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MENADIONE AND CUMENE HYDROPEROXIDE INDUCED CYTOTOXICITY IN BILIARY EPITHELIAL CELLS ISOLATED FROM RAT LIVER

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Abstract—Biliary epithelial cells (BEC) and parenchymal cells isolated from normal rat liver were exposed *in vitro* to a number of toxic compounds. BEC were found to be highly sensitive to concentrations of menadione (100 μM) and cumene hydroperoxide (10 μM) that are usually not effective as toxic agents in hepatocytes under normoxic conditions. On the other hand, BEC were not affected by concentrations of carbon tetrachloride or 7-ethoxycoumarin that are known to exert toxic effects on hepatocytes. The development of both menadione- and cumene hydroperoxide-induced toxic injury in BEC followed a common and time-correlated pattern, and included a strong depletion of GSH, depletion of protein thiols and an increase in the extent of cell death. The damage induced by cumene hydroperoxide was found to be independent of lipid peroxidative processes and was prevented by a pre-incubation with desferrioxamine. The cytotoxicity of menadione was further exacerbated by dicoumarol but was not prevented by desferrioxamine or promethazine. The mechanisms underlying BEC injury and death induced by the quinone and by the organic hydroperoxide are discussed in relation to the known biochemical characteristics of BEC.

The biliary tree in mammalian liver consists of a system of ductules and ducts that interpenetrate the liver to connect the canaliculi between hepatocytes with large bile ducts in the portal tracts [1, 2]. Such a ramifying system is lined by small cells, known as biliary epithelial cells (BEC), with a characteristic ultrastructure: a large nucleus surrounded by a scanty cytoplasm with very few cellular organelles [1, 2]. Disturbances of the BEC are known to be involved in several pathological conditions affecting the hepatobiliary system in humans, such as primary biliary cirrhosis, chronic active- as well as persistent-hepatitis, drug induced hepatitis and cholestasis (see Ref. 3). Proliferation of BEC or BEC-derived cells has also been observed in experimental cholestasis [4] and in several models of chemical hepatocarcinogenesis [5, 6]. Moreover, the literature concerning liver toxicology offers several examples of chemicals that produce liver injury associated with variable degrees of cholestatic injury, or of chemicals that specifically induce mainly ductular and/or canalicular injury like α-naphthyl-isothiocyanate and the mycotoxin sporidesmin [7–10] in the rat.

Despite all these previous studies the evaluation of the role of the BEC in pathological as well as toxicological conditions has long been restricted by the lack of biochemical and functional data on these cells. Such a limitation has been overcome only very recently when several groups have developed isolation procedures that allow the separation of BEC suspensions [11–14] essentially free from hepatocytes and other non parenchymal cells. In our hands freshly isolated BEC are always more than 90% viable, retain typical *in vivo* characteristics and thus represent a useful model to investigate biochemical properties of BEC as well as their susceptibility to toxic agents.

In the present paper we describe the results that we have obtained by exposing BEC *in vitro* to a number of toxic compounds known to exert cytotoxicity in liver parenchymal cells by different mechanisms.

MATERIALS AND METHODS

Collagenase type I, Protease E (Pronase), Hyaluronidase type IS, Deoxyribonuclease type I, cytochrome *c* and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from the Sigma Chemical Co. (St Louis, MO). Glutathione reductase, reduced and oxidized glutathione as well as NADPH were purchased from Boehringer (Mannheim, F.R.G.). Percoll was from Pharmacia (Uppsala, Sweden), 7-ethoxycoumarin was from the Aldrich Chemical Co. (Gillingham, U.K.) whereas trypsin and Dulbecco's modified Eagle medium (DMEM) were obtained from Flow (Milano, Italy). Menadione (2-methyl-1,4-naphthoquinone) as well as all the other reagents were of the highest purity grade and were purchased from Merck (Darmstadt, F.R.G.).

Male albino Wistar rats (approx. 250 g of body weight) were obtained from Nossan (Correnzana, Italy) and fed *ad lib.* with a semisynthetic diet (diet n. 48, F.lli Piccioni, Brescia, Italy).

