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EFFECTS OF METHOXSALEN ON THE METABOLISM OF ACETAMINOPHEN IN HUMANS

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Abstract—We reported recently that the drug methoxsalen, a potent suicide inhibitor of hepatic cytochrome P-450, decreases the metabolic activation of acetaminophen and prevents its hepatotoxicity in mice. We have now studied the effects of methoxsalen on the metabolism of acetaminophen in humans. *In vitro*, 100 μM methoxsalen decreased by 40% the covalent binding of a [^{3}H]acetaminophen metabolite to microsomal proteins after incubation of [^{3}H]acetaminophen with human liver microsomes and an NADPH-generating system. *In vivo*, a single oral dose of methoxsalen (30 mg), given 3 hr before acetaminophen (1 g), decreased by 38% the partial apparent oral salivary clearance of acetaminophen into glutathione-derived conjugates (the end products of its oxidative metabolism) in nine human volunteers. These observations demonstrate that methoxsalen decreases the metabolic activation of acetaminophen in humans.

Methoxsalen is a natural furocoumarin derivative present in many plant species, including some edible ones, such as figs, celery, parsley or parsnip [1]. Oral administration of methoxsalen, followed by exposure to ultraviolet A light has photosensitizing effects, which are used in the treatment of psoriasis [2]. Methoxsalen is a potent suicide inhibitor of hepatic cytochrome P-450 in rats [3]. Its potency is similar to, or greater than, that of piperonyl butoxide or SKF 525-A (two potent experimental inhibitors not used in humans) and much greater than that of the drug cimetidine [3]. Recently, we have observed that the destruction of cytochrome P-450 by a methoxsalen metabolite was much greater in human liver microsomes than in rat liver microsomes [4].

Acetaminophen hepatotoxicity is related to its oxidation by hepatic cytochrome P-450 into a highly reactive metabolite (probably, N-acetyl *p*-benzoquinone imine) and to the resulting depletion of liver glutathione [5]. Recently we have observed that the administration of methoxsalen decreases the metabolic activation of acetaminophen to its reactive metabolite, limits the depletion of liver glutathione and prevents the hepatotoxicity of high doses of acetaminophen in mice [6].

This prompted us to test the possible relevance of these results in humans. As a preliminary step, we have determined the effects of methoxsalen on the *in vitro* and *in vivo* metabolism of acetaminophen in humans.

MATERIALS AND METHODS

In vitro *studies*. Human liver microsomes were obtained and prepared as described previously [4, 7, 8]. A liver specimen was obtained by surgical biopsy in six patients undergoing laparotomy for various reasons (digestive carcinoma, gall-bladder stones and liver tumor). We excluded alcoholic patients or those who had taken drugs known to induce microsomal enzymes [9] during the two weeks preceding surgery; patients taking other drugs were not excluded inasmuch as drugs had to be taken for premedication and anesthesia anyway. Patients were premedicated with alimemazine, atropine and hydroxyzine, and were anesthetized with droperidol, enflurane, fentanyl, thiopental sodium and pancuronium bromide. Part of the liver specimen was placed in Bouin's fluid and sent to the pathologist. The remaining part of the liver sample was stored at −20° until the conclusion of the pathologist was known. Only those liver fragments with a normal liver histology were used in the present study. The liver fragment was thawed, blotted dry, weighed and homogenized in 3 vol. of ice-cold 0.154 M KCl, 0.01 M sodium/potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was centrifuged at 100,000 *g* for 60 min. Microsomal pellets were stored at −20° until analysed, 1–8 days later. [^{3}H]Acetaminophen (0.5 mM, 0.5 μCi/ml) was incubated in 1 ml of 0.154 M KCl, 0.1 M sodium/potassium phosphate buffer, pH 7.4, containing microsomes from 125 mg of blotted dry liver and a reduced nicotamide adenine dinucleotide phosphate (NADPH)-generating system including NADP (1 mM), glucose 6-phosphate (6 mM), glucose 6-phosphate dehydrogenase (3 enzyme units), and EDTA (1.5 mM). 100 μM Methoxsalen was added in some incubation mixtures. An aliquot was taken at zero-time and the mixture was incubated with shaking under air, at 37° for 15 min. The amount of [^{3}H]metabolite irreversibly bound to microsomal proteins was determined as previously described [6, 10]. Briefly, pro-

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teins were precipitated with trichloroacetic acid (TCA), washed three times with TCA and repeatedly extracted with various solvents (methanol, twice; n-heptane, twice; ethyl ether, once). Radioactivity could not be removed further by introducing additional solvent extractions [6]. Proteins were then dissolved in 1 M NaOH, and after neutralization with HCl, were counted for ^{3}H-radioactivity. Radioactivity in the zero-time sample was subtracted from that in the incubated sample.

