Biochemical Pharmacology, Vol. 37, No. 16, pp. 3109–3117, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

NITROFURANTOIN-MEDIATED OXIDATIVE STRESS CYTOTOXICITY IN ISOLATED RAT HEPATOCYTES

LUISA ROSSI,* JOSE M. SILVA, LARRY G. MCGIRR and PETER J. O'BRIEN†
Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada M5S 1A1

(*Received* 14 *July* 1987; *accepted* 7 *January* 1988)

Abstract—Freshly isolated rat hepatocytes were used to study the mechanism(s) of toxicity of the antimicrobial drug nitrofurantoin. This 5-nitrofuran derivative stimulated hepatocyte oxygen uptake in the presence of the mitochondrial respiration inhibitors KCN or antimycin A. This could indicate the formation of $O_2^{\cdot-}$ and H_2O_2, following intracellular nitrofurantoin reduction. Addition of nitrofurantoin to suspensions of isolated rat hepatocytes produced a dose- and time-dependent decrease of cell viability. H_2O_2 probably plays a significant role in the cytotoxic effects of nitrofurantoin as the catalase inhibitors azide or aminotriazole markedly enhanced cytotoxicity. The loss of cell viability was preceded by glutathione (GSH) depletion and a concomitant and nearly stoichiometric formation of oxidised glutathione (GSSG) that did not occur in hepatocytes lacking glutathione peroxidase activity isolated from rats fed a low-selenium diet. This indicates that H_2O_2 and the seleno-enzyme glutathione peroxidase are responsible for GSH oxidation. Furthermore, addition of nitrofurantoin to isolated rat hepatocytes produced a reversible inactivation of hepatocyte glutathione reductase activity and explains the maintenance of high GSSG levels. The compromised hepatocytes were also highly susceptible to H_2O_2. The hepatocyte toxicity of nitrofurantoin may, therefore, be attributed to oxidative stress caused by redox-cycling mediated oxygen activation.

Partially reduced oxygen derivatives such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) have been implicated in the occurrence of cell damage in several pathological circumstances. Recently, *in vitro* studies carried out with cultured rat hepatocytes demonstrated that H_2O_2, either added directly to the medium or slowly generated *in situ*, can cause irreversible cell injury, resulting in cell death [1–3]. Endogenous $O_2^{\cdot-}$ and H_2O_2 are occasionally produced in low amounts during normal oxidative metabolism in aerobic cells. The production of $O_2^{\cdot-}$ can be further stimulated by compounds, e.g. menadione or paraquat, undergoing intracellular one-electron reduction, followed by auto-oxidation in the presence of oxygen. This process, referred to as redox-cycling, can give rise to large amounts of $O_2^{\cdot-}$, which can subsequently undergo spontaneous or enzymatic dismutation to produce H_2O_2 [4]. The metal-catalysed reaction between H_2O_2 and $O_2^{\cdot-}$ can generate the hydroxyl radical ($OH^{\cdot}$), a much more reactive oxygen species, which may be responsible for the H_2O_2-induced oxidative injury leading to cell death [3]. Therefore, the enhancement of intracellular levels of partially reduced oxygen species has often been proposed as one of the mechanisms of toxicity of a variety of foreign compounds.

In the present study, the role of H_2O_2 in the toxic effects of the redox-cycling compound nitrofurantoin (*N*-[5-nitro-2-furfurylidine]-1-amino-hydantoin) has been investigated, using isolated rat hepatocytes as the target system. This 5-nitrofuran derivative is widely utilised as a urinary antimicrobial drug and its long-term use has been associated with pulmonary fibrosis in humans [5], neuropathy [6], hepatitis [7] and hemolytic anemia in patients with glucose-6-phosphate dehydrogenase‡ (EC 1.1.1.49) deficiency [8].

Nitrofurantoin intracellular activation may proceed via one-electron reduction of the nitro-group to the nitro-anion radical, catalysed by several intracellular flavoprotein reductases, including enzymes located in the cytosol and microsomal fractions [9] and in the outer mitochondrial membrane [10]. High concentrations of ascorbate can also reduce nitrofurantoin to the nitro-anion radical [11]. Under aerobic conditions, the nitro-anion radical readily auto-oxidises to the parent compound, and concomitant $O_2^{\cdot-}$ formation has been shown upon nitrofurantoin activation by liver [12] and lung [12, 13] microsomes. Hyperoxia has also been shown to exacerbate acute pulmonary injury in the rat [14].

In addition, evidence supporting the hypothesis of the involvement of H_2O_2 in nitrofurantoin-induced damage comes from studies in experimental systems in which the defence mechanisms against oxidative damage have been impaired. Deficiency of the antioxidant Vitamin E, obtained by dietary manipulation, strongly increases the acute pulmonary toxicity of nitrofurantoin in the rat [14]. Furthermore, a decrease in the activity of the seleno-enzyme glutathione peroxidase (EC 1.11.1.9), produced by selenium deficiency, potentiates chick mortality induced by nitrofurantoin up to 3-fold [15].

* Permanent address: Department of Biology, 2nd University of Rome, "Tor Vergata", via O. Raimondo, I-00173 Rome, Italy.
† To whom correspondence should be addressed.
‡ Enzymes: catalase (EC 1.11.1.6); glucose-6-phosphate dehydrogenase (EC 1.1.1.49); glutathione peroxidase (EC 1.11.1.9); glutathione reductase (EC 1.6.4.2); and superoxide dismutase (EC 1.15.1.1).

Glutathione peroxidase, together with catalase (EC 1.11.1.6), plays a determinative role in protecting mammalian cells from H_2O_2 [16]. Metabolism of H_2O_2 by glutathione peroxidase and glutathione reductase (EC 1.6.4.2) activities is responsible for the intracellular glutathione redox-cycle leading to NADPH oxidation. However, the accumulation and efflux of glutathione disulfide (GSSG†) from the cell can be observed following prolonged H_2O_2 production, resulting in reduced glutathione (GSH) depletion. Increased GSSG levels have also been observed in the perfusate of isolated rabbit lung perfused with nitrofurantoin [17] and in the plasma of rats following *in vivo* nitrofurantoin administration [18].

