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THE CONTENT AND ACTIVITY OF CYTOCHROME P-450 IN LONG-TERM CULTURE OF HEPATOCYTES FROM MALE AND FEMALE RATS

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Abstract—The content of cytochrome P-450 and the capacity for *O*-demethylation have been measured in cultures of hepatocytes from male and female rats for a period of 21 days. The effect of dexamethasone, insulin, glucagon, phenobarbital and hemin was investigated. In hepatocytes from female rats the content of cytochrome P-450 was unchanged after one day of culture. From day 1 to day 3 the content of cytochrome P-450 decreased by 65% and only the combined addition of dexamethasone, phenobarbital and hemin diminished the fall. After the initial fall, addition of 0.1 μM dexamethasone resulted in a stable value. Addition of 1 μM dexamethasone or 1 mM phenobarbital gave rise to an induction of cytochrome P-450 (285%). The high level of cytochrome P-450 was maintained for 3 weeks. In hepatocytes from male rats the content of cytochrome P-450 decreased by 40% after one day of culture. From day 1 to day 3 the content decreased by 45% and the decrease continued irrespective of the presence of hormones and/or phenobarbital. The *O*-demethylase activity in cultures of hepatocytes from female rats correlated to the cytochrome P-450 content independent of medium composition and age of the cultures, whereas no correlation was found in cultures from male rats. The present study demonstrates that hepatocytes from female rats in cultures retain *O*-demethylase activity for at least 3 weeks and that, with the experimental conditions used, the response to the hormones and inducers is different for hepatocytes from male and female rats.

A major property of hepatocytes in the intact organism is the ability to metabolise xenobiotics by oxidation/reduction reactions catalyzed by the enzymes of the cytochrome P-450 system (phase I). This reaction is followed by the conversion of the product to water soluble products by conjugation (phase II) [1]. In cultured rat hepatocytes the activity of the transferases, which catalyze the conjugations seems more stable, more stimulated by inducers and far more active than the enzymes of the cytochrome P-450 system [2–5]. Several studies have shown that the content of cytochrome P-450 and the associated activities decline rapidly when hepatocytes are cultured in a simple medium [6, 7]. A variety of attempts have been made to maintain either the content of cytochrome P-450, the activities of associated enzymes or both [8–16]. These studies have used either post-natal hepatocytes or adult hepatocytes from male rats. One reason for using hepatocytes from male rats could be that the activity of cytochrome P-450 with most substrates is higher than in female rats [17–20]. In a recent study [21] we have demonstrated that it is possible to maintain several liver-specific parameters, such as albumin secretion, urea synthesis, enzymes activities, and the content of cytochrome P-450, in cultures of hepatocytes from adult female rats for up to 3 weeks.

The purpose of the present investigation is to compare the time-course of the content of cytochrome P-450 and the capacity for *O*-demethylation of *p*-nitroanisol in cultures of hepatocytes from male and female rats for a period of 21 days. Cytochrome P-450 activity was measured as *O*-demethylation of *p*-nitroanisol, since this activity shows no sex difference in contrast to several other substrates [19]. Also the effect of addition of glucocorticoid, insulin, glucagon, phenobarbital and hemin was investigated.

MATERIALS AND METHODS

Cell isolation and cultures. Hepatocytes were isolated from 16 hr starved adult male (300 g) or female (200 g) Wistar rats and plated in 60 mm collagen-coated Petri dishes (2.5 million cells) as previously described [22]. Twenty to twenty-two hours after plating, on day 1, the medium was changed to a modified Waymouth medium [23] with addition of 1% defatted albumin, 250 μM oleate, 175 μM palmitate and 75 μM linoleate. The fatty acids were dissolved in dimethylsulfoxide, the final concentration of which was 0.1% and identical in all incubations. Except in the control experiments the culture medium contained dexamethasone (1 or 0.1 μM), insulin (0.1 μM) and glucagon (0.01 μM). Hemin (1 μM) and/or phenobarbital (1 mM) were added as indicated. The medium was changed every 48 hr. The content of cytochrome P-450 and the *O*-demethylase activity were determined at day 0 (freshly isolated cells), 1, 3, 7, 13 and 21.