Biliary epithelial cells from normal rat liver were isolated essentially according to the procedure recently described by us [14] with the exception that

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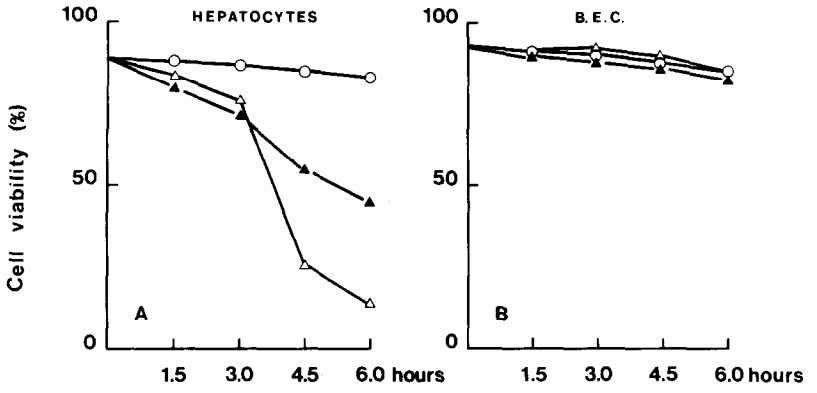

Fig. 1. Cell viability in isolated hepatocytes (A) and biliary epithelial cells (B.E.C.) exposed to carbon tetrachloride and 7-ethoxycoumarin. The results are expressed as the per cent of viable cells over a 6 hr incubation period and represent the means of three experiments. The symbols represent: (○) control cells; (△) cells exposed to 129 μM carbon tetrachloride; (▲) cells exposed to 100 μM 7-ethoxycoumarin.

the proteolytic digestion of the biliary tree, which remained undigested after collagenase–hyaluronidase perfusion of the liver, was obtained by incubating it at 37° for 60 min in the presence of 0.25% trypsin and 0.1% pronase instead of trypsin alone. This modification completely abolished contamination of the final suspension with hepatocytes and with a yield of approx. 15×10^6 BEC from each liver. Usually 90% of cells (or more) of the final cell suspension were identified as BEC by means of positive staining for the histochemical procedure for γ-glutamyltranspeptidase or for an immunofluorescence technique by using antibodies to bovine hoof prekeratin as already described [14]. Normal hepatocytes were also isolated during the same procedure as already described [14, 15] with a final yield of approx. $3–4 \times 10^8$ cells from each liver and a viability always greater than 85%.

Exposure to toxic agents *in vitro* was achieved by incubating BEC (2×10^6 cells/mL) and hepatocytes (10^6 cells/mL) in a final volume of 2–3 mL in medium C as described by Poli *et al.* [15] for experiments with isolated hepatocytes. Menadione was dissolved in DMSO at a final concentration of 1%, whereas relative controls received only DMSO. Cumene hydroperoxide was prepared in distilled water just before use. Carbon tetrachloride was added to the centre well of specially designed stoppered flasks as described by Poli *et al.* [15].

Cell viability was routinely assessed by the Trypan blue exclusion test. GSH and GSSG content were evaluated with the enzymatic procedure developed by Tietze [16]. Protein thiols were determined as described by Di Monte *et al.* [17]. The enzymes of the glutathione redox cycle were assayed in cell homogenates obtained by sonicating either BEC or hepatocyte suspensions with a MSE sonicator as described by Poli *et al.* [15]. Glutathione peroxidase was determined by following the oxidation of NADPH at 340 nm [18] and using either H_2O_2 or *t*-butyl-hydroperoxide (0.25 mM and 1.5 mM final concentration respectively) as substrates. Glutathione transferase was measured by the method of Habig *et al.* [19] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Glutathione reductase was evaluated as described by Babson *et al.* [20]. The protein content of cell samples was measured by the method of Lowry *et al.* [21] with bovine serum albumin as a standard. Statistical analysis was performed by using Student's *t*-test.

