INFLUENCE OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ON THE METABOLISM OF AMINOPYRINE IN ISOLATED HEPATOCYTE SYSTEM

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The effect of phenobarbital (PB) and 3-methylcholanthrene (3-MC) on the metabolic behavior of aminopyrine (AM) was studied using an isolated hepatocyte system prepared from male Wistar rats. The formation of 4-formylaminopyrine (FAA) was increased after pretreatment with PB, but not 3-MC. However, the total amounts of AM and its main metabolites recovered from hepatocyte system after the incubation for 30 min were considerably reduced both by PB and 3-MC-pretreatment. Furthermore, when using 4-monomethylaminopyrine (MAA), the first metabolite of AM, as a substrate, 3-MC-pretreatment resulted in a more significant decrease in the total amounts of MAA and its further metabolites recovered than did PB.

Present observation suggests the participation of cytochrome P-448 as well as cytochrome P-450 in the metabolism of AM.

Keywords—isolated hepatocyte; aminopyrine; 4-monomethylaminoantipyrine; phenobarbital; 3-methylcholanthrene

The metabolism and urinary excretion of aminopyrine (AM) in man have been reported. Previous papers in this laboratory have reported the excretion of 4-formylaminopyrine (FAA) as a new metabolite of AM.1-4)

Houston et al.5,6 reported that the total urinary excreted amounts of AM and its metabolites decreased after pretreatment with phenobarbital (PB), though the demethylation step (AM to 4-aminoantipyrine (AA) via 4-monomethylaminopyrine (MAA)) increased. As to the paradox, they postulated the existence of alternative pathways of AM other than the main pathway.6)

In the present studies, isolated hepatocyte system was used since it has the advantage of eliminating the influences of many extrahepatic variables and it enable us to examine phase II transformation processes, such as acetylation7) and glucuronidation,8) as well as phase I reactions, i.e., aromatic hydroxylation,9) alkyl-hydroxylation10) and N- and O-demethylation.11)

In order to examine the problem described above, the quantitative determination of AM and its main metabolites, such as MAA, AA, FAA and 4-acetylaminoantipyrine (AcAA), was performed after the incubation with AM and isolated hepatocytes prepared from control, PB-pretreated and 3-MC-pretreated rats. From the quantitative data, the effect of PB or 3-MC on the alternative pathways was discussed. In addition, the effect of PB or 3-MC on the formation of FAA was also discussed.

MATERIALS AND METHODS

Chemicals — Aminopyrine (AM), 4-
monomethylamineoantipyrine (MAA), 4-aminooantipyrine (AA), 4-formylamineoantipyrine (FAA) and 4-acetylaminoantipyrine (AcAA) used in this work were obtained as described in the previous paper. Deuterium-labeled AM (d₅-AM), MAA (d₅-MAA) and AA (d₅-AA) were synthesized according to the method of Goromaru et al. 8-Chlorotheophylline was purchased from Tokyo Chemical Ind. Co. Ltd., and EGTA from Nakarai Chemical Ind. Co. Ltd., collagenase was obtained from Boehringer Mannheim, bovine serum albumin, fraction V, was from Sigma Chemical Co., N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (Hepes) was from Nakarai Chemical Ind. Co. Ltd., and Penicillin G potassium was from Meiji Seika Co. All other chemicals were of special reagent grade.

Preparation of Isolated Rat Hepatocytes — Wistar strain male rats (11 weeks old) weighing 300–350 g were maintained on a commercially balanced stock diet (Oriental Yeast Co. Ltd.). The rats were divided into three groups consisting of 6 animals each. The groups of rats were injected (i.p.) with 3-MC, 20 mg/kg/d, in 1 ml of corn oil and PB, 50 mg/kg/d, in physiological saline for 3 d. The control rats received either corn oil or physiological saline solution. The cell preparation was performed according to the method described by Moldèus et al. Each rat was anesthetized with ether, and heparin (0.1 ml, 1000 unit/ml) was injected via the caval vein to prevent blood coagulation in the liver during cannulation. The yield of each preparation was 4–6 × 10⁸ cells/liver, and the viability of fresh cells was 94–98% according to the lactic dehydrogenase latency test. The values remained unchanged for 5 h if the cells were suspended in albumin containing Krebs-Henseleit buffer at 4°C saturated with 95% O₂-5% CO₂.

