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Effects of salicylate on rat liver in short-term toxicity studies

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It has become increasingly evident that salicylates can cause acute hepatic damage in human beings, albeit usually mild and often revealed by elevated serum transaminase levels without jaundice or other clinical manifestations [1, 2]. Biopsies have revealed a variable picture but usually show focal hepatocellular injury including vacuolation, ballooning degeneration and focal necrosis, and mild periportal inflammation. Severe acute damage and chronic injury are relatively rare events.

In experimental animals, acute or multiple high dose regimens of salicylates have been associated with mild liver toxicity, but little overt histopathologic evidence of hepatocellular degeneration or necrosis [3–8]. Damage has also been inferred from increases in transaminases or other enzyme activities in the blood. The purpose of the present study was to assess the acute and subacute effects of high doses of salicylate on rat liver by biochemical, including hepatic and more liver-specific plasma markers of damage, as well as morphologic evaluations. In addition, these experiments were corroborated by short-term studies in which salicylate was incubated at high concentrations with suspensions of isolated rat hepatocytes.

Materials and methods

Male albino Wistar rats (approximately 250 g) were obtained from Charles River Canada Inc. and supplied with water and Purina Certified Rodent Chow No. 5002 *ad lib*. Salicylic acid (analytical grade, BDH Chemicals Canada Limited) in distilled water was adjusted to pH 7.2 and administered orally by gavage.

Heparinized blood was collected at sacrifice and the plasma analyzed for alanine and aspartate aminotransferase (Worthington Statzyme Kits), for 5′-nucleotidase and sorbitol dehydrogenase (Sigma Diagnostic Kits) using a Rotochem CFA 2000 centrifugal analyzer, and for ornithine carbamyl transferase using a manual method [9]. For histopathologic evaluation, tissue was fixed in formalin and embedded in paraffin; then sections were stained with periodic acid-Schiff or hematoxylin and eosin. The remaining liver was homogenized in 0.25 M sucrose (pH 7.4). Reduced glutathione (sulfosalicylic acid non-precipitable sulfhydryl) was determined in the liver homogenate [10]. Microsomes were prepared with calcium chloride [11] and assayed for cytochrome(s) P-450 [12], cytochrome *c* reductase [13], aniline hydroxylase [13], aminopyrine demethylase [13], glucose-6-phosphatase [14], and protein [15].

Isolated hepatocytes were obtained by perfusion of the liver via the portal vein with 0.5 mM ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA) in Hanks' balanced salt solution (without Ca^{2+}) followed by 0.05% collagenase (Sigma Type IV or Boehringer Mannheim) dissolved in Williams' Medium E (Gibco Laboratories). The incubation medium consisted of Waymouth MB 752/1 medium supplemented with 17.5% heat-inactivated horse serum (Gibco Laboratories), 25 mM *N*-2-hydroxyethylpiperazine-*N*^{1}-2-ethanesulfonic acid (HEPES), and heparin (10 I.U./ml). Isolated hepatocytes were incubated at 37° under 95% O_2:5% CO_2 using a rotary evaporator fitted with five round bottom flasks [16]. The pH of each flask was adjusted to 7.5 after addition of salicylate but before the introduction of hepatocytes. Viability of hepatocytes was assessed by trypan blue exclusion [17] and the lactate dehydrogenase latency test [18]. After 4 hr of incubation, the contents of each flask were centrifuged at low speed. The pelleted cells were analyzed for glutathione levels [19], while the medium (supernatant) was analyzed for transaminase activities as an index of enzyme leakage from cells.

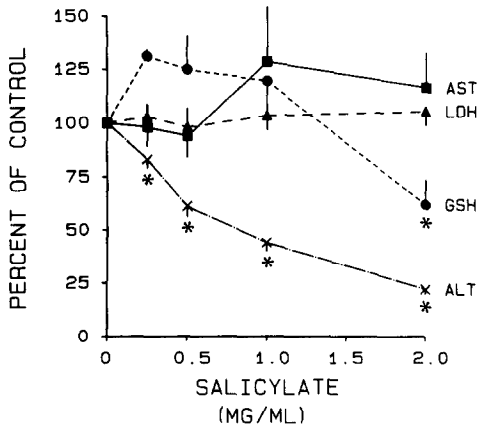

Fig. 1. Effects of a range of salicylate concentrations after 4 hr of incubation on suspensions of isolated rat hepatocytes. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined in the incubation medium; lactate dehydrogenase latency (LDH) was measured in aliquots of cells and medium; and glutathione (GSH) levels were determined in the cells. Results are presented as percentages (mean ± SE, N = 4) of control incubations in which salicylate was absent. Absolute control values after a 4-hr incubation were: AST, 209 I.U.; ALT, 73 I.U./L; LDH, 35%; and GSH, 4.44 μmol/g liver cells. Key: (*) statistically significant difference ($P < 0.05$) from controls.