RESULTS

The data in Fig. 1 refer to the development of cell injury and the associated cell death when BEC and parenchymal cells isolated from normal rat liver were exposed *in vitro* to carbon tetrachloride (CCl_4) and 7-ethoxycoumarin (7-EC).

The incubation of control hepatocytes in the presence of 129 μM CCl_4 is followed by cell death that becomes evident between 3.0 and 4.5 hr of incubation (Fig. 1A). No evidence for a similar increase in Trypan blue uptake was found for BEC exposed to the same CCl_4 concentration over 6 hr of incubation (Fig. 1B). A very similar picture was also observed after exposure *in vitro* to 100 μM 7-EC over the same period of incubation (Fig. 1A and B).

In contrast, BEC were extremely sensitive to concentrations of menadione (MEN) and cumene hydroperoxide (CHP) that were completely ineffective in parenchymal cells under normoxic conditions (e.g. the conditions employed in this study, see Figs 2A and B; 3A and B). Cell death of BEC produced by these two compounds followed a progressive time-dependent and dose-dependent pattern (Figs. 2B and 3B). The analysis of the time course of lethality led us to choose concentrations of 100 μM for menadione and 10 μM for CHP for subsequent experiments with BEC, mainly because these doses were able to induce approx. 50% cell death in BEC suspensions after only 3 hr of incubation (see also Tables 1 and 2).

Table 1 summarizes the results obtained in a number of experiments in which BEC were exposed to toxic concentrations of MEN in the presence or in the absence of a number of exogenously added agents. After 3 hr of incubation in the presence of 100 and 200 μM menadione only 43 and 26% of BEC,

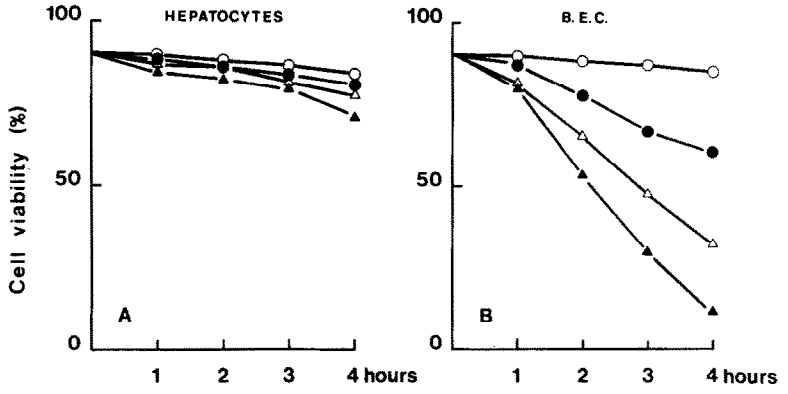

Fig. 2. Cell viability in isolated hepatocytes (left panel) and biliary epithelial cells (B.E.C., right panel) exposed to different concentrations of menadione. The results are expressed as the per cent of viable cells over a 4 hr incubation period and represent the means of three experiments. The symbols represent: (○) control cells; (●) cells exposed to 50 μM menadione; (△) cells exposed to 100 μM menadione; (▲) cells exposed to 200 μM menadione.

