Metabolism of the Food Mutagen 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in Isolated Liver Cells from Guinea Pig, Hamster, Mouse, and Rat

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The metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), the most abundant compound of the aminomimidazoaarene (AIA) group of mutagens/carcinogens isolated from the crust of fried and broiled meat, was examined in freshly isolated hepatocytes from untreated rat, mouse, hamster, and guinea pig. Activation was evaluated by the total level of covalent binding of PhIP to macromolecules. Rat hepatocytes had the lowest rate of metabolism, both to reactive and detoxified metabolites. The products were identified as 4'-PhIP-sulfate, PhIP-glucuronide, and N(OH)-PhIP-glucuronide. The ring hydroxylation rate was much greater in mouse hepatocytes, the main products being 4'-PhIP-sulfate and 4-hydroxy-PhIP. The level of covalent binding in the mouse hepatocytes exceeded those of the rat and guinea pig at high doses of PhIP. An extensive metabolism was seen in guinea pig hepatocytes, the major products being 4'-PhIP-sulfate, 4'-O-PhIP glucuronide, PhIP-glucuronide, and N(OH)-PhIP-glucuronide. In addition, several other unknown metabolites were formed. However, the amount of covalent binding in guinea pig hepatocytes was similar to that in rat hepatocytes. Covalent binding of PhIP metabolites was highest in hamster hepatocytes. Three of the main metabolites were identified as 4'-PhIP-sulfate, 4'-O-PhIP-glucuronide, and PhIP-glucuronide, but several unknown PhIP metabolites also were formed. Only minor amounts of N(OH)-PhIP-glucuronide were produced in the hamster. The present study shows that both the direct detoxification of PhIP and further conjugation of the 2-hydroxylamino-PhIP to reactive and/or detoxified metabolites are important for the resulting covalent binding. — Environ Health Perspect 102(Suppl 6):109-114 (1994)

Key words: food carcinogen, PhIP, 2-amino-1-methyl-6-phenylimidazo pyridine, hepatocytes, metabolism, species differences, rat, mouse, hamster, guinea pig

Introduction

Among the carcinogenic and mutagenic aminomimidazoaarene (AIA) group of compounds isolated from the crust of fried meat, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant (1-6). Although PhIP is less potent in the Salmonella test than the quinoline- and the quinoxaline-AIA compounds, it has been shown to be an equally potent genotoxin in mammalian cells both in vitro and in vivo (7-10). PhIP has recently been shown to cause colon cancer in rats (11) and abdominal lymphomas in mice (12). In a series of previous studies, we have characterized the metabolic pathways of PhIP leading to mutagenic activation and detoxification (10,13-16). In these studies we have used whole rats, isolated rat hepatocytes, subcellular fractions, and purified enzymes. Other groups have studied PhIP activation in liver and colon tissues from humans and PhIP metabolism in mice (17-19).

We have previously reported that 2-hydroxylamino PhIP appears to be the principal metabolite leading to mutations in Salmonella and DNA damage in mammalian cells (10). The proximate metabolite can be further conjugated to glucuronic acid (15). Ring hydroxylation of the phenyl ring in position 4' followed by sulfation appears to be the main detoxifying pathway in the rat and the mouse (14,19). Both oxidations are P450 dependent, the rat liver P450IA2 being most active in the N'-hydroxylation, while the P450A1 is the principal enzyme hydroxylating the ring in position 4' (14,18).

The metabolism of PhIP will influence its genotoxic and carcinogenic effects in vitro. Thus, characterization of the metabolism is important for the extrapolation of effects of PhIP in animal experiments to the human situation. We have found the use of hepatocytes in studies on metabolism particularly valuable because this experimental system takes into account the role of possible competing pathways and phase 2 metabolism (20,21).

In the present study, we have examined the metabolism and covalent binding to macromolecules of PhIP in hepatocytes isolated from four different rodent species, and we find large quantitative as well as qualitative differences between species.

Materials and Methods

Chemicals

PhIP was donated by Dr. Errol Zeiger, National Institute of Environmental Health (Research Triangle Park, NC). PhIP was labeled with tritium by Amersham International plc. (Little Chalfont, UK), using their TR7 catalytic tritiation protocol. The crude reaction mixture was purified by Lars Dragsted, Institute of Toxicology, National Food Administration (Copenhagen, Denmark). The resulting 3H-PhIP had a specific activity of 55 mCi/mmol with a purity of >99%. Other chemicals were obtained...
from the following sources: collagenase (CLSII, 150 μg/mg) from Worthington Biochemical Corp. (Freehold, NJ); Insta-Gel from Packard (Groningen, The Netherlands); diethylamine from BDH Chemical Ltd (Poo1, UK); bovine serum albumin (fraction V) from Sigma Chemical Co. (St. Louis, MO). All other chemicals were commercially obtained and of p.a. quality. Solvents for HPLC were of HPLC quality.

