Biochemical Pharmacology, Vol. 36, No. 24, pp. 4263–4267, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

INDUCTION OF CYTOCHROME P-450 AND RELATED DRUG-OXIDIZING ACTIVITIES IN MUSCONE (3-METHYLCYCLOPENTADECANONE)-TREATED RATS

EINOSUKE TANAKA, NORIMITSU KURATA*, MAYUMI KOHNO, TAKEMI YOSHIDA* and YUKIO KUROIWA*

Institute of Community Medicine, University of Tsukuba, Ibaraki 305, and
*School of Pharmaceutical Science, Showa University, Tokyo 142, Japan

(*Received* 6 *April* 1987; *accepted* 30 *June* 1987)

Abstract—In the present study, we investigated the effects of muscone on both *in vitro* and *in vivo* parameters of the hepatic microsomal drug-metabolizing enzyme system and other enzyme activities in rats. In the *in vivo* study, the serum dimethadione (DMO)/trimethadione (TMO) ratios at 2 hr after oral administration of TMO (100 mg/kg) were significantly increased in both male and female rats treated with 75 and 150 but not 40 mg muscone/kg. Antipyrine metabolite profile in 24 hr urine of rats pretreated with muscone (150 mg/kg) was examined. The results showed that the excretion of norantipyrine was significantly increased as compared to the control group. In the *in vitro* study, we found that the content of cytochrome P-450, and activities of aminopyrine *N*-demethylase, aniline hydroxylase and δ-aminolevulinic acid (ALA) synthetase were significantly increased as compared to the controls in both male and female rats treated with muscone (75 and 150 mg/kg). This type of induction of the hepatic metabolizing enzymes was similar to that seen after treatment with a prototype drug, phenobarbital.

Muscone (3-methylcyclopentadecanone) is a product usually made from the musk glands of male musk deer. Recently, Peng *et al.* [1] reported that pretreatment of male Sprague–Dawley rats (body weight 50–60 g) with muscone (75 mg/kg, single i.p. injection) induced a cytochrome P-450 species that was similar to the phenobarbital-inducible cytochrome P-450 isozymes, and to many "type I" substrates of the monooxygenase system in binding to P-450, and causing a low- to high-spin transition of the heme group.

In a series of experiments carried out in rats by using trimethadione (TMO), we showed that the plasma or serum concentration ratio of dimethadione (DMO) to TMO measured at 1 or 2 hr after oral administration of TMO correlated well with hepatic microsomal drug-oxidizing capacity which was measured *in vitro* and *in vivo* [2–5]. A similar correlation is seen in normal rats as well as in rats pretreated with some chemicals such as hepatotoxic agents [2–5] and inducers of an enzyme system [6]. The present study was undertaken to investigate the disposition kinetics of TMO and its only metabolite, DMO, and to determine the changes in some drug-metabolizing activities, and urinary amounts of antipyrine and its metabolite in male or female rats pretreated with muscone.

MATERIALS AND METHODS

Chemicals. Muscone was purchased from Ogawa & Co. Ltd (Tokyo, Japan) and TMO was obtained from Dainippon Pharmaceutical Co. Ltd (Osaka, Japan). Antipyrine and phenacetin were purchased from Wako Pure Chemical Ind. (Osaka, Japan), and 4-hydroxyantipyrine, norantipyrine and 3-hydroxymethylantipyrine were obtained from Chikoh Co. Ltd. (Tokyo, Japan). All other chemicals were of the highest grade commercially available.

Animals and treatment. Adult male and female Wistar (W) rats (Doken, Ibaraki, Japan) weighing 200–240 g were used for most of the experiments. Before starting the experiment, the rats were kept in an air-conditioned room (25 ± 1°, 50–60% humidity) with a 12-hr light–dark cycle (8:00–20:00) and maintained on free access to commercial rat chow (Oriental-MF, Tokyo, Japan) and water.