Analyses. The culture medium was changed at the day of measurement and *p*-nitroanisol, dissolved in

dimethylsulfoxide, was added to the cultures to a final concentration of 0.5 mM *p*-nitroanisol and 0.5% dimethylsulfoxide. This concentration of dimethylsulfoxide had no influence on the content of cytochrome P-450 during the incubation. After 2 hours of incubation at 37° the culture medium was removed and frozen at −20° until assayed for *p*-nitrophenol. The cells were used for determination of the content of cytochrome P-450, after harvesting in 0.8 ml 20% (v/v) glycerol in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol and 2% Lubrol PX [24]. The *O*-demethylase activity of freshly isolated hepatocytes was measured by adding *p*-nitroanisol to cells suspended in the culture medium and incubated in a shaking water bath at 37°. After 2 hours of incubation the cells were removed and the medium was analyzed for *p*-nitrophenol. The content of cytochrome P-450 decreased by 30–40% during the incubation of freshly isolated cells.

The concentration of cytochrome P-450 was determined by the method of Omura and Kato [25]. The spectrum of each dithionite reduced sample was recorded and subtracted from the spectrum obtained after bubbling with CO. The extinction coefficient ($\varepsilon = 91\ mM^{-1} \times cm^{-1}$) for the wavelength pair 450 minus 490 nm were used to estimate the concentration of cytochrome P-450. This procedure can cause up to 10% underestimation of cytochrome P-450 due to interference from cytochrome a_3 [26].

The metabolism of *p*-nitroanisol was measured by quantitating the accumulation of the glucuronide and sulfate conjugates of *p*-nitrophenol by a modification of the method of Netter and Seidel [27]. To 0.9 ml of the culture medium was added 0.1 ml 1 M glycine buffer, giving a final pH of 4.5 and the mixture was incubated for 2 hr at 37° with 45 milliunits β-glucuronidase and 140 milliunits arylsulfatase. pH was adjusted to 9.8 and a spectrum was recorded from 500 to 350 nm. Medium removed immediately after addition of *p*-nitroanisol to a parallel dish and treated as the sample, was used as a blank. The concentration of *p*-nitrophenol was calculated from the absorbance at 400 nm ($\varepsilon = 16.8\ mM^{-1} \times cm^{-1}$). No *p*-nitrophenol was found in the medium before treatment with glucuronidase and sulfatase, which indicated that the transferase activity in the cells was large enough to catalyze the conjugation of all *p*-nitrophenol produced. V_{max} and K_m for *O*-demethylase were estimated in day 1 cultures, and in a microsomal fraction prepared according to [28]. The substrate concentration was varied from 0.125 to 8 mM.

DNA was determined in parallel dishes treated as mentioned above. The cells were harvested by ultrasonication [22] in 1.5 ml 25 mM glycylglycine, 150 mM KCl, 5 mM $MgSO_4$, 5 mM EDTA, 1 mM dithiothreitol and 0.2% defatted albumin, pH 7.4 and kept at −20° until analysis [29].

Materials. Enzymes and coenzymes were from Boehringer (Mannheim, F.R.G.) except collagenase (type II) which was from Worthington Biochemical Corp. (Freehold, NY). Horse serum and albumin was from Difco (Glasgow, U.K.) and Armour (Sussex, U.K.) respectively. Collagen was prepared from rat tails [30]. Insulin and glucagon were purchased from NOVO (Copenhagen, Denmark) and dexamethasone from Merck, Sharp & Dohme (Haarlem, The Netherlands). Phenobarbital was from Meco-Benzon (Copenhagen, Denmark) and hemin from BDH (Poole, U.K.).

Calculations. The content and activity of cytochrome P-450 was related to the amount of DNA. The data obtained on the same day of culture were compared by one-way analysis of variance. Comparison between day 0 and day 1 was carried out by Student's *t*-test. $P < 0.05$ was considered statistically significant.

