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The cytotoxicity of menadione in hepatocytes isolated from streptozotocin-induced diabetic rats

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Alterations in the activity of both the cytochrome P-450 dependent mixed function oxidase (MFO*) system [1, 2] and the enzymes of conjugation [3, 4] have been shown to occur in experimentally-induced diabetes. In male streptozotocin treated diabetic rats the glucuronic acid conjugation of several substrates is deficient and the activity of glutathione-*S*-transferase is decreased [4]. These alterations in detoxification pathways will alter the balance between the processes of activation and detoxification of xenobiotics in diabetic animals and may result in increased susceptibility to xenobiotic-induced cytotoxicity. To investigate this possibility the cytotoxicity of menadione (2-methyl-1,4-naphthoquinone) was assessed in hepatocytes isolated from streptozotocin-induced diabetic rats.

Quinones can undergo either one-electron reduction to yield semiquinone free radicals or two electron reduction directly to the more stable hydroquinone which is then readily conjugated and excreted from the cell. Many semiquinones are readily re-oxidised in aerobic conditions and can enter redox cycles with molecular oxygen forming deleterious reactive oxygen species, causing oxidation of reduced glutathione (GSH) and ultimately cell death [5, 6]. The toxicity of menadione in isolated hepatocytes is influenced by the activities of the competing one- and two-electron reduction pathways, the glucuronic acid and glutathione conjugation reactions and the enzymes which afford cellular protection from oxidative challenge (e.g. glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase) and by intracellular GSH concentrations. The flavoprotein NAD(P)H:(quinone acceptor) oxidoreductase (also known as DT-diaphorase) catalyses the two-electron reduction of menadione directly to the hydroquinone, whereas NADPH-cytochrome *c*- and NADH-cytochrome b_5- reductases catalyse the one-electron reduction to the semiquinone radical [5].

* *Abbreviations*: MFO, mixed function oxidase; GSH, reduced glutathione.

Materials and methods

1. *Treatment of animals and assessment of the induced diabetes.* Male Sprague–Dawley rats (180–220 g) were used and were starved for 24 hr prior to receiving 60 mg/kg streptozotocin, intravenously, in acetate buffer, pH 4.5, on day 1. The animals were used for experiments on day 6 after treatment. The induced diabetes was assessed as described previously [3, 4]. On day 6 after treatment, streptozotocin-treated rats had blood glucose concentrations over 250 mg/100 ml compared with 80–100 mg/100 ml in control rats. Between 4 and 6 days after treatment diabetic rats excreted approximately 16 g glucose/24 hr in the urine.

2. *Preparation and incubation of hepatocytes.* Hepatocytes were prepared by collagenase perfusion as described previously [7] and viability was assessed by Trypan Blue exclusion. Control rat preparations were 90 ± 1% (N = 12) viable and diabetic rat preparations 87 ± 2% (N = 14). Incubations were carried out at 10^6 viable cells/ml in Krebs–Henseleit buffer, pH 7.4, containing 10 mM Hepes, in 50 ml round bottomed flasks at 37° under 95% O_2/5% CO_2. Cytotoxicity was evaluated by cell membrane damage (Trypan Blue exclusion) and depletion of GSH.

3. *Preparation of hepatic cytosol and microsomal fractions.* Rat livers were washed in ice-cold 0.01 M Tris buffer, pH 7.4, containing 1.15% (w/v) KCl, homogenised in 4 vol. of ice-cold 0.1 M Tris buffer, pH 7.4, containing 1.15% (w/v) KCl and 15% (v/v) glycerol and centrifuged at 15,000 *g* for 20 min at 0–4°. The supernatant was centrifuged again at 105,000 *g* for 50 min at 0–4° to separate the cytosolic (supernatant) and microsomal fractions. The cytosol was stored in 1 ml aliquots at −80° until required. The microsomal pellet was washed with homogenising buffer, recentrifuged at 105,000 *g* for 30 min at 0–4° and finally resuspended at 1 ml/g liver in 0.1 M Tris buffer, pH 7.4. Aliquots (0.5 ml) of the microsomal fraction were stored at −80° until required.

4. *Analytical methods.* GSH was determined by the method of Saville [8]. Cytosolic DT-diaphorase activity was measured by following the reduction of cytochrome *c* spectrophotometrically at 550 nm as described by Lind and Hogberg [9]. Glutathione peroxidase and reductase activities in cytosol were measured by following the oxidation of NADPH as described previously [10, 11]. Cumene hydroperoxide was used as the substrate for glutathione peroxidase as it is metabolised by both the selenium-dependent and -independent enzymes [10]. Microsomal NADH-cytochrome b_5 reductase activity was measured by following the reduction of potassium ferricyanide [12] and microsomal NADPH-cytochrome *c* reductase activity by the reduction of cytochrome *c* [13]. Catalase activity was measured as described by Aebi [14]. Protein was quantified by the method of Lowry and coworkers [15] using bovine serum albumin as the standard.