Muscone was dissolved in corn oil and administered by a single intraperitoneal (i.p.) injection at different dose levels (40, 75 and 150 mg/kg) 24 hr prior to the administration of TMO (100 mg/kg, p.o.) or antipyrine (50 mg/kg, i.p.). The rats in the control group received corn oil only. In additional induction studies, the rats received daily i.p. injections of phenobarbital (PB, 80 mg/kg in saline) or 3-methylcholanthrene (3-MC, 20 mg/kg in corn oil) for 3 days.

The control and muscone-treated rats were fixed on a plate (CFK Lab., Tokyo, Japan) only at the time of blood samplings from the jugular vein after oral administration of TMO or i.p. administration of antipyrine without anesthesia. Serum fractions separated from the blood samples by centrifugation were stored at −20° until used for the determination of TMO and DMO levels. For collection of urine after antipyrine administration, control rats and muscone (150 mg/kg)-, PB- and 3-MC-treated-rats were individually placed in metabolic cages (Natsume Seisakusho Co. Ltd, Tokyo, Japan) and were given only water without food, and 24-hr urine samples were collected.

Preparation of the liver microsomal fraction. Following the above-mentioned blood sampling, the rats

were sacrificed 2 hr after TMO which was administered at 9:30 to 10:00. Then, the livers were perfused *in situ* with cold 0.9% NaCl solution via the portal vein, removed and homogenized in 4 vol. of 1.15% KCl by using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 9000 *g* for 20 min. The resulting supernatant fraction was recentrifuged at 105,000 *g* for 60 min. The microsomal pellet was washed once and resuspended in 0.05 M phosphate buffer (pH 7.6) containing 1 mM ethylenediaminetetraacetic acid (EDTA).

Enzyme assay. Heme oxygenase activity was assayed by the method of Tenhunen *et al.* [7] by determining the formation of bilirubin in the presence of cytosolic biliverdin reductase with and without adding NADPH. δ-Aminolevulinic acid (ALA) synthetase activity was assayed by the method of Marver *et al.* [8] using a total liver homogenate as enzyme source. The ALA produced was estimated colorimetrically after condensation with acetylacetone and isolation of the pyrrole compound formed on a Dowex-1-acetate column [9]. The cytochrome P-450 content was determined from the CO difference spectrum of dithionite-treated microsomes as described by Omura and Sato [10]. The cytochrome b_5 content was determined from the difference spectrum between NADH-reduced and air-saturated microsomes as described by Omura and Sato [10].

The activity of aminopyrine *N*-demethylase was measured by determining the formaldehyde released according to the method of Nash [11].

The activity of aniline hydroxylase was estimated by the method of Imai *et al.* [12]. Protein content was determined by the method of Lowry *et al.* [13] using bovine serum albumin as the standard.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Hepatic microsomal proteins from control and muscone-treated rats were dissolved in a solution containing 2% sodium dodecylsulfate, 10% glycerol, 5% β-mercaptoethanol and 62.5 mM Tris–HCl (pH 6.8), and the prepared protein solution was heated at 100° for 3 min before electrophoresis. Samples containing 0.01 mg protein were run on slab gels (2 mm thick) of 10% polyacrylamide in the presence of 0.1% sodium dodecylsulfate at 25 mA/slab for 15 hr according to the method of Laemmli [14]. The gels were stained with 0.25% Coomassie brilliant blue R and dried on thick filter paper.

TMO and DMO assay. Serum TMO and DMO levels were determined by gas–liquid chromatography (GLC) using maleinimide as an internal standard [15].

