

Demethylation capacity of human fetal adrenal mitochondrial cytochrome P-450 *in vitro*¹

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KEY WORDS cytochrome P-450; adrenal glands; fetus; mitochondria; troleandomycin; erythromycin; benzphetamine; aminopyrine; microsomes; polyacrylamide gel electrophoresis

ABSTRACT

AIM: To explore the capacity and characteristics of adrenal mitochondria to metabolize xenobiotics *in vitro* in human fetus. **METHODS:** Subcellular fractions of fetal adrenal were prepared by differential centrifugation. Mitochondrial P-450 system was proved by spectral analyses and SDS-PAGE. The formaldehyde formation contents were measured with Nash reagent. **RESULTS:** The erythromycin *N*-demethylation linearly increased in the protein concentration (1-4 mg)- and incubation time (10-30 min)-dependent manners. A typical concentration-effect relationship appeared with erythromycin 0.067-1 mmol·L⁻¹ and a positive correlation ($r = 0.641$, $P < 0.05$) existed between erythromycin *N*-demethylation and gestation months. The *N*-demethylation values (nmol·s⁻¹/g protein) of erythromycin (2.7 ± 0.8), benzphetamine (1.1 ± 0.5), and aminophenazone (0.9 ± 0.4) in mitochondria were 89% ($P > 0.05$), 162% ($P < 0.01$), and 62% ($P < 0.01$), respectively, of those in microsomes. There was correlation between mitochondria and microsomes in the *N*-demethylation of erythromycin ($r = 0.708$, $P < 0.05$) and benzphetamine ($r = 0.707$, $P < 0.05$). Troleandomycin stimulated erythromycin *N*-demethylation in adrenal mitochondria as well as in adrenal and liver microsomes *in vitro*.

CONCLUSION: Fetal adrenal mitochondria, with multiple P-450 isoforms and greater capacity of demethylation, play a role in drug-metabolism during fetal development.

INTRODUCTION

The multiple forms of P-450 existed in many extrahepatic organs. Adrenal gland contains P-450 enzymes which are mainly involved in the steroidogenesis^[1]. We previously demonstrated the existence and higher activities of aniline hydroxylase and aminophenazone *N*-demethylase in adrenal microsomes of human fetus in comparison with liver microsomes^[2]. In this paper, we studied the characteristics and the demethylation capacity of adrenal mitochondrial P-450 system *in vitro* in human fetus, to further understand the biotransformation function of adrenal gland.

MATERIALS AND METHODS

Biological samples Human fetal specimens were obtained from therapeutic abortions and legal abortions during 23-38 wk of gestation, approved by the Academic Committee and the Ethics Committee of Hubei Medical University.

Chemicals Erythromycin, benzphetamine, aminophenazone, isocitric acid, and isocitric acid dehydrogenase were from Sigma. All other chemicals and reagents were of AR.

Subcellular fractions The isolation procedure of subcellular fractions was described in detail^[3]. In brief, adrenal and liver homogenates were spun at 9000 × *g* for 20 min to get mitochondrial pellets. The postmitochondria supernatants (S₀) were spun at 105 000 × *g* for 60 min to get microsomes. The

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mitochondrial and microsomal pellets were suspended in sucrose $0.25 \text{ mol} \cdot \text{L}^{-1}$ corresponding to 1 g of adrenal per mL and stored at $-30 \text{ }^\circ\text{C}$.

Enzyme assays The total P-450 content, spectral analysis and NADPH-cytochrome (Cyt) C reductase activity were assayed as described before^[2] with a Shimadzu (UV-3000) recording spectrophotometer.