Results and discussion

The effect of 25 μM menadione on the viability and GSH content of control and diabetic rat hepatocytes is shown on Fig. 1A and B. In the presence of menadione the viability of diabetic rat hepatocytes declined markedly between 60 and 120 min (28.8 ± 9.0% viable in menadione treated cells compared with 82.1 ± 3.6% in untreated cells after 120 min). In contrast, the viability of control rat hepatocytes was not affected by this concentration of menadione (81.1 ± 1.9% viable in menadione treated cells compared with 86.1 ± 1.1% in untreated cells after 120 min), although higher concentrations (100 μM) caused loss of viability (data not shown). GSH depletion preceded loss in viability and this effect was also more marked in the diabetic rat cells (see Fig. 1B). Figure 2 illustrates that the threshold concentration of menadione which caused GSH depletion in diabetic rat hepatocytes was lower than that in control rat hepatocytes. It has been established that depletion of GSH and protein thiol groups by oxidative stress is critically associated with a perturbation of calcium homeostasis and loss of viability in menadione-treated hepatocytes [16].

The increased susceptibility of diabetic rat hepatocytes to menadione could be due to several factors including: (i) an alteration in the balance between the activities of the one- and two-electron reduction pathways, (ii) a decrease in the ability to detoxify the products of oxidative stress, (iii) a decrease in the intracellular GSH content, and/or (iv) a deficiency in the formation of conjugates with menadione metabolites.

In order to determine which of these factors contribute to altered menadione toxicity in diabetic rat cells the activities of several enzymes were measured in control and

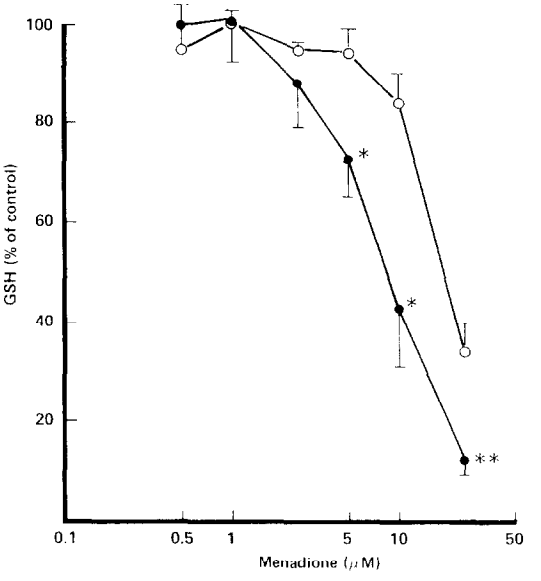

Fig. 2. Threshold concentration of menadione for GSH depletion in control (○—○, N = 5) and diabetic (●—●, N = 6) rat hepatocytes. Cells were incubated with menadione for 15 min. GSH depletion is expressed as a percentage of the GSH levels in incubations carried out in the absence of menadione. Results are means and error bars represent SE mean. * $P < 0.05$, ** $P < 0.01$, by non-paired Student's *t*-test. Significance values refer to differences between diabetic and control rat hepatocytes.

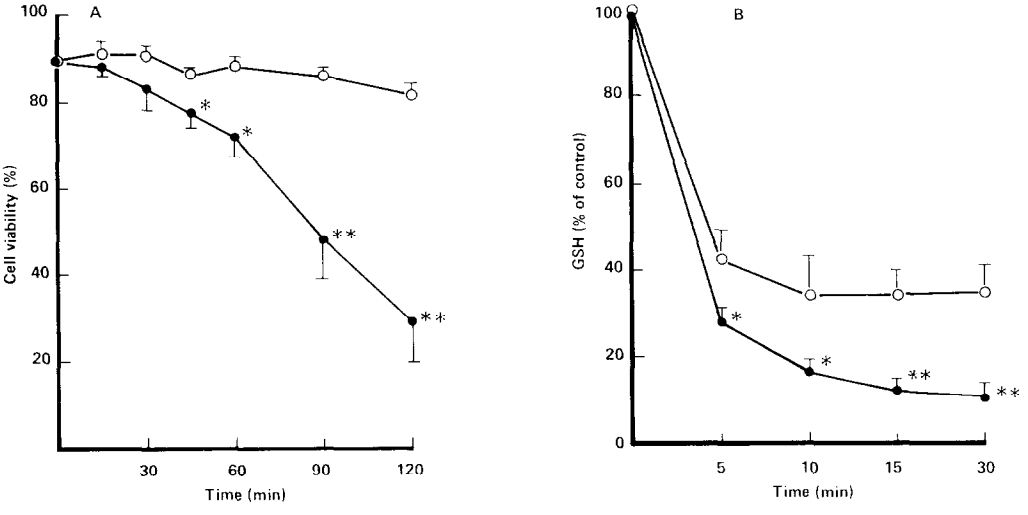

Fig. 1. Viability (A) and GSH depletion (B) in hepatocytes isolated from control (○—○, N = 6) and diabetic (●—●, N = 8) rats treated with 25 μM menadione. GSH depletion is expressed as a percentage of the initial GSH levels in the freshly isolated cells. Results are means and the error bars represent SE mean. * $P < 0.05$, ** $P < 0.01$, by non-paired Student's *t*-test. Significance values refer to differences between diabetic and control rat hepatocytes.

