Pergamon

Biochemical Pharmacology, Vol. 47, No. 4, pp. 625–632, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0009-U

EFFECT OF OXIDATIVE STRESS AND DISRUPTION OF Ca^{2+} HOMEOSTASIS ON HEPATOCYTE CANALICULAR FUNCTION *IN VITRO*

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(*Received* 19 *August* 1993; *accepted* 19 *October* 1993)

Abstract—Isolated rat hepatocyte couplets were used to study the effects of menadione and a rise in the intracellular concentration of calcium on biliary canalicular function. Canalicular function was assessed by counting the percentage of couplets which were able to accumulate the fluorescent cholephile, cholyl lysyl fluorescein (CLF) into the canalicular vacuole between the two cells. Menadione induced a concentration-dependent inhibition of the canalicular vacuole accumulation (CVA) of CLF reaching 7.6 ± 1.8% of control at 100 μM menadione. This disruption was not prevented by blocking receptor-operated calcium channels with Ni^{2+} (300 μM). The concentration range of menadione used did not deplete cellular ATP content. In contrast glutathione content was reduced to 52% of its control value by 100 μM menadione. A rise in cytosolic calcium induced by the calcium ionophore, A23187 (up to 30 μM) also disrupted CVA in a concentration-dependent manner. Release of endoplasmic reticulum calcium stores by thapsigargin (50 nM) affected the retention of canalicular contents to a much lesser extent, although it was able to stimulate a reduction in canalicular area to 40% of its original value, assumed to be due to canalicular contraction. Menadione (30 and 100 μM) reduced the fluorescence of phalloidin-FITC-labelled F-actin in both the total and pericanalicular cytoskeleton. Canalicular function was therefore disrupted by non-lethal concentrations of menadione via a mechanism which does not appear to involve ATP depletion or the entry of extracellular calcium, but is associated with a depletion of both cellular glutathione and F-actin. An increase in the concentration of intracellular calcium can stimulate canalicular contraction, and at relatively high concentrations calcium can also disrupt canalicular function.

Key words: hepatocyte couplet; menadione; canaliculus; cholyl lysyl fluorescein; A23187; thapsigargin

The effect of toxic agents on hepatic canalicular primary bile formation is difficult to study *in vivo* due to the inaccessibility of the bile canaliculus. It is possible however, to sample bile which has been subject to modification by ductular epithelial cells via bile duct cannulation [1]. A variety of *in vitro* hepatocyte models have now been developed to allow access to primary bile, either using hepatocyte couplets in short-term culture in which canaliculi remain functional [2, 3], or more long-term cultures of embryonic- [4] or dexamethasone-treated [5] hepatocytes in which canaliculi are formed *de novo*.

The isolated rat hepatocyte couplet provides an accessible functional biliary unit in which the polarity of bile transport is established within approximately 4 hr of isolation [2, 6]. Canalicular function can be assessed in the couplet by monitoring its ability to take up, transport, secrete and accumulate the fluorescent cholephile, CLF‡ [7]. The couplet has thus proved to be a useful tool in the investigation of the cholestatic effects of various reagents [8–10].

Induction of oxidative stress by menadione (2-methyl-1,4-naphthoquinone) has been studied extensively in relation to cell death [11], but little is known about the mechanism by which it induces cholestasis [12]. The lethal toxic mechanisms induced by this redox cycling quinone are known to involve a depletion of intracellular reduced GSH [11], an increase in cytosolic calcium concentration [13], a reduction in cellular ATP content [14] and disruption of the cytoskeleton [15]. The aim of this investigation was to study the disturbance of canalicular function in relation to these four parameters using the isolated rat hepatocyte couplet as an *in vitro* biliary model.

MATERIALS AND METHODS

Materials. The following reagents were obtained from the sources stated: collagenase from *Clostridium hystolyticum* (0.651 U/mg) (Boehringer Mannheim, Lewes, U.K.), Leibovitz-15 (L-15) tissue culture medium (Gibco, Paisley, U.K.), CLF (synthesized as described by Mills *et al.* [7]), menadione, A23187, GSH, ATP, firefly lantern extract, *o*-phthaldialdehyde and phalloidin-FITC (Sigma Chemical Co., Poole, U.K.), formaldehyde (Fisons Scientific Equipment, Loughborough, U.K.), thapsigargin (Calbiochem Novabiochem (U.K.) Ltd, Nottingham, U.K.). All other chemicals were of reagent grade.