Demethylation was determined using erythromycin, benzfetamine, and aminophenazone as substrates. The assay mixture (pH 7.4, $37 \text{ }^\circ\text{C}$) contained Tris·HCl $50 \text{ mmol} \cdot \text{L}^{-1}$, MgCl_2 $10 \text{ mmol} \cdot \text{L}^{-1}$, KCl $150 \text{ mmol} \cdot \text{L}^{-1}$ and an NADPH-generating system (including NADP^+ $0.4 \text{ mmol} \cdot \text{L}^{-1}$, isocitric acid $10 \text{ mmol} \cdot \text{L}^{-1}$, and isocitric acid dehydrogenase 0.6 units). Erythromycin $0.4 \text{ mmol} \cdot \text{L}^{-1}$, benzfetamine $2 \text{ mmol} \cdot \text{L}^{-1}$, or aminophenazone $8 \text{ mmol} \cdot \text{L}^{-1}$ was added. The reaction was initiated with the NADPH-generating system. The supernatant was incubated with the Nash reagent at $60 \text{ }^\circ\text{C}$ for 20 min and the color was measured at 415 nm in a Shimadzu (UV-120) spectrophotometer.

The formaldehyde formation rates from erythromycin, benzfetamine, and aminophenazone were defined under the above selected incubation conditions. The effects of troleandomycin on the formaldehyde formation were determined at $0 - 200 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$ by preincubation with the mitochondria (or microsome) and NADPH-generating system at $37 \text{ }^\circ\text{C}$ for 30 min before adding substrate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) The SDS-PAGE of adrenal mitochondrial and microsomal protein was performed on a 10 %-acrylamide separating gel. The sample ($30 \text{ } \mu\text{g}$ protein) was loaded onto each of gel lanes for the detection of total P-450. Standard proteins and their molecular weights (M_r) included lysozyme (14 400), carbonic anhydrase (31 000), ovalbumin (42 700), bovine serum albumin (66 200), and phosphorylase b (97 400).

Data analysis Data were expressed as $\bar{x} \pm s$. Difference between two groups of the adrenal mitochondrial and microsomal fractions was detected using the paired comparison of *t* test.

RESULTS

Characteristics of adrenal mitochondrial

P-450 When dithionite-reduced difference spectral analyses were employed, most of the adrenal mitochondria (13/15 fetuses, 87 %) yielded a maximal absorption of P-450 at 448 nm and the ratio between the highest and lowest mitochondrial P-450 content was about 7.0-fold. Demonstrable tendency of P-450 levels to increase as a function of fetal age was observed ($r = 0.773$, $P < 0.01$). The rate-limiting enzyme — NADPH-Cyt C reductase activity in adrenal mitochondria was also detected. No marked sex difference was observed in P-450 content and NADPH-Cyt C reductase activity. (Tab 1)

Tab 1. The total Cyt P-450 content and NADPH-Cyt C reductase activity in adrenal mitochondria of human fetus. $\bar{x} \pm s$.

Indices	Mean (15)	Male (7)	Female (8)
P-450/ $\mu\text{mol} \cdot \text{g}^{-1}$	0.22 ± 0.13	0.23 ± 0.15	0.22 ± 0.12
Cyt C reductase/ $\mu\text{mol} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$	1.2 ± 0.6	1.4 ± 0.7	1.1 ± 0.6
Protein/ $\text{g} \cdot \text{L}^{-1}$	19 ± 6	15 ± 5	22 ± 4

The existence of adrenal mitochondrial P-450 was also shown using SDS-PAGE (Fig 1).

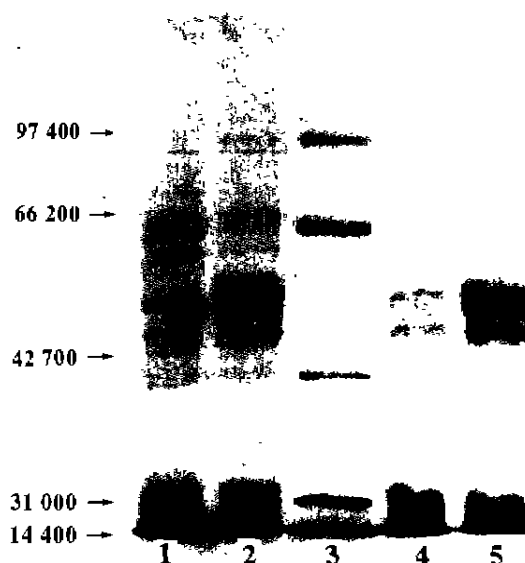


Fig 1. SDS-PAGE electrophoresis on a 10 %-acrylamide separating gel. Lane 1 and 4, $30 \text{ } \mu\text{g}$ of adrenal mitochondrial protein of 2 human fetuses. Lane 2 and 5, $30 \text{ } \mu\text{g}$ of adrenal microsomal protein of 2 human fetuses. Lane 3, $20 \text{ } \mu\text{g}$ of standard proteins.