Table 1. Enzyme activities and glutathione content of diabetic rat liver

	NADH-cyt b_5 reductase	NADPH-cyt c reductase	DT-diaphorase
Control	830.3 ± 86.6 (6)	62.5 ± 3.6 (6)	529.9 ± 95.8 (5)
Diabetic	656.2 ± 95.6 (6)	53.3 ± 10.0 (6)	559.2 ± 45.6 (7)

	Reduced glutathione content	Glutathione reductase	Glutathione peroxidase	Catalase
Control	39.6 ± 3.2 (7)	167.7 ± 8.0 (4)	159.0 ± 29.4 (4)	72.8 ± 8.7 (4)
Diabetic	29.2 ± 1.9* (12)	142.5 ± 9.2 (7)	242.3 ± 22.2* (7)	71.8 ± 8.5 (6)

Results are expressed as the mean ± SE mean, with the number of experiments in parentheses. NADH-cytochrome b_5- and NADPH-cytochrome c-reductase activities are nmol/min/mg microsomal protein; DT-diaphorase activity is nmol/min/mg cytosolic protein; reduced glutathione content is nmol/10^6 hepatocytes; glutathione reductase and peroxidase are μmol/min/mg cytosolic protein and catalase is nmol/min/mg cytosolic protein.

* $P < 0.02$, by non-paired Student's t-test. Significance values refer to differences between control and diabetic animals.

diabetic rat liver microsomal and cytosolic fractions. Table 1 shows that the activities of NADH-cytochrome b_5 reductase, NADPH-cytochrome c reductase, DT-diaphorase, glutathione reductase and catalase, were not significantly altered in diabetic rat liver, whereas the activity of glutathione peroxidase was increased ($P < 0.02$). The activity of superoxide dismutase has been investigated previously in streptozotocin-induced diabetic rats [17, 18]. Crouch and coworkers found that 5 days after streptozotocin treatment superoxide dismutase activity was unaltered in diabetic rat liver [17], although in chronic streptozotocin-induced diabetes (12 weeks after streptozotocin treatment) there is a decrease in liver catalase, glutathione peroxidase and superoxide dismutase activities [18]. Under the conditions of our study the balance between one- and two-electron reduction and the ability to detoxify the products of oxidative stress are unaltered.

Intracellular GSH was decreased in diabetic rat hepatocytes (Table 1) and this decrease will contribute to the increased susceptibility of diabetic rat cells to menadione-induced toxicity. Hassing and coworkers [19] have shown that hepatic glutathione levels are lower in female streptozotocin-treated rats and that the diabetic animals are more susceptible to the GSH-depleting effects of high doses of paracetamol. Decreased intracellular GSH may be responsible, at least partly, for the increased susceptibility to chloroform, carbon tetrachloride and 1,1,2-trichloroethane hepatotoxicity observed in alloxan- and streptozotocin-induced diabetic rats [20–22].

The activity of UDP-glucuronyltransferase towards several substrates including 1-naphthol, 4-nitrophenol and phenolphthalein has been shown to be decreased in hepatic microsomes, perfused livers and isolated hepatocytes from streptozotocin-induced diabetic rats [3, 4, 23]. The V_{max} of the transferase enzyme is decreased in diabetic animals [3, 23] and the hepatic concentration of UDP-glucuronic acid, the essential cofactor for glucuronidation, is decreased [4]. It is therefore likely that detoxification of menadione hydroquinone by this pathway will be defective in diabetic rats. The defect in glutathione conjugation which has been previously described in diabetic rats [4] may also contribute to the increase in menadione-induced toxicity observed in streptozotocin diabetes.

Hepatocytes from diabetic rats are more susceptible to menadione-induced GSH depletion and loss in viability than are those from control rats. This is not due to an alteration in the balance between one- and two-electron reduction of the quinone, or to a decrease in the enzymes which afford cellular protection from the products of oxidative stress. Intracellular GSH levels in diabetic rat hepatocytes are lower than those in control rat hepatocytes and this factor together with diabetes-induced defects in glucuronic acid and glutathione conjugation may explain, at least partially, the increased susceptibility of diabetic rats to menadione-induced cytotoxicity.

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