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ELEVATION OF CONJUGATION CAPACITY IN ISOLATED HEPATOCYTES FROM BHA-TREATED MICE

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Abstract—Using harmol and paracetamol as the substrates, the elevation of conjugation reactions by BHA feeding and their significance towards the protective effects of this antioxidant has been studied with hepatocytes obtained from mice. With both substrates, an almost five fold elevation of the glucuronidation was observed. However, there was no change in the rate of sulfate conjugation. The rate of glutathione conjugate formation with paracetamol was also not enhanced, even though both the GSH level and glutathione *S*-transferase activities were increased. Finally, BHA administration afforded no protective effect against the hepatotoxic effects of paracetamol, even when the production of reactive paracetamol metabolites was increased by 3-methylcholanthrene pretreatment.

The production of experimental tumors by a variety of chemical carcinogens (environmental, nutritional and other exogenous factors) may be inhibited or prevented by dietary administration of certain chemical compounds [1, 2]. One of the most widely studied classes of protective agents includes the antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoulene and ethoxyquin. Because these agents are commonly used as preservatives in human and animal diets [3, 4], it is of special interest to understand the mechanisms involved in the protective actions of these compounds. In particular BHA has been noted for its low toxicity [3, 4] and previous studies on dietary administration of this compound has led to the observation that it enhanced the activities of various enzymes which have key roles in the deactivation of reactive metabolites [5–10]. Thus, it has been suggested that the protective effects of BHA may in fact be largely due to this ability to enhance the metabolic detoxification processes.

However, these observations were made with subcellular fractions of liver tissue and, consequently, little is known about their importance in the intact cell where the interaction and balance between metabolic activation and deactivation reactions are maintained. Towards this goal, the use of isolated hepatocytes can offer several distinct advantages, even though being an *in vitro* system the isolated hepatocytes still retain most of their *in vivo* properties and have been shown to catalyse various reactions involved in drug metabolism (activation as well as deactivation) at rates comparable to those occurring *in vivo*.

We have, thus, in the present study, used isolated hepatocytes from female mice for investigations of the effect of BHA treatment on conjugation reactions using harmol and paracetamol as substrates. The effect of BHA on the acute toxicity of paracetamol in these cells is also evaluated.

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MATERIALS AND METHODS

Animal treatment and hepatocyte preparation. Female NMRI mice (25–30 g) were allowed free access to tap water and food pellets (Anticimex avelsfoder, Astra–Ewos, Södertälje, Sweden) for a week of acclimation. Thereafter, mice were fed either powderized pellets or BHA diet (7.5 g/kg food) for two weeks. For experiments involving the 3-methylcholanthrene (MC), several mice from both groups were given intraperitoneal injections of MC (35 mg/kg) dissolved in corn oil (0.2 ml/mouse) for three consecutive days from the tenth day, and were killed 40 hr after the last injection.

Hepatocytes were isolated essentially according to the collagenase perfusion procedure as described previously [11]. As the hepatocytes of BHA-treated mice appeared more fragile during normal isolation procedures, the buffer salt, as well as the albumin concentrations, not only in the perfusion but also in the suspending media, were increased by 50% (buffer salts) and 100% (up to 4% albumin). This favored the recovery of intact viable cells from both control and BHA groups and about 30×10^6 hepatocytes were routinely harvested from one mouse liver ($1 \sim 2$ g). The viability of cells, isolated by this modified procedure, was >80% as judged by the trypan blue and NADH exclusion tests [11, 12].

Analytical methods. Incubations were performed at 37° in rotating, round-bottomed flasks under a stream of 95% O_2–5% CO_2 gas mixture. The incubation medium was either concentrated Krebs–HEPES buffer (see above) or Krebs–HEPES buffer supplemented with an amino acid mixture [13]. The concentration of hepatocytes were generally 1 or 2×10^6 per ml of incubation medium.