Taken the adrenal microsomal protein as the P-450 positive control, two mitochondrial protein bands of M_r about 47 400 and 54 200, and three microsomal protein bands of M_r about 45 100, 48 700, and 52 300 were detected on the 10 %-acrylamide separating gel. In the range of P-450 molecular weights, the total microsomal P-450 peak area was nearly 2 times of those of mitochondria by thin layer chromatography.

Mitochondrial demethylation The formaldehyde formation rates from erythromycin catalyzed by adrenal mitochondria were found to be linear with 1–4 mg of mitochondrial protein and 10–30 min of incubation time, which meant that the capacity of mitochondrial demethylation *in vitro* increased in the protein concentration- and reaction time-dependent manners. The relationship of demethylation velocity and substrate concentration was observed and a concentration-effect relationship appeared over the range of erythromycin 0.067–1 mmol·L⁻¹ (Fig 2).

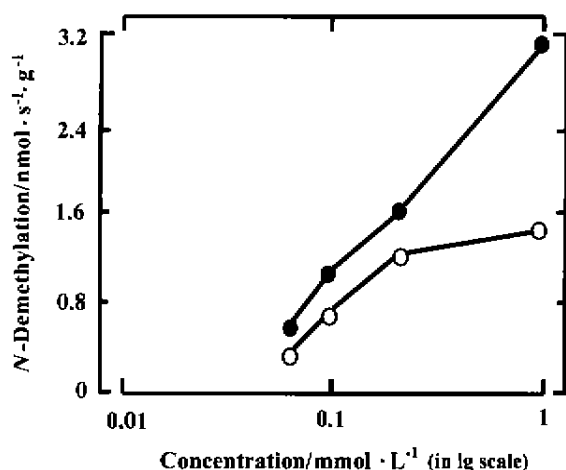


Fig 2. Effect of erythromycin *N*-demethylation by human fetal adrenal mitochondria. Two mg of mitochondrial protein was incubated with erythromycin at 37 °C for 30 min. $n = 2$ fetuses.

The highest formaldehyde formation was adrenal mitochondrial demethylation level of erythromycin, and the lowest was that of aminophenazone. Erythromycin, benzfetamine, and aminophenazone *N*-demethylations in adrenal mitochondria were 89 % ($P > 0.05$), 162 % ($P < 0.01$), 62 % ($P < 0.01$), respectively, of the corresponding ones in adrenal microsomes. (Tab 2)

Tab 2. *N*-Demethylations (nmol·s⁻¹·g⁻¹) of erythromycin, benzfetamine, and aminophenazone in adrenal mitochondria and microsomes of human fetus. $n = 10$ fetuses. $\bar{x} \pm s$. ^a $P > 0.05$, ^c $P < 0.01$ vs the corresponding microsomal group.

Groups	Erythromycin	Benzfetamine	Aminophenazone
Mitochondria	2.7 ± 0.8 ^a	1.1 ± 0.5 ^c	0.9 ± 0.4 ^c
Microsomes	3.0 ± 0.8	0.7 ± 0.5	1.4 ± 0.4

Correlation analyses There was a positive correlation between mitochondrial erythromycin *N*-demethylation and gestation months ($r = 0.641$, $P < 0.05$). Meanwhile, a correlation also existed between mitochondrial P-450 content and benzfetamine *N*-demethylation ($r = 0.744$, $P < 0.01$). The good positive correlation occurred between adrenal mitochondrial and microsomal demethylation (erythromycin; $r = 0.708$, $P < 0.05$; benzfetamine; $r = 0.707$, $P < 0.05$).

