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Biochemical Pharmacology, Vol. 29, pp. 845–847.

0006-2952/80/0301–0845 $02.00/0

Hepatic mitochondrial cholesterol hydroxylase activity—a cytochrome P-450-catalyzed mono-oxygenation refractory to cobaltous chloride

(*Received* 7 *May* 1979; *accepted* 14 *September* 1979)

It is well known that the administration of cobaltous chloride to rats results in an inhibition of hepatic heme biosynthesis [1–3] and a marked decrease in the concentration of cytochromes P-450 in hepatic microsomes [1–6]. Since hepatic mitochondria have been shown recently to contain a form of cytochrome P-450 which catalyses the 25- and 26-hydroxylations of cholesterol* [7–11], the present study was undertaken to determine if mitochondrial and microsomal cytochromes P-450 are affected similarly by cobaltous chloride.

Glucose-6-phosphate, yeast glucose-6-phosphate dehydrogenase (type VII), NADP, NADPH (type III) and cholesterol (chromatography standard grade) were obtained from the Sigma Chemical Co. (St. Louis, MO). [7(*n*)-^{3}H]Cholesterol (specific activity, 9.5 Ci/mmole) was obtained from Amersham/Searle (Arlington Heights, IL), and the radioactive purity was confirmed to be greater than 98 per cent by thin-layer chromatographic analysis. Ethylmorphine hydrochloride was purchased from Merck & Co., Inc. (Rahway, NJ); thin-layer plastic sheets (5 × 20 cm), precoated with silica gel G, were obtained from Brinkman Instruments, Inc. (Westbury, NY). Metyrapone and aminoglutethimide were gifts from the Ciba–Geigy Corp. (Summit, NJ). Goat anti-bovine adrenal ferredoxin and goat preimmune immunoglobulin (Ig) fractions were obtained as described previously [12]. Sheep anti-rat hepatic microsomal NADPH-cytochrome *c* (P-450) reductase and sheep preimmune IgG fractions were prepared as described previously [13]. All other chemicals employed were of the highest purity available.

Male albino Holtzman rats, 150–250 g, were used throughout these studies. Cobaltous chloride ($CoCl_2 \cdot 6H_2O$) was administered subcutaneously once daily for 4 days at a dose of 60 mg/kg. Control rats received

* Trivial names used are: cholesterol, 5-cholesten-3β-ol; 25-hydroxycholesterol, 5-cholesten-3β,25-diol; 26-hydroxycholesterol, 5-cholesten-3β,26-diol; and Ig, immunoglobulin.

Table 1. Effects of mono-oxygenase inhibitors and adrenal ferredoxin on rat hepatic mitochondrial cholesterol hydroxylase activity

Addition	Concentration	% Conversion in 30 min*
None		1.30 ± 0.06 (100)
Metyrapone	0.1 mM	0.61 ± 0.11† (46)
	1.0 mM	0.18 ± 0.03† (14)
Aminoglutethimide	1.0 mM	0.70 ± 0.12† (53)
	10.0 mM	0.04 ± 0.04† (3)
Adrenal ferredoxin	2.0 mM	5.40 ± 0.37† (392)
Goat anti-adrenal ferredoxin Ig	0.1 mg/mg mitochondrial protein	0.31 ± 0.08† (23)
Goat preimmune Ig	0.1 mg/mg mitochondrial protein	1.03 ± 0.08‡ (80)
Sheep anti-NADPH-cytochrome *c* (P-450) reductase IgG	0.1 mg/mg mitochondrial protein	1.04 ± 0.05‡ (80)
Sheep preimmune IgG	0.1 mg/mg mitochondrial protein	1.08 ± 0.14 (83)

* Each value represents the mean ± S.E. for four determinations with the percentage of control activity given in parentheses.
† $P < 0.001$, compared with the control value.
‡ $P < 0.02$, compared with the control value.

Table 2. Effect of cobaltous chloride pretreatment on rat hepatic microsomal cytochrome P-450 content, microsomal ethylmorphine *N*-demethylase activity and mitochrondrial cholesterol hydroxylase activity*

Pretreatment	Microsomal cytochrome P-450 content (nmoles/mg protein)	Ethylmorphine *N*-demethylase activity (nmoles/min/mg protein)	Cholesterol hydroxylase activity† (% conversion in 30 min)
Distilled water	0.84 ± 0.05	15.9 ± 0.7	4.35 ± 0.62
Cobaltous chloride	0.15 ± 0.03‡ (18)	0.9 ± 0.3‡ (6)	4.73 ± 0.69 (111)

* Each value represents the mean ± S.E. of eight determinations with the percentage of control activity or content given in parentheses.

† It should be noted that the rats used to obtain the results presented in Table 1 were not fasted, and the percentage of tritiated products formed was considerably less than the values reported in this table where the rats had been fasted for 5 days. The difference in activity is presumably due to differences in the endogenous cholesterol contents in mitochondria isolated from the livers of fed and fasted rats.