Microsomal protein concentration was determined by the method of Lowry *et al.* [11].

In vivo *studies*. The metabolism of acetaminophen was studied on two occasions in nine volunteers. The nine volunteers (five authors and four other members of the medical staff) were healthy males, 25 to 40 years-old. None had evidence of past or present liver disease. All were non-smokers. They took no other drug for two weeks before the study and throughout the test, and drank no alcohol on the day of acetaminophen administration and sample collection. All gave their informed consent for the study. After a light breakfast, taken between 7:30 and 8:00 a.m., each subject received a single oral dose of 1 g acetaminophen in commercially available tablets (Doliprane®) with 100 ml of water at 10:00 a.m. Five minutes later, the mouth was rinsed three times with water to avoid any direct contamination from the tablets. Saliva samples (approximately 1 ml) were collected by direct salivation into plastic vials, 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 8 hr after acetaminophen administration, and were stored at −20° until analysed. Urine was collected for 24 hr, and analysed the day after collection. Seven to fourteen days later, each subject received a single oral dose of 30 mg of methoxsalen (Meladinine®) at 7:00 a.m., followed, 3 hr later, by acetaminophen. The same experimental protocol was then repeated.

The concentration of acetaminophen in saliva were measured by a high performance liquid chromatographic (HPLC) assay as described by Knox and Jurand [12] after an extraction performed after Miners [13]. The concentrations of acetaminophen and its metabolites in urine were measured by two different HPLC assays [10, 12] because we found that human urine naturally contained some contaminants which interfered with the determination of some acetaminophen metabolites (different for each method). Therefore, unchanged acetaminophen and its cysteine and mercapturic acid conjugates were determined by the HPLC assay of Knox and Jurand [12], while conjugates to glucuronic acid and sulfate were determined as described elsewhere [10]. Acetaminophen and its metabolites were quantitatively measured in human urine by the methods employed.

Acetaminophen kinetics were estimated from the salivary acetaminophen concentration–time curve. After reaching a peak, the natural logarithm of the salivary concentration of acetaminophen decreased linearly with time. The slope of the linear portion (β) was calculated by least square regression analysis. The half-life was calculated as $0.639/\beta$. The area under the curve of the salivary acetaminophen concentration as a function of time (AUC) was calculated using the trapezoidal rule and extrapolation to infinity [14]. The apparent oral salivary clearance (Cl_{os}) was calculated as described by Miners [13] as: Cl_{os} = dose/(AUC × body weight). The partial apparent oral salivary clearance of acetaminophen (Cl_m) to a given metabolite was calculated as: Cl_m = fm × Cl_{os}, where fm is the fraction of the total acetaminophen-derived products recovered in urine, excreted as the metabolite. Glutathione-derived conjugates were calculated as the sum of cysteine and mercapturic acid conjugates.

Statistical analysis. Results were expressed as means ± SEM. The *t* test for paired data was used to compare the differences between means.

RESULTS

Effects of methoxsalen on the in vitro *covalent binding of an acetaminophen metabolite to microsomal proteins*

Incubation of [^{3}H]acetaminophen with a NADPH-generating system and microsomes prepared from fragments of six human livers resulted in the *in vitro* covalent binding of a reactive acetaminophen metabolite to microsomal proteins. This covalent binding was decreased by 17% to 90% when methoxsalen (100 μM) was added to the incubation mixture; the mean decrease was 40% (Fig. 1).

Effects of methoxsalen on the in vivo *metabolism of acetaminophen*

The salivary concentrations of acetaminophen (Fig. 2), the apparent oral salivary clearance of acetaminophen (mean ± SEM: 4.82 ± 0.35 ml·min^{-1}·kg^{-1} before methoxsalen, and 4.42 ± 0.17 after methoxsalen) and its half-life (127 ± 9 min before, and 137 ± 7 min after) were not significantly modified when measured again after the administration of methoxsalen.