Incubation of Isolated Hepatocytes with AM or MAA — The hepatocytes were diluted with incubation buffer to a final concentration of 5 × 10⁸ cells/ml. Four ml of hepatocytes were incubated in a rotating round-bottom flask at 37°C under a stream of 95% O₂-5% CO₂ unless otherwise stated. After 3 min of temperature equilibration, reactions were initiated by the addition of substrate (AM or MAA in a final concentration of 0.2 mM). Incubation was continued at 37°C for 5, 10, 20 and 30 min. After the addition of 1 ml of phosphate buffer (pH 7.4) containing 8-chlorotheophylline (5 μg), d₅-AM (200 μg), d₅-MAA (50 μg) and d₅-AA (25 μg) as internal standards, the samples of incubation mixture were added to 1 ml of 40% zinc sulfate and 2 ml of saturated barium hydroxide. The resulting precipitate was sedimented by centrifugation, and an aliquot of 5 ml of supernatant fluid was extracted twice with 20 ml of chloroform. The combined extract was used for metabolite measurements. AcAA and FAA derived from AM or MAA were determined by a high performance liquid chromatography (HPLC). AM, MAA and AA were determined by a modification of the method of Goromaru et al. using gas chromatography-mass spectrometer (GC-MS).

HPLC Assay — The elution took place with a 2-component, 2-phase system, consisting of acetonitrile and 10 mM potassium dihydrogen phosphate solution (15:85) on an octadecyl silane bonded silica column (Unisil®, Gasukuro Kogyo Co., Ltd.) at a flow rate of 2 ml/min. The apparatus used was a Shimadzu model LC-3A liquid chromatograph with a UV-detector, set at 261 nm (SPD-2A, Shimadzu, Kyoto). The concentration of AcAA or FAA was calculated from the relative peak height ratio of AcAA or FAA to 8-chlorotheophylline by reference to a calibration curve.

GC-MS Assay — The instrument used was a Shimadzu model GCMS 7000 equipped with a multiple ion detector. A glass column, 1 m × 3 mm i.d., containing 1.5% OV-17 coated onto 80 to 100 mesh Shimalite W was used. The operating temperatures were as follows; injection port, 250°C; Column oven for AM and AA, 230°C, and for MAA, 170°C. The mass spectrometer conditions were as follows; accelerating voltage, 3 kV; ionizing current, 300 μA; ionizing energy, 23 eV; separator and ion chamber temperature, 250°C.
In order to determine the amount of AM, the relative peak height of the molecular ion peak of AM at m/z 231 (M^+) to d₈-AM at m/z 234 (M^+) was measured. In the case of MAA, the relative peak height of MAA at m/z 217 (M^+) to d₈-MAA at m/z 220 (M^+) was measured. For the determination of AA, derivatization with acetone was performed. The acetonide formed was analyzed at m/z 243 and 246. The concentration of AM, MAA and AA was calculated from the relative peak height of each compound to its deuterium labeled compound by reference to a calibration curve.

**Statistical Treatment** — The statistical significance was evaluated at 0.05 or 0.02 levels by two-sided t-test.

**RESULTS**

Fig. 1 shows the time course of the concentration of AM and its metabolites after incubation of 0.2 mM of AM with isolated hepatocytes prepared from control, PB-pretreated and 3-MC-pretreated rats. Using HPLC and GC-MS, AM and its four kinds of metabolites (MAA, AA, AcAA and FAA) were successfully detected in isolated hepatocytes. AM was metabolized so rapidly that its four metabolites were all detected within 5 min after incubation. PB-pretreatment resulted in a rapid disappearance of AM and in an increase in FAA level. The disappearance half-life of AM was 3.0 ± 0.6 min, 13.6 ± 3.1 min, and 17.3 ± 2.9 min for PB-pretreated, 3-MC-pretreated and control rat hepatocytes, respectively. The pretreatment with PB shortened the half-life by about half or one-fourth in comparison with 3-MC-pretreatment or the control. Furthermore, the level of FAA at 30 min in PB-pretreated rat hepatocytes was significantly higher than that in the control (p < 0.05). However, no significant difference was observed between those from 3-MC-pretreated