Animals. Male Wistar rats bred in the University

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‡ Abbreviations: CLF, cholyl lysyl fluorescein; CVA, canalicular vacuole accumulation; GSH, glutathione; ER, endoplasmic reticulum.

of Birmingham (220–240 g) fed with standard laboratory chow (41B maintenance diet, Pilsbury, Birmingham, U.K.) and tap water *ad lib*, were used throughout. Anaesthesia was obtained using Ketalar (Ketamine hydrochloride 6 mg/100 g body weight) with Domitor (Medetomidine 25 μg/100 g body weight). Surgery was commenced between 8 a.m. and 10 a.m.

Hepatocyte couplet isolation and centrifugal elutriation. Hepatocyte couplets were isolated according to a two-step collagenase perfusion method [16] adapted from Gautam *et al.* [17]. Tissue remaining from the initial digest was reincubated in collagenase solution at 37° [16] to liberate a second cell preparation with a high viability as assessed by Trypan blue (99.0 ± 1.6%), and hence these cells were used for all experiments. Hepatocyte preparations were quantified using an improved Neubauer haemocytometer expressing results in terms of units, where a unit could consist of a singlet, couplet, triplet or larger multiple [16]. If any cell within the unit stained positively with Trypan blue then the whole unit was counted as non-viable.

For the analysis of ATP and GSH content, and for image analysis of canalicular size, hepatocyte preparations containing a relatively high percentage of couplets were required. Couplets were enriched using centrifugal elutriation as described by Wilton *et al.* [16] to yield a preparation containing 71.1 ± 2.3% couplets (N = 12).

Culture and treatment of hepatocyte couplets. For microscopic observation, hepatocytes were incubated at a density of 1×10^5 U/2 mL. For biochemical analysis, hepatocytes were incubated at a density of 4×10^5 U/2 mL. All hepatocytes were incubated in L-15 medium on plastic culture dishes, in air atmosphere for 4.5 hr at 37°.

Menadione, A23187 and thapsigargin were added as 10 μL doses dissolved in DMSO to give various final concentrations. $NiCl_2$ (300 μM final concentration) in distilled water (10 μL) was added 30 min prior to addition of menadione and CLF (see below).

Analysis of canalicular function. To assess canalicular function, the number of couplets able to undergo CVA of CLF was counted and expressed as a percentage of control couplets exhibiting this phenomenon. CLF (5 μM final concentration) was added to each 2 mL plate and incubated at 37° for 15 min either (i) prior to drug addition (thapsigargin and menadione), or (ii) simultaneous with drug treatment (menadione and A23187). Cells were washed twice with 2 mL of L-15 medium either (i) before drug addition, or (ii) before observation at 37° using an Olympus IMT2-RFL inverted fluorescence microscope. Measurement of canalicular area and breadth was accomplished using an image analysis system (Applied Imaging, Sunderland U.K.) to computate the fluorescent images. The percentage of couplets exhibiting plasma membrane blebs was assessed by light microscopy as used by Nicotera *et al.* [18].

Confocal microscopic study of phalloidin-FITC-stained actin. Hepatocyte couplets were plated onto glass coverslips and incubated in L-15 for 4.5 hr at 37°. Cells were treated with menadione for 15 min

