDTC to induce a pro-oxidant state in HepG2 cells, as evidenced by a significant decrease in the GSH:GSSG ratio. A prominent role for certain ROS, such as hydrogen peroxide, the peroxyl radical and the hydroxyl radical, as pro-oxidant PDTC signalling intermediates was discounted by flow cytometric analysis of DHR oxidation and by lack of inhibition with catalase. We hypothesize that PDTC induction of the GCS_h and GCS_l genes involves the oxidation and activation of an, as yet unidentified, regulatory protein(s) by a reversible sulphydryl disulphide exchange mechanism, secondary to the formation of PDTC thiuram disulphides or metal–PDTC complexes. The thiol compound NAC partially blocked PDTC-induced *GCS* gene expression by reducing the oxidized form of PDTC back to the parent compound, thereby indirectly preventing the oxidation of the postulated regulatory protein(s), or by reducing the regulatory protein(s) directly. Precedents for this model include the dithiocarbamate oxidation of the transcription factors nuclear factor κB and p53 [33,47], the apoptotic signalling molecules caspase 1 and caspase 3 [34,35] and the enzymes hexokinase [48] and D-amino acid oxidase [49]. This working model for the PDTC induction of the GCS_h and GCS_l genes is under further investigation.

We thank Dr. James P. Thomas, Jerry Gipp and Helen Moinova for their valuable comments and discussion of the work presented in this manuscript. This work was supported by National Institutes of Health Grants RO1-CA57549 (to R. T. M.) and 5T32-CA09471 (to A. C. W.).