Table 1. Effect of hormones and inducers on DNA content in hepatocyte cultures from male and female rats

Sex	Addition (μM) Dex	Ins	Glu	Pb	H	DNA (content per dish, % of day 1) days of culture 3	7	13	21
Male	—	—	—	—	—	98 ± 5 (4)	68 ± 7 (4)	46 ± 9 (4)	—
Female						76 ± 4 (6)	66 ± 5 (6)	22 ± 3 (6)	—
Male	0.1	0.1	0.01	—	—	91 ± 4 (4)	74 ± 9 (4)	50 (2)	17 ± 1 (3)
Female						83 ± 4 (7)	80 ± 4 (7)	70 ± 4 (7)	63 ± 5 (6)
Male	0.1	0.1	0.01	1000	—	96 ± 6 (4)	79 ± 7 (4)	50 ± 3 (4)	23 ± 5 (4)
Female						88 ± 4 (7)	82 ± 3 (7)	76 ± 4 (7)	64 ± 10 (4)
Male	1.0	0.1	0.01	—	—	97 ± 6 (4)	82 ± 9 (4)	53 ± 2 (4)	36 ± 6 (4)
Female						88 ± 3 (4)	82 ± 4 (7)	75 ± 3 (7)	69 ± 5 (7)
Male	1.0	0.1	0.01	1000	—	95 ± 3 (4)	82 ± 6 (4)	55 ± 5 (4)	34 ± 8 (4)
Female						87 ± 2 (7)	82 ± 2 (7)	78 ± 3 (7)	68 ± 5 (6)
Male	1.0	0.1	0.01	1000	1.0	91 ± 7 (4)	85 ± 5 (4)	60 ± 3 (4)	31 ± 7 (4)
Female						87 ± 4 (6)	77 ± 3 (6)	76 ± 3 (6)	75 ± 4 (4)

Hepatocytes were cultured in a modified Waymouth medium. At day 1 medium containing hormones, phenobarbital (Pb) and/or hemin (H) was added and changed every 48 hr. The results are mean ± SEM with the number of hepatocyte preparations shown in parentheses. The DNA content of the hepatocyte cultures at day 1 was: 28 ± 3 (4) μg DNA/plate for male and 32 ± 3 (7) μg DNA/plate for female. Dex, Ins, Glu, Pb and H indicate dexamethasone, insulin, glucagon, phenobarbital and hemin, respectively.

RESULTS

DNA

The DNA content per dish was determined as a measure of cell number. In cultures from female rats the content of DNA decreased by 10–20% from day 1 to day 3, regardless of the presence of hormones (Table 1). Without hormones present the initial fall continued and only 20% was left at day 13. The content at day 21 was not determined as earlier experiments had shown that no DNA was present at this time of culture [21]. When hormones were added to the medium the DNA content declined only slowly after day 3 and was still about 70% of the initial value at day 21.

In hepatocyte cultures from male rats the time-course of the DNA content differed from that of female rats. Firstly, no fall was observed from day 1 to day 3 (Table 1). Secondly, when no hormones were added to the medium the content of DNA decreased from day 3 to day 13, but to a lesser extent than was observed in cultures from female rats. Thirdly, addition of hormones did not prevent the fall in the content of DNA, the amount present at days 7 and 13 being identical to that measured without hormones. At day 21 the DNA content was higher with a medium containing 1.0 μM dexamethasone. However, significantly more DNA was retained at day 13 and day 21 in cultures of female hepatocytes than in male hepatocytes, provided that hormones were added.

Cytochrome P-450

The content of cytochrome P-450 measured in freshly isolated cells immediately after isolation showed that female hepatocytes contained 25% less cytochrome P-450 than hepatocytes from male rats (Table 2). After one day of culture the content was unchanged in hepatocytes from female rats, whereas only 40% was left in male hepatocytes, resulting in a higher content of cytochrome P-450 at day 1 in female hepatocytes.