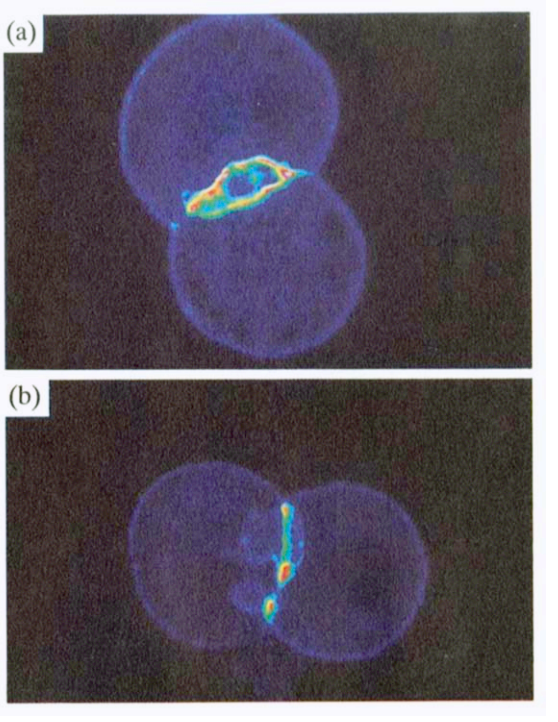

Fig. 1. Phalloidin-FITC-labelled F-actin localization in the mid *z*-plane of rat hepatocyte couplets by confocal microscopy (magnification ×16). (a) DMSO (10 μL) 15 min, (b) menadione (final concentration of 100 μM; administered as a 10 μL dose dissolved in DMSO) 15 min.

before fixing with 3% formalin in PBS. Fixed cells were then stored at 4° until permeabilized with 0.1% Triton X-100 in PBS and labelled with phalloidin-FITC according to the method of Knutton *et al.* [19]. To observe the stained cells, coverslips were inverted onto citifluor mounting solution.

Using a Bio Rad 500 confocal laser scanning system attached to a Leitz SM-LUX microscope, it is possible to obtain *xy*-images at any level of the *z*-axis through the specimen. Due to the short depth of focus obtained using this instrument, out-of-focus flare is reduced, thus improving image definition and precision. For the purpose of this study the central section through each couplet (Fig. 1) was selected for analysis of fluorescence location and integrated intensity (area × mean intensity) within the cell. Choosing the central section allowed unprejudiced measurements to be taken from the most representative *z*-section of the specimen. Thibault *et al.* [8] measured canalicular fluorescence by designating an elliptical area occupying 10% of the total couplet area as the region of the pericanalicular cytoskeleton. Under the conditions of our experiments the pericanalicular cytoskeleton rarely formed a uniform ellipse, therefore we found it more satisfactory to define the clear edge of the high intensity fluorescence using the image analysis system.

Analysis of GSH and ATP. GSH was measured fluorometrically according to the method of Hissin and Hilf [20]. ATP was measured using the

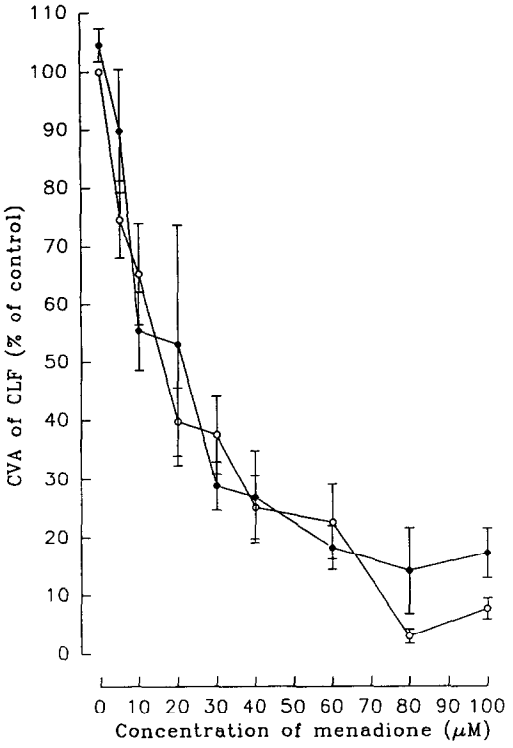

Fig. 2. Couplet CVA of CLF during simultaneous treatment with menadione (0–100 μM) for 15 min, pretreated either with 10 μL of distilled water (vehicle) (○), or $NiCl_2$ (300 μM) (●). Control couplets accumulated CLF in 68.39 ± 5.12% of units. Each value is the mean ± SEM (N = 6 experiments). There was no significant protection against CVA disruption when couplets were pretreated with Ni^{2+} (ANOVA).

substrate enzyme system, luciferin–luciferase [21]. Fluorescence and bioluminescence were quantified using a Perkin Elmer Luminescence Spectrometer (LS 50B; Buckinghamshire, U.K.).