REFERENCES

1 Meister, A. and Anderson, M. E. (1983) Annu. Rev. Biochem. **52**, 711–760
2 Mulcahy, R. T., Wartman, M. A., Bailey, H. H. and Gipp, J. J. (1997) J. Biol. Chem. **272**, 7445–7454
3 Wild, A. C., Gipp, J. J. and Mulcahy, R. T. (1998) Biochem. J. **332**, 373–381
4 Shi, M. M., Kugelman, A., Iwamoto, T. and Forman, H. J. (1994) J. Biol. Chem. **269**, 26512–26517
5 Shi, M. M., Iwamoto, T. and Forman, H. J. (1994) Am. J. Physiol. **267** (Lung Cell. Mol. Physiol. **11**), L414–L421
6 Rahman, I., Bel, A., Mulier, B., Lawson, M. F., Harrison, D. J., MacNee, W. and Smith, C. A. D. (1996) Biochem. Biophys. Res. Commun. **229**, 832–837
7 Borroz, K. I., Buetler, T. M. and Eaton, D. L. (1994) Toxicol. Appl. Pharmacol. **126**, 150–155
8 Eaton, D. L. and Hamel, D. M. (1994) Toxicol. Appl. Pharmacol. **126**, 145–149
9 Woods, J. S. and Ellis, M. (1995) Biochem. Pharmacol. **50**, 1719–1724
10 Li., S., Thompson, S. A., Kavanagh, T. J. and Woods, J. S. (1996) Toxicol. Appl. Pharmacol. **141**, 59–67
11 Sekhar, K. R., Long, M., Long, J., Xu, Z., Summar, M. L. and Freeman, M. L. (1997) Radiat. Res. **147**, 59–67
12 Cai, J., Huang, Z.-Z. and Lu, S. C. (1997) Biochem. J. **326**, 167–172
13 Liu, R., Hu, H., Robison, T. W. and Forman, H. J. (1996) Am. J. Respir. Cell. Mol. Biol. **14**, 186–191
14 Galloway, D. C., Blake, D. G., Shepherd, A. G. and McLellan, L. I. (1997) Biochem. J. **328**, 99–104
15 Urata, Y., Yamamoto, H., Goto, S., Tsushima, H., Akazawa, S., Yamashita, S., Nagataki, S. and Kondo, T. (1996) J. Biol. Chem. **271**, 15146–15152
16 Morales, A., Garcia-Ruiz, C., Miranda, M., Mari, M., Colell, A., Ardite, E. and Fernandez-Checa, J. C. (1997) J. Biol. Chem. **272**, 30371–30379
17 Yao, K.-S., Godwin, A. K., Ozols, R. F., Hamilton., T. C. and O'Dwyer, P. J. (1993) Cancer Res. **53**, 3662–3666
18 Iwanaga, M., Mori, K., Iida, T., Urata, Y., Matsuo, T., Yasunaga, A., Shibata, S. and Kondo, T. (1998) Free Radicals Biol. Med. **24**, 1256–1268
19 Morales, A., Miranda, M., Sanchez-Reyes, A., Colell, A., Biete, A. and Fernandez-Checa, J. C. (1998) FEBS Lett. **427**, 15–20
20 Moinova, H. R. and Mulcahy, R. T. (1998) J. Biol. Chem. **273**, 14683–14689
21 Tian, L., Shi, M. M. and Forman, H. J. (1997) Arch. Biochem. Biophys. **342**, 126–133
22 Orrenius, S., Nobel, C. S. I., van den Dobbelsteen, D. J., Burkitt, M. J. and Slater, A. F. G. (1996) Biochem. Soc. Trans. **24**, 1032–1038
23 Mankhetkorn, S., Abedinzadeh, Z. and Houee-Levin, C. (1994) Free Radicals Biol. Med. **17**, 517–527
24 Liu, J., Shigenaga, M. K., Yan, L., Mori, A. and Ames, B. N. (1996) Free Radicals Res. **24**, 461–472
25 Bartoli, G. M., Muller, A., Cadenas, E. and Sies, H. (1993) FEBS Lett. **164**, 371–374
26 Zanocco, A. L., Pavez, R., Videla, L. A. and Lissi, E. A. (1989) Free Radicals Biol. Med. **7**, 151–156
27 Hosni, M., Meskini, N., Prigent, A.-F., Anker, G., Joulain, C., Habib, R. E. and Lagarde, M. (1992) Biochem. Pharmacol. **43**, 1319–1329
28 Nobel, C. S. I., Kimland, M., Lind, B., Orrenius, S. and Slater, A. F. G. (1995) J. Biol. Chem. **270**, 26202–26208
29 Burkitt, M. J., Bishop, H. S., Milne, L., Tsasng, S. Y., Provan, G. J., Nobel, C. S. I., Orrenius, S. and Slater, A. F. G. (1998) Arch. Biochem. Biophys. **353**, 73–84
30 Schreck, R., Meier, B., Mannel, D. N., Droge, W. and Baeuerle, P. A. (1992) J. Exp. Med. **175**, 1181–1194
31 Meyer, M., Schreck, R. and Baeuerle, P. A. (1993) EMBO J. **12**, 2005–2015
32 Brennan, P. and O'Neill, L. A. J. (1995) Biochim. Biophys. Acta **1260**, 167–175
33 Brennan, P. and O'Neill, L. A. J. (1996) Biochem. J. **320**, 975–981
34 Nobel, C. S. I., Burgess, D. H., Zhivotovsky, B., Burkett, M. J., Orrenius, S. and Slater, A. F. G. (1997) Chem. Res. Toxicol. **10**, 636–643
35 Nobel, C. S. I., Kimland, M., Nicholson, D. W., Orrenius, S. and Slater, A. F. G. (1997) Chem. Res. Toxicol. **10**, 1319–1324
36 Tietze, F. (1969) Anal. Biochem. **27**, 502–522
37 Griffith, O. W. (1980) Anal. Biochem. **106**, 207–212
38 Miles, A. M., Bohle, D. S., Glassbrenner, P. A., Hansert, B., Wink, D. A. and Grisham, M. B. (1996) J. Biol. Chem. **271**, 40–47
39 Mancini, M., Anderson, B. O., Caldwell, E., Sedghinasab, M., Paty, P. B. and Hockenbery, D. M. (1997) J. Cell Biol. **138**, 449–469
40 Moldeus, P. and Cotgreave, I. A. (1994) Methods Enzymol. **234**, 482–492
41 Trombetta, L. D., Toulon, M. and Jamall, I. S. (1988) Toxicol. Appl. Pharmacol. **93**, 154–164
42 Royall, J. A. and Ischiropoulos, H. (1993) Arch. Biochem. Biophys. **302**, 348–355
43 Moellering, D., McAndrew, J., Jo, H. and Darley-Usmar, V. M. (1998) Free Radicals Biol. Med., in the press
44 Ishikawa, T., Bai, J., Yamane, Y., Akimaru, K., Frindrich, K., Wright, C. D. and Kuo, M. T. (1996) J. Biol. Chem. **271**, 14981–14988
45 Stephen, D. W. S., Rivers, S. L. and Jamieson, D. J. (1995) Mol. Microbiol. **16**, 415–423
46 Halliwell, B. and Gutteridge, J. M. C. (1990) Methods Enzymol. **186**, 1–85
47 Wu, H.-H. and Momand, J. (1998) J. Biol. Chem. **273**, 18898–18905
48 Stromme, J. H. (1963) Biochem. Pharmacol. **12**, 157–166
49 Neims, A. H., Coffey, D. S. and Hellerman, L. (1966) J. Biol. Chem. **241**, 5941–5948

Received 17 September 1998/16 November 1998; accepted 17 December 1998