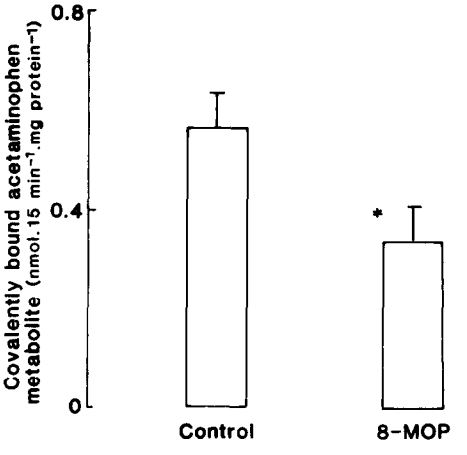

Fig. 1. Effects of methoxsalen on the *in vitro* covalent binding of an acetaminophen metabolite to hepatic microsomal proteins. [^{3}H]acetaminophen (0.5 mM, 0.5 μCi/ml) was incubated at 37° for 15 min with an NADPH-generating system, EDTA (1.5 mM) and hepatic microsomes in the presence ("8-MOP") or in the absence ("Control") of 100 μM methoxsalen. Results are means ± SEM for six human livers. The asterisk indicates a significant difference from the incubation made without methoxsalen (*t* test for dependent data), $P < 0.01$.

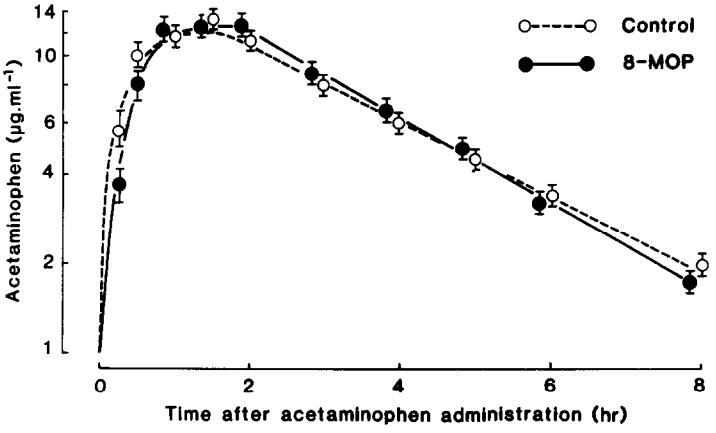

Fig. 2. Salivary concentration of acetaminophen. Acetaminophen (1 g) was ingested alone ("Control") or 3 hr after the oral administration of 30 mg of methoxsalen ("8-MOP"). Results are means ± SEM for nine volunteers.

Table 1. Effects of methoxsalen on the urinary excretion of acetaminophen and its metabolites and on the partial clearance of acetaminophen to various metabolites

	Unchanged acetaminophen	Glucuronide conjugate	Sulfate conjugate	Glutathione-derived conjugates	Total
Urinary excretion (% of recovered dose of acetaminophen)					
Control determination	4.3 ± 2.4	59.5 ± 2.6	30.7 ± 2.4	5.4 ± 0.5	101 ± 3
After methoxsalen	5.1 ± 0.3	60.3 ± 2.2	30.8 ± 1.8	3.8 ± 0.6*	99 ± 3
Partial oral salivary clearance of acetaminophen to various metabolites (ml/min/kg)					
Control determination	0.21 ± 0.03	2.87 ± 0.25	1.48 ± 0.14	0.26 ± 0.03	4.82 ± 0.35
After methoxsalen	0.22 ± 0.02	2.68 ± 0.17	1.36 ± 0.08	0.16 ± 0.02†	4.42 ± 0.17

Glutathione-derived conjugates were measured as the sum of the cysteine and mercapturic acid conjugates. Partial apparent oral salivary clearance of acetaminophen to various metabolites (Cl_m) was calculated as: $Cl_m = fm \times Cl_{os}$, fm being the percent of the recovered dose of acetaminophen in urine excreted as the metabolite, and Cl_{os} being the apparent oral salivary clearance of acetaminophen. Results are means ± SEM for nine subjects.

* Significantly different from that in the control determination (Student's *t* test for dependent data), $P < 0.05$.

† $P < 0.01$.