Statistical analysis. Each individual observation (N) consisted either of the total number of couplets observed within one field of view (approx. 40–60), or one couplet (confocal microscopy). Each set of data consisted of individual observations taken from a minimum of six rats.

A Student's *t*-test was used to distinguish significant differences between individual data sets, and ANOVA was used to determine whether a significant difference occurred between two groups of data.

RESULTS

The concentration-dependent effects of menadione on couplet canalicular function

A 15-min incubation with menadione (up to 100 μM) induced a concentration-dependent inhibition of the CVA of CLF (added simultaneously) (Fig. 2). These results are consistent with those of Wilton *et al.* [3]. The largest dose of quinone used (100 μM) was sufficient to reduce CVA to 7.61 ± 1.82% of control. The disruption of canalicular function was coincident with an increase in plasma membrane blebbing, although within the lower concentration range (0–40 μM), CVA was the most sensitive of the two parameters. The concentration of menadione able to inhibit CVA by 50% (IC_{50}) was 19 μM, which was much less than the concentration of menadione effective at inducing 50% maximal blebbing (EC_{50}; 43 μM).

Table 1. Canalicular accumulation of CLF (added for 15 min prior to observation) in rat hepatocyte couplets after treatment with menadione for 15 min and subsequent cell washing

Time after menadione removal (min)	Menadione concentration		
	0 μM	30 μM	100 μM
0	100	27.07 ± 7.39	6.02 ± 2.51
30	100	71.63 ± 9.50	41.33 ± 11.24
90	100	70.20 ± 5.33	58.90 ± 23.49

All values are expressed as mean % of control ± SEM (N = 4–6). Untreated couplets accumulate CLF in 73.64 ± 6.23, 61.12 ± 7.52 and 46.81 ± 5.92% of units at time 0, 30 and 90 min, respectively.

Removal of menadione (30 and 100 μM) by washing twice with 2 mL of fresh L-15 medium (37°) allowed canalicular function to recover to 70.2 ± 5.3% of control and 58.9 ± 23.4% of control, respectively, within 90 min of menadione removal (CLF was added for 15 min prior to observation only; Table 1). Couplets treated with 30 μM menadione were able to recover their CVA to 105.2 ± 16.5% of control within 230 min of drug removal, therefore CVA was recoverable and the doses of menadione used were not lethal. Plasma membrane blebbing returned to control levels 90 min after treatment with both concentrations of menadione (30 and 100 μM) (data not shown).

Treatment of couplets with CLF for 15 min, and subsequent exposure to menadione (0–100 μM) for 10 min after washing twice with 2 mL of fresh L-15 medium (37°), revealed that menadione (10 μM) was sufficient to reduce the proportion of couplets retaining CLF within the canaliculus to 54.76 ± 7.40% of control (Fig. 3). At a final concentration of 100 μM, menadione decreased the percentage of couplets retaining CLF to 34.42 ± 6.19% of control. The path by which CLF leaves the canaliculus cannot be determined via this experimental method.

The manipulation of intracellular calcium by various reagents and its effects on canalicular function

To study the mechanisms of CVA disruption, the calcium ionophore A23187 [18] was used to identify the consequences of a relatively large rise in cytosolic calcium concentration on couplet canalicular function. As with menadione, a 15 min concurrent incubation with CLF and A23187 (up to 30 μM) resulted in a concentration-dependent reduction in CVA (Fig. 4). In contrast with the findings using