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**FIG. 1. Time Course of AM Metabolism in Isolated Rat Hepatocytes at 37°C**

0.2 mM of AM was used as a substrate. Each value is the mean of four experiments. Vertical bars represent standard errors. (-●-) AM, (-○-) MAA, (-□-) AA, (-△-) FAA, (-▲-) AcAA. a) p < 0.05 when compared with FAA in the control.
FIG. 2. Mass Balance for AM and Its Metabolites recovered, and for the Unknown Metabolites. Each value is the mean of four experiments. Vertical bars represent standard errors.

(•—•) total, (○—○) unknown.
a) p < 0.05, b) p < 0.02 when compared with the control.

FIG. 3. Time Course of MAA Metabolism in Isolated Rat Hepatocytes at 37°C. 0.2 mM of MAA was used as a substrate. Each value is the mean of four experiments. Vertical bars represent standard errors.

(○—○) MAA, (□—□) AA, (Δ—Δ) FAA, (▲—▲) AcAA.
a) p < 0.02 when compared with FAA in the control.
rats and from the control.

From the time course studies, it is clear that the substrate which disappears is unaccounted for, if only MAA, AA, FAA and AcAA formation is taken into account. The same phenomenon was observed by Bast et al.\textsuperscript{15} using rat liver microsomes. Fig. 2 shows the mass balance for AM and its metabolites recovered, and for the unknown metabolites. The percentage of unknown metabolites to the substrate added was calculated by subtracting the amount of MAA, AA, AcAA and FAA from the disappeared amount of AM. It is clear that the pretreatment with PB or 3-MC caused an increase in unknown metabolites. More than 60% of metabolites were not recovered after incubation for 30 min in the case of PB-pretreated rat hepatocytes, and 50 and 25% in 3-MC-pretreated rat hepatocytes and control rat hepatocytes, respectively.

In order to study the effect of inducers, such as PB and 3-MC, on the metabolism of AM in more detail, MAA, the first metabolite of AM, was submitted to isolated hepatocytes as a substrate. Fig. 3 shows the time course of concentration of MAA and further metabolites, i.e., AA, AcAA and FAA after incubation of 0.2 mM of MAA with hepatocytes prepared from the control, PB-pretreated and 3-MC-pretreated rats.

The disappearance rate of MAA was slower than that of AM in all cases. The disappearance half-life of MAA in the control rat hepatocytes was significantly shortened from 44.3 ± 7.7 to 25.7 ± 1.5 min by PB-pretreatment (p < 0.05). When pretreated with 3-MC, the disappearance half-life of MAA was more significantly shortened by less than half from 44.3 ± 7.7 min in the control to 19.0 ± 4.7 min by 3-MC-pretreatment (p < 0.02). However, there was no significant difference in the level of AA, the first metabolite of MAA, among these three groups.

With reference to the level of FAA, PB-pretreatment caused a significant increase (p < 0.02) in the level of FAA similar to the case using AM as a substrate. The FAA level was

FIG. 4. Mass Balance of MAA and Its Metabolites recovered, and for the Unknown Metabolites
Each value is the mean of four experiments. Vertical bars represent standard errors.

(– ● –) total, (– ○ –) unknown.

a) p < 0.02 when compared with the control.
increased by more than three times when compared with the control.

Fig. 4 shows the mass balance for MAA and its metabolites recovered, and for the unknown metabolites. 3-MC-pretreatment increased the percentage of unknown metabolites significantly ($p < 0.02$). More than 40% was not recovered in the case of 3-MC-pretreatment, while about 20% was not accounted for when pretreated with PB and 15% was lost in the control.