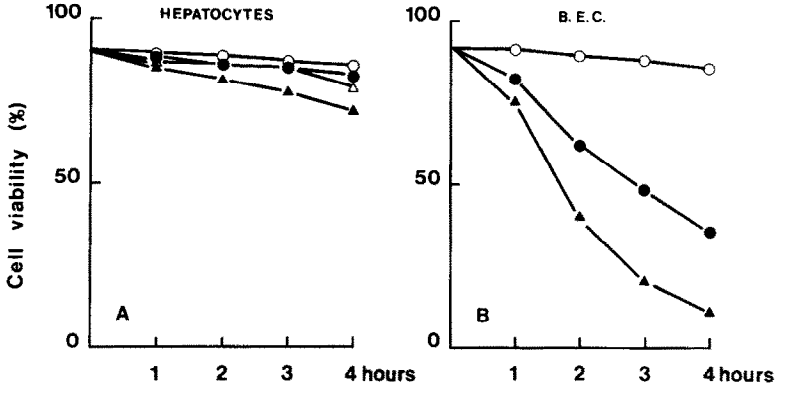

Fig. 3. Cell viability in isolated hepatocytes (left panel) and biliary epithelial cells (B.E.C., right panel) exposed to different concentrations of cumene hydroperoxide. The results are expressed as the percent of viable cells over a 4 hr incubation period and represent the means of three experiments. The symbols represent: (○) control cells; (●) cells exposed to 10 μM cumene hydroperoxide; (△) cells exposed to 50 μM cumene hydroperoxide; (▲) cells exposed to 100 μM cumene hydroperoxide.

Table 1. Menadione toxicity in biliary epithelial cells isolated from normal rat liver

	Viable cells (%)
Control	87.1 ± 3.1
Menadione 100 μM	43.0 ± 5.2
Menadione 200 μM	25.8 ± 6.7
Menadione 100 μM + dicoumarol 30 μM	21.6 ± 4.9
Menadione 100 μM + desferrioxamine 200 μM	56.9 ± 5.2
Menadione 100 μM + promethazine 100 μM	40.9 ± 3.9
Menadione 100 μM + SOD 300 units/mL	54.8*
Menadione 100 μM + catalase 800 units/mL	42.9*

Cell viability was assessed after 3 hr exposure to menadione. The cells were also pre-incubated for 30 min in the presence or in the absence of the mentioned protective agents. Values refer to the means ± SD of 3–5 experiments or * the means of two experiments and represents % of cell excluding Trypan blue.

SOD, superoxide dismutase.

respectively, were still viable. The simultaneous addition to BEC of 30 μM dicoumarol and 100 μM menadione resulted in an even more pronounced cell death at the end of the incubation period. No significant protection against menadione toxicity was obtained by pre-incubating BEC (30 min) with a number of agents.

The results in Table 2 show that after 3 hr of exposure to 10 and 100 μM CHP only 44 and 20% of BEC, respectively, are still viable. A significant and almost complete protection was obtained by pre-incubating cells with desferrioxamine. No protection was observed by pre-treating BEC with promethazine, superoxide dismutase or catalase. No significant increase in malondialdehyde (MDA) production (data not shown) was observed in BEC exposed to CHP.

Figures 4 and 5 provide evidence that MEN and CHP exert their toxic effects on BEC by first depleting cellular GSH (Fig. 4) and then cellular protein thiols (Fig. 5) before an increase in cell death could be seen. An almost complete depletion of GSH occurs within the first 15 min after the addition of both the quinone compound and the organic hydro-

Table 2. Cumene hydroperoxide (cumene-OOH) toxicity in biliary epithelial cells isolated from normal rat liver

	Viable cells (%)
Control	89.2 ± 2.1
Cumene-OOH 10 μM	44.0 ± 2.5
Cumene-OOH 100 μM	19.5 ± 5.0
Cumene-OOH 10 μM + desferrioxamine 200 μM	82.4 ± 3.9
Cumene-OOH 10 μM + promethazine 100 μM	44.7 ± 9.9
Cumene-OOH 10 μM + SOD 300 units/mL	52.4*
Cumene-OOH 10 μM + catalase 800 units/mL	36.2*

Cell viability was assessed after 3 hr exposure to cumene hydroperoxide. The cells were also pre-incubated for 30 min in the absence or in the presence of the mentioned protective agents. Values refer to the means ± SD of 3–5 experiments or * the means of two experiments and represent % of cell excluding Trypan blue.

SOD, superoxide dismutase.

peroxide (Fig. 4). It is interesting to note that in both cases the consumption of GSH is not accompanied by a complete recovery of the thiol compound as GSSG. The depletion of protein thiols became evident after 45 min and then progressively increased with time with either MEN or CHP (Fig. 5). A direct correlation between depletion of protein thiols and the increase in cell death was observed.