In cultures of hepatocytes from female rats the content of cytochrome P-450 decreased by 65% from day 1 to day 3. Only the combined addition of dexamethasone (1 μM), phenobarbital (1 mM) and hemin (1 μM) diminished this fall significantly. Without hormones present the initial fall continued and no cytochrome P-450 was present at day 13. Addition of hormones (dexamethasone, 0.1 μM) resulted in a stable value of cytochrome P-450 after day 3. Addition of phenobarbital or a higher concentration of dexamethasone (1 μM) resulted in a significant increase of the content of cytochrome P-450 at day 7, to twice that with the lower concentration of dexamethasone. Further addition of phenobarbital or phenobarbital plus hemin to the medium with 1 μM dexamethasone enhanced this increase significantly, resulting in a 2–3 times higher level of cytochrome P-450 at day 7 and at day 21 compared to values with 0.1 μM dexamethasone.

In hepatocyte cultures from male rats the content of cytochrome P-450 decreased 45% from day 1 to day 3. Without addition of hormones the initial fall continued and no cytochrome P-450 was left at day 13 (Table 2). Addition of hormones with 0.1 μM dexamethasone had no effect, whereas 1 μM dexamethasone delayed significantly the decrease in the cytochrome P-450 content at day 7. Addition of phenobarbital to a medium with 0.1 μM dexamethasone caused a significant increase in the cytochrome P-450 content at day 7. At day 13, a high concentration of dexamethasone with or without phenobarbital resulted in a significantly higher cytochrome P-450 content than in control cultures. No detectable cytochrome P-450 was left at day 21.

Table 2. Effect of hormones and inducers on cytochrome P-450 content in hepatocyte cultures from male and female rats

Sex	Addition (μM) Dex	Ins	Glu	Pb	H	Cytochrome P-450 content (nmol/mg DNA) days of culture: 3	7	13	21
Male	—	—	—	—	—	2.6 ± 0.4 (4)	1.0 ± 0.4 (4)	ND (3)	—
Female						2.6 ± 0.5 (6)	1.1 ± 0.3 (6)	ND (5)	—
Male	0.1	0.1	0.01	—	—	2.4 ± 0.3 (4)	1.9 ± 0.3 (3)	0.7 (2)	ND (4)
Female						2.7 ± 0.4 (8)	2.1 ± 0.4 (8)	2.6 ± 0.3 (6)	1.9 ± 0.7 (6)
Male	0.1	0.1	0.01	1000	—	2.8 ± 0.4 (4)	2.3 ± 0.3 (4)	1.4 (2)	ND (4)
Female						3.8 ± 0.4 (8)	4.5 ± 0.7 (8)	4.5 ± 0.9 (8)	3.3 ± 0.3 (7)
Male	1.0	0.1	0.01	—	—	2.6 ± 0.4 (4)	2.9 ± 0.2 (4)	1.6 ± 0.4 (3)	ND (4)
Female						2.9 ± 0.4 (7)	3.8 ± 0.4 (8)	4.8 ± 0.8 (6)	3.9 ± 0.6 (7)
Male	1.0	0.1	0.01	1000	—	3.3 ± 0.3 (4)	3.3 ± 0.2 (4)	2.6 ± 0.4 (3)	ND (4)
Female						3.8 ± 0.4 (8)	5.8 ± 0.6 (8)	5.2 ± 0.7 (7)	4.5 ± 0.8 (7)
Male	1.0	0.1	0.01	1000	1.0	4.1 ± 0.4 (4)	2.7 ± 0.4 (4)	1.9 ± 0.3 (3)	ND (4)
Female						4.3 ± 0.6 (7)	6.2 ± 0.9 (7)	5.9 ± 1.1 (5)	6.0 ± 0.8 (5)
Male	day 0: 11.8 ± 0.8 (6); day 1: 4.9 ± 0.4 (4)								
Female	day 0: 8.7 ± 0.7 (4); day 1: 7.4 ± 0.8 (8)								

Hepatocytes were cultured in a modified Waymouth medium. At day 1 medium containing hormones, phenobarbital (Pb) and/or hemin was added and changed every 48 hr. The results are mean ± SEM with the number of hepatocyte preparations shown in parentheses. Dex, Ins, Glu, Pb and H indicate dexamethasone, insulin, glucagon, phenobarbital and hemin, respectively. ND indicates not detectable.

