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Glycogenolysis in the Rat Isolated Perfused Liver as a Measure of Chemically Induced Liver Toxicity

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Abstract—The relationship between chemically induced glycogenolysis and decreased thiol content in the rat isolated, perfused liver has been examined. Chemicals such as 2,4-dinitrophenol (DNP), diethyl maleate, alchohols and anti-inflammatory agents (except for sodium salicylate) accelerated glycogenolysis. Protein thiol loss correlated well with a marked increased rate of glucose release. Non-protein thiol loss, without significant loss of protein thiol, caused by a slight increase in the rate of glycogenolysis compared with controls. Since it has been reported that protein thiol loss rather than non-protein thiol loss is correlated to liver cell injury, a marked glucose release from the perfused liver may be a convenient measure of hepatic toxicity for a variety of chemicals.

Isolated hepatocytes have been extensively used as an experimental model to investigate mechanisms of liver injury with a variety of toxic compounds (Orrenius et al 1983). Using isolated hepatocytes, it has been reported that loss of protein thiol and/or non-protein thiol induces cytotoxicity in hepatocytes (Mitchell et al 1973; Jewell et al 1982; Di Monte et al 1984). Moore et al (1985) have shown that the induction of hepatocyte toxicity through protein thiol loss is mediated by an increase in cytosolic Ca^{2+} concentrations; i.e. protein thiol loss in isolated hepatocytes elevates cytosolic Ca^{2+} concentrations by inhibition of microsomal Ca^{2+} uptake and by inhibition of plasma membrane Ca^{2+}-ATPase activity.

The isolated, perfused liver technique has been widely used to investigate glucose metabolism (Woods et al 1974; Jakob et al 1980; Kleinek & Soling 1985; Nishihata et al 1986b). The liver is a major organ involved in regulating blood glucose concentration by an insulin-independent mechanism (Woods et al 1974). The liver stores glucose as glycogen. It has been reported that glycogenolysis in rat isolated, perfused liver was increased in the presence of toxic chemicals such as 2,4-dinitrophenol (DNP) (Jakob et al 1980; Kleinek & Soling 1985). Thus, it is of interest to investigate the effect of toxic chemicals on both thiol content and glycogenolysis in the perfused liver in terms of developing a simple method for evaluating chemical toxicity in the intact liver.

In the present report, we investigated the effect of various chemical agents on thiol content in the isolated liver to estimate toxicity. We also demonstrated the relationship between thiol loss and glycogenolysis.

Materials and Methods

Materials

Indomethacin and sodium diclofenac were supplied by Sumitomo Pharmaceutical Industry Co. (Osaka, Japan) and Ciba Geigy Japan (Takarazuka, Japan), respectively. Sodium salicylate was obtained from Nakarai Chemicals Co. (Kyoto, Japan). Diethylmaleate and 2,4-dinitrophenol (DNP) were purchased from Sigma Inc. (St. Louis, USA). Ethanol, methanol and glucose were obtained from Wako Pure Chemicals Co. (Osaka, Japan). Other reagents used were of analytical grade.

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Rat isolated, perfused liver study

Male Wistar rats (200–230 g), with free access to food and water, were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} i.p.). The isolated, perfused liver was prepared according to Soling et al (1968) and perfused with control buffer system for 5 min and then with the Ca^{2+}-free medium containing the experimental agents. The perfusate through the liver was collected at 2 min intervals for 30 or 60 min. Krebs-Henseleit bicarbonate buffer (pH 7·4) (Krebs & Henseleit 1932) containing 1% bovine serum albumin was used as the control buffer and was saturated with O_2:CO_2, 95:5, during the experiments, according to Kleinek & Soling (1985). The flow rate was 28 mL min^{-1}. After perfusing for 30 min, the liver was homogenized to measure both non-protein thiol and protein thiol.

Assays

The glucose assay with the perfusate was performed using an analytical kit (Glucose Test Wako, Wako Pure Chemicals Co., Osaka, Japan). Assays of non-protein and protein thiol were carried out by the method of Di Monte et al (1984), using glutathione as the standard thiol (Szabo et al 1981). Protein content in the homogenate was determined by the method of Lowry et al (1951), with a minor modification for sample preparation (Nishihata et al 1986a).