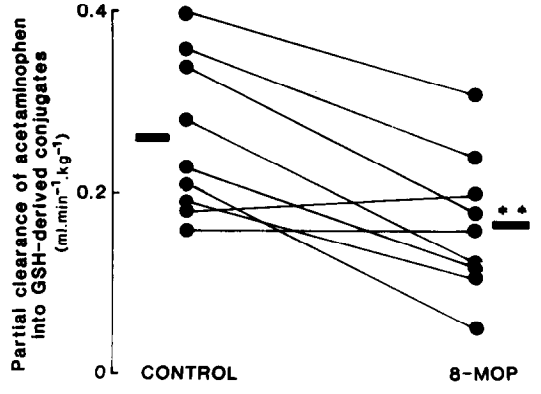

Fig. 3. Partial apparent oral salivary clearance of acetaminophen into glutathione-derived conjugates. Acetaminophen (1 g) was ingested alone ("Control") or 3 hr after the oral administration of 30 mg of methoxsalen ("8-MOP"). Results are means ± SEM for nine volunteers. The asterisks indicate a significant difference from the control clearance (*t* test for dependent data), $P < 0.01$.

The recovery of the dose of acetaminophen in 24 hr urine as unchanged acetaminophen or as metabolites, was similar before and after methoxsalen administration (Table 1). The urinary excretion of acetaminophen and that of its glucuronide and sulfate conjugates were unchanged after methoxsalen administration (Table 1). In contrast, the urinary excretion of glutathione-derived conjugates was decreased by 30% after administration of methoxsalen (Table 1). The mean value for the partial apparent oral salivary clearance of acetaminophen into glutathione-derived conjugates was decreased by 38% in the whole group (Table 1), although two subjects failed to respond (Fig. 3).

DISCUSSION

We have shown previously that methoxsalen, a suicide substrate for cytochrome P-450 [3], decreases the metabolic activation of acetaminophen in mice [6]. The present results show that similar effects are observed in humans.

Indeed, *in vitro*, 100 μM methoxsalen decreased by 40% the covalent binding of a reactive acetaminophen metabolite to human microsomal proteins (Fig. 1). It is noteworthy that such concentrations of methoxsalen are unlikely to occur in the liver after administration of doses of methoxsalen used in PUVA therapy ($0.6\,mg \cdot kg^{-1}$ or $3\,\mu mol \cdot kg^{-1}$). However, because of its irreversible nature, a slow inactivation of cytochrome P-450 may, with time, become quite noticeable. Indeed, methoxsalen has been shown to decrease the clearance of antipyrine in patients with psoriasis [15]. In the present study, administration of methoxsalen (30 mg) to human volunteers decreased by 30% the 24 hr-urinary excretion of glutathione-derived metabolites, which are the end products of the conjugation of the reactive acetaminophen metabolite with glutathione (Table 1). The 24 hr-urinary excretion of metabolites does not indicate at which speed these metabolites are formed. We therefore calculated the partial apparent oral salivary clearance of acetaminophen into various metabolites [13], i.e. the part of the apparent oral clearance which can be accounted for by the transformation of acetaminophen into any given metabolite. When calculated in this way, the partial clearance into glutathione-derived metabolites was decreased by a mean of 38% after methoxsalen (Table 1, Fig. 3).

Metabolite activation is only a quantitatively minor pathway in the metabolism of acetaminophen. Methoxsalen did not modify the formation of the main metabolites of acetaminophen (the glucuronide and the sulfate) and, therefore, did not modify the salivary concentration–time curve of acetaminophen (Fig. 2). Therefore methoxsalen appears to decrease the formation of the reactive metabolite of acetaminophen without hampering its elimination from the body. Similar observations have been made in mice [6]. In mice, administration of methoxsalen up to two hours after acetaminophen prevented the hepatotoxocity of acetaminophen [6]. It is noteworthy that the hepatotoxicity of acetaminophen occurs at a faster rate in mice than in humans. Indeed, the half-life of acetaminophen after a hepatotoxic dose is 90 min in mice [6] but 7 hr in humans [16]. The maximum increase in SGPT activity occurs at 24 hr in mice [6] but after three days or more in humans [16]. Therefore, the 2 hr time limit for efficient prevention observed in mice [6] might correspond to longer periods of time in humans. Because *N*-acetylcysteine has been shown to be highly efficient in humans seen shortly after ingestion of an overdose of acetaminophen [16], it would seem unethical not to administer this glutathione precursor. Interestingly, however, with lower doses of methoxsalen and a low dose of *N*-acetylcysteine, an additive protective effect was observed when both protective agents were given concomitantly 2 hr after acetaminophen in mice [6]. Whether such an additive effect might be of use in subjects admitted after an overdose of acetaminophen remains to be determined. The present results, however, indicate that methoxsalen administration does decrease the metabolic activation of therapeutic doses of acetaminophen in humans.

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