**DISCUSSION**

We performed the quantitative determination of AM and its metabolites after incubation with isolated hepatocytes prepared from the control, PB-pretreated and 3-MC-pretreated rats. AM is demethylated to give MAA which in turn is demethylated to AA. These two reactions are mediated by cytochrome P-450. MAA may be metabolized to FAA, which was proven to be a new metabolite of AM by our laboratory in 1975. The formation of FAA from AM has not yet been characterized completely. The evaluation of FAA in hepatic microsomes was performed by some researchers using HPLC. Nigam et al. reported that when AM or MAA was submitted to hepatic microsomes prepared from rats and guinea pigs, FAA could not be detected in the microsome system at all and the pretreatment with PB resulted in only a trace level of FAA. However, they could detect a considerable amount of FAA from rat urine after MAA administration. In the present study, a reasonable amount of FAA was successfully detected even in the control hepatocytes. Since the metabolism of drugs in isolated hepatocytes is closely correlated to *in vivo* metabolism, the formation of FAA, which could not be detected in microsome system, can be identified in hepatocyte system.

As to the effect of PB or 3-MC-pretreatment on FAA formation, PB-pretreatment increased the FAA level more than twice that in the control, while 3-MC-pretreatment did not. When AM was used as a substrate, the level of FAA was increased from 0.8 ± 0.1 nmol/10⁸ cells/30 min in

**CHART 1. Metabolic Pathways of AM in Rat**
the control to 1.5 ± 0.1 nmol/10⁶ cells/30 min in PB-pretreatment ($p < 0.05$). In the case of MAA, it caused a more significant increase in FAA level ($p < 0.02$). The level increased from 1.0 ± 0.2 nmol/10⁶ cells/30 min in the control to 3.8 ± 0.2 nmol/10⁶ cells/30 min. These observations suggest that the formation of FAA is dependent not on cytochrome P-448, but on cytochrome P-450.

Houston et al.⁵,⁶ reported the effect of PB on the urinary excretion products of AM, that is, a significant reduction in the urinary excretion of AM and AcAA was observed after PB-pretreatment, though the demethylation step from AM to AA via MAA was induced. In regard to this apparent paradox, they proposed alternative pathways, and concluded that under the normal conditions the pathways must be minor routes, but under the induced conditions the pathways become important. Chart 1 shows the main metabolic pathway of AM (solid line) and its alternative pathways (dotted line).

In the present studies, we determined the amounts of AM and its four metabolites formed from the main metabolic pathway.

From the present observation, it was clearly recognized that the total amounts of AM and its four metabolites were significantly decreased both by PB and 3-MC-pretreatment ($p < 0.02$ for PB-pretreatment, $p < 0.05$ for 3-MC-pretreatment). In addition, when using MAA as a substrate, 3-MC-pretreatment resulted in a more significant decrease ($p < 0.02$) in the total amounts of MAA and its further metabolites (AA, FAA and AcAA) than did PB-pretreatment. This finding is of particular interest because there are no reports that cytochrome P-448 takes part in the metabolism of AM. The data presented in Fig. 2 and 4 confirm the assumptions that the alternative pathways are induced both by cytochrome P-450 and cytochrome P-448 and that the increase in unknown metabolites resulted in the reduction of total amounts recovered.

Studies relating to unknown metabolites have already been reported by some researchers.¹⁸-²¹ Večerková et al.¹₆ reported 4-methylacetyl-aminoantipyrine and rubazonic acid. Furthermore, Yoshimura et al.¹⁹ reported that a part of ingested AM was excreted as 4-dimethyl-amino-3-hydroxymethyl-2-methyl-1-phenyl-3-pyrazolin-5-one (AM-3-CH₃OH) in rat urine. Recently, Goromaru et al.²⁰ identified the presence of MAA-3-CH₃OH, AA-3-CH₂OH, AcMAA-3-CH₂OH and AcAA-3-CH₂OH. In addition, the formation of 4'-dimethylamino-5,5'-dioxo-1,1'-diphenyl-2,2',3'-trimethyl-N-(3'-pyrazolin-3'-ylmethylidene)-3-pyrazolin-4-ylamine (Schiff base) was also reported by Niwa et al.²¹ These metabolites are thought to be derived from the alternative pathways. However, there are probably many unknown metabolites such as pyrazolone cleavages, glucuronide and so on.

In the present work, we did not detect the unknown metabolites. Further investigations are in progress to assess the effect of PB or 3-MC on the unknown metabolites.

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