Assay of antipyrine and its metabolites. Urine concentrations of unchanged antipyrine and its three main metabolites (norantipyrine, 3-hydroxymethylantipyrine and 4-hydroxyantipyrine) were carried out according to Teunissen *et al.* [16] and Blyden *et al.* [17]. To 1 ml of urine, 1.5 ml of 1 M sodium acetate buffer (pH 5.0), 80 mg of sodium metabisulfite ($Na_2S_2O_5$) and 25 mg of limpet acetone powder (β-glucuronidase-sulphatase: type 1) were added. The mixture was incubated for 3 hr in a water bath at 37°. After cooling, 4 ml of chloroform–ethanol (9:1, v/v) containing 40 μg of phenacetin (as an internal standard) and 400 mg of sodium chloride

Table 1. Effects of muscone, phenobarbital (PB) and 3-methylcholanthrene (3-MC) pretreatment on hepatic microsomal cytochrome contents and activities of drug-oxidizing enzyme, heme oxygenase and ALA-synthetase activities in rats

Treatment	Sex	Cytochrome P-450 (nmol/mg protein)	Cytochrome b_5 (nmol/mg protein)	Aminopyrine *N*-demethylase (nmol/mg protein/min)	Aniline hydroxylase (nmol/mg protein/min)	Heme oxygenase (nmol/mg protein/hr)	ALA synthetase (nmol/g liver/hr)
Control	M	0.88 ± 0.08*	0.26 ± 0.02	4.66 ± 0.46	1.17 ± 0.05	2.12 ± 0.22	12.41 ± 0.74
	F	0.66 ± 0.06	0.14 ± 0.01	4.01 ± 0.18	0.91 ± 0.03	2.83 ± 0.17	10.22 ± 0.72
Muscone							
40 mg/kg	M	1.04 ± 0.08	0.28 ± 0.02	6.11 ± 0.23	1.31 ± 0.09	2.07 ± 0.21	15.19 ± 1.82
	F	0.78 ± 0.04	0.12 ± 0.01	5.25 ± 0.16	1.02 ± 0.05	2.79 ± 0.19	11.14 ± 0.98
75 mg/kg	M	1.22 ± 0.05*	0.29 ± 0.02	7.39 ± 0.19*	1.49 ± 0.08*	2.02 ± 0.19	24.86 ± 0.51*
	F	0.92 ± 0.03†	0.14 ± 0.02	6.38 ± 0.22*	1.16 ± 0.04†	2.68 ± 0.12	16.43 ± 0.66*
150 mg/kg	M	1.52 ± 0.04*	0.31 ± 0.02	9.84 ± 0.54*	1.67 ± 0.04*	1.98 ± 0.25	32.70 ± 1.63*
	F	1.14 ± 0.03†	0.16 ± 0.02	8.46 ± 0.26*	1.30 ± 0.03*	2.22 ± 0.23	22.32 ± 0.99*
PB	M	1.66 ± 0.09*	0.33 ± 0.02†	9.66 ± 0.32*	1.62 ± 0.06*	2.23 ± 0.18	26.33 ± 0.88*
3-MC	M	1.23 ± 0.08*	0.28 ± 0.02	6.87 ± 0.17†	1.52 ± 0.04†	2.16 ± 0.23	18.01 ± 0.91†

The values presented are the mean ± SD of 4–5 rats. M: male, F: female.
* $P < 0.01$ compared to control group.
† $P < 0.05$ compared to control group.

(NaCl) were added and the mixture was extracted by mixing on a vortex mixer for 2 min. After centrifugation (10 min, 1800 *g*), the organic phase was evaporated to dryness at 40° under a gentle stream of nitrogen. The residue was dissolved in 0.1 ml of methanol and diluted with 0.1 ml of 0.01 M acetate buffer containing 12.5 mg of $Na_2S_2O_5$ (freshly prepared). Of this solution 20 μl were injected into the HPLC system. HPLC (Model 114 M, Beckman Instrument, Inc, CA) with a variable-wavelength ultraviolet (UV) spectrophotometer (870-UV, Japan Spectroscopic Co. Ltd, Tokyo, Japan) was employed. The column employed was of reverse-phase type (μBondapack C-18, 30 cm long × 4 mm i.d., 5-μm particles). The detector was set at 254 nm, and 7.5% acetonitrile in 0.1 M sodium acetate buffer (pH 5.0) was used as mobile phase. Flow rate was set at 1.5 ml/min. All the analyses were performed at room temperature.