In the following, the effects of nitrofurantoin on isolated rat hepatocytes have been investigated, with particular regard to the fate of GSH. The involvement of H_2O_2 in nitrofurantoin-induced toxicity and the changes in GSH levels have been assessed by the use of catalase inhibitors and of glutathione peroxidase deficient hepatocytes and by comparing these effects with that found with H_2O_2 directly added to the cell medium under the same experimental conditions.

MATERIALS AND METHODS

Chemicals. Nitrofurantoin, Trypan blue, GSH, GSSG, sodium azide, fluoro-2,4-dinitrobenzene, iodoacetic acid, antimycin A (type III) and H_2O_2 (as a 30% solution) were obtained from Sigma (St. Louis, MO). Collagenase (from *Clostridium histoliticum*) and Hepes were purchased from Boehringer-Mannheim (Montreal, Canada). 3-Amino-1,2,4-triazole was obtained from Mann Research Laboratories (New York, NY), sodium selenite from BDH Chemicals (Poole, England), and KCN from the Fisher Scientific Co. (Fair Lawn, NJ). Other chemicals were of the highest grade available commercially.

Animals. Male Sprague–Dawley rats (body wt 180–250 g) fed a standard chow diet and tap water *ad lib*. were used to prepare hepatocytes.

When inactivation of selenium-dependent glutathione peroxidase was required, 50-g rats were fed for 7–9 weeks on a low-selenium diet (ICN Biomedicals, Canada) supplemented with 100 I.U./kg diet of Vitamin E as alpha-tocopherol acetate and distilled water. Paired controls received 0.2 ppm of selenium as sodium selenite in the drinking water.

Isolation and incubation of the hepatocytes. Hepatocytes were obtained by collagenase perfusion of the liver as previously described [19]. Routinely, 85–95% of the freshly isolated hepatocytes excluded Trypan blue (Trypan blue concentration: 0.16%, w/v). Cells were suspended in Krebs–Henseleit buffer containing 12.5 mM Hepes at pH 7.4, in round-bottom 50-ml flasks, rotating in a water bath at 37°, under an atmosphere of 95% O_2, 5% CO_2, essentially according to Moldeus *et al.* [19]. The final incubation volume was 20 ml with a cell concentration of 10^6/ml.

Nitrofurantoin (0.5 M stock solution in DMSO) was prepared immediately prior to use and added to the cells after approximately 15 min of preincubation. All controls received the vehicle DMSO alone. At the concentration used, DMSO (8 μl) was not cytotoxic and did not alter nitrofurantoin-induced cytotoxicity.

To inactivate catalase, sodium azide (final concentration: 5 mM) or aminotriazole (final concentration: 25 mM) was added to the cells after about 15 min of preincubation and maintained in the cell medium throughout the experiment. Nitrofurantoin or H_2O_2 was added 10 or 30 min after sodium azide or aminotriazole addition respectively.

Microsomal preparation. Microsomes were isolated from rat liver as previously described [20] and kept cold on ice before use.

Assays. Hepatocyte viability was assessed by the Trypan blue dye exclusion test in a Neubauer chamber, by a light microscope.

Total GSH and GSSG content of hepatocytes was measured in deproteinated samples (5% metaphosphoric acid) after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene, by HPLC, using a μBondapak NH_2 column (Waters Associates, Milford, MA) [21]. GSH and GSSG were used as external standards. A Waters 6000A solvent delivery system, equipped with a model 660 solvent programmer, a WISP 710A automatic injector and a Data Module, was used for analysis.

Glutathione peroxidase activity was measured according to Lawrence and Burk [22], with 0.25 mM H_2O_2 as a substrate, in 105,000 *g* supernatant fractions of livers obtained from rats fed with a selenium-deficient diet or a selenium-supplemented diet, for 7 weeks. The liver cytosol prepared from selenium-deficient rats had 2% of the glutathione peroxidase activity of the liver cytosol prepared from selenium-supplemented rats [0.65 ± 0.10 μmol NADPH oxidized·min^{-1}·(mg protein),$^{-1}$ N = 4]. Enzymatic activities in cell incubation samples were determined spectrophotometrically after cell disruption by sonication in an ice bath for a total of 2 min with a Branson probe-type sonicator at a power of 50 W; catalase activity was determined spectrophotometrically by following the decrease in absorbance of H_2O_2 at 240 nm [23]. Catalase activity after treatment of hepatocytes with azide or aminotriazole accounted for 0–5% of the activity of control samples ($0.73 \pm 0.14 \times 10^3$ units/mg protein, N = 4). Glutathione reductase activity was measured according to Goldberg and Spooner [24]. Protein content was assayed with a modified Lowry method, including a precipitation step, according to Peterson [25].

Oxygen consumption was measured by a Clark-type electrode (Yellow Springs Instrument Co., Inc., model 5300) in a 2-ml chamber, maintained at 37°. Before use, hepatocytes were kept at 37° in Krebs–Henseleit buffer, plus Hepes (12.5 mM), pH 7.4, under a stream of 95% air, 5% CO_2. KCN (2 mM, neutralized with HCl) or antimycin A (100 μM, in 4 μl absolute ethanol) was added to inhibit mitochondrial respiration. The results described have

† Abbreviations: aminotriazole, 3-amino-1,2,4-triazole; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; DMSO, dimethyl sulfoxide; GSH, glutathione; GSSG, oxidised glutathione; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

been taken from typical experiments, unless otherwise stated in the figure legends. All experiments were repeated at least three times with different cell preparations.

RESULTS

The addition of nitrofurantoin concentrations as low as 0.1 mM stimulated oxygen consumption by suspensions of isolated rat hepatocytes both in the absence and in the presence of KCN or antimycin A, added to prevent spontaneous mitochondrial respiration (Table 1). The rate of oxygen uptake increased with nitrofurantoin concentration, when KCN or antimycin A was also present, but not proportionally. This suggests that nitrofurantoin can undergo redox-cycling under these experimental conditions, thus producing $O_2^{\cdot-}$ and consequently H_2O_2. Control incubations, which received only the DMSO vehicle, did not show any increased oxygen uptake.