The procedure for conjugation of harmol was the

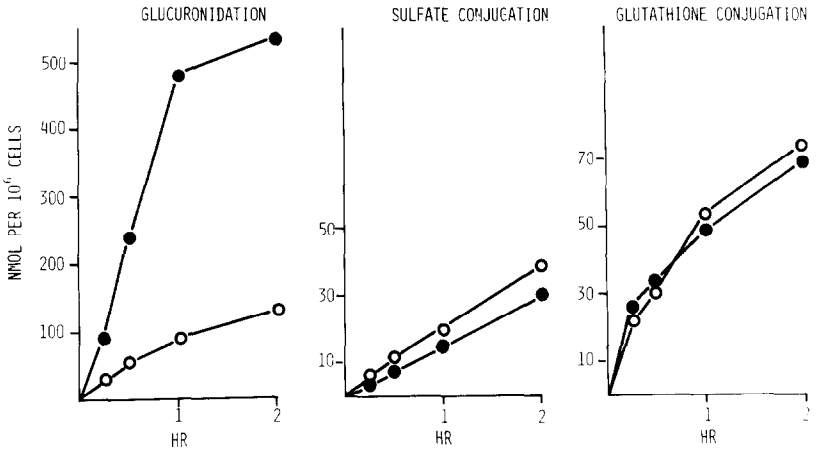

Fig. 1. Paracetamol metabolite formation in isolated hepatocytes from control (○——○) and BHA fed (●——●) female mice. Incubations were performed at a paracetamol concentration of 2 mM, and in a incubation medium supplemented with amino acids as described in Materials and Methods. Values are from one of three independent experiments. The ratio between the conjugation reactions in control and BHA hepatocytes varied not more than 5% in the different experiments.

same as described previously [14]. The concentration of harmol used was 400 μM and the incubation was carried out for 20 min. The metabolites of paracetamol were determined according to the modified HPLC method described by Moldéus [15]. The levels of reduced (GSH) and oxidized glutathione (GSSG) were measured by the HPLC method as described by Reed and Beatty [16].

Collagenase was obtained from Boehringer Mannheim, GmbH (West Germany) and bovine serum albumin fraction V was purchased from Sigma Chemical Co. (St. Louis, MO). Paracetamol metabolite standards were obtained from the Chemical Division, Sterling Winthrop (Newcastle, U.K.). All other chemicals and solvents were of analytical grade and were obtained from local commercial sources.

RESULTS

Harmol is conjugated with sulfate and glucuronic acid in rat hepatocytes [17]. The same two conjugation products were formed by cells from a control mouse (Table 1). However, whereas the rate of glucuronidation was about the same as that found with normal rat hepatocytes, the rate for sulfate conjugation was considerably lower for mouse.

Table 1. Effect of BHA feeding on sulfation and glucuronidation of harmol in isolated hepatocytes from female mice

	nmol per 10^6 cells per 20 min		
	Harmol glucuronide	Harmol sulfate	Conjugate total
Control	24.1*	3.3	27.4
BHA fed	93.0	2.9	95.9

* Values are from one of four independent experiments. The ratio between the conjugation reactions in control and BHA hepatocytes varied not more than 7% in the different experiments.

In agreement with observations made using liver microsomes [5], the rate of glucuronidation was enhanced about 4-fold by BHA feeding (Table 1). On the other hand, the rate of sulfate ester formation was not increased by the same treatment. The level appeared to be even smaller in BHA cells.

Paracetamol is a widely used drug which, like harmol, can be directly conjugated with sulfate and glucuronic acid, but unlike harmol can also undergo a cytochrome P-450 dependent activation reaction forming a reactive metabolite. This metabolite may be conjugated with GSH either non-enzymatically or by the catalytic action of glutathione *S*-transferase [18]. In agreement with the results obtained using

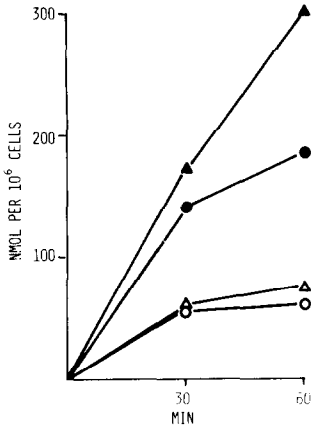

Fig. 2. Paracetamol glutathione conjugate formation in isolated hepatocytes from MC treated (○——○) or MC + BHA treated (△——△) female mice. Incubations were performed as described in Materials and Methods at a paracetamol concentration of 2 mM, in either salt buffer (open symbols) or in the amino acids containing medium (filled symbols). Values are from one or three independent experiments. The ratio of GSH conjugation in the hepatocytes after the various treatments varied not more than 7% between the different experiments.