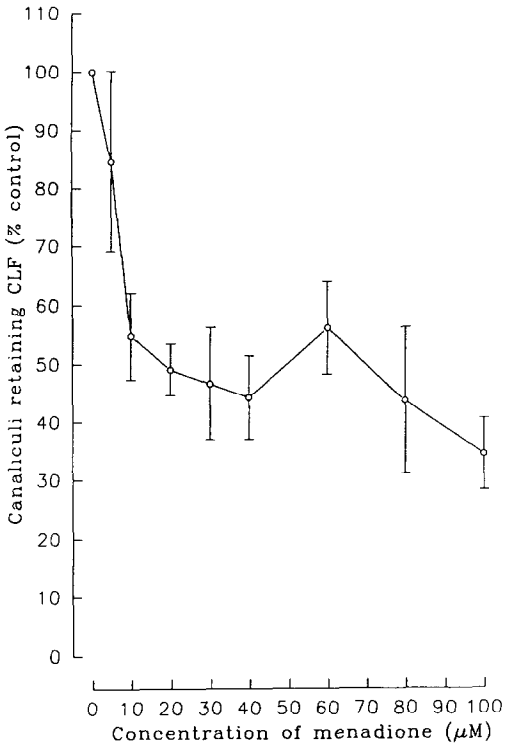

Fig. 3. Retention of CLF (15 min incubation) within the couplet canalicular vacuole after subsequent treatment with menadione (0–100 μM) for 10 min. Control couplets retain within the canaliculi of 57.11 ± 3.93% of units. Each value is the mean ± SEM (N = 6 experiments).

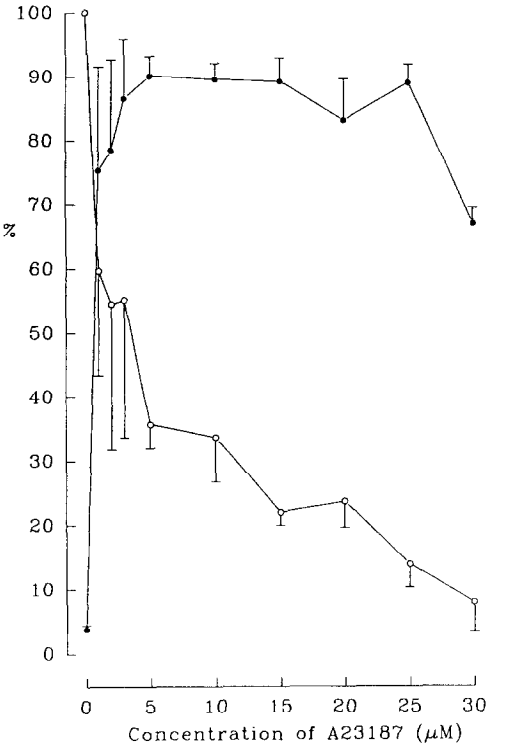

Fig. 4. Couplet CVA of CLF (○) and plasma membrane blebbing (●) during simultaneous treatment with the calcium ionophore A23187 (0–30 μM). Control couplets accumulate CLF in 69.08 ± 7.48% of units. Each value is the mean ± SEM (N = 4 experiments).

menadione, plasma membrane blebbing was more extensive than the inhibition of canalicular function at low A23187 concentrations (up to 10 μM).

Thapsigargin is a tumour-promoting lactone which specifically inhibits the Ca^{2+}-ATPase of the ER [22]. Inhibition of the Ca^{2+}-ATPase prevents sequestration of calcium into the ER, resulting in an increase in cytosolic calcium within seconds. Treatment of couplets with thapsigargin (50 nM; a concentration capable of fully releasing ER calcium stores [22]) for 10 min reduced the proportion of canaliculi retaining pre-loaded CLF by only 26.37 ± 8.71% of control (Table 2). Canaliculi of couplets treated with thapsigargin were visually smaller than those of control couplets. Image analysis of the fluorescent image proved that thapsigargin (5–50 nM) decreased both canalicular area (% of couplet area) and breadth (Table 2).

Oxidative stress-induced rises in cytosolic calcium are thought to involve both the entry of extracellular calcium via plasma membrane receptor-operated calcium channels and the release of internal calcium stores [23, 24]. Receptor-operated channels are not blocked by the voltage-gated channel inhibitors such as verapamil, instead they can be blocked using Ni^{2+} [25]. Pretreatment of couplets with $NiCl_2$ (300 μM) for 30 min prior to addition of menadione and CLF did not protect CVA against oxidative stress (Fig. 2) (data analysed using ANOVA), therefore the entry of extracellular calcium does not appear to be important in the mechanism of cholestasis induced by concentrations of menadione up to 100 μM.

