Induction of Cytochrome P-450c and P-450d by Metyrapone in the Primary Culture of Rat Hepatocytes

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Accepted June 15, 1989

Abstract—The effects of metyrapone on qualitative changes in cytochrome P-450-dependent drug metabolizing activities in primary cultures of rat hepatocytes were investigated. Metyrapone apparently increased benzo(a)pyrene hydroxylation and maintained both ethoxycoumarin-O-deethylation and propoxycoumarin-O-depropylation, whereas it had little effect on methoxycoumarin-O-demethylation. Furthermore, P-450d (high spin type of P-448) as well as P-450c (low spin type of P-448) were induced by metyrapone, while P-450b and P-450e were not. In conclusion, metyrapone act as a 3-methylcholanthrene-like inducer in the primary cultures of rat hepatocytes.

A primary culture of hepatocytes is regarded as a suitable system for studying the metabolic fates of drugs, the mechanism of their toxic effects and the regulation of drug metabolizing enzymes. However, the capacities of drug metabolizing activities are normally diminished within the first few hours of culture and, sometimes, they are not sufficient for toxicological and biochemical studies (1). Until now, several modifications have been attempted to maintain the level of drug metabolizing activities, but most of the systems reported still do not exactly mimic the profile of drug metabolism in vivo. Lake and Paine (1) have recently shown that the addition of pyridines prevent the decrease in drug metabolizing activities in cultured hepatocytes. The substrate specificity and population of P-450s have also been suggested to change in hepatocytes during the periods of culture. Therefore, we have investigated in the present study the effect of metyrapone on changes in individual forms of P-450 and several P-450-dependent drug metabolizing activities in cultured hepatocytes to understand the mechanism of the metyrapone-induced increases in drug metabolizing activities.

Cytochrome P-450o and P-450e were purified from phenobarbital-treated male Sprague-Dawley rats as described previously (2), while cytochrome P-450c and P-450d were from polychlorinated biphenyl-treated male Sprague-Dawley rats (3). Polyclonal antibodies to P-450b/e, P-450c and P-450d isoforms were raised in rabbits. In the Western blotting experiments (data not shown), the antibody raised to P-450b recognized it and P-450e, and the antibody to P-450d recognized it and P-450c among the four purified proteins. Antibody to P-450c recognized only P-450c. Because these four P-450 isoforms can be separated by SDS-polyacrylamide gel electrophoresis, we could distinguish between these isoforms by using these antibodies.

Hepatocytes were prepared from male Sprague-Dawley rats (9–12 weeks) by the
method of Moldéus et al. (4). Aliquots (2×10⁶ cells/4 ml) of hepatocytes were placed in collagen-coated dishes (60 mm in diameter, CORNING) and incubated at 37°C in 4 ml of William's E medium (Flow Laboratories) containing 10% fetal calf serum (Flow Laboratories), 10⁻⁹ M insulin (bovine, Choay Chimie Reactifs), 10⁻⁶ M dexamethasone (Roussel Uclaf), 10⁻⁴ M δ-aminolevulinic acid (Sigma) and antibiotics (50 IU/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml amphotericin B; Flow Laboratories) under 5% CO₂ and 95% air. The medium was changed after 2 hr of incubation to remove the unattached cells and every 24 hr thereafter. Metyrapone (0.5 mM, Sigma) was added at 2 hr of incubation and thereafter.

Cells were harvested with a Cell Lifter® (Coaster), suspended in ice-cold isotonic potassium chloride solution (1.19 w/v %, 2 ml/dish), and subsequently homogenized in an ice bath with an ultrasonic cell disrupter (Microson®, Heat Systems-Ultrasoundics, Inc.). The homogenates were centrifuged at 100,000 x g for 1 hr. The pellets were stored at -80°C and then resuspended in isotonic potassium chloride solution just before use. Benzo(a)pyrene hydroxylation was measured by the method of Nebert and Gelboin (5); and 7-methoxycoumarin-O-demethylation, 7-ethoxycoumarin-O-deethylation and 7-propoxycoumarin-O-depropylation were determined by the method of Kamataki et al. (6). Estradiol-2-hydroxylation was measured by the method of Ryan et al. (7). Protein was determined by the method of Lowry et al. (8) with bovine serum albumin as a standard. The immunochemical determination of P-450 isozymes was performed according to the method of Guengerich et al. (9) using a semi-dry blotting apparatus (Horize-Blot, Atto) and a computer-aided densitometer (Shonic-GA, Showa Denko).

Table 1 shows the changes in drug metabolizing activities in the rat hepatocytes cultured with or without metyrapone. The rates of methoxycoumarin-O-demethylation, ethoxycoumarin-O-deethylation and propoxycoumarin-O-depropylation were decreased similarly in the absence of metyrapone. Their activities decreased to approximately 60% and 30% of the zero-hr value at 24 and 48 hr of culture, respectively. Benzo(a)pyrene hydroxylation decreased more rapidly, and approximately 35% of the zero-hr value was detected at 24 hr. However, the activity did not decrease further at 48 hr. The total P-450 contents, determined spectrometrically (10), decreased to approximately 60% of its initial value at 48 hr.