Results

Glucose release from the isolated, perfused liver was at a constant rate during experimental periods for 60 min, with only a slight decrease in glucose concentration over the time course (Fig. 1). Therefore, glucose release from the liver was determined at 10 and 30 min as shown in Tables 1 and 2.

The addition of DNP to the perfusate accelerated glucose release in a dose-dependent fashion. A significant effect from DNP was observed at a concentration of > 5 μM (Table 1,

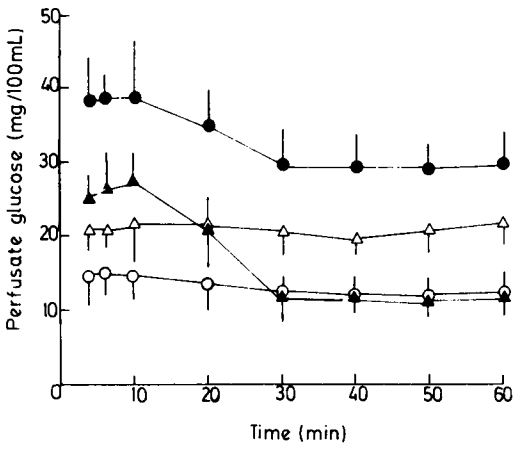

FIG. 1. The effects of various agents in the perfusate on glucose concentrations as a function of time in the perfusate after passing through the rat isolated, perfused liver. Key: ○, no additive; ●, 50 μM DNP; △, 1 mM diethyl maleate; ▲, 50 mM salicylate. Each value represents the mean ± s.d. (n = 3).

Table 1. Glucose concentrations in perfusate after passing through the rat isolated, perfused liver, and non-protein thiol and protein thiol concentrations in liver tissue after a 30 min-perfusion.

	Glucose concentration mg/100 mL		Thiols, μmol g^{-1} protein	
Agents	10 min	30 min	Non-protein	Protein
No additive (control)	14·1 ± 3·2	12·4 ± 1·7	31·9 ± 4·2	107·6 ± 10·1
DNP				
0·5 μM	14·9 ± 3·0	13·1 ± 2·5	32·6 ± 2·9	106·5 ± 8·1
5 μM	38·4 ± 7·6*	29·7 ± 4·5*	31·7 ± 3·2	79·8 ± 6·9*
50 μM	43·2 ± 7·7*	36·1 ± 4·2*	19·2 ± 4·8	69·7 ± 7·1*
Diethyl maleate				
0·2 mM	19·7 ± 3·4	17·2 ± 1·9**	20·9 ± 4·2*	106·3 ± 11·7
1·0 mM	21·5 ± 4·7	20·6 ± 3·2**	16·8 ± 4·0*	102·4 ± 10·1
5·0 mM	33·2 ± 5·6*	29·7 ± 3·4*	9·3 ± 1·4*	75·6 ± 4·2*
Ethanol				
10 mM	16·1 ± 5·4	13·2 ± 2·4	35·2 ± 5·6	111·6 ± 17·9
100 mM	16·6 ± 3·1	16·7 ± 5·2	28·2 ± 7·1	97·2 ± 8·4
500 mM	22·63 ± 3·2**	21·8 ± 2·5**	22·6 ± 4·1*	99·3 ± 6·9
1 M	24·5 ± 4·2**	22·9 ± 4·6**	21·1 ± 2·7*	95·6 ± 7·3
Methanol				
1 mM	12·7 ± 1·6	13·6 ± 2·9	30·6 ± 2·5	101·6 ± 11·9
10 mM	24·4 ± 6·8**	20·6 ± 3·1**	32·2 ± 5·6	90·4 ± 6·3
100 mM	39·2 ± 7·1*	32·4 ± 4·6*	27·9 ± 2·4	79·6 ± 4·5*

Each value represents the mean ± s.d. (n > 4).
*, $P < 0.01$ versus no additive (Student's t-test); **, $P < 0.05$ versus no additive.

Table 2. Glucose concentrations in perfusate after passing through the rat isolated, perfused liver, and non-protein thiol and protein thiol concentrations in the liver after a 30 min perfusion.