The effect of nitrofurantoin at causing hepatocyte loss of viability, expressed as the percentage of cells permeable to Trypan blue, is shown in Fig. 1. Nitrofurantoin concentrations as low as 0.2 mM did not produce a very pronounced decrease in cell viability within 4 hr of incubation. However, a marked enhancement of cell toxicity was obtained with 0.5 mM nitrofurantoin, accounting for a 43% loss of cell viability after 2 hr of incubation and 100% cell death after 4 hr. Higher concentrations of the drug (1.0 and 2.0 mM) produced a further decrease in cell viability (Fig. 1). Concentrations higher than 2 mM could not be used owing to the poor solubility of nitrofurantoin. Early signs of toxicity, represented by deformation of the cellular shape and appearance of surface blebs, were observed with 0.5 mM nitrofurantoin at 30 min of incubation (data not shown), prior to the occurrence of appreciable cell toxicity. Some variation in the rate at which toxicity manifested itself in response to the addition of nitrofurantoin was also observed in various experiments. This could be due to variation in the rate of nitrofurantoin intracellular metabolism.

Table 1. Nitrofurantoin-induced oxygen uptake by isolated rat hepatocytes*

Addition	O_2 consumed ($nmol \cdot min^{-1} \cdot 10^6$ cells^{-1}) None	KCN (2 mM)	Antimycin A (100 μM)
None	21.8 ± 2.2	1.3 ± 0.7	12.0 ± 2.0
NF, 0.1 mM	27.8 ± 0.2	12.2 ± 2.1	ND†
NF, 0.2 mM	31.2 ± 2.4	16.7 ± 3.5	19.4 ± 2.1
NF, 0.5 mM	30.0 ± 2.6	22.1 ± 3.7	24.1 ± 1.0
NF, 1.0 mM	32.7 ± 5.4	26.2 ± 7.3	28.7 ± 0.7

* O_2 uptake was measured using a Clark electrode in 2 ml Krebs-Henseleit buffer, containing Hepes (12.5 mM) at a cell density of 10^6 cells/ml and 37° in the absence or in the presence of KCN or antimycin A. Nitrofurantoin (NF) was added in 8 μl DMSO. Results (mean ±SD) are the average of at least four determinations with two different batches of cells.

† Not determined.

In Fig. 2, alterations of the cellular glutathione redox status on incubating isolated rat hepatocytes with increasing concentrations of nitrofurantoin are shown. A time- and dose-dependent decrease in the total GSH content occurred within 30 min (Fig. 2A) and preceded the onset of cytotoxicity. The extent of GSH depletion correlated with the degree of cell injury: the highest nitrofurantoin concentrations tested (1 and 2 mM) were the most toxic and markedly depleted cellular soluble thiols levels so that after 5 min of incubation the GSH level reached almost undetectable values. However, even nontoxic nitrofurantoin concentrations (0.2 mM) produced significant GSH depletion, reaching a level of approximately 70% of the starting control values after 30 min of incubation. A corresponding and nearly stoichiometric increase in GSSG was also observed with all nitrofurantoin concentrations (Fig. 2B), thus explaining the disappearance of GSH as an oxidation to GSSG. Inactivation of hepatocyte

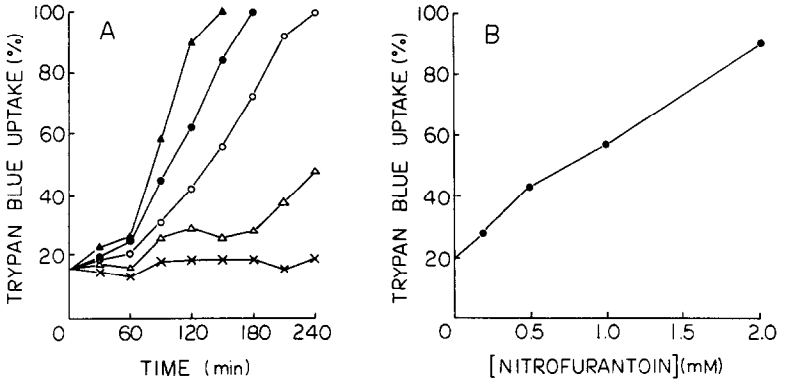

Fig. 1. Nitrofurantoin cytotoxicity in isolated rat hepatocytes. (A) Hepatocytes, 10^6 cells/ml, were incubated alone (×) or in the presence of 0.2 mM (△), 0.5 mM (○), 1 mM (●) and 2 mM (▲) nitrofurantoin. Cell toxicity was determined as the percentage of cells taking up Trypan blue. (B) Percentage of cells permeable to Trypan blue at 120 min of incubation with various nitrofurantoin concentrations.

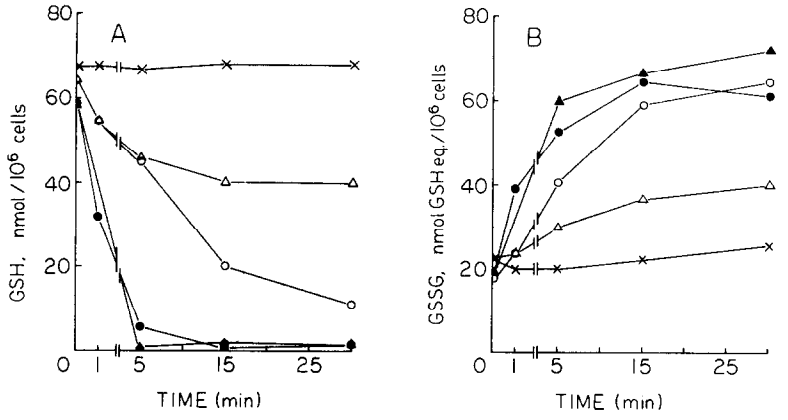

Fig. 2. GSH depletion (A) and GSSG formation (B) induced by nitrofurantoin in isolated hepatocytes. Nitrofurantoin at differing concentrations was incubated with hepatocytes, 10^6 cells/ml, with and without the catalase inhibitor azide. Total GSH and GSSG levels were determined by HPLC analysis, as described under Materials and Methods. Key: (×) no addition, or 4 mM azide; (△) 0.2 mM nitrofurantoin; (○) 0.5 mM nitrofurantoin; (●) 1.0 mM nitrofurantoin or 0.2 mM nitrofurantoin + 4 mM azide; and (▲) 2.0 mM nitrofurantoin.

catalase activity with 4 mM azide resulted in an approximate 2- to 3-fold increase in the rate and extent of GSH oxidation by 0.2 mM nitrofurantoin.

