Menadione increases hepatic tight-junctional permeability

Its effect can be decreased by butylated hydroxytoluene and verapamil

Kwok S. KAN and Roger COLEMAN

School of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

Infusion of menadione at two different doses [2.7 mg and 5.5 mg in 100 μ l of dimethyl sulphoxide (DMSO)] into perfused rat livers for 30 min caused no or a 6-fold increase respectively in junctional permeability to horseradish peroxidase as compared with controls receiving 100 μ l of DMSO alone. The total glutathione (GSH) contents in these livers measured at the end of the experiments were 115% and 53%, compared with the controls. The free-radical scavenger butylated hydroxytoluene (BHT) (final concn. 5 μ M) protected against the GSH depletion caused by the higher dose of menadione and partially decreased the menadione-induced increase in junctional permeability. Verapamil, a Ca^{2+} -channel blocker which was added into the perfusion medium (final concn. 40 μ M) 10 min before the infusion of 5.5 mg of menadione, completely abolished the effect of menadione on junctional permeability. Menadione exposure therefore increases tight-junctional permeability in the liver; this may involve a depletion of GSH and a subsequent increase in intracellular Ca^{2+} .

INTRODUCTION

Tight-junctional permeability in the liver can be increased by a number of compounds (vasopressin, adrenalin, angiotensin) which, by stimulating the generation of inositol phosphates, result in an increase in intracellular $[\text{Ca}^{2+}]$ (Lowe *et al.*, 1988). The Ca^{2+} ionophore A23187, which increases the movement of Ca^{2+} across the plasma membrane and the membranes of intracellular organelles, has also been shown to increase tight-junctional permeability in the isolated perfused rat liver (Kan & Coleman, 1988).

Several hepatotoxic compounds, e.g. menadione (Monte *et al.*, 1984; Orrenius *et al.*, 1988), are thought to mediate their cellular effects ultimately via disturbances of intracellular Ca^{2+} homeostasis. Whether such compounds influence tight-junctional permeability has yet to be explored.

Menadione is reduced in the liver to a semiquinone radical and, in the presence of oxygen, it is reoxidized back to menadione; this redox cycling continuously generates superoxide anion, which is rapidly removed by the glutathione peroxidase system, leading to the formation of GSSG. This substantial loss of total GSH in the liver during menadione metabolism may ultimately lead to an increase in intracellular Ca^{2+} , probably as a consequence of damage to the thiol proteins involved in gating the Ca^{2+} channels (see Monte *et al.*, 1984).

The present study examines the consequences to hepatic tight-junctional permeability of exposure of the liver to menadione, and also investigates the effects of menadione on junctional permeability in the presence of a free-radical scavenger (butylated hydroxytoluene) to prevent glutathione depletion, and of a Ca^{2+} -channel blocker (verapamil).

MATERIALS AND METHODS

Materials

Menadione, dimethyl sulphoxide (DMSO), butylated hydroxytoluene (BHT), tetramethylbenzidine, BSA, horseradish peroxidase (HRP), verapamil and other fine-grade chemicals were purchased from Sigma Chemical Co., Poole, Dorset, U.K. H_2O_2 solution (100 vol.) was obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K. The plastic cannula

tubing (PP 10) was supplied by Portex, Hythe, Kent, U.K., and the aspartate aminotransferase assay kits were obtained from Boehringer, Lewes, Sussex, U.K.

Liver perfusion

Male Wistar rats (approx. 250 g body wt. each) were used throughout the study. The bile duct was cannulated with PP 10 tubing during pentobarbital anaesthesia. The liver was isolated *in situ* as described by Hems *et al.* (1966) and perfused with 150 ml of Krebs–Ringer bicarbonate solution (Krebs & Henseleit, 1932) containing 1% (w/v) BSA, 5 mM-glucose, 1.2 mM- CaCl_2 , a physiological amino acid mixture (see Barnwell *et al.*, 1983) and 20% (v/v) packed ox red cells. The solution was continuously gassed with O_2/CO_2 (19:1) and was maintained at 37 °C in a thermostatically controlled cabinet.

Menadione infusion

This was carried out at 10 min after initial liver perfusion; 100 μ l of DMSO containing 0, 2.7 mg or 5.5 mg of menadione was infused into the liver via the portal line for a period of 30 min.

Drug pretreatments

(i) BHT, dissolved in 10 μ l of DMSO, was added to the perfusion medium to give a final concentration of 5 μ M, 5 min before the infusion of menadione (5.5 mg in 100 μ l of DMSO) or DMSO (100 μ l) alone. (ii) Verapamil, dissolved in 0.5 ml of 0.9% NaCl, was added to the perfusion medium to give a final concentration of 40 μ M, 10 min before the infusion of 5.5 mg of menadione (dissolved in 100 μ l of DMSO).

HRP pulsing

At the end of the exposure period to menadione or DMSO (controls), the liver perfusion was converted to a single-pass condition, and 3 mg or HRP, dissolved in 1 ml of Krebs–Ringer bicarbonate solution, was then infused, within 1 min, into the livers at the same portal infusion point (Kan & Coleman, 1988). At the end of 5 min as a single-pass perfusion, the liver was converted back to recycling perfusion for the rest of the experiment.

