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SHORT COMMUNICATION

Hormonal Regulation of Microsomal Flavin-containing Monooxygenase Activity by Sex Steroids and Growth Hormone in Co-Cultured Adult Male Rat Hepatocytes

Sandra Coecke, Gilda Debast,* Ian R. Phillips,† Antoine Vercruysse,* Elizabeth A. Shephard‡ and Vera Rogiers*§*

*Department of Toxicology, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium; †Department of Biochemistry, Queen Mary & Westfield College, University of London, Mile End Road, London E1 4NS, U.K.; and ‡Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.

ABSTRACT. To investigate the hormonal control of the expression of flavin-containing monooxygenase (FMO; EC 1.14.13.8) under defined *in vitro* conditions, adult male rat hepatocytes were isolated by collagenase perfusion and co-cultured with rat liver epithelial cells of primitive biliary origin. The direct effect of 17β-estradiol, testosterone, 5α-dihydrotestosterone (5α-DHT) and human growth hormone (hGH) on FMO activity was studied using this *in vitro* model. Optimal, non-cytotoxic hormonal concentrations were determined by measuring the lactate dehydrogenase (LDH) index. In addition, the microsomal protein content of the cultured hepatocytes was determined as a function of culture time. The female sex hormone 17β-estradiol caused a significant decrease in FMO as a function of culture time. After 14 days of exposure, FMO activity decreased by 56%. Neither of the male sex hormones or human growth hormone had an effect on FMO activity. These results in co-cultured male rat hepatocytes support *in vivo* observation that 17β-estradiol is a potent hormone involved in the negative regulation of the expression of FMO in male rat liver. BIOCHEM PHARMACOL **56**;8: 1047–1051, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. flavin-containing monooxygenase; 17β-estradiol; testosterone; 5α-dihydrotestosterone; human growth hormone; rat hepatocytes

Xenobiotics containing nucleophilic nitrogen and sulphur atoms are often substrates for both FMO‖ (EC 1.14.13.8) and the cytochrome P450-dependent monooxygenase system [1]. While the latter has been extensively investigated, FMOs have been relatively neglected.

There is compelling evidence in rat that gender-related differences exist in hepatic microsomal FMO, with male animals usually showing a higher enzymatic activity than their female counterparts [2–4]. Sex hormones seem to be the primary regulating factor for these differences [5], GH is believed to exert a feminizing effect [6]. Despite the increasing amount of data implicating hormones as major factors regulating FMO metabolism [7, 8], the precise mechanisms remain unknown.

Due to the complexity of hormone effects *in vivo*, the development of a well-defined *in vitro* system capable of maintaining several *in vivo* metabolic capacities would be a useful tool to investigate the direct effect of potential hormone modulators on FMO activity. Primary cultures of rat hepatocytes have been used to study the hormonal regulation of some phase I [9] and phase II enzymes [10]. It is, however, well known that this *in vitro* system quickly loses liver-specific functions and in particular phase I biotransformation capacity [11–15]. In our previous study, it was demonstrated that hepatocytes, co-cultured with rat liver epithelial cells, maintain FMO activity better and longer than hepatocytes cultured in the absence of these cells [16]. In the latter system, FMO activity declines continuously until the cells detach after approximately 6 days. In contrast, in co-cultured hepatocytes a steady-state level of FMO activity is maintained for at least one week from the 7th day of culture, making this a good system to study the hormonal regulation of FMO expression. Therefore, this co-culture system of male rat hepatocytes with rat liver epithelial cells was used to determine the individual effects, on FMO activity, of testosterone, 5α-DHT, 17β-estradiol and hGH. Because it has been previously reported that high doses of steroids are required because of extensive hormonal metabolism in hepatocytes [17], the highest,

§ Corresponding author: Prof. Vera Rogiers, Department of Toxicology, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium. Tel. (32)2 4774516; FAX (32)2 4774582.

‖ *Abbreviations.* 5α-DHT, 5α-dihydrotestosterone; FMO, flavin-containing monooxygenase; hGH, human growth hormone; and LDH, lactate dehydrogenase.

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noncytotoxic concentration of each hormone was determined by measuring its effect on the LDH index and microsomal protein content of the cultured cells.