	Glucose concentration mg/100 mL		Thiols, μmol g^{-1} protein	
Agents	10 min	30 min	Non-protein	Protein
Sodium diclofenac				
1 μM	15·9 ± 3·6	13·7 ± 1·9	29·6 ± 3·1	102·4 ± 10·9
10 μM	17·2 ± 4·1	13·6 ± 3·8	31·2 ± 4·2	101·2 ± 8·7
100 μM	21·5 ± 1·4**	22·6 ± 2·4**	24·1 ± 2·8*	92·7 ± 8·6
indomethacin				
1 μM	16·7 ± 3·1	14·2 ± 4·2	31·4 ± 2·7	104 ± 10·1
10 μM	20·3 ± 4·6**	23·6 ± 4·2**	28·6 ± 3·7	82·1 ± 5·3**
100 μM	29·2 ± 5·6*	32·1 ± 4·2*	25·1 ± 4·6**	79·7 ± 4·2*
sodium salicylate				
1 mM	14·1 ± 4·9	12·7 ± 4·2	30·6 ± 3·7	106·4 ± 16·2
10 mM	15·3 ± 4·1	12·8 ± 3·6	32·5 ± 4·2	98·7 ± 9·1
25 mM	17·4 ± 5·1	14·6 ± 2·7	28·0 ± 2·6	99·2 ± 8·5
50 mM	26·3 ± 4·6**	10·1 ± 3·7	27·4 ± 4·2	95·4 ± 0·8

Each value represents the mean ± s.d. (n > 4).
*, $P < 0.01$ versus no additive in Table 1; **, $P < 0.05$ versus no additive in Table 1.

Fig. 1). DNP at 5 μM caused a significant decrease in protein thiol concentration without affecting non-protein thiol (Table 1). DNP at 50 μM caused loss of both thiol types. The glucose release in the presence of DNP at 5 μM or at 50 μM was 2·5 to 3 times greater compared with control value.

Diethyl maleate at 0·2 or 1 mM also accelerated glucose release by a factor of 1·5 compared with controls (Table 1). This was significantly less than in the presence of 5 μM DNP. Diethyl maleate at 0·2 or 1 mM caused a significant loss of non-protein thiol, but did not affect protein thiol (Table 1). When the concentration of diethyl maleate in the perfusate increased to 5 mM, the increased glucose release was similar to that seen with 5 μM DNP. There was also a significant loss of protein thiol.

The presence of ethanol, up to 1 M, did not affect the concentration of protein thiol in the liver homogenate, but non-protein thiol was observed when the ethanol concentration was more than 500 mM (Table 1). An increase in glucose release was observed in the presence of 500 mM or 1 M ethanol, as well as in the presence of 1 mM diethyl maleate. These losses were less than those induced by 5 μM DNP (Table 1).

Methanol at 100 mM caused a significant release of glucose similar to that observed using 5 μM DNP, along with decreased protein thiol concentrations, but without significant changes in non-protein thiol (Table 1).

Among the anti-inflammatory agents used, the effect of indomethacin at 100 μM on preparation was similar to that of 5 μM DNP (i.e. a significant increase in glucose release and protein thiol loss were observed without a significant change of non-protein thiol, Table 2). Indomethacin, < 10 μM had no effect.

Sodium diclofenac had no effect at concentrations up to 10 μM. Sodium diclofenac at 100 μM caused a significant loss of non-protein thiol and an increase in rate of glucose release, as was observed with 0·2 mM diethyl maleate. There was no change in protein thiol content (Table 2).

The presence of salicylate in the perfusate did not markedly influence glucose release nor the concentration of either thiol types, when salicylate concentrations were less than 25 mM. Salicylate at 50 mM induced a slight decrease in both thiols (but not significant) (Table 2). An increase in glucose release was observed at an early stage after perfusion of 50 mM salicylate, but later rapidly approached control value (Fig. 1 and Table 2).

Discussion

It has been documented that glucose release from the rat isolated perfused liver occurs by glycogenolysis (Kleinek & Soling 1985), because the perfusate did not contain any precursors for gluconeogenesis (Woods et al 1974). Glycogenolysis in the liver is mediated by an activation of phosphorylase kinase (Klee et al 1979). It has been reported that an activation of phosphorylase kinase is induced possibly by two different mechanisms (Jakob et al 1980): (i) the activation is correlated with a decrease in the tissue ATP content and an increase in AMP, or (ii) the activation is induced by an increase in cytosolic Ca^{2+} concentration, independent of AMP.

It has been demonstrated (Jakob et al 1980; Kleinek &

Soling 1985) that the increase in glycogenolysis by DNP occurs by the first mechanism.