Table 3. Effect of hormones and inducers on *O*-demethylase activities in hepatocyte cultures from male and female rats

Sex	Addition (μM) Dex	Ins	Glu	Pb	H	*O*-demethylase activity (nmol/min mg DNA) days of culture 3	7	13	21
Male	—	—	—	—	—	0.8 ± 0.3 (4)	0.8 ± 0.4 (3)	ND (4)	—
Female						1.3 ± 0.9 (4)	0.6 ± 0.1 (4)	0.2 ± 0.2 (5)	—
Male	0.1	0.1	0.01	—	—	1.2 ± 0.3 (4)	0.9 (2)	1.3 (2)	ND (2)
Female						0.6 ± 0.2 (5)	1.3 ± 0.4 (4)	1.2 ± 0.5 (7)	0.3 ± 0.1 (6)
Male	0.1	0.1	0.01	1000	—	1.4 ± 0.1 (4)	1.4 ± 0.2 (4)	1.1 ± 0.5 (4)	ND (4)
Female						1.7 ± 0.2 (5)	4.1 ± 0.5 (6)	4.4 ± 0.8 (7)	3.3 ± 1.1 (6)
Male	1.0	0.1	0.01	—	—	1.1 ± 0.3 (4)	1.1 ± 0.2 (4)	0.4 ± 0.2 (4)	ND (4)
Female						1.0 ± 0.6 (5)	2.9 ± 0.4 (6)	2.2 ± 0.7 (7)	2.2 ± 1.3 (6)
Male	1.0	0.1	0.01	1000	—	1.5 ± 0.2 (4)	1.1 ± 0.2 (4)	1.2 ± 0.3 (4)	ND (4)
Female						1.1 ± 0.2 (4)	3.9 ± 0.9 (7)	4.9 ± 1.0 (7)	2.7 ± 1.0 (6)
Male	1.0	0.1	0.01	1000	1.0	1.3 ± 0.3 (4)	1.5 ± 0.0 (4)	1.7 ± 0.5 (4)	ND (4)
Female						2.6 ± 1.1 (5)	4.0 ± 1.0 (5)	5.1 ± 0.9 (6)	6.1 ± 0.8 (3)
Male	day 0: 3.1 ± 0.6 (4); day 1: 1.5 ± 0.4 (4)								
Female	day 0: 2.0 ± 0.2 (5); day 1; 3.0 ± 0.3 (5)								

Hepatocytes were cultured in a modified Waymouth medium. At day 1 medium containing hormones, phenobarbital (Pb) and/or hemin (H) was added and changed every 48 hr. The results are mean ± SEM with the number of hepatocyte preparations shown in parentheses. Dex, Ins, Glu, Pb and H indicate dexamethasone, insulin, glucagon, phenobarbital and hemin, respectively. ND indicates not detectable.

O-demethylase activity

The maximal oxidation rate of *p*-nitroanisol, determined in microsomes isolated from freshly isolated hepatocytes and in day 1 cultures were 0.32 ± 0.10 and 0.70 ± 0.09 mol *p*-nitrophenol/mol cytochrome P-450 × min, respectively and K_m for *p*-nitroanisol was 0.85 ± 0.24 and 0.15 ± 0.03 mM, respectively (N = 3, female rats). The maximal oxidation rate in microsomes has been reported to 0.39 mol *p*-nitrophenol/mol cytochrome P-450 × min [19] and K_m to 0.23 mM [27].

In cultures of hepatocytes from female rats the *O*-demethylase activity (Table 3) correlated with the cytochrome P-450 content (Table 2) throughout the experimental period with a correlation coefficient of 0.5602 (N = 118, $P < 0.001$) and a slope of 0.55 mol *p*-nitrophenol/mol cytochrome P-450 × min. In suspensions of freshly isolated hepatocytes from female rats a similar ratio of 0.51 ± 0.06 min^{-1} (N = 5) was measured.

In cultures of hepatocytes from male rats no correlation between *O*-demethylase activity (Table 3) and the cytochrome P-450 content (Table 2) was found. In suspensions of freshly isolated hepatocytes from male rats the turnover rate was 0.45 ± 0.06 min^{-1} (N = 4), which is similar to the rate found in hepatocytes from female rats.