MATERIALS AND METHODS

Materials

Glucose-6-phosphate, $NADP^+$, methimazole, hGH (2 IU/mg) and dithiotreitol were obtained from Sigma Chemical Co. 5α-DHT was purchased from Janssen Chimica, 17β-estradiol from Fluka Chemie, and testosterone from K&K Rare and Fine Chemicals. Glucose-6-phosphate dehydrogenase was obtained from Boehringer Mannheim and 5,5′-dithiobis(2-nitrobenzoate) from Aldrich-Chemie.

Preparation of Hepatocyte Cultures and Enzymatic Assays

Hepatocytes were isolated from male outbred OFA Sprague–Dawley rats (I.O.P.S. caw, 15 weeks old, 350 g, Iffa-Credo) as described by Rogiers *et al.* [18]. Cell integrity was tested by trypan blue exclusion and ranged between 85 and 95%. Rat liver epithelial cells obtained by trypsinisation of 10-day-old Sprague–Dawley rat livers [19] were subcultured and applied between the 20th and the 30th passage. For the determination of FMO activity in co-cultured cells, 10^7 parenchymal cells were brought into culture using 75% minimal essential medium and 25% medium 199, supplemented with 1 mg/mL of BSA, 10 μg/mL of bovine insulin, 7×10^{-6} M of hydrocortisone, and 10% fetal calf serum as described previously [16]. After 4 hr, co-cultures were prepared by adding 1.5×10^7 rat liver epithelial cells in fresh medium containing 10% fetal calf serum. Twenty-four hours after plating, co-cultures were fed serum-free medium supplemented with the appropriate hormones (0.1, 1, 10 or 100 μM of testosterone, 5α-DHT or 17β-estradiol in a final concentration of 0.1% ethanol and 0.01, 0.1 or 1 μg/mL of hGH in a final concentration of 0.1% PBS). Microsomal fractions were prepared from freshly isolated (To) and co-cultured hepatocytes as described by Hales *et al.* [20]. They were stored at −80°, without any loss of FMO activity [21], until required for enzymatic activity measurements and microsomal protein determination.

FMO activity was estimated spectrophotometrically as described previously [21] by measuring the rate of methimazole oxygenation via the reaction of the oxidized product with nitro-5-thiobenzoate (TNB) to generate 5,5′-dithiobis(2-nitrobenzoate) (DTNB). The disappearance of the yellow coloured nitro-5-thiobenzoate was followed spectrophotometrically at 412 nm. The microsomal protein content of the cultured cells was measured using the Bio-Rad Protein Assay based on the principle of Bradford [22], using BSA as a standard. Cytotoxicity was assessed by measuring LDH leakage into the medium (Merck–Belgolabo). The LDH leakage index was determined as the percentage of LDH release in the medium versus the total LDH in the medium and cells.

Western Blot Analysis

Microsomal membrane proteins were separated by electrophoresis through a 10% polyacrylamide SDS gel and then electroblotted onto a nitrocellulose filter (Hybond C extra, Amersham). FMO protein was detected using an antisera to pig FMO1 (polyclonal rabbit antibodies against pig FMO1, kindly provided by Dr. D. Ziegler) and a goat-anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Bio-Rad Laboratories). The FMO protein was quantified using a CAMAG Scanner 3 with a CAMAG TLC evaluation software.

Determination of Hormonal Concentrations

In order to have an idea of the rate of metabolism of the hormones used and to determine the frequency for culture medium renewal, hormonal concentrations in the media were determined as a function of culture time. Hormone-containing media were renewed every 24 hr and samples were taken from freshly prepared media and from media present in the co-cultures. Samples were taken 24 hr after medium renewal and after 2, 3, 4, 7, 10 and 14 days in co-culture. Hormonal physiological circulating concentrations were determined using blood serum from the adult male Sprague–Dawley rats from which the hepatocytes were isolated. Dosages of testosterone and 5α-DHT were performed with a ^{3}H-testosterone and ^{3}H-dihydrotestosterone radioimmunoassay (RIA) kit (Bio Mérieux), respectively. Estradiol concentration was measured using a Estradiol-2 Clinical Assay kit (Sorin Biomedica), and hGH using an hGH radioimmunoassay kit (Pharmacia).