To investigate if such GSH oxidation was the result of glutathione peroxidase-catalysed H_2O_2 catabolism, hepatocytes isolated from rats fed for a minimum of 7 weeks with a low selenium diet were used. This treatment has been reported previously to impair the activity of the seleno-dependent glutathione peroxidase, which has high specificity for the substrate H_2O_2 [22]. As shown in Table 2, almost no depletion of GSH or GSSG formation occurred after exposure of glutathione peroxidase-deficient hepatocytes to 0.5 mM nitrofurantoin for 30 min. On the contrary, cells isolated from rats fed with the same diet but supplemented with selenium (i.e. with normal glutathione peroxidase activity) had no GSH after 30 min of incubation with 0.5 mM nitrofurantoin, thus confirming the trend shown in Fig. 2. Surprisingly, the cytotoxicity induced was similar for both groups (results not shown). The starting amount of GSH reported in Table 2 is much lower than that shown in Fig. 2 (approximately 17 nmol/10^6 cells vs about 65 nmol/10^6 cells). This presumably reflects that the commercial selenium-deficient diet used is deficient in sulfur amino acids.

To investigate the role of H_2O_2 in nitrofurantoin-induced cell toxicity and to estimate the contribution of catalase to H_2O_2 metabolism, experiments were carried out using two catalase inhibitors, namely azide and aminotriazole. Incubation of hepatocytes with 4 mM azide for 10 min or with 25 mM aminotriazole for 30 min caused almost complete inhibition of the catalase activity (measured as described in Materials and Methods), without causing cytotoxicity. However, as shown in Fig. 3, azide or aminotriazole pretreatment of hepatocytes potentiated hepatocyte sensitivity to nitrofurantoin. At 90 min 0.5 mM nitrofurantoin caused about 60% cytotoxicity with azide or aminotriazole and only 25% in their absence. Furthermore, 0.2 mM nitrofurantoin caused about 50% cytotoxicity at 180 min with azide but was not cytotoxic in the absence of azide even at 240 min. It should be noted that the

Table 2. Effect of dietary selenium on nitrofurantoin-induced alteration of GSH:GSSG redox ratio in isolated rat hepatocytes

Hepatocyte treatment		GSH (nmol GSH eq./10^6 cells) 0 min	GSSG (nmol GSH eq./10^6 cells) 0 min	GSH (nmol GSH eq./10^6 cells) 30 min	GSSG (nmol GSH eq./10^6 cells) 30 min
+Se	No addition	12.0 ± 2.2	4.6 ± 0.2	12.0 ± 3.1	5.8 ± 0.7
	+0.5 mM NF	12.8 ± 1.8	6.0 ± 0.3	0 ± 0.2	10.4 ± 1.2
−Se	No addition	21.5 ± 4.2	1.5 ± 0.5	22.1 ± 7.4	1.8 ± 0.6
	+0.5 mM NF	20.6 ± 5.7	1.7 ± 0.8	17.7 ± 4.8	2.7 ± 1.6

* Hepatocytes were isolated from livers of rats fed with a selenium-deficient diet (−Se) or with the selenium-deficient diet supplemented with selenium (+Se), as described in Materials and Methods. Cells (10^6/ml) were incubated in Krebs–Henseleit buffer supplemented with 12.5 mM Hepes, at pH 7.4 and 37°, in the presence or in the absence of 0.5 mM nitrofurantoin, and total GSH and GSSG were measured at the indicated times. Values are the means ± SD of three separate measurements.