The data in Table 3 refer to an analysis of GSH content and of the efficiency of the enzymes of the glutathione redox cycle in BEC as compared with parenchymal cells. BEC, which are characterized by a very low level of intracellular glutathione, have also a very low level of GSH-transferase as well as GSSG-reductase. Concerning GSH-peroxidase activity no significant difference from hepatocyte values was detected in BEC samples.

DISCUSSION

BEC isolated from normal rat liver are not affected by carbon tetrachloride and 7-ethoxycoumarin, two

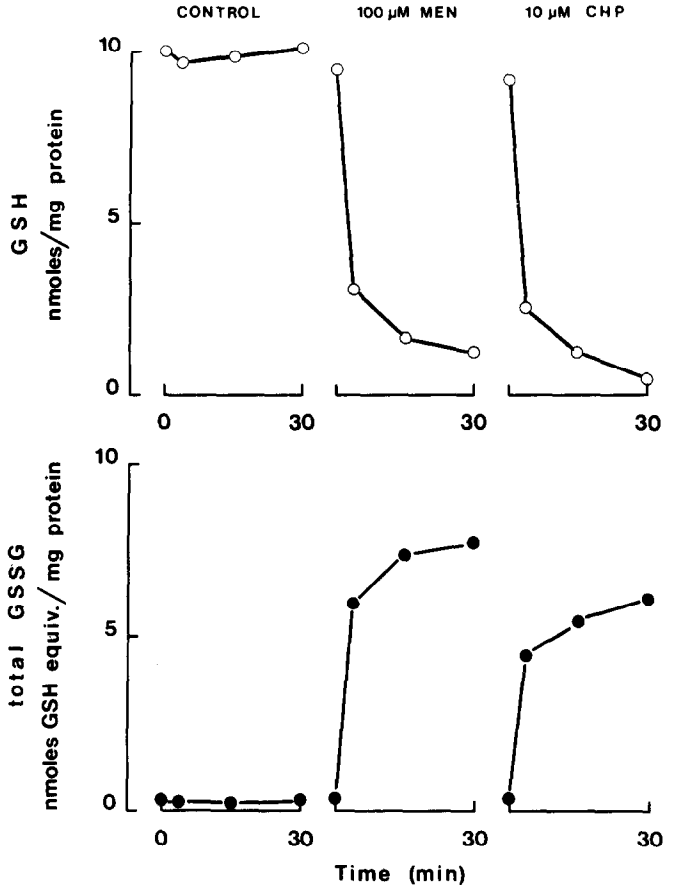

Fig. 4. Time course of GSH depletion and GSSG formation in biliary epithelial cells exposed to 100 μM menadione (MEN) or to 10 μM cumene hydroperoxide (CHP). At the indicated time samples were collected and processed for quantitation of GSH (○, upper panel) and GSSG (●, lower panel). One experiment typical of three is shown.