Table 1. Effect of various treatments on bile flow rate and on junctional permeability

Each value shown in the first column represents the bile flow rate at the first biliary peak of HRP (5 min), and each in the second column represents the height of the first biliary peak of HRP 5 min after pulsing with 3 mg of HRP (see the Materials and methods section). All values are means $\pm$ S.E.M. of at least four experiments. The bile flow values in rows *b*, *c*, *d* and *e* are not significantly different from that in row *a*. The asterisk by the values of the HRP peak height indicates statistical significance at $P < 0.05$ as compared with the control (*c* versus *a*) or compared with 5.5 mg of menadione (*d* and *e* versus *c*).

Treatment	Bile flow rate (μ l/min)	HRP peak height (A_{370} units/min)
(a) DMSO (100 μ l)	12.5 $\pm$ 0.6	1.1 $\pm$ 0.3
(b) Menadione (2.7 mg)	11.3 $\pm$ 0.5	1.2 $\pm$ 0.6
(c) Menadione (5.5 mg)	11.1 $\pm$ 1.1	5.9 $\pm$ 1.2*
(d) BHT (5 μ M)/menadione (5.5 mg)	11.7 $\pm$ 0.4	2.3 $\pm$ 0.8*
(e) Verapamil (40 μ M)/menadione (5.5 mg)	12.3 $\pm$ 1.2	1.0 $\pm$ 0.3*

Bile collection and analysis

Bile was collected into pre-weighed tubes, and the flow rates were determined gravimetrically (assuming a density of 1 g/ml). The HRP in the collected bile was assayed spectrophotometrically as described by Gallati & Pract (1975). At the end of the experiment, a sample of the perfusion fluid was taken for the measurement of aspartate aminotransferase by a commercial assay kit; in all cases the amount of aminotransferase in the perfusion fluid was scarcely detectable, amounting only to less than 0.014 % of the liver activity.

Liver analysis

At the end of each perfusion, the liver was removed, weighed, and homogenized in 50 ml of Krebs–Ringer bicarbonate solution. The homogenate was deproteinized in 4 % (w/v) HClO₄ and centrifuged at 10000 *g* min; the acid-soluble glutathione in the supernatant was then measured by reaction with 5,5'-dithiobis-(2-nitrobenzoate) (Tietze, 1969).

RESULTS AND DISCUSSION

Under single-pass perfusion conditions, an increase in the first peak height of biliary HRP correlates with an increase in junctional permeability (Lowe *et al.*, 1985; Kan & Coleman, 1986, 1988; Kan *et al.*, 1989); this peak height is therefore used in the present series of experiments as an indicator of junctional permeability change. In all cases of treatment described below, the biliary HRP peak occurred at 5 min and the bile-flow rates were all comparable (Table 1).

When 2.7 mg of menadione was infused into the liver no increase in HRP peak height was observed compared with the control (Table 1, rows *a* and *b*) whereas infusion of a higher dose (5.5 mg) significantly increased HRP peak height by 6-fold (Table 1, rows *a* and *c*), suggesting an increase in junctional permeability as a consequence of exposure to the higher dose.

Monte *et al.* (1984) have demonstrated that menadione causes a decrease in the total amount of cytosolic GSH in the isolated hepatocytes, and therefore GSH content was measured in the livers used above (Table 2). The GSH content of livers exposed to the lower dose of menadione was similar to that of the control (DMSO), but those exposed to the higher dose of menadione, and which showed enhanced junctional permeability, had a substantial decrease in GSH content (to 53 % of the control). Clearly, the lower dose of menadione did not overcome the ability of the liver to regenerate GSH, but had done so at the higher dose.

BHT pretreatment, which protected against menadione-induced GSH loss and restored GSH values to those of controls

Table 2. GSH content of liver after various treatments

100 % represents 3.7 μ mol/g of fresh liver. All values are means $\pm$ S.E.M. of three experiments.

Treatment	GSH content (%)
DMSO (100 μ l)	100 $\pm$ 4.8
Menadione (2.7 mg)	115 $\pm$ 12.3
Menadione (5.5 mg)	53 $\pm$ 7.5
BHT (5 μ M)/menadione (5.5 mg)	99 $\pm$ 10.7
Verapamil (40 μ M)/menadione (5.5 mg)	87 $\pm$ 10

(Table 2), also decreased the effect of menadione on junctional permeability (Table 1). However, the decrease in junctional permeability is not complete, even though the GSH content is fully restored. There are two possibilities for this: (i) the increase in junctional permeability caused by menadione is not solely due to a decrease in cytosolic GSH, but may include a further effect of menadione or its metabolites on junctions; (ii) the residual increase in permeability after BHT/menadione may be due to a complete abolition of the menadione effect, but BHT itself may have been responsible for the 2-fold increase in peak height; in this connection, studies by Billington *et al.* (1990) have demonstrated that higher doses of BHT cause substantial increase in junctional permeability in intact animals.

In other studies (Lowe *et al.*, 1988; Kan & Coleman, 1988), an increase in intracellular [Ca²⁺] has been suggested to lead to an increase in hepatic junctional permeability. The complete prevention of the 6-fold increase in junctional permeability by the Ca²⁺ blocker verapamil (Table 1, rows *c* and *e*) indicated that the effect of menadione on junctional permeability may also involve an increase in intracellular [Ca²⁺] and that this increase can therefore be prevented by blocking the [Ca²⁺] elevation.

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