‡ $P < 0.001$, compared to control (distilled water pretreated).

appropriate volumes of vehicle (distilled water). Rats were fasted throughout the treatment period and were killed 24 hr after the last injection. Livers were perfused with ice-cold 0.9% NaCl, and mitochondria and microsomes were prepared from 10% (w/v) homogenates by differential centrifugation, as described previously [12, 14]. The mitochondrial pellets were suspended in distilled water at a protein concentration of 10 mg/ml, and the mitochondrial membrane structure was disrupted by freezing and thawing five times, using a dry ice–acetone bath [12]. Cholesterol hydroxylase activity catalysed by the frozen and thawed mitochondria was determined employing minor modifications of the method described previously [15]. Each 0.5-ml reaction mixture contained 3 mg of mitochondrial protein, 1 mM NADP, 4.25 mM glucose-6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase, 1 mM KCN, 10 mM $MgCl_2$, and 0.1 M Tris–HCl buffer, pH 7.4. The reactions were initiated by the addition of 1.3 μCi [7(*n*)-^{3}H]cholesterol in 0.01 ml acetone, and were terminated after a 30-min incubation at 37° by the addition of 2 ml methanol. Cholesterol hydroxylase activity was expressed as the percentage of conversion of [7(*n*)-^{3}H]cholesterol to ^{3}H-labeled 25- and 26-hydroxycholesterol during the 30-min incubation.

Microsomal pellets were resuspended in 0.25 M sucrose, containing 50 mM Tris–HCl buffer, pH 7.4, to a protein concentration of 10 mg/ml. Ethylmorphine *N*-demethylase activity catalysed by hepatic microsomes was determined at 37° by measuring formaldehyde production according to the method of Nash [16], as modified by Cochin and Axelrod [17]. Each 7-ml reaction mixture contained 7–14 mg of microsomal protein, 8 mM ethylmorphine, 150 mM KCl, 10 mM $MgCl_2$, and 50 mM Tris–HCl buffer, pH 7.4. Ethylmorphine *N*-demethylase activity was initiated by the addition of 200 μM NADPH, and 1-ml aliquots were removed every 30 sec for the determination of formaldehyde.

The contents of cytochrome P-450 in hepatic microsomal preparations were determined from carbon monoxide difference spectra, employing an extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorbance between 450 and 490 nm [18]. Protein was determined by the biuret method [19], using bovine serum albumin as a standard.

The hydroxylations of cholesterol at carbons 25 and 26 catalysed by hepatic mitochondrial preparations have been demonstrated to be ferredoxin and cytochrome P-450 dependent [7–11, 15], and the results presented in Table 1 are consistent with findings reported previously. Both metyrapone and aminoglutethimide, inhibitors of cytochrome P-450-catalysed mono-oxygenations [20–22], inhibited cholesterol hydroxylation in a concentration-dependent manner (Table 1). Furthermore, the addition of homogeneous bovine adrenal ferredoxin markedly stimulated cholesterol hydroxylation, while the addition of goat antibody produced against homogeneous bovine adrenal ferredoxin significantly inhibited cholesterol hydroxylation, thereby confirming the previously reported ferredoxin requirement [7, 8, 15]. Antibody produced against rat hepatic microsomal NADPH-cytochrome *c* (P-450) reductase exerted a weak inhibitory effect on cholesterol hydroxylase activity (Table 1). The observation that similar degrees of inhibition were produced by the sheep anti-reductase IgG fraction and the preimmune goat Ig and sheep IgG fractions indicates that this inhibitory effect was nonspecific and that the determined cholesterol hydroxylase activity was not of microsomal origin. This observation is in agreement with the report by Björkhem and Gustafsson [23] that cholesterol is not hydroxylated at carbons 25 and 26 by hepatic microsomes.

In contrast to the observation of Okuda *et al.* [11], cytochrome P-450 could not be detected spectrophotometrically in our mitochondrial preparations. Since the content of mitochondrial cytochrome P-450 could not be determined directly, possible alterations in the level of this hemoprotein in hepatic mitochondria following cobaltous chloride administration were assessed indirectly by determining the cytochrome P-450-dependent cholesterol hydroxylase activity. The data presented in Table 2 demonstrate that, while pretreatment of rats for 4 days with cobaltous chloride resulted in an 82 per cent decrease in hepatic microsomal cytochrome P-450 content and a 94 per cent decrease in the associated ethylmorphine *N*-demethylase activity, hepatic mitochondrial cholesterol hydroxylase activity was not affected. Although the percentage of conversion of cholesterol to hydroxylated products is dependent on the amount of endogenous cholesterol present, differences were not seen when the contents of cholesterol were determined in hepatic mitochondria prepared from cobaltous choride- and vehicle-pretreated rats.*

The results presented in this communication demonstrate that rat hepatic microsomal and mitochondrial cytochome P-450-catalysed mono-oxygenations are differentially affected by pretreatment with cobaltous chloride. These findings suggest that the half-life of mitochondrial cytochrome P-450 is greater than the half-lives of microsomal cytochromes P-450.

Acknowledgements—The authors would like to thank Dr. T. R. Tephly for helpful discussions and critical evaluation of this work. This study was supported by USPHS Grant 12675 from the National Institute of General Medical Sciences.

* G. Kapke, T. Kaduce, A. Spector and J. Baron, unpublished observations.

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* Recipient of Research Career Development Award 00091 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.