harmol the BHA feeding resulted in a marked increase in formation of the glucuronide conjugate, but not of the sulfate conjugate of paracetamol (Figs. 1a and b). Formation of the paracetamol glutathione conjugate, which reflects the extent of cytochrome P-450 dependent activation, was not affected by BHA feeding either (Fig. 1c). It should also be noted that the incubation conditions were such that cells were able to synthesize GSH [19].

It has previously been shown that BHA feeding causes an elevation of non-protein thiol levels [9] along with increases in the activities of GSSG reductase [20] and glutathione *S*-transferases [8, 9]. It is evident that the increase in the level of non-protein thiols can be ascribed to an elevation of GSH specifically determined by the HPLC method (Table 2). The level of GSSG was slightly lower in BHA treated mouse liver. This, although not marked, may well be due to the increased activity of GSSG reductase. However, despite these enhancements in the level of GSH and in the activities of glutathione *S*-transferase and GSSG-reductase, no difference in paracetamol–glutathione conjugate formation was observed (Fig. 1c).

Thus, in an effort to elevate the rate of cytochrome P-450 dependent activation of paracetamol and then to determine the significance of accelerated GSH conjugation by BHA, the BHA fed mice were also treated with MC. This treatment is known to elevate the metabolic activation of paracetamol and increase its hepatotoxic effects [18].

The results shown in Fig. 2 were obtained by incubating the hepatocytes isolated from mice pretreated with either MC alone or in combination with BHA. Due to the enhanced rate of metabolic activation of paracetamol by the MC treatment, the level of paracetamol glutathione conjugate formed in 60 min (Fig. 2) was several times greater than those shown in Fig. 1c. Both of these results were obtained by incubating cells in the amino acid supplemented medium to allow *de novo* synthesis of GSH. When MC cells were suspended in just the salt buffer medium, a condition which does not permit synthesis of GSH, this co-substrate appears to have been depleted within 30 min. Under such incubation conditions, the BHA feeding did not have any effect on the paracetamol glutathione conjugate formation. However, under the incubation conditions allowing for GSH synthesis, where the formation of paracetamol glutathione conjugates is severalfold higher, BHA feeding seemed to have a stimulating effect. In these cells the formation of paracetamol glutathione conjugates was almost linear for 60 min.

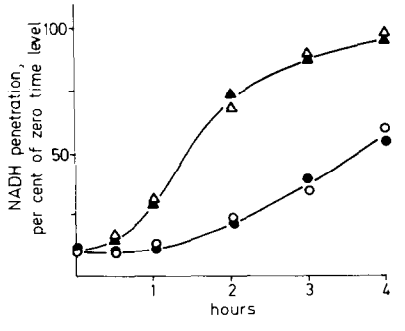

Fig. 3. Paracetamol induced loss of viability of isolated hepatocytes from mice with the following treatments: controls (○——○), BHA (●——●), MC (△——△), MC + BHA (▲——▲). Incubations were performed as described in Materials and Methods using a paracetamol concentration of 2 mM and in a simple salt buffer. Values present the means of two independent experiments.

Paracetamol incubated only in the absence but not in the presence of amino acids has previously been shown to cause the loss of viability of isolated mouse hepatocytes within a 5 hr period [15]. Thus, it was considered of interest to examine if BHA feeding would exert a protective effect on the hepatocytes under these conditions. For these experiments, hepatocytes were isolated from control and BHA fed mice which were treated with corn oil vehicle alone or with MC.

The loss of cell viability, as determined by the NADH-penetration assay [11], was much more pronounced in MC cells. Thus, after only 2 hr incubation, severe loss of viability was observed (Fig. 3). The BHA feeding, however, did not appear to protect either the control or MC cells from the toxicity of paracetamol.