Statistical analysis. The results were statistically analyzed by Student's *t*-test.

RESULTS

We studied the serum DMO/TMO ratios at 2 hr after TMO (100 mg/kg, p.o.) administration in rats treated with muscone (40, 75 or 150 mg/kg), PB or 3-MC. Thus, the DMO/TMO ratios were increased in the rats treated with muscone 75 mg/kg (male: 1.21 ± 0.09 vs 0.96 ± 0.05, mean ± SE, $P < 0.05$; female: 0.56 ± 0.07 vs 0.43 ± 0.03, $P < 0.05$), muscone 150 mg/kg (male 1.48 ± 0.06 vs 0.96 ± 0.05, $P < 0.01$; female 0.66 ± 0.08; 0.43 ± 0.03, $P < 0.01$) and PB (male: 1.75 ± 0.12 vs 0.96 ± 0.05, $P < 0.01$; female: 0.77 ± 0.09 vs 0.43 ± 0.03, $P < 0.01$) as compared to the controls.

However, the serum DMO/TMO ratios were comparable between controls and the rats treated with muscone 40 mg/kg (male: 0.96 ± 0.05 vs 1.07 ± 0.11, NS; female: 0.43 ± 0.03 vs 0.46 ± 0.09, NS) or 3-MC (male: 0.96 ± 0.05 vs 1.08 ± 0.11, NS; female: 0.43 ± 0.03 vs 0.46 ± 0.09 NS).

As shown in Table 1, the contents of cytochrome P-450 and activities of aminopyrine *N*-demethylase, aniline hydroxylase and ALA synthetase were significantly increased in both male and female rats treated with muscone (75 or 150 mg/kg), PB or 3-MC.

On the other hand, in the other groups the contents of cytochrome b_5 and activities of heme oxygenase