Disruption of cytoskeletal actin by menadione

Phalloidin-FITC-labelling of fixed, permeabilized cells allows visualization of the F-actin cytoskeleton [8]. During the 4.5 hr incubation period, the actin cytoskeleton of the couplet reorganizes to form a polarized structure concentrated below the canalicular membrane [8, 26] as was observed by confocal microscopy (Fig. 1). Re-establishment of couplet biliary polarity is known to be microfilament dependent [6]. Image analysis of the confocal image allows quantification of the intensity of fluorescence within the total couplet and specifically in the location of the pericanalicular cytoskeleton. Both 30 and 100 μM menadione were able to reduce total couplet fluorescence (Fig. 5). Since phalloidin binds F-actin only [27] this can be interpreted as a menadione-induced decrease in the total quantity of polymerized actin within the hepatocytes. On treatment with 30 and 100 μM menadione, 31.54 and 41.3% of the decrease in fluorescence/μm^2, respectively, can be accounted for by a reduction in the pericanalicular cytoskeleton fluorescence (Fig. 5), therefore menadione appears to disrupt the pericanalicular actin cytoskeleton.

Table 2. Effect of thapsigargin treatment (10 min) on retention of CLF in couplet canaliculi, and on couplet breadth and canalicular area (% of couplet area) as distinguished by CLF fluorescence

Thapsigargin (μM)	Canalicular accumulation (% of control)	Breadth (pixels)	Canalicular area (% couplet area)
0	100	13.43 ± 0.81	12.95 ± 2.62
5	81.48 ± 13.46	9.68* ± 0.79	7.57* ± 0.77
50	73.62 ± 8.72	8.01* ± 0.69	5.20* ± 0.57

Control couplets retain CLF in 63.28 ± 10.35% of units after treatment with thapsigargin (N = 6). *Canalicular breadth and area (% of couplet area) were both significantly reduced by 5 and 50 nM thapsigargin ($P < 0.001$) (N = 40, 28 and 31, respectively).

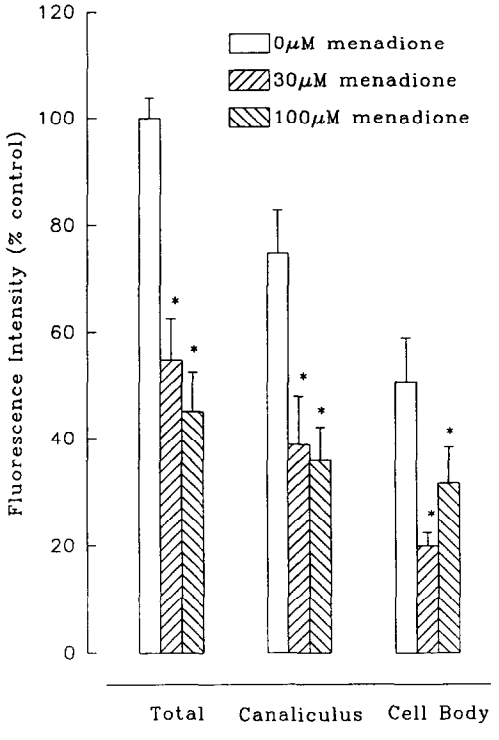

Fig. 5. Phalloidin-FITC-labelled F-actin fluorescence intensity in hepatocyte couplets treated with menadione (0, 30 and 100 μM) for 15 min (*$P < 0.001$). Each value is the mean ± SEM (N = 16, 9 and 13 couplets, respectively).