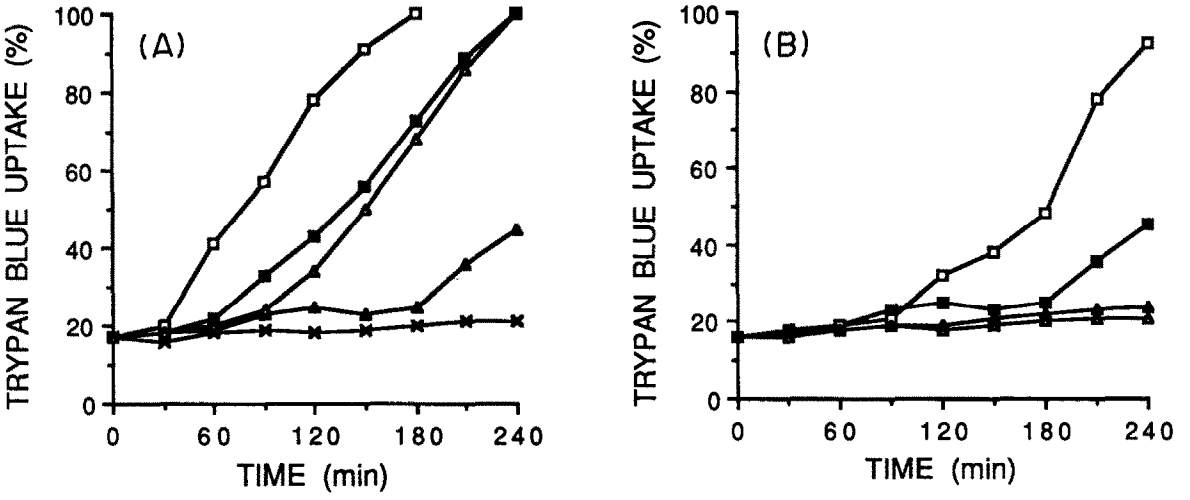

Fig. 3. Potentiation of nitrofurantoin-induced cytotoxicity in hepatocytes by (A) catalase inhibitors and (B) hydrogen peroxide. Hepatocytes, 10^6 cells/ml, were preincubated for 10 or 30 min with the catalase inhibitors azide or aminotriazole, respectively, prior to the addition of nitrofurantoin. Cell toxicity was determined by Trypan blue uptake. (A) □, 0.2 mM nitrofurantoin + 4 mM axide or 25 mM aminotriazole, ■, 0.5 mM nitrofurantoin; △, 0.2 mM nitrofurantoin +4 mM azide; ▲, 0.2 mM nitrofurantoin; ×, no addition or 4 mM azide or 25 mM aminotriazole; (B) □, 0.2 mM nitrofurantoin + 0.5 mM H_2O_2; (■) 0.2 mM nitrofurantoin; (△) 0.5 mM H_2O_2; (×) no addition or 2 mM H_2O_2.

hepatocytes used in the experiments for Fig. 3 were more resistant to nitrofurantoin than the hepatocytes used in Fig. 1.

Previous works with isolated hepatocytes have shown that redox-cycling bipyridilium compounds like diquat [26] or quinonoid drugs like duroquinone [27] causes cytotoxicity and significant alteration of intracellular soluble thiols only when cellular defence against oxidative stress is compromised. In particular, inhibition of the intracellular glutathione reductase activity by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) was required for cytotoxicity and thiol oxidation induced by these redox-cycling compounds. BCNU is, therefore, a useful tool to demonstrate intracellular H_2O_2 production by these compounds [26–28]. In contrast, as shown in Figs. 1 and 2, nitrofurantoin produced cell toxicity and irreversible GSH oxidation even in hepatocytes not pretreated with BCNU.

Nitrofurantoin compromises the hepatocyte cellular defence system against oxidative stress. Thus in Fig. 3, it can be seen that H_2O_2 (0.5 mM) increased the cytotoxicity of nitrofurantoin even though H_2O_2 (2.0 mM) was not cytotoxic in the absence of nitrofurantoin. In addition, as shown in Fig. 4A, when 0.5 mM H_2O_2 was added directly to the hepatocyte suspension, a drop in the level of GSH in seconds (from 60 to 5 nmol/10^6 cells) was accompanied by a corresponding increase in GSSG. The rapidity of the GSH oxidation suggests that catalysis by glutathione peroxidase was involved. However, within 1 min, GSH levels returned to control values (about 50 nmol GSH/10^6 cells), presumably as a result of glutathione reductase activity. When azide was also present (Fig. 4B), GSH levels

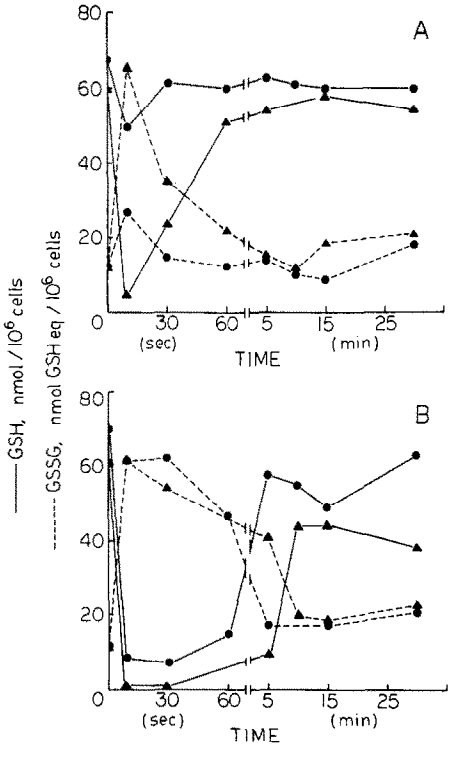

Fig. 4. H_2O_2-induced changes in hepatocyte GSH/GSSG levels. H_2O_2 was added directly to isolated hepatocytes (10^6 cells/ml) both in the absence (A) and in the presence (B) of 4 mM azide. GSH (solid line) and GSSG (dashed line) levels were determined by HPLC. Key: (●) 0.1 mM H_2O_2; and (▲) 0.5 mM H_2O_2.

Table 3. H_2O_2-induced cytotoxicity in hepatocytes with inactivated catalase*

Addition	Cytotoxicity (% Trypan blue uptake) 30 min	60 min	120 min
None	12	16	17
H_2O_2 (2 mM)	13	18	15
Azide	16	15	18
Azide + H_2O_2 (0.1 mM)	12	13	25
Azide + H_2O_2 (0.5 mM)	20	35	90
Azide + H_2O_2 (1.0 mM)	75	100	

* Isolated rat hepatocytes were preincubated in Krebs–Henseleit buffer, pH 7.4, supplemented with 12.5 mM Hepes, at a concentration of 10^6 cells/ml with 4 mM azide for 10 min before H_2O_2 addition. Azide was kept in the incubation medium throughout the experiment. The precentage of cells permeable to Trypan blue was measured at 30, 60 and 120 min from H_2O_2 addition.