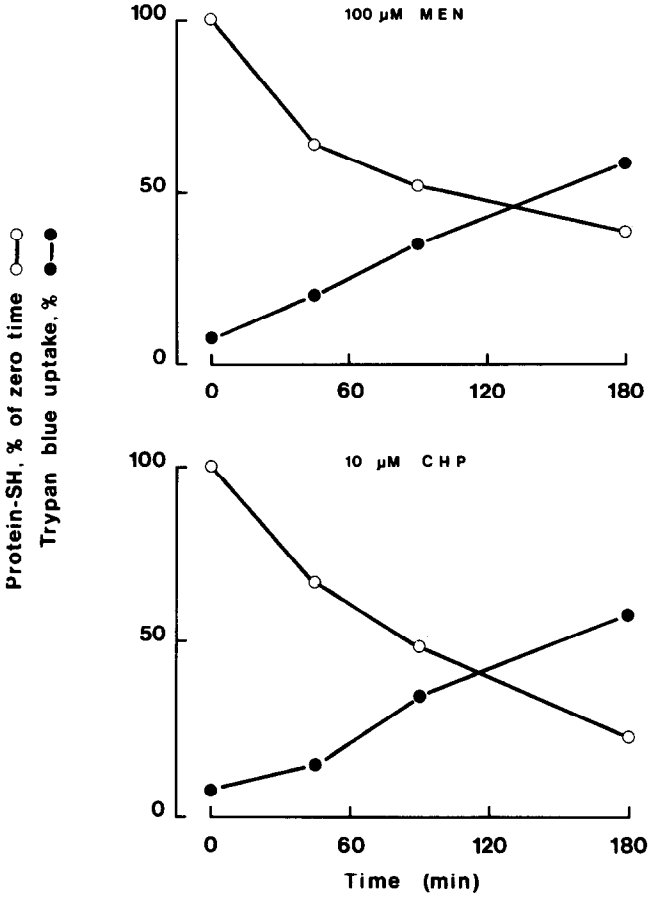

Fig. 5. Oxidation of protein thiols (○—○) and increase in Trypan blue uptake (●—●) in biliary epithelial cells exposed to 100 μM menadione (MEN, upper panel) or 10 μM cumene hydroperoxide (CHP, lower panel). Values represent the means of three separate experiments for each chemical.

Table 3. Enzymes of the glutathione redox cycle and glutathione content in normal parenchymal cells and in biliary epithelial cells (B.E.C.) isolated from rat liver

		Hepatocytes	B.E.C.
GSH-peroxidase	(a)	166.97 ± 11.60	164.92 ± 15.94
(nmol NADPH/min/mg protein)		(6)	(4)
	(b)	148.20 ± 8.11	148.39 ± 7.60
		(3)	(3)
GSH-transferase		882.95 ± 225.28	185.89 ± 43.76†
(nmol adduct/min/mg protein)		(3)	(3)
GSSG-reductase		49.49 ± 7.46	32.64 ± 4.45*
(nmol NADPH/min/mg protein)		(5)	(5)
GSH		28.43 ± 3.44	9.82 ± 0.83†
(nmol/mg protein)		(5)	(7)
GSSG		0.56 ± 0.02	0.35 ± 0.05†
(nmol/mg protein)		(5)	(7)

The assays were performed on disrupted cell suspensions. The values are expressed as means ± SD. The number of experiments is shown in parenthesis.
* $P < 0.05$; † $P < 0.01$; (a) H_2O_2; (b) *t*-butyl-hydroperoxide.

agents that are known to produce hepatocyte damage and death [15, 22, 23]. Despite the differences in the mechanisms by which these two chemicals exert their toxic effects on parenchymal cells they both need, as a common first step, to be metabolically activated by cytochrome P450 or by cytochrome P450-related enzymes [15, 22–25]. In a previous paper [14] we have shown that isolated BEC seem to be virtually devoid of cytochrome P450 and of 7-ethoxycoumarin deethylase activity; moreover we obtained

no indication of aminopyrine demethylase activity in BEC samples (data not shown). This metabolic picture, which is also supported by immuno-histochemical studies performed *in vivo* [26] and by a study performed on BEC isolated from livers during cholestasis [27], clearly suggests that the resistance of BEC to CCl_4 and 7-EC may be simply the consequence of a lack of metabolic activation of the former compounds to very toxic intermediates.

An almost opposite picture is observed when the cytotoxicity of MEN and CHP is investigated in BEC and hepatocytes. BEC are extremely sensitive to concentrations of MEN (100 μM) and CHP (10 μM) that, at least under the normoxic conditions employed in this study, are practically ineffective on parenchymal cells. The cytotoxicity of CHP and MEN against isolated hepatocytes has been ascribed mainly to metabolic activation of the two compounds to very toxic intermediates. Concerning MEN it is well known that quinone metabolism in parenchymal cells can occur by either one electron reduction catalysed by flavoenzymes, on which cytotoxicity mainly depends, or by two-electron reduction catalysed by DT diaphorase, and enzyme that serves an important protective function [28–32]. The one-electron reductive pathway leads to an extensive formation of the superoxide anion radical and then hydrogen peroxide which in turn leads to GSH depletion, pyridine nucleotide oxidation, oxidation of protein thiols, alterations of calcium homeostasis and of the cytoskeleton [28–31]. In this connection BEC show an easily detectable activity of the flavoenzyme NADPH-cytochrome *c* reductase; thus the same one-electron reductive route of metabolic activation of MEN so far described for hepatocytes may also apply to the BEC. This possibility is also supported by the fact that the simultaneous addition of dicoumarol, a potent inhibitor of DT-diaphorase [32], markedly increased BEC susceptibility to menadione.