Animals
Male Wistar rats (MOL:WIST, 200–300 g) and male C57BL/6J Bom mice (20–30 g) were obtained from Mellegaard (Ejby, Denmark). Male Duncan-Hartley guinea pigs (350–450 g) were obtained from J.A. Sahlin’s Laboratory Animal Farm (Malmö, Sweden). Male Syrian golden hamsters (80–100 g) were from Wrights (Essex, Chelmsford, England). Mice, hamsters, and rats were given Ewos R3 standard pelleted feed (Astra Ewos AB, Södertälje, Sweden) and water ad libitum. Guinea pigs were given Ewos guinea pig pellets and water containing 25 mg ascorbate/100 ml.

Isolation and Incubations of Hepatocytes
Liver cell suspensions were prepared by the collagenase perfusion technique (22–24). Pooled hepatocytes from three to four mice were used in each experiment. The hepatocytes were suspended in Hank’s HEPES buffer with 1% albumin, pH 7.4, and purified by low-speed centrifugation (50g for 30 sec). The viability was always >90%, as determined by trypan blue exclusion. Hepatocytes (4 × 10⁶ cells/ml) in suspensions of 2 ml were incubated for various time intervals in Hank’s HEPES buffer with 1% albumin, pH 7.4.

Covalent Binding
Covalent binding of PhIP to macromolecules was determined as the glass filter paper method (25). Aliquots of 75 μl from the hepatocyte suspensions were transferred to Whatman GF/C glass filters (Maidstone, UK) and macromolecules were precipitated by immersing the filters in 95% ethanol, followed by washing in organic solvents. Binding was determined using liquid scintillation counting.

HPLC Analysis
After removal of cells and precipitation of proteins with ethanol in an argon atmosphere, the volume of the supernatant was reduced using a Speed Vac. The metabolites were analyzed by HPLC on a Perkin-Elmer Series 4 system using a Waters Nova-Pak phenyl column. Metabolites were eluted by a linear gradient from 1.5% acetonitrile and 1% methanol to 15% acetonitrile and 10% methanol in water with 0.1% vol/vol diethylamine adjusted to pH 7.0 by acetic acid during 60 min. Metabolite profile of guinea pig was eluted with a super-linear gradient. Peak assignment: 1) 4′-PhIP-sulfate; 2) 4′-O-glucuronide-PhIP; 3) 4′-hydroxy-PhIP; 4) PhIP-glucuronide3; 5) NOH-PhIP-glucuronide; 6) PhIP-glucuronide2; 7) phenyl-substituted-PhIP; and PI PhIP. See “Note Added in Proof.”

Results
As previously reported (13,15), hepatocytes from untreated rats metabolized PhIP at a relatively low rate and to few metabolites (Figure 1). Hepatocytes from mouse only produced one major metabolite, the 4′-PhIP-sulfate. Low levels of metabolites with unknown structure were also recorded. Hamster hepatocytes and in particular guinea pig hepatocytes transformed PhIP into numerous metabolites. Some of these were identical to those isolated after incubation of PhIP with hepatocytes from PCB-pretreated rats (13,15); unpublished data. In all species 4′-PhIP-sulfate was the dominating detoxification product. However, the 4′-PhIP-O-glucuronide (previously identified in PCB-pretreated rat hepatocytes (unpublished data)) was isolated from untreated and PCB-pretreated rat hepatocytes (unpublished data).

Hamster and guinea pig hepatocytes produced one of these—PhIP-glucuronide3 (Figure 2). In the guinea pig hepatocytes another PhIP-glucuronide was formed, PhIP-glucuronide2, and minor amounts of a third one, PhIP-glucuronide1 (Figure 2). PhIP-glucuronide2 was the dominating PhIP glucuronide in PCB rat hepatocytes (unpublished data). Several unidentified metabolites of PhIP were also produced by the hamster and guinea pig hepatocytes. Traces of two of these metabolites were also seen in the mouse hepatocyte incubate (not shown). Unconjugated 4′-hydroxy-PhIP was seen in all incubates. The levels of unconjugated metabolites did not seem to increase markedly with time, whereas the levels of conjugates increased linearly with time. Larger relative amounts of 4′-hydroxy-PhIP were seen in the hamster hepatocytes indicating saturation of conjugation reactions in these cells (Figures 2, 3).

The analytical method used in these experiments did not allow determination of the proximate metabolite 2-hydroxylamino-PhIP. However, the glucuronide conjugate of this metabolite was a major metabolite in all species except in the hamster, where only minor amounts could be detected (Figures 1–3).

Metabolic activation to reactive intermediates that bound covalently to macromolecules in the hepatocytes differed greatly among the species examined (Figure
Figure 2. Time course of formation of PhIP metabolites of PhIP (0.1 mM) in suspensions of hepatocytes (4 × 10⁶ cells/ml) from rat, mouse, hamster, and guinea pig. Values are mean of three to five experiments.