Effect of troleandomycin on human fetal demethylation *in vitro* Troleandomycin was initially employed to identify involvement of P-450 3A4 in mitochondrial demethylation. However, erythromycin, benzfetamine, and aminophenazone *N*-demethylation were unexpectedly enhanced by preincubation of troleandomycin 25–200 μmol·L⁻¹ with adrenal mitochondria at 37 °C for 30 min. The maximal stimulation of erythromycin, benzfetamine, and aminophenazone *N*-demethylation by troleandomycin was 1.25–1.26, 1.37–10.58, and 1.37–1.82-fold, respectively, of control (Tab 3).

Tab 3. Effects of troleandomycin on *N*-demethylation (nmol·s⁻¹·g⁻¹) by adrenal mitochondria of human fetus. $n = 2$ fetuses. \bar{x} of duplicates.

Troleandomycin /μmol·L ⁻¹	Erythromycin n1	Erythromycin n2	Benzfetamine n1	Benzfetamine n2	Aminophenazone n1	Aminophenazone n2
0	2.65	3.79	3.80	0.36	1.75	2.35
25	2.70	3.31	5.22	1.94	2.00	3.45
50	3.00	3.45	5.03	2.16	2.28	4.27
100	3.33	3.88	4.93	3.81	2.00	3.41
200	3.08	4.75	4.37	3.74	2.40	3.31

The stimulation of troleandomycin was also found in adrenal and liver microsomes, where the increasing

velocities of erythromycin *N*-demethylation were 1.08 – 1.33 and 1.15 – 1.33 fold, respectively, of control. (Tab 4)

Tab 4. Effects of troleandomycin on erythromycin (0.4 nmol·L⁻¹) *N*-demethylations (nmol·s⁻¹·g⁻¹) in liver and adrenal microsomes of human fetus. *n* = 2 fetuses. \bar{x} of duplicates.

Troleandomycin / $\mu\text{mol}\cdot\text{L}^{-1}$	Adrenal microsomes		Liver microsomes	
	n 1	n 2	n 1	n 2
0	3.82	3.84	5.33	3.67
25	4.17	4.07	ND	4.12
50	4.23	4.15	5.37	4.46
100	4.45	3.96	5.72	4.82
200	5.08	3.96	6.13	4.88

ND = no determination.

DISCUSSION

The demethylation is an important catalyzing function of P-450 monooxygenase system. Our results showed that the adrenal mitochondrial P-450 contents were almost at the same range of the adrenal microsomal ones^[2]. Meanwhile, the adrenal mitochondria catalyzed *N*-demethylation of erythromycin, benzfetamine, and aminophenazone. The demethylation showed a substrate-dependent manner and the capacity was comparable with that of adrenal microsome. Furthermore, the demethylation was enhanced by pretreatment with troleandomycin. Benzfetamine and erythromycin *N*-demethylation were catalyzed by P-450 2B and 3A4, respectively, in human and animal liver microsomes^[4,5]. Our results suggested the existence of multiple P-450 isoforms in adrenal mitochondria of human fetus. P-450 3A4 is the most abundant isoform of human hepatic P-450 enzyme and capable of oxidizing many important drugs^[6]. In our study, erythromycin *N*-demethylation was also the most potent among the 3 compounds.

Troleandomycin as a potent and mechanism-based P-450 3A4 inhibitor has been applied in drug-metabolism *in vitro*^[7] for the fractional inhibition of a reaction in microsome (or another crude preparation) expressed the extent to which a particular P-450 (or set of P-450) is responsible for a reaction^[8]. However, our results showed the enhanced demethylation of adrenal mitochondria catalyzed by troleandomycin *in*

vitro, and further proved similar effects in adrenal and liver microsomes. Troleandomycin is the single inducer of hepatic microsomal P-450 3A and can stimulate hepatic erythromycin *N*-demethylase *in vivo* in rat^[9]. Where the difference is from has not been eventually ascertained and needs further investigating. In a word, the matter of P-450 3A4 requires special attention since its active site has some unusual features^[8].

The present study indicated the higher capacity of mitochondrial demethylation and possible existence of mitochondrial multiple P-450 isoforms in fetal adrenal gland, which supported our previous conclusion^[2,3] that adrenal gland served as an important drug-metabolizing organ during fetal development.