DISCUSSION

As evident from the results presented in this study, the activity of glucuronidation is markedly elevated in hepatocytes from BHA treated mice. The extent of this increase (3–4 fold) demonstrable with intact isolated cells is similar to that observed with BHA microsomes when optimized with an excess of UDP-glucuronic acid [5]. From these results, it is also evident that the synthesis of the cofactor UDPGA is not limiting even at the enhanced rate of glucuronidation. As the level of endogenous UDPGA is low, and the glucuronidation in isolated hepatocytes is dependent on the synthesis of this cofactor [15, 21, 22], the result also implied an enhancement of its synthesis. In support of this, the dietary administration of BHA was demonstrated to increase the activity of UDP glucose dehydrogenase 6 fold [5].

However, an increase in the sulfate conjugation reaction in BHA cells could not be demonstrated, even with harmol and paracetamol incubated in the presence of excess inorganic sulfate (Table 2 and Fig. 1b). It was suggested that the synthesis of activated sulfate (PAPS), a cofactor required for sulfate

Table 2. Levels of GSH and GSSG in liver from control and BHA fed female mice

	μmol/g liver	
	GSH	GSSG
Control	9.5 ± 0.8	0.94 ± 0.13
BHA fed	13.8 ± 0.7	0.85 ± 0.14

Values represents mean ± S.E.M. of results obtained from three different livers.

conjugation, may have been limiting in the BHA hepatocytes. To eliminate this possibility, the hepatic cytosol from both control and BHA treated mice were compared for their sulfate transferase activity, using either a PAPS generating system or commercially acquired PAPS. The enzyme activity was not elevated in BHA treated mouse liver (unpublished observation). Thus, this enzyme appears to be the only one, among several conjugation enzymes so far investigated, which is not elevated by BHA treatment.

Many toxic chemicals undergo cytochrome P-450 dependent oxidation to form reactive metabolites. For the protection of cells from the toxic effects of these reactive metabolites, the conjugation with GSH has a more direct implication. Although the intracellular concentration of GSH and of glutathione *S*-transferase are high in normal cells, the BHA feeding elevated these constituents even further [7–9]. Thus, as expected, the previously observed elevation of non-protein thiol levels by BHA feeding [7] was supported by specific measurement of GSH in the present study (Table 2). Also the marked (10 fold) enhancement of glutathione *S*-transferase activity [8, 9] was confirmed, by using BP-4,5-oxide as the substrate, in a previous study [6].

The reactive metabolite resulting from the oxidation reactions of paracetamol is most likely the *N*-acetyl quinoneimine. This reactive product may then be conjugated with GSH. Since GSH and glutathione *S*-transferases were increased by BHA feeding the formation of GSH-conjugates from paracetamol was expected to be increased. However, the results shown in Fig. 1c demonstrate that the rate of GSH-conjugate formation was not enhanced. Thus, it is evident that the capacity of GSH conjugation was quite sufficient, even in the normal state (i.e. prior to elevation by BHA feeding), for the detoxification of reactive products arising from paracetamol. An increased capacity of GSH-conjugate formation in BHA cells was demonstrable only under conditions in which the rate of metabolic activation of paracetamol was enhanced by MC treatment (Fig. 2). Even under such conditions, the synthesis of GSH, rather than the elevation of GSH or the dramatically enhanced glutathione *S*-transferase activities might have been responsible for the increased formation of GSH-conjugates. Experiments dealing with the implication that the rate of GSH synthesis was enhanced in BHA cells are presently in progress in the laboratory.

Furthermore, the paracetamol induced hepatotoxicity was not lowered in hepatocytes isolated from BHA fed mice under conditions which did not allow the *de novo* synthesis of GSH. This would imply that the elevated GSH level and glutathione *S*-transferase activities by the BHA treatment could not provide protection in the absence of intracellular GSH synthesis. Unfortunately no toxic effects of paracetamol, even after MC treatment, could be observed in the hepatocytes, within the 4 hr of incubation under conditions allowing GSH synthesis. The effect of BHA under such conditions could thus not be evaluated in this system. In addition, it remains to be established if BHA treatment has any protective effect *in vivo* against acute toxic drugs like paracetamol.

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