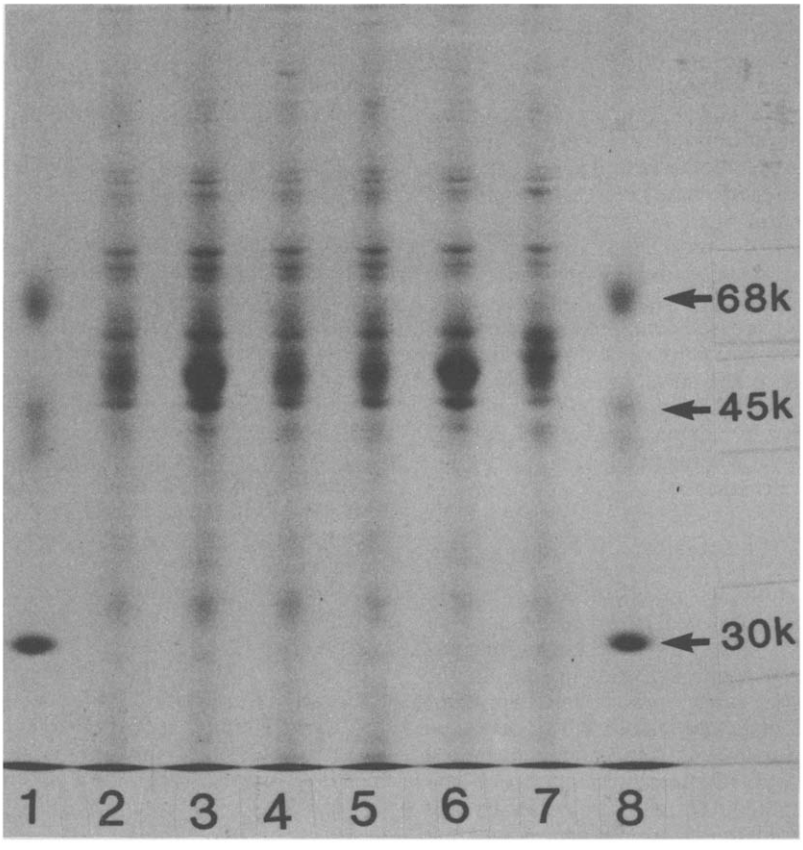

Fig. 1. SDS–polyacrylamide gel electrophoresis of hepatic microsomal protein of control, muscone (150 mg/kg)-, PB- and 3-MC-treated rats. Electrophoresis was performed as described in Materials and Methods. Well 1 and 8 contained protein standards of known molecular weights: bovine serum albumin (MW 68,000) and ovalbumin (MW 45,000), carbonic anhydrase (MW 30,000). Well 2–7 contained microsomal preparations from control rats (well 2 and 4), PB-(well 3; male), 3-MC-(well 5; male) and muscone-(well 6; male: well 7; female) treated rats.

Table 2. Excretion of antipyrine and its metabolites expressed as percentages of dose in 24 hr urine in rats pretreated with muscone, phenobarbital (PB) and 3-methylcholanthrene (3-MC)

Treatment	Sex	Antipyrine	3-Hydroxymethyl-antipyrine	4-Hydroxy-antipyrine	Norantipyrine	Total
Control	M	1.6 ± 1.3	17.1 ± 2.5	26.6 ± 3.6	6.7 ± 0.7	52.0 ± 4.2
Muscone						
75 mg/kg	M	0.8 ± 0.4	15.9 ± 2.7	27.6 ± 1.6	11.2 ± 1.0*	55.6 ± 3.4
150 mg/kg	M	0.9 ± 0.3	15.3 ± 2.6	28.2 ± 2.1	12.5 ± 0.8*	57.0 ± 2.5†
PB	M	0.5 ± 0.4	12.1 ± 3.2*	35.6 ± 2.6†	10.7 ± 1.2*	58.9 ± 3.2†
3-MC	M	1.3 ± 0.8	10.0 ± 1.1†	24.5 ± 1.7	8.5 ± 0.8†	44.2 ± 3.5†

The values presented are the means ± SD of 6–7 rats and are expressed as % dose. M: male.
* $P < 0.01$ compared to control group.
† $P < 0.05$ compared to control group.

were not changed as compared to the controls except for increase of content of cytochrome b_5 in PB-treated group.

The electrophoretic profiles of liver microsomal preparations from control rats and, muscone (150 mg/kg)-, PB- and 3-MC-treated rats are shown in Fig. 1.

It was found that the treatment with muscone and PB induced protein molecules with mol. wt. of *ca.* 52,000.

The data of antipyrine and its main metabolites in 24-hr urine in the four groups are summarized in Table 2, where the results are expressed as percentages of the dose (50 mg/kg, i.p.) of antipyrine. In the muscone (75 and 150 mg/kg)-treated rats, the amount of norantipyrine and total amount of antipyrine and its metabolite (conjugated + free) in the 24 hr urine was significantly increased compared to that of the control rats.

In the PB-treated group, the amounts of 4-hydroxyantipyrine, and norantipyrine and total amounts of antipyrine and its metabolite in urine were significantly increased compared to the control group, but the amount of 3-hydroxyantipyrine decreased. On the other hand, in rats treated with 3-MC, the amounts of 3-hydroxyantipyrine, and total amount of antipyrine and its metabolite were significantly decreased, whereas that of norantipyrine was significantly increased.

DISCUSSION

In the present study, we investigated the effects of muscone on both *in vitro* and *in vivo* parameters of the hepatic microsomal drug-metabolizing enzyme system and other enzyme activities in rats. In the preliminary study, male and female Sprague–Dawley rats, and male and female Wistar rats were pretreated with muscone 150 mg/kg at 24 hr before administration of TMO, and serum DMO/TMO ratios were estimated. Serum DMO/TMO ratios were significantly increased in Wistar rats whereas in Sprague–Dawley rats, an increase from the control was seen only in the high dose of 350 mg/kg. Accordingly, Wistar rats were employed in the main study.