Results are presented as mean ± standard error of at least four animals. Statistical significance was determined by Student's *t*-test for *in vivo* experiments or by repeated measures one-way analysis of variance and subsequent Newman–Keuls multiple range test where appropriate for the isolated hepatocyte data.

Results and discussion

The effects of salicylate on selected hepatic and plasma variables were determined 4 hr after a single 800 mg/kg dose (acute study), and 18 hr after the last of three 500 mg/kg/day doses (subacute study); the results are shown in Tables 1 and 2 respectively. The effects of different salicylate concentrations on suspensions of isolated rat hepatocytes are shown in Fig. 1. The lower concentrations (0.25 and 0.50 mg/ml) are similar to blood levels in human beings showing toxicity [2], whereas the higher concentrations would represent severe intoxication and are probably not achievable *in vivo* for any appreciable length of time.

The liver/body weight ratio was reduced significantly by salicylate in the acute study and was probably due, at least in part, to the marked glycogen depletion which was seen microscopically and occurred primarily in periportal and mid-zonal regions. Transient decreases in liver glycogen concentration after high acute doses of salicylates have been reported previously [5, 7, 20]. There were no other microscopic changes in either the acute or subacute study.

Table 1. Effect of a single 800 mg/kg dose of salicylate on hepatic and plasma variables at 4 hr post-dose

Variable	Salicylate	Control
Liver/body weight (g/100 g)	3.75 ± 0.11*	4.53 ± 0.15
Cytochrome P-450 (nmol/mg protein)	0.60 ± 0.06	0.60 ± 0.05
Cytochrome *c* reductase (nmol/mg protein/min)	2.78 ± 0.12	2.42 ± 0.10
Aniline hydroxylase (nmol/mg protein/min)	1.22 ± 0.19	1.16 ± 0.09
Aminopyrine demethylase (nmol/mg protein/min)	2.30 ± 0.63	2.37 ± 0.14
Glucose-6-phosphatase (μmol/mg protein/min)	0.190 ± 0.020	0.170 ± 0.004
Glutathione (μmol/g liver)	6.20 ± 0.44*	7.66 ± 0.07
Plasma alanine aminotransferase (I.U./L)	53.3 ± 4.1*	28.5 ± 1.9
Plasma aspartate aminotransferase (I.U./L)	148 ± 26*	72 ± 2
Plasma sorbitol dehydrogenase (I.U./L)	0.73 ± 0.10	0.70 ± 0.14

Values are means ± SE, N = 4.
* Statistically significant difference ($P \leq 0.05$) from control.

Table 2. Effect of three 500 mg/kg/day doses of salicylate on hepatic and plasma variables 18 hr after the last dose

Variable	Salicylate	Control
Liver/body weight (g/100 g)	5.64 ± 0.17	5.15 ± 0.18
Cytochrome P-450 (nmol/mg protein)	0.70 ± 0.7	0.60 ± 0.04
Cytochrome *c* reductase (nmol/mg protein/min)	62.4 ± 7.1	55.5 ± 3.0
Aniline hydroxylase (nmol/mg protein/min)	1.27 ± 0.11*	0.83 ± 0.10
Glucose-6-phosphatase (μmol/mg protein/min)	0.258 ± 0.013*	0.332 ± 0.008
Glutathione (μmol/g liver)	8.44 ± 0.21*	7.09 ± 0.23
Plasma aspartate aminotransferase (I.U./L)	75 ± 6*	55 ± 1
Plasma alanine aminotransferase (I.U./L)	45 ± 2*	33 ± 2
Plasma sorbitol dehydrogenase (I.U./L)	10.0 ± 1.3	11.4 ± 1.3
Plasma ornithine carbamyl transferase (I.U./L)	0.37 ± 1.0	0.35 ± 0.07
Plasma 5'-nucleotidase (I.U./L)	12.8 ± 1.1	13.5 ± 0.3

Values are means ± SE, N = 4.
* Statistically significant difference ($P \leq 0.05$) from control.