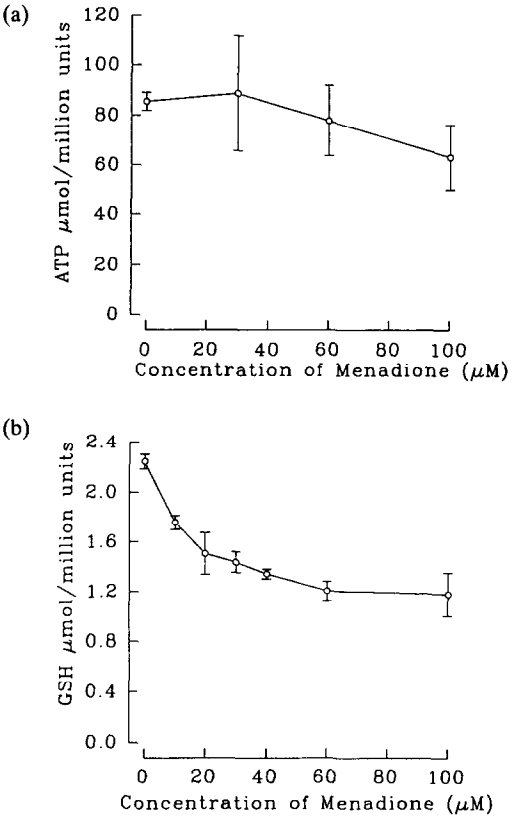

Fig. 6. Couplet GSH and ATP content after treatment with menadione (0–100 μM) for 15 min. Control couplets contain 85.65 ± 3.68 μmol ATP/10^6 U and 2.25 ± 0.06 μmol GSH/10^6 U. Each value is the mean ± SEM (N = 4 experiments).

The concentration-dependent effects of menadione on couplet ATP and GSH content

Treatment with menadione (100 μM) for 15 min did not significantly reduce the content of ATP ($P > 0.05$) from that of control couplets (85.65 ± 3.68 μmol/10^6 U) (Fig. 6a). In contrast, the GSH content was reduced in a concentration-dependent manner from 2.25 ± 0.05 μmol GSH/10^6 U in control couplets to 1.18 ± 0.18 μmol GSH/10^6 U (Fig. 6b).

DISCUSSION

Effect of menadione on canalicular function

The metabolism of menadione has been well studied using isolated hepatocytes [11] and more recently using precision cut liver slices [28]. Menadione metabolism is known to involve redox

cycling of the parent quinone molecule and its 3-glutathionyl conjugate, resulting in the liberation of various reactive oxygen species [29]. Detoxification of the reactive oxygen species results in the oxidation and depletion of NADPH and glutathione allowing oxidative damage to ensue [reviewed in 30]. Menadione is also able to arylate cellular protein thiol groups [11].

Consequences of oxidative stress include oxidation of the thiol group of Ca^{2+}-ATPases in the plasma membrane, ER and mitochondria leading to a rapid rise in cytosolic calcium and cell toxicity [31–33]. In addition, disturbance of mitochondrial function, results in a depletion of cellular ATP and ultimately to cell death [34].

Akerboom *et al.* [12] studied the effects of menadione on bile flow in the isolated perfused rat liver, and found low doses (20 μM) to be choleretic and high doses (100 μM) to cause a temporary choleresis followed by a sustained cholestasis. The choleresis can partly be accounted for by the increased output into bile of GSSG [12, 35] acting as an osmotic driving force for bile formation. The cause of the sustained cholestasis remains unexplained.

The present study indicates that the ability of couplets to accumulate the fluorescent cholephile CLF is severely affected by the action of menadione (Fig. 2). The doses of menadione which inhibit CVA are relatively low in comparison with cytotoxic doses reported in the literature [11, 31]. All concentrations of menadione used proved to be insufficient to cause significant cell death as indicated by the maintenance of cellular ATP levels (Fig. 6). This evidence was strengthened by the demonstration that disruption of canalicular accumulation is reversible (Table 1).

Doses of menadione that disrupted canalicular function were, however sufficient to deplete cellular GSH levels in a concentration-dependent manner (Fig. 6). Observations made by Akerboom *et al.* [12] and Redegeld *et al.* [14] support the lack of mortality induced by these thiol-depleting doses of quinone used in the present experiments. Inhibition of canalicular function therefore appears to occur at non-cytotoxic concentrations of menadione and by a mechanism which is independent of ATP depletion.

Addition of menadione to couplets which had previously taken up and accumulated the fluorescent cholephile illustrated that the quinone was able to disrupt the mechanism which allows retention of the canalicular contents (Fig. 3). The accumulated CLF could conceivably exit via the tight junctions, alternatively it could re-enter one or both of the hepatocytes.

Susceptibility of couplet canalicular function to manipulations of intracellular calcium

The calcium ionophore, A23187 allows calcium to flow from the external medium and internal stores into the cytoplasm [18]. A23187 caused a concentration-dependent inhibition of couplet CLF canalicular accumulation and retention, illustrating the susceptibility of canalicular function to a large rise in cytosolic calcium.