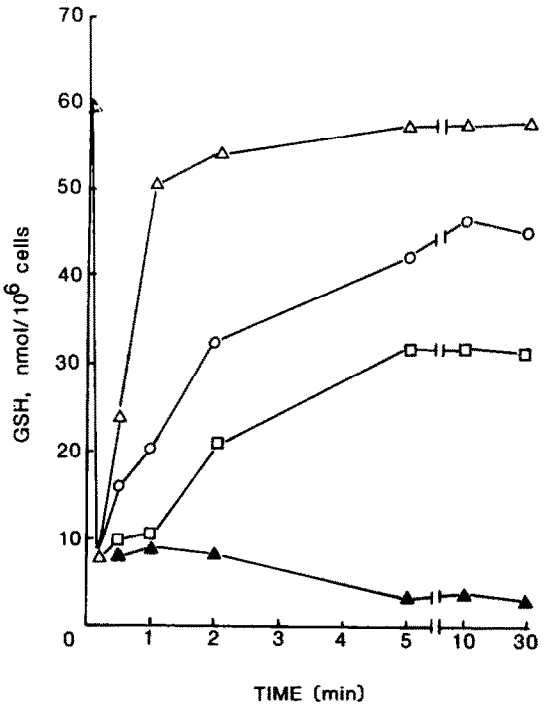

Fig. 5. Prevention of hepatocyte GSH recovery by nitrofurantoin after H_2O_2-induced GSH depletion. H_2O_2 was added to the isolated hepatocytes (10^6 cells/ml) immediately after the addition of nitrofurantoin and, at various times, samples were processed to measure GSH content. Key: (△) 0.5 mM H_2O_2; (○) 0.1 mM nitrofurantoin + 0.5 mM H_2O_2; (□) 0.2 mM nitrofurantoin + 0.5 mM H_2O_2; and (▲) 0.5 mM nitrofurantoin + 0.5 mM H_2O_2.

fell to almost non-detectable values and a lag period was observed before its recovery. Furthermore, 0.1 mM H_2O_2, which did not affect cellular GSH content under control conditions (Fig. 4A), strongly depleted GSH to levels as low as 5 nmol/10^6 cells when catalase was inhibited (Fig. 4B). As reported in Table 3, H_2O_2 concentrations as high as 2 mM did not produce cytotoxicity in isolated hepatocytes. However, when hepatocyte catalase was inactivated with 4 mM azide, a time- and dose-dependent cytotoxicity occurred, with 1.0 mM H_2O_2 being more toxic than 0.5 mM H_2O_2 (100% Trypan blue uptake vs 35% within 60 min). No cytotoxicity was observed with 0.1 mM H_2O_2 and azide. A comparison of Table 3 and Fig. 4 shows that cytotoxicity only occurred when the cellular GSH level was depleted to at least 5–10% of the control value and stayed depleted for several minutes.

Several reports have indicated that nitrofurantoin is an inhibitor of glutathione reductase [29, 30]. To investigate whether such an inhibition could also occur in isolated hepatocytes, thereby providing a possible explanation for nitrofurantoin-induced changes in the GSH:GSSG ratio, experiments were designed in which the effects of H_2O_2 on glutathione levels could be studied in the presence of nitrofurantoin. The results of these experiments are shown in Fig. 5. A rapid and extensive depletion of GSH occurred when 0.5 mM H_2O_2 was added to the hepatocytes. A stoichiometric oxidation to GSSG was found (results not shown). GSH levels were fully restored within 2 min and no increased cytotoxicity occurred in a 4 hr incubation period. However, if the H_2O_2 were added to the hepatocytes immediately after 0.5 mM nitrofurantoin, the GSH was rapidly oxidised, but no reduction to GSH then ensued. This indicates that glutathione reductase activity had been impaired by nitrofurantoin. Furthermore, direct measurements of glutathione reductase activity in sonicated suspensions of isolated rat hepatocytes which had been treated with 0.5 mM nitrofurantoin for only 5 min showed that reductase activity was only 10% of that of untreated hepatocytes [0.040 μmol NADPH oxidised·min^{-1}(mg protein)$^{-1}$]. Assaying glutathione reductase activity in a liver cytosol fraction in the presence of various concentrations of nitrofurantoin *in vitro* showed that the K_i for nitrofurantoin was 52 μM. Reductase activity was restored after extracting the nitrofurantoin with ethyl acetate from the cytosol fraction, indicating that the inactivation was reversible and required the presence of nitrofurantoin (results not shown).

To investigate the intracellular mechanism of nitrofurantoin activation and reconstitute the intracellular mechanism of GSH oxidation, the activities of subcellular fractions were compared (Table 4). Microsomes and NADPH were found to be much more active than mitochondria in catalysing cyanide-resistant respiration with nitrofurantoin. If GSH was present, stoichiometric oxidation to GSSG occurred. Carrying out the reaction under an argon atmosphere or adding catalase prevented the oxidation of GSH, indicating that H_2O_2 was responsible. Azide stimulated GSH oxidation presumably by inhibiting endogenous catalase. The redox-cycling effectiveness of nitrofurantoin in carrying out oxygen activation catalysed by microsomal NADPH reductase was also found to be more effective than paraquat but less effective than menadione, diquat or duroquinone.

Table 4. Nitrofurantoin-mediated oxygen activation and GSH oxidation with liver microsomal or mitochondrial fractions

	O_2 uptake* ($nmol \cdot min^{-1} \cdot mg^{-1}$)	GSSG† (nmol GSH equiv.)	
		10 min	30 min
Microsomes	8.1 ± 1.2	43	52
+ NF (0.025 mM)	18.2 ± 1.5	82	110
+ NF (0.05 mM)	24.3 ± 0.8	112	162
+ NF (0.1 mM)	32.8 ± 0.9	163	215
+ NF (0.2 mM)	48.1 ± 2.6	281	355
+ NF (0.2 mM) + azide	60.8 ± 5.1	456	625
+ NF (0.2 mM) + catalase	43.4 ± 4.1	128	158
+ paraquat (0.025 mM)	11.5 ± 1.2		
+ paraquat (0.05 mM)	19.4 ± 1.5		
+ diquat (0.025 mM)	23.8 ± 3.2		
+ duroquinone (0.025 mM)	38.3 ± 4.2		
+ duroquinone (0.05 mM)	47.0 ± 4.6		
+ menadione (0.025 mM)	57.4 ± 5.6		
+ menadione (0.05 mM)	68.4 ± 7.1		
Mitochondria + CN^-	1.2 ± 0.2		
Mitochondria + CN^- + NF (0.1 mM)	3.2 ± 0.8		

* Reaction conditions: 2 ml of 0.1 M Tris (pH 7.4) buffer contained microsomes or mitochondria (1 mg/ml), NADPH (0.2 mM) and, where indicated, nitrofurantoin (NF), paraquat, duroquinone, menadione, cyanide (1 mM), azide (1 mM) or catalase (2600 units) at 20°. The initial rate of oxygen uptake was recorded with an oxygen electrode. The values are expressed as the means (±SE) of three separate experiments.