REFERENCES

- 1 Nagaya M, Arai M, Widmaier EP. Ontogeny of immunoreactive and bioactive microsomal steroidogenic enzymes during adrenocortical development in rats. *Mol Cell Endocrinol* 1995; 114: 27–34.
- 2 Peng RX, Wang YS, Lei SB, Fu LS, Chen JH, Li QX, *et al.* Characterization of monooxygenase system in Chinese fetal adrenal gland. *Asian Pac J Pharmacol* 1994; 9: 195–200.
- 3 Peng RX, Wang H, Wang YS, Fu LS, Ding H. Glutathione-related enzyme activities in human fetal adrenal, liver, and kidney. *Acta Pharmacol Sin* 1998; 19: 167–71.
- 4 Wrighton SA, Thomas PE, Willis P, Maines SL, Watkins PB, Levin W, *et al.* Purification of a human liver cytochrome P-450 immunochemically related to several cytochromes P-450 purified from untreated rats. *J Clin Invest* 1987; 80: 1017–22.
- 5 Yamazaki H, Urano T, Hiroki S, Shinnada T. Effects of erythromycin and roxithromycin on oxidation of testosterone and nifedipine catalyzed by CYP3A4 in human liver microsomes. *J Toxicol Sci* 1996; 21: 215–26.
- 6 Inaba T, Nebert DW, Burchell B, Watkins PB, Goldstein JA, Bertilsson L, *et al.* Pharmacogenetics in clinical pharmacology and toxicology: a tribute to Werner Kalow. Toronto, Ontario, Canada, 1994 Jul 20. *Can J Physiol Pharmacol* 1995; 73: 331–8.
- 7 Schmider J, Greenblatt DJ, Fogelman SM, von Moltke LL, Shader RI. Metabolism of dextromethorphan *in vitro*; involvement of cytochromes P450 2D6 and 3A3/4, with a possible role of 2E1. *Biopharm Drug Dispos* 1997; 18: 227–40.
- 8 Halpert JR, Guengerich FP, Bend JR, Correia MA.

Contemporary issue in toxicology. Selective inhibitors of cytochromes P450.

Toxicol Appl Pharmacol 1994; 125: 163-75.

9 Delaporte E, Cribb AE, Renton KW. Modulation of rat hepatic CYP3A1 induction by the interferon inducer polyinosinic acid-polycytidylic acid (polyic).

Drug Metab Dispos Biol Fate Chem 1993; 21: 520-3.

微粒体; 聚丙烯酰胺凝胶电泳

目的: 了解胎肾上腺线粒体代谢外源性化合物的能力和特征. 方法: 制备亚细胞组分. 酶学检测脱甲基反应代谢产物—甲醛的含量. 结果: 在光谱分析和 SDS-PAGE 证实线粒体存在 P-450 的基础上, 进一步证明线粒体 P-450 具有脱甲基功能. 其脱甲基作用呈蛋白浓度(1-4 mg)和反应时间(10-30 min)依赖性增加, 与底物浓度间有良好的量效关系, 并与胎龄呈正比. 线粒体中红霉素、苯非他明和氨基比林的脱甲基反应分别为微粒体中的 89%, 162% 和 62%. 醋竹桃霉素增强肾上腺的红霉素脱甲基反应. 结论: 胎肾上腺线粒体有较强的脱甲基功能. 提示胎儿肾上腺线粒体兼有药物代谢功能.

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人胎肾上腺线粒体细胞色素 P-450
体外脱甲基功能¹

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关键词 细胞色素 P-450; 肾上腺; 胎儿; 线粒体;
醋竹桃霉素; 红霉素; 苯非他明; 氨基比林;

脱甲基功能

药理学进展 (1998)

金正均 王永铭 苏定冯, 主编

本书介绍了近几年来国内外药理学研究的某些进展. 内容涉及药理学的各个领域, 包括神经药理学、心血管药理学、肿瘤化疗、免疫药理学、临床药理学和毒理学等. 从药物作用的分子机制到临床应用均有论述, 颇有一定的深度和广度. 本书可供从事药理学及其相邻学科的科研、教学人员学习参考.

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