In contrast, thapsigargin induces a rise in intracellular calcium concentration by inhibition of the ER Ca^{2+}-ATPase [22]. Emptying of the inositol (1,4,5)triphosphate-sensitive store may stimulate the entry of extracellular calcium across the plasma membrane via receptor-operated channels leading to a potentiation of the increase in cytosolic calcium [36]. A concentration of thapsigargin (50 nM) known to release the ER calcium store fully [22] caused substantially less disruption of canalicular function in hepatocyte couplets compared with A23187 (Table 2). Release of ER calcium has also been reported by Farrell *et al.* [37] to be insufficient to cause hepatoxicity.

Calcium has been shown to stimulate couplet canalicular contractions in the presence of ATP [38, 39]. Release of ER calcium stores by thapsigargin induced a reduction in couplet canalicular area (% of couplet area) and breadth, assumed to be due to contraction of the canaliculus (Table 2).

Based on the above findings, it appears that for calcium to be involved in the disruption of CVA there would need to be an influx of extracellular calcium. Blockage of the receptor-operated calcium channels [40] using Ni^{2+} [30] proved to be non-toxic, but did not prevent the disruptive effects of menadione on couplet canalicular function (Fig. 1). Therefore the entry of extracellular calcium does not appear to be important in disruption of canalicular CLF accumulation.

Disruption of the pericanalicular actin arrangement by menadione

The hepatocyte has a polar cytoskeletal arrangement characteristic of transporting epithelial cells [41]. Immediately adjacent to the canalicular membrane is a dense network of actin filaments surrounded by a pericanalicular sheath of cytokeratin [5]. Microtubules are arranged throughout the cytoplasm but are particularly well organized around the pericanalicular sheath [5]. Disruption of the canalicular cytoskeletal elements by phalloidin [42], cytochalasin [5] and colchicine [5], respectively, has been shown to induce cholestasis. Actin is thought to be involved in the process of canalicular contraction proposed to be necessary for bile propulsion within the biliary tree [26]. Cytoskeletal disruption could therefore be a mechanism by which menadione prevents the accumulation and/or retention of CLF within the canaliculus. In particular actin microfilament disruption could inhibit canalicular motility and disrupt tight junction stability.

Our results clearly show the relatively high intensity of phalloidin-FITC-labelling at the pericanalicular cytoskeleton in comparison with the remainder of the cytoplasm and plasma membrane (Fig. 1). Menadione (30 and 100 μM) reduced both the total couplet and the pericanalicular fluorescence indicative of F-actin disruption (Fig. 5). Treatment of hepatocytes [15] and platelets [13] with menadione has been reported elsewhere to reduce cellular F-actin content and to increase the occurrence of large molecular weight aggregates. Mirabelli *et al.* [15] suggest that these results are due to actin being cross-linked with itself and other cytoskeletal proteins possibly by a mechanism involving thiol oxidation and ATP depletion [15]. These experiments

demonstrate that menadione (up to 100 μM) depleted GSH but not ATP, therefore the most probable mechanism of disruption of F-actin integrity is by thiol oxidation. The effect of oxidative stress on other cytoskeletal proteins is now under investigation.

Thibault *et al.* [8] have also used phalloidin-FITC to observe the actin cytoskeleton of couplets and the toxic effects of various agents on this structure. In these experiments all drug incubations lasted for 2 hr, which induced an increase in pericanalicular fluorescence intensity as a ratio of couplet intensity. Under these conditions, the elevation of actin labelling may be a secondary stress-response to oxidative damage possibly involving new protein synthesis [43].

Isolated rat hepatocyte couplets have proved to be a useful *in vitro* tool to investigate canalicular disruption by menadione and agents that modulate intracellular calcium concentrations. Canalicular function appears to be disrupted by non-lethal concentrations of menadione via a mechanism which does not involve ATP depletion or the entry of extracellular calcium, but is associated with a depletion of both cellular GSH and F-actin.

Acknowledgements—This work was supported by The Wellcome Trust. We would like to thank David J. Lankester for kind assistance in the production of hepatocyte couplets, and Dr F. Michelangeli for advice concerning thapsigargin.

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