The picture appears to be more complicated for CHP-induced cell death in BEC. In parenchymal cells two possible mechanisms may be involved: (i) cytochrome P450-mediated activation of CHP leading to lipid peroxidation [33] or (ii) metabolism of CHP through glutathione peroxidase leading to a deleterious shift in the cell redox status. In BEC, the first mechanism cannot be responsible since these cells are devoid of cytochrome P450, no MDA production is detected and the powerful free radical scavenger promethazine [34] is not protective. However, BEC death induced by 10 μM CHP is prevented by the iron chelator desferrioxamine suggesting the involvement of low molecular weight iron complexes in catalysing or exacerbating CHP-induced cell damage [35, 36]. Nevertheless, since CHP is also a good substrate for GSH peroxidase [37, 38], which activity is present at high levels in these cells, and since the basal concentration of GSH is low it seems possible that CHP induced BEC death is due to metabolism of CHP by the peroxidase leading to an oxidation of GSH and a compromised cellular redox status.

It should be noted, at this point, that both MEN and CHP are able to induce GSH depletion and oxidation of protein thiols leading to conditions of oxidative stress. As already shown for MEN-induced hepatocyte injury and death [28], with both CHP and MEN the depletion of GSH in BEC occurs as soon as within the first 15 min of incubation. The oxidation of protein thiols then follows and always precedes the appearance of the earlier evidence of cell death in BEC. The role of cytosolic glutathione in preserving protein -SH groups from oxidative damage thus seems to be critical. Moreover the analysis of these events seems to suggest the involvement of GSH redox cycling in which the rate of GSH consumption presumably exceeds the capacity of the cell to maintain its normal thiol status [28]. It is therefore of primary importance that the GSH content per mg of protein in BEC amounts to only one third of what is usually found in parenchymal cells. A picture observed with both MEN and CHP is represented by the fact that at the end of the first 30 min of incubation (e.g. when the cell content of GSH is already down to minimal values) the recovery of total (intracellular + extracellular) GSSG is not complete. These results may suggest the involvement of some other minor alternatives that may be not mutually exclusive: (i) GSH depletion may not be totally related to redox cycling through the GSH-peroxidase/GSSG-reductase system; this could be true at least for MEN that can react directly with GSH giving a menadione–GSH conjugate [17, 29, 30, 38–40]. It has been estimated that this conjugate may represent approx. 15% of the total hepatocellular disappearance of GSH in parenchymal cells [17]: in BEC, where the activity of the flavoprotein NADPH cytochrome *c* reductase is only approx. one third of the activity found in hepatocytes [41], this alternative route may be more pronounced. It should be noted, however, that in parenchymal cells the menadione–GSH conjugate has been reported to be either transported out of the cell or to redox cycle itself [17]: we actually do not know if the same events apply also to BEC and to what extent. (ii) GSSG formed through the redox cycle may not all be available to GSSG reductase since it can interact with intracellular protein to form glutathione–protein mixed disulphides as already suggested for MEN and for *t*-butyl-hydroperoxide [42, 43]. According to Bellomo *et al.* [42] the amount of mixed disulphides produced in toxic conditions and the kinetics of their formation are strictly dependent mainly on the intracellular GSH/GSSG ratio and the actual level of available GSH. In BEC, where the level of cytosolic GSH is particularly low even in control cells and the rate of GSH disappearance after MEN and CHP addition seems extremely fast, this alternative may also occur. We are currently investigating these points as well as several other critical biochemical and functional parameters during the course of BEC injury and death induced by MEN and CHP.

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