**Table 1. Effects of metyrapone on various cytochrome P-450-mediated drug metabolizing activities**

| Culture time (hr) | Activities (% of 0 hr)⁹ |
|-------------------|-------------------------|
|                   | methoxycoumarin O-demethylation | ethoxycoumarin O-deethylation | propoxycoumarin O-depropylation | benzo(a)pyrene hydroxylation |
| Control | | | | |
| 0 | 100.0⁹⁵ | 100.0 | 100.0 | 100.0 |
| 24 | 64.7±6.9 | 66.0±8.1 | 58.4±1.0 | 48.1±1.3 |
| 48 | 31.3±6.2 | 34.4±5.8 | 27.3±4.9 | 37.7±6.7 |
| 72 | 17.3±10.1 | 16.9±8.0 | 9.4±3.5 | 21.5±6.2 |
| Metyrapone | | | | |
| 0 | 100.0 | 100.0 | 100.0 | 100.0 |
| 2 | 91.0±9.0 | 97.3±6.8 | 93.7±3.1 | 86.3±14.0 |
| 24 | 62.5±7.1 | 93.3±22.0 | 106.9±21.2 | 93.2±36.4 |
| 48 | 47.3±4.7 | 108.1±14.9 | 113.4±13.7 | 267.8±46.9 |
| 72 | 25.4±8.7 | 86.6±3.8 | 104.1±11.2 | 248.7±105.7 |

⁹mean±S.E. ⁵⁵The activities at 0 hr are the following (mean±S.E.; nmol/min/mg protein, n=3): methoxycoumarin, 0.19±0.06; ethoxycoumarin, 0.23±0.05; propoxycoumarin, 0.22±0.05; benzo(a)pyrene, 0.16±0.07.
suggests that there are differences in stability among the P-450 isozymes.

Metyrapone maintained the activities of ethoxycoumarin-O-deethylation and propoxycoumarin-O-depropylation up to three days, and it increased the activity of benzo(a)-pyrene hydroxylation to 250% of the zero-hr value at 48 hr. However, the rates of methoxycoumarin-O-demethylation and 17β-estradiol-2-hydroxylation (data not shown) were not affected by the addition of metyrapone.

The changes in the levels of P-450 isozymes in the rat hepatocyte cultures were immunochemically determined. As shown in Fig. 1, P-450b, P-450c and P-450e were not detectable in the freshly isolated hepatocytes, whereas P-450d was present at significant amounts. In the presence of metyrapone, the contents of P-450c was not detected at 24 hr, but was increased at 48 hr, and the level was maintained to 96 hr. On the other hand, P-450d increased more rapidly within 24 hr and continued to increase up to 96 hr in cultured hepatocytes. The sum of P-450c and P-450d was about 15% of the total P-450 contents, determined spectrometrically (10), at 48 hr. The amounts of phenobarbital-inducible P-450b and P-450e were also measured by Western blots. Metyrapone, however, did not increase P-450b and P-450e.

The P-450 concentration has been shown to decline rapidly in the first few hours of the cultures of rat hepatocytes with unsupplemented media (1). Impaired synthesis and enhanced degradation are suggested as the mechanisms underlying the loss of P-450 in cultured hepatocytes (11). Although metyrapone is considered to maintain the level of P-450 by affecting both processes (11), the qualitative changes in the population of P-450 isozymes also occur in hepatocytes cultured with metyrapone (1).

As shown in Table 1, metyrapone enhanced the rate of benzo(a)pyrene hydroxylation, and it maintained the levels of ethoxycoumarin-O-deethylation and propoxycoumarin-O-depropylation in cultured hepatocytes. These activities are known to be induced by the treatment of rats with 3-methylcholanthrene in vivo. In contrast, methoxycoumarin-O-demethylation and 17β-estradiol-2-hydroxylation, which are not increased by the treatment with 3-methylcholanthrene in vivo (6), were not affected by the addition of metyrapone to cultured hepatocytes (data not shown). Furthermore, the contents of P-450c and P-450d, but not those of P-450b and P-450e, were induced by the addition of metyrapone in this experiment. Both P-450c and P-450d are induced by 3-methylcholanthrene, and both P-450b and P-450e are induced by phenobarbital in vivo. These results suggest that metyrapone acts as a 3-methylcholanthrene-type inducer in the primary cultures of rat hepatocytes on collagen-coated dishes.

It is considered that P-450c and P-450d belong to the same gene subfamily, but that the expressions of these isozymes differ (12). Although it was reported that only P-450c, but not P-450d, was inducible in cultured

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**Fig. 1.** Effects of metyrapone on the concentration of P-450 isozymes in the primary culture of rat hepatocytes. ●, 0.5 mM metyrapone; ○, control. “P-450b+e” means the sum of P-450b and P-450e contents.
hepatocytes on collagen (12) and on matrigel (13), prepared from an extract of extracellular matrix of murine Engelbreth-Holm-Swarm sarcoma, our results showed that P-450d is inducible as well as P-450c in cultured hepatocytes. P-450d is inducible in the liver, but not in extrahepatic tissues in vivo by TCDD (14). Therefore, the rat hepatocytes cultured under our conditions may favor the maintenance of this type of liver specificity.

Lake and Paine (15) reported that metyrapone acts as a phenobarbital-type inducer in vivo. The reason for the differences in vivo and in vitro induction by metyrapone is unknown. However, it is important that not only P-450c but also P-450d were inducible by metyrapone in the primary cultures of rat hepatocytes, since these enzymes are considered to play an important role in carcinogenesis in vivo, and metyrapone does not bind P-450c and P-450d (7). The addition of metyrapone to the primary culture of hepatocytes seems to be useful for in vitro studies of toxicity related to these P-450 species.

Acknowledgment: This work was supported by the Japan Health Sciences Foundation.

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