The second mechanism involves the activation of phosphorylase kinase through α-adrenergic agonists and vasopressin (Assimacopoulos et al 1977; Kleinek & Soling 1985). Kleinek & Soling (1985) have also demonstrated that 4·5 μM DNP accelerated glucose release by about three times compared with controls. The injury of mitochondria or microsomes by chemical substances reduces the capability of these organelles to store Ca^{2+}, and caused a release of Ca^{2+} from storage pools (Dabinsky & Cockrell 1975; Kleinek & Stratman 1975; Chen et al 1978; Blackmore et al 1982; Murphy et al 1980).

In the present study, the effects of DNP (5 μM) and diethyl maleate (5 mM) on glucose release, were accompanied by a significant protein thiol loss. Diethyl maleate (5 mM) also caused significant non-protein thiol loss in contrast to 5 μM DNP. We therefore consider protein thiol loss to be more closely correlated with significant increased glycogenolysis, rather than non-protein thiol loss.

Uncouplers such as DNP caused a decrease in ratio of ATP/AMP by inhibition of ATP production and also induction of NAD(P)H oxidation (Mannella & Persons 1977). It has been reported (Di Monte et al 1984; Moore et al 1985) that NAD(P)H oxidation resulted in thiol loss and a rapid depletion of the mitochondrial Ca^{2+} pool. Thus, a marked increase in glycogenolysis induced by DNP is correlated either with a decrease in the ratio of ATP/AMP as reported (Jakob et al 1980; Kleinek & Soling 1985), or with protein thiol loss, as was observed in the present study. However, it is not clear at the present time why 5 μM DNP induced only protein thiol loss without loss of non-protein thiol. It has been reported that protein thiol loss inhibited Ca^{2+}-ATPase along with an increase in the cytosolic Ca^{2+} concentration in hepatocytes (Nicotera et al 1985). Thus, the greatly increased release of glucose along with protein thiol loss may partly be due to high cytosolic Ca^{2+} concentrations. It has also been reported that the calcium ionophore A23187 caused a release of Ca^{2+} from the extramitochondrial Ca^{2+} pool, but only caused a small increase in glucose release (DiMonte et al 1984; Kleinek & Soling 1985). This suggests that Ca^{2+} release from the intracellular Ca^{2+} pool, induced by A23187, was followed by the release of Ca^{2+} from the cells. It has also been reported that non-protein thiol loss, induced by diethyl maleate in isolated hepatocytes, not only caused a release of Ca^{2+} from the intracellular pool (Thor et al 1984), but also caused a rapid release of Ca^{2+} from the cells (Jewell et al 1982). Thus, the slight increase in glucose release by 0·2 mM diethyl maleate or 1 mM diethyl maleate may be due to a slight increase in cytosolic Ca^{2+}, without loss of protein thiol.

It has been suggested that hepatocyte injury accompanied by glutathione depletion, may be the result of some change secondary to such glutathione depletion (Di Monte et al 1984). Protein thiol oxidation, following significant glutathione depletion, as observed in the presence of 5 mM diethyl maleate (Table 1), might induce a marked increase in cytosolic Ca^{2+} concentration, resulting in a decrease in cell viability as reported by Moore et al (1985). Protein thiol loss rather than non-protein thiol loss is more closely linked to hepatic injury (Moore et al 1985). Thus, a marked increase in glycogenolysis, correlated with protein thiol loss, may indicate sever chemical toxicity.

The toxic effects of ethanol seem to occur in a manner similar to that seen with diethyl maleate (up to 1 mM). However, methanol may cause hepatic toxicity through a rapid depletion of protein thiol as with DNP (more than 5 μM).

Hepatic toxicity induced by anti-inflammatory drugs, at concentrations greater than therapeutic concentrations, seems to be different, depending on the individual drug. Indomethacin caused a rapid protein thiol loss, but sodium diclofenac produced a rapid non-protein thiol loss. The toxicity of salicylate is not clear in the present study at concentrations less than 50 mM. Salicylate at 50 mM accelerated glucose release only during the early stages of the perfusion.

Glycogenolysis, which was correlated with protein thiol loss, was more pronounced than that which was correlated with non-protein thiol loss. Thus, we conclude that the degree of accelerated glycogenolysis in the perfused liver is a measure of both thiol loss and hepatic injury.

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