† GSH oxidation was measured using HPLC (as described under Materials and Methods) with 1-ml reaction mixtures containing 1 mM GSH, after an incubation period of 10 or 30 min.

DISCUSSION

The results obtained in the present study suggest a cytotoxic role for the H_2O_2 produced intracellularly as a consequence of nitrofurantoin metabolism. Evidence that H_2O_2 was formed includes the induction of cyanide-resistant respiration; the stoichiometric oxidation of intracellular GSH; the marked increase in intracellular GSH oxidation by catalase inhibitors; and the absence of GSH oxidation in hepatocytes lacking GSH peroxidase activity. The marked increase in respiration induced by nitrofurantoin was cyanide and antimycin resistant, indicating that nitrofurantoin was not a substrate for the mitochondrial electron transport chain. The oxygen activation induced by nitrofurantoin is probably the result of futile redox-cycling of nitrofurantoin to the auto-oxidisable nitro-anion radical [11] by reductases located in the microsomes and outer mitochondrial membrane. However, cyanide- or antimycin-sensitive respiration was also inhibited by nitrofurantoin probably as a result of inhibiting states 3 and 4 mitochondrial respiration [31].

Mammalian cells are normally well equipped with defence mechanisms against "active" oxygen species. The defence mechanisms include enzymes like superoxide dismutase (EC 1.15.1.1), catalase, glutathione peroxidase and glutathione reductase, as well as reducing agents like GSH and antioxidants like Vitamin E. Previously we have shown that this defence system is particularly efficient in isolated rat hepatocytes treated with duroquinone which undergoes intracellular futile redox-cycling to generate H_2O_2 but is not cytotoxic if all the components of the defence system are fully active [26, 27]. However, duroquinone becomes highly cytotoxic if catalase is inhibited by aminotriazole or azide or if glutathione reductase is inhibited by BCNU [27]. H_2O_2 directly added to the incubation medium of isolated rat hepatocytes was toxic only in the presence of catalase inhibitors. Others have shown that H_2O_2 can be cytotoxic to cultured rat hepatocytes if continuously generated by glucose oxidase and that aminotriazole or BCNU markedly enhances toxicity [2].

Impairment of hepatocyte catalase activity by azide potentiated and prolonged intracellular GSH oxidation produced by added H_2O_2, and H_2O_2 became cytotoxic to hepatocytes. Toxic concentrations of nitrofurantoin also caused GSH oxidation that was completed within the first few minutes of incubation and was potentiated by catalase inhibition. Akerboom *et al.* [32] and Dunbar *et al.* [17, 33] also found extensive production and excretion of GSSG in isolated rat liver and rabbit lung on perfusion with nitrofurantoin. The nearly stochiometric oxidation to GSSG suggests that little alkylation of GSH occurs during nitrofurantoin metabolism in isolated rat hepatocytes or in other mammalian systems [33]. Unlike other toxic xenobiotics, such as the quinone menadione [34], parental nitrofurantoin does not directly interact with soluble thiols [35]. The nitro-anion free radical, as monitored by ESR spectroscopy, also does not react with GSH [36]. Presumably, the GSH is oxidised by H_2O_2, generated by the auto-oxidation of the nitro-anion

free radical, and catalysed by the seleno-enzyme glutathione peroxidase. This would explain the lack of GSH oxidation or depletion in hepatocytes isolated from selenium-deficient rats. In confirmation of this, GSH underwent stoichiometric oxidation to GSSG when nitrofurantoin was aerobically reduced by liver microsomes and NADPH. Carrying out the reaction under an argon atmosphere or adding catalase prevented this oxidation, indicating that GSH was oxidised by H_2O_2 and not superoxide anion radicals [37] or nitro-anion radicals.

Further reduction of nitrofurantoin to highly reactive intermediates such as the nitroso-derivative and hydroxylamine may be produced under anaerobic conditions upon reductive metabolism [38, 39]. Covalent binding of alkylating metabolites of nitrofurantoin to tissue macromolecules is prevented by exogenous GSH [38], and it has been shown that nitroso-compounds can form GSH conjugates in an *in vitro* system [40]. Direct conjugation with thiols under anaerobic conditions could represent an alternative mechanism of cytotoxicity. The lack of GSH conjugation found suggests that the futile redox-cycling was not rapid enough to cause anaerobiosis in the hepatocyte before the GSH was fully oxidised.

GSSG formed 1 min after the addition of H_2O_2 to hepatocytes was rapidly reduced back to GSH within 1 min but was not reduced back in the case of nitrofurantoin-treated hepatocytes. This phenomenon cannot be due to an additional effect of nitrofurantoin on the GSH level, since nitrofurantoin produced only a small amount of GSH depletion in this time period. H_2O_2 also became cytotoxic to hepatocytes if nitrofurantoin was present. Three mechanisms could explain why intracellular GSSG reduction is prevented by nitrofurantoin: (1) the intracellular redox cycling of nitrofurantoin and continuous glutathione reductase action could lead to the depletion of intracellular NADPH, (2) GSSG once formed is rapidly released from the hepatocyte [41] and would therefore become inaccessible to glutathione reductase, and (3) glutathione reductase is reversibly inhibited by nitrofurantoin [29, 30]. Dunbar *et al.* [33] did not find a decrease of glutathione reductase activity in homogenates obtained from rabbit lungs previously isolated and perfused with nitrofurantoin for 30 min. This is presumably because glutathione reductase inhibition is reversed by the removal of nitrofurantoin.

In conclusion, nitrofurantoin caused intracellular H_2O_2 formation in isolated rat hepatocytes. Catalase inhibitors increased this cytotoxicity which suggests that H_2O_2 is responsible. The susceptibility of hepatocytes to H_2O_2 was markedly enhanced, presumably as a result of the reversible inactivation of glutathione reductase. Whether nitrofurantoin alkylating metabolites also contribute to cytotoxicity is not known. GSSG formation in hepatocytes in the presence of nitrofurantoin appeared to be due to glutathione peroxidase catalysed GSH oxidation, since it did not occur in hepatocytes lacking glutathione peroxidase (isolated from selenium-deficient rats). However, cytotoxicity still occurred with the latter hepatocytes, indicating that GSSG or mixed protein disulfides, formed by the interaction of GSSG with protein thiols, is not likely to be the cause of nitrofurantoin cytotoxicity.