DISCUSSION

The present study shows that addition of dexamethasone, glucagon and insulin was more effective in maintaining the number of cells (DNA content per dish) in cultures of hepatocytes from female than from male rats. Addition of dexamethasone (0.1 μM) to cultures from female rats was able to maintain about 70% of the initial content of DNA for 2–3 weeks [21]. Cultures of male hepatocytes did not respond to media containing hormones, after 13 days of culture only about 50% of the initial content of DNA was left, in accordance with earlier observations [31, 32]. Phenobarbital had no effect on the DNA content, which is in contrast to earlier observations that phenobarbital (3 mM) in the presence of dexamethasone (10 μM) preserves 60% of the cells for 4 weeks in cultures of male hepatocytes [31].

The cytochrome P-450 content in freshly isolated hepatocytes from male Wistar rats was higher than in cells from female rats, which is in accordance with one reported observation in Sprague–Dawley rats [18], but in contrast to another [19]. The cytochrome P-450 content was more labile in cultures of male than of female hepatocytes. The fall during the first 24 hours was significantly less in cultures of female hepatocytes (15%) than in those of male (58%), which is in contrast to a reported loss of 50% at day 1 in cultures of both female and male hepatocytes from Fisher rats [5]. The fall in the content of cytochrome P-450 from day 1 to day 3 was observed in hepatocytes from both male and female rats. Hormones had no effect on this fall, in accordance with observations that dexamethasone is unable to induce a synthesis of cytochrome P-450 during the first days of culture [33]. In short-term experiments, various additions to the medium, such as dexamethasone [11], ascorbate [13], delta-aminolevulinic acid [12], heme [34], and nicotinamide [14] have been reported to reduce the initial loss of cytochrome P-450. With one exception [34], these experiments were carried out with hepatocytes from male rats.

Addition of hormones to cultures from female rats stabilized or increased the content of cytochrome P-450 after day 3, whereas in cultures of hepatocytes from male rats only a high concentration of dexamethasone was able to stabilize the content of cytochrome P-450 during the rest of the first week. The high level of cytochrome P-450 in the presence of a

high concentration of dexamethasone may represent induction of isoenzyme P-450p [35], which is induced by phenobarbital as well [36]. However the isoenzyme pattern of cytochrome P-450 was not studied in the present work. Only addition of metyrapone (0.5 mM) plus a high concentration of hydrocortisone (0.1 mM) has been reported to maintain the cytochrome P-450 content for 7 days in cultures of male hepatocytes [15].

Inducers of cytochrome P-450 such as methylcholanthrene and phenobarbital, often in combination with a high concentration of glucocorticoid, have been reported to reduce the loss of cytochrome P-450 in cultured hepatocytes [9, 15, 34]. In the present study phenobarbital only slightly delayed the fall of cytochrome P-450 content in cultures of hepatocytes from male rats, in agreement with results obtained in 7 day cultures [15]. In cultures of hepatocytes from female rats addition of 1 mM phenobarbital with or without 1 μM hemin caused an increase in the content of cytochrome P-450 after the initial fall from day 1 to day 3. The higher concentration was maintained throughout the experimental period. The ability of a hemin- and phenobarbital-containing medium to maintain cytochrome P-450 in culture for 4 days has been reported by others [34]. The time-course of induction found in the present study agrees with the *in vivo* situation, in which the concentration reaches a maximum after 4–5 days of phenobarbital administration [37].

The maximal oxidation rate of *p*-nitroanisol per cytochrome P-450 in day 1 cultures was significantly larger than the maximal oxidation rate in isolated microsomes. This difference was not due to product inhibition in isolated microsomes (unpublished results), suggesting that use of hepatocyte cultures is superior to microsomes in studies of the metabolism of xenobiotics. The ratio between the *O*-demethylase activity and the cytochrome P-450 content was the same in suspension and in day 1 cultures of male and female hepatocytes, confirming that this activity shows no sex difference [19]. However, during the 3 weeks of culture the ratio was only maintained in the hepatocytes from female rats.

The present study demonstrates that with the experimental conditions used the responses to the hormones and inducers are different for hepatocytes from male and female rats, and that the cytochrome P-450 in culture of adult female rat hepatocytes retains its *O*-demethylase activity for at least 3 weeks.

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