While glucose-6-phosphatase activity was reduced in rats given salicylate in the subacute study, there were no decreases in cytochrome(s) P-450 levels or microsomal mixed-function oxidase activities in either *in vivo* study which might be indicative of hepatotoxicity. Salicylate has been reported to cause a small change in the difference spectrum of cytochrome P-450 *in vitro* [6].

Plasma transaminase activities in the salicylate-treated groups were slightly, but significantly, higher than control activities in both the acute and subacute studies, which is in agreement with previously reported work [3, 20]. However, sorbitol dehydrogenase and ornithine carbamyl transferase which are considered more hepato-specific markers of injury [1], were unchanged compared to control activities. Possible explanations for these findings are that the increased transaminase activities may reflect leakage from non-hepatic sources or that transaminases, while somewhat less liver-specific than sorbitol dehydrogenase and ornithine carbamyl transferase, are more sensitive markers of hepatic injury, particularly at the low levels shown in the present study. Indeed the lack of change in ornithine carbamyl transferase and 5'-nucleotidase may be a result of their primarily non-cytosolic cellular locations (mitochondrial and plasma membrane, respectively); however sorbital dehydrogenase is mainly a cytoplasmic enzyme.

In the suspensions of isolated hepatocytes, salicylate caused a dose-dependent decrease in alanine aminotransferase activity in the medium, presumably due to direct

enzyme inhibition. Alanine aminotransferase is the most sensitive of the transaminases to the general inhibitory action of high concentrations (10 mM) of salicylate on this group of enzymes [20]. The initial concentrations of salicylate in the isolated hepatocyte incubation medium (0.25 to 2.0 mg/ml) would have corresponded to levels of 0.14 to 1.12 mM in the final assay volume for alanine aminotransferase activity. However, at slightly higher assay concentrations of 1.25 and 2.5 mM, salicylate did not reduce rat serum alanine aminotransferase activity significantly [21], a finding consistent with our own (data not shown) in which salicylate was added to control rat plasma at the concentrations to which the isolated hepatocytes were exposed; therefore, it is considered unlikely that salicylate-induced elevations of alanine aminotransferase were underestimated significantly in the *in vivo* experiments.

The reason why salicylate at similar concentrations reduced alanine aminotransferase activity in the medium of the isolated hepatocytes, but not in plasma, is probably related, at least in part, to the much higher albumin levels in the plasma to which salicylate binds extensively.

The small increase in aspartate aminotransferase in the medium of the isolated hepatocyte incubations at the two highest salicylate concentrations is suggestive of mild hepatotoxicity; however, there were no morphologic alterations (trypan blue-stained cells) or changes in lactate dehydrogenase latency of the isolated hepatocytes. These results differ from those of Tolman *et al.* [22] who reported that, under similar conditions of oxygenation and time course, salicylate at concentrations up to 0.4 mg/ml damaged liver cells in monolayer culture, determined by lactate dehydrogenase activity in the medium. Therefore, it appears that, under conditions of cell culture, hepatocytes appear to be more susceptible to injury by salicylate than in cell suspensions or *in vivo*.

The glutathione content of the liver was decreased in the acute study, but increased in the subacute study. In isolated hepatocytes, the glutathione level increased at all but the highest salicylate concentration where it was significantly lower. Kaplowitz *et al.* [8] also showed that liver glutathione is lowered by a high dose of acetylsalicylic acid *in vivo* as well as a high concentration of acetylsalicylic acid in liver slices *in vitro*, and it was suggested that the cause was leakage from hepatocytes. Conceivably, the increased hepatic glutathione noted in our subacute study may represent a response to repeated acute loss of glutathione, whatever the mechanism.

In summary, while the results of the isolated hepatocyte experiments appear somewhat equivocal in relation to the *in vivo* findings, these studies indicate that, at least in the short term, healthy rat liver is relatively resistant to overt salicylate-induced hepatotoxicity.

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