Figure 3. Concentration-dependent formation of PhIP metabolites in suspensions of hepatocytes (4 × 10⁶ cells/ml) incubated for 4 hr with 0.05, 0.1, and 0.5 mM PhIP. Values are mean of three to five experiments. Hepatocytes from rat, mouse, hamster, and guinea pig were used. Metabolite assignment as in Figure 2. See "Note Added in Proof."
At the two lower doses (0.05 and 0.1 mM) of PhIP, binding was lowest in the rat hepatocytes, closely followed by hepatocytes from the mouse and guinea pig, whereas a much higher binding was seen in the hamster. At the higher dose (0.5 mM), the rat and guinea pig hepatocytes had comparable levels of binding, whereas the binding in the mouse hepatocytes was doubled. The hamster hepatocytes showed the highest binding of all species studied (Figure 4).

Discussion

The resulting covalent binding of PhIP to macromolecules in the hepatocytes is determined by several factors. First, activation to the proximate metabolite 2-hydroxylamino-PhIP has to take place. This is a P450-dependent reaction, primarily involving the P4501A2, but also P4501A1, P4502C11, and a P4501A-like enzyme (14,18,26). However, 2-hydroxyamino-PhIP apparently has, unlike the 2-hydroxylamino derivatives of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), and 2-amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQx), a very low reactivity towards proteins and DNA in vitro (17,27,28; unpublished data). Nevertheless, in microsomal incubations with PhIP, protein binding occurs (19,33). One may speculate that this may be a result of further oxidation of 2-nitro-PhIP which reacts directly with protein sulfhydryl groups ([15]; unpublished data). Further activation of 2-hydroxylamino-PhIP, presumably by esterification to form a leaving group, is needed in the formation of the reactive nitrenium derivative (28,29). These pathways have not yet been characterized for PhIP in living cells. In comparison with the quinolines IQ and MeIQ, PhIP apparently is not dependent on the O-acetylation reaction (10). Sulfation and other reactions may be as important (29). It is also important to note that 2-hydroxylamino-PhIP can also be conjugated to glucuronic acid and thus escape further activation (15). This pathway apparently operates with low efficiency in the hamster compared with the other species (Figures 2,3).

Metabolism by uninduced liver microsomes from rat showed nearly no detectable oxidative activity, whereas marked N-hydroxylating activity was seen in the guinea pig and the hamster liver microsomes, the former being more active (not shown). Thus, lack of further activation or increased detoxification of 2-hydroxylamino-PhIP may explain the low level of covalent binding in guinea pig hepatocytes. In addition, the ability to detoxify directly PhIP is also important for the resulting covalent binding and such pathways seem to be dominant in the guinea pig in relation to its large total metabolic activity.

Similar results were obtained in a previous study using acetylaminofluorene as a substrate (24). In this study it was found that with acetylaminofluorene, guinea pig hepatocytes had the largest ability to detoxify acetylaminofluorene to water-soluble metabolites, and that covalent binding was highest in the hamster hepatocytes.

In PCB-pretreated rat hepatocytes, we have previously identified a glutathione (GSH) derivative of PhIP whose exocyclic amino group had been substituted by GSH probably as a result of the formation of 2-nitro-PhIP (15). Furthermore, we also identified the PhIP-cysteinylglycin derivative in the incubations as a result of the γ-glutamyltransferase activity on PhIP-GSH. Although GSH in general does not appear to be an important detoxification pathway of reactive PhIP metabolites, PhIP-cysteinyl-
glycine was identified in the guinea pig hepatocyte incubations, indicating formation of 2-nitro-PhIP by the guinea pig.

PhIP apparently does not induce liver cancer either in rats or in mice (11,12), although hepatic adenoma in neonatal B6C3F1 mice (30) have been reported. Variable results on the appearance of PhIP-induced enzyme-altered foci in rat liver have been found (31,32). Carcinogenicity of PhIP has not been examined in hamsters and guinea pigs. The present study on PhIP metabolism suggests that the guinea pig would be resistant to PhIP related to liver carcinogenesis, as it is towards acetylaminofluorene (20), because the rate of activation relative to detoxification of PhIP is low. In contrast, it might be expected that PhIP might cause liver cancer in the hamster.

NOTE ADDED IN PROOF: Recent studies have shown that the metabolite originally identified as PhIP-glucuronide and designated PhIP-glucuronide2 (Figure 1, peak 6; Figures 2,3, PhIP-glu2) is a glucuronide of 2-hydroxyamino-PhIP different from N(OH)-PhIP-glucuronide (Figure 1, peak 5; Figures 2,3, N(OH)-PhIP-glu).
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