Metabolic profiles in Wistar rats pretreated with muscone 40, 75 and 150 mg/kg were investigated both *in vivo* and *in vitro*.

In the *in-vivo* study, we determined serum DMO/TMO ratios at 2 hr after administration of TMO 100 mg/kg to male and female rats, and 24-hr urinary excretion of antipyrine metabolites after antipyrine administration to male rats. Induction of metabolic activity was observed both for TMO and antipyrine. A large production of norantipyrine by muscone was characterized by induction of muscone as well as PB (Table 2). This induction occurred in the doses of 75 and 150 but not 40 mg/kg.

In-vitro experiments produced results similar to those obtained *in vivo*. Cytochrome P-450 contents, and activities of aminopyrine *N*-demethylase, aniline hydroxylase, and ALA synthetase, the last being a key enzyme in heme biosynthesis, were increased from the controls in both male and female rats treated with muscone 75 and 150 but not 40 mg/kg. This pattern of induction was similar to those found after treatment with PB or 3-MC (Table 1).

The results of SDS-polyacrylamide gel electrophoresis of hepatic microsomal protein from the muscone-treated rats were rather similar to those from the PB-treated rats. The induction in females was not so marked as in males (Fig. 1).

Peng *et al.* [1] investigated hepatic microsomal enzymes from young (body weight: 50–60 g) Sprague–Dawley rats *in vitro*, and showed that either induction or inhibition of the drug-metabolizing enzyme was observed, depending on the substrates used. The difference from our findings might be related to the age, strain, or some other factors of the rats used.

In the previous report [6], we suggested that TMO metabolism is induced by PB treatment but not 3-MC.

In summary, male rats were more sensitive to the induction of drug-metabolizing enzymes by muscone than females, and it appears that muscone induces a cytochrome P-450 component that may be similar to the PB-sensitive cytochrome P-450 isozyme.

REFERENCES

1. R. Peng, X-Y. Zhu and C. S. Yang, *Biochem. Pharmac.* **35**, 1391 (1986).
2. E. Tanaka, H. Kinoshita, T. Yoshida and Y. Kuroiwa, *J. Pharmaco-Dyn.* **4**, 961 (1981).

3. E. Tanaka, H. Kinoshita, T. Yoshida and Y. Kuroiwa, *Jap. J. Pharmac.* **32**, 1182 (1982).
4. E. Tanaka, H. Kinoshita, T. Yoshida and Y. Kuroiwa, *Pharmacology* **25**, 202 (1982).
5. E. Tanaka, T. Nakamura, S. Misawa, T. Yoshida and Y. Kuroiwa, *Res. Commun. Chem. Pathol. Pharmac.* **45**, 137 (1984).
6. E. Tanaka, H. Kinoshita, T. Yoshida and Y. Kuroiwa, *J. Pharmaco-Dyn.* **5**, 162 (1982).
7. R. Tenhunen, H. S. Marver and R. Schmid, *J. Lab. clin. Med.* **75**, 410 (1970).
8. H. S. Marver, D. P. Tschudy, M. G. Perlroth and A. Collins, *J. biol. Chem.* **241**, 2803 (1966).
9. G. Urata and S. Granick, *J. biol. Chem.* **238**, 811 (1963).
10. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
11. T. Nash, *Biochem. J.* **55**, 416 (1953).
12. Y. Imai, A. Ito and R. Sato, *J. Biochem. (Tokyo)* **60**, 417 (1966).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 263 (1951).
14. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
15. E.Tanaka and S. Misawa, *J. Chromatogr.* **413**, 376 (1987).
16. M. W. E. Teunissen, J. E. Meerberg-van Der Torren, N. P. E. Vermeulen and D. D. Breimer, *J. Chromatogr.* **278**, 367 (1983).
17. G. T. Blyden, B. W. LeDuc and D. I. Greenblatt, *Pharmacology* **32**, 226 (1986).