Acknowledgements—We would like to thank G. Rinaldi for drawing the figures. This work was supported by the Medical Research Council of Canada.

REFERENCES

1. R. Rubin and J. L. Farber, *Archs Biochem. Biophys.* **228**, 450 (1984).
2. P. E. Starke and J. L. Farber, *J. biol. Chem.* **260**, 86 (1985).
3. P. E. Starke and J. L. Farber, *J. biol. Chem.* **260**, 10099 (1985).
4. G. Rotilio, I. Mavelli, L. Rossi and M. R. Ciriolo, *Envir. Hlth Perspect.* **64**, 259 (1985).
5. E. C. Rosenow, in *Immunologic and Infection Reactions in the Lung* (Eds. C. H. Kirkpatrick and H. Y. Reynolds), p. 261. Marcel Decker, New York (1976).
6. C. J. Rubinstein, *J. Am. med. Ass.* **187**, 647 (1964).
7. H. J. Zimmerman, *Med. Clins. N. Am.* **63**, 574 (1979).
8. K. J. Lavelle, K. F. Atkinson and S. A. Klit, *Am. J. med. Sci.* **272**, 201 (1976).
9. S. Swaminathan and G. M. Lower Jr., in *Carcinogenesis* (Ed. G. T. Bryan), p. 59. Raven Press, New York (1978).
10. S. N. J. Moreno, R. P. Mason and R. Docampo, *J. biol. Chem.* **259**, 6298 (1984).
11. D. N. R. Rao, L. Harman, A. Motten, J. Schreiber and R. P. Mason, *Archs Biochem. Biophys.* **255**, 419 (1987).
12. R. Mason and J. L. Holtzmann, *Biochem. biophys. Res. Commun.* **67**, 1267 (1975).
13. H. A. Sasame and M. R. Boyd, *Life Sci.* **24**, 1091 (1979).
14. M. Boyd, H. Sasame, J. Mitchell and G. Catignani, *Fedn. Proc.* **36**, 405 (1977).
15. F. J. Peterson, G. F. Combs, Jr., J. L. Holtzmann and R. P. Mason, *J. Nutr.* **112**, 1741 (1982).
16. D. P. Jones, L. Eklöw, H. Thor and S. Orrenius, *Archs Biochem. Biophys* **210**, 505 (1981).
17. J. R. Dunbar, A. J. DeLucia and L. R. Bryant, *Res. Commun. Chem. Path. Pharmac.* **34**, 485 (1981).
18. B. H. Lauterburg, C. V. Smith, H. Hughes and J. R. Mitchell, *J. clin. Invest.* **73**, 124 (1984).
19. P. Moldeus, J. Högberg and S. Orrenius, in *Methods in Enzymology* (Eds. P. A. Hoffee and M. E. Jones), Vol. 51, pp. 60–71. Academic Press, New York (1978).
20. L. Ernster, P. Siekevitz and G. E. Palade, *J. Cell Biol.* **15**, 541 (1962).
21. D. J. Reed, J. R. Babson, B. W. Beatty, A. E. Brodie, W. W. Ellis and D. N. Potter, *Analyt. Biochem.* **106**, 55 (1980).
22. R. A. Lawrence and R. F. Burk, *Biochem. biophys. Res. Commun.* **71**, 952 (1976).
23. H. Lück, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), pp. 885–94. Verlag-Chemie, West Germany (1965).
24. D. M. Goldberg and R. J. Spooner, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), Vol. III, pp. 258–65. Verlag-Chemie, West Germany (1985).
25. G. L. Peterson, *Analyt. Biochem.* **83**, 346 (1977).
26. L. Eklöw-Lastbom, L. Rossi, H. Thor and S. Orrenius, *Free Rad. Res. Commun.* **2**, 57 (1986).
27. L. Rossi, G. A. Morre, S. Orrenius and P. J. O'Brien, *Archs. Biochem. Biophys.* **251**, 25 (1986).
28. L. Eklöw, P. Moldeus and S. Orrenius, *Eur. J. Biochem.* **138**, 459 (1984).
29. J. A. Buzard and F. Kopko, *J. biol. Chem.* **238**, 464 (1963).

30. M. C. Beatrice, D. L. Stiers and D. R. Pfeiffer, *J. biol. Chem.* **259**, 1279 (1984).
31. L. O. Lim, R. Bortell and A. H. Neims, *Toxic. appl. Pharmac.* **84**, 493 (1986).
32. T. P. M. Akerboom, M. Bilzer and H. Sies, *J. biol. Chem.* **257**, 4248 (1982).
33. J. R. Dunbar, A. J. De Lucia and L. R. Bryant, *Biochem. Pharmac.* **33**, 1343 (1984).
34. D. Di Monte, D. Ross, G. Bellomo, L. Eklöw and S. Orrenius, *Archs Biochem. Biophys.* **235**, 334 (1984).
35. E. Boyland and B. E. Speyer, *Biochem. J.* **119**, 463 (1970).
36. C. F. Polnaszek, F. J. Peterson, J. L. Holtzmann and R. P. Mason, *Chem. Biol. Interact.* **51**, 263 (1984).
37. H. Wefers and H. Sfer, *Eur. J. Biochem.* **137**, 29 (1983).
38. M. R. Boyd, A. W. Stiko and H. A. Sasame, *Biochem. Pharmac.* **28**, 601 (1979).
39. R. F. Minchin, P. C. Ho and M. R. Boyd, *Biochem. Pharmac.* **35**, 575 (1986).
40. P. Eyer, *Chem. Biol. Interact.* **24**, 227 (1979).
41. L. Eklöw, H. Thor and S. Orrenius, *Fedn Eur. Biochem. Soc. Lett.* **127**, 125 (1981).