Statistical Analysis

Statistical analysis was done by general ANOVA procedures and Student's *t*-tests, wherever suitable, using the Software Package of the Social Sciences, SPSS/PC^+ [23]. $P < 0.05$ was the accepted level of significance.

RESULTS AND DISCUSSION

Prior to investigating the effect of individual hormones on FMO activity in the co-culture system, the noncytotoxic concentration of each hormone was determined. Twenty-four hours after plating (day 1), different concentrations of hormones were added to the co-cultures. Table 1 shows the effects of the various hormones on microsomal protein content after 7 days of treatment. Similar results were obtained after 10 and 14 days, (data not shown). For testosterone, 5α-DHT and hGH, the concentrations used had no significant effect on the microsomal protein content. However, on exposure to 100 μM 17β-estradiol, the microsomal protein content of co-cultures was significantly

TABLE 1. Effects of hormonal concentration on microsomal protein content of co-cultured cells and on LDH index into the medium after 7 days of exposure

	Hormonal concentration							
	0.1 μM		1 μM		10 μM		100 μM	
Treatment	Protein	LDH	Protein	LDH	Protein	LDH	Protein	LDH
Testosterone	99 ± 4	111 ± 27	108 ± 10	121 ± 28	108 ± 12	117 ± 15	108 ± 12	211 ± 13§
5alpha-DHT	97 ± 9	102 ± 17	94 ± 5	94 ± 12	111 ± 9	100 ± 24	90 ± 9	280 ± 61§
17β-Estradiol	98 ± 8	98 ± 18	103 ± 11	99 ± 13	123 ± 14	102 ± 13	75 ± 10§	236 ± 36§
	0.01 μg/mL		0.1 μg/mL		1 μg/mL			
hGH	100 ± 11	101 ± 20	106 ± 12	89 ± 11	115 ± 9	152 ± 27		

Each value represents the mean (expressed as a percentage of the nontreated control value) ± SD. The mean is an average of three separate isolation and culture experiments. Microsomal protein contents (LDH index) of the controls containing 0.1% PBS and 0.1% ethanol were 0.86± 0.14 and 0.91 ± 0.21 mg/mL (3.1 ± 0.5 and 2.8 ± 0.3%), respectively.

§Significantly different from the control value according to a paired Student's *t*-test ($P < 0.05$).

lower than that of the corresponding control value (75 ± 10%, $P < 0.05$).

To determine whether the lower cellular protein content of cells exposed to 100 μM of 17β-estradiol was due to an increased permeability of the cell membranes as a result of cellular damage, the LDH index was measured. The results shown in Table 1 illustrate that cells exposed for 7 days to 17β-estradiol, testosterone and 5α-DHT at concentrations of 100 μM all show an increased LDH index.

Based on the results shown in Table 1, it was decided to add testosterone, 5α-DHT, and 17β-estradiol at a concentration of 10 μM and hGH at a final concentration of 0.1 μg/mL to the culture medium when their effect on FMO activity was studied.

Hormonal Metabolism in Co-Cultures as a Function of Culture Time

It is known that steroids are extensively metabolised in hepatocytes [17]. It was therefore necessary to determine how often the hormone-containing media needed to be changed. Hormonal concentrations were determined in freshly prepared media and in media taken from co-cultures as a function of culture time. Testosterone, 5α-DHT and 17β-estradiol concentrations decreased significantly within 24 hr of medium renewal (results not shown). They remained, however, at a concentration higher than the physiological serum concentrations. The results showed that in co-cultured cells steroid hormones are extensively metabolised within a 24-hr period, making it necessary to renew the hormone-containing media daily. Although the hGH concentration remained practically unchanged after addition to the co-cultures, this hormone was also renewed every 24 hr.

Effect of Hormones on FMO Activity

No significant difference was observed between the FMO activities of epithelial cells measured before and after exposure to the different hormonal media. To determine the FMO activity in epithelial cells co-cultured with the hepatocytes, the latter were removed using a 0.07% in PBS (w/v) collagenase treatment as described in [24]. The FMO activity of the epithelial cells was never higher than 3.4 ± 2.0% of the total FMO activity of the co-culture system. Consequently, it seems safe to conclude that correction of FMO results obtained from the co-cultures for FMO activity in the helper cells is not required. Because co-cultures of rat hepatocytes maintain a steady-state situation with respect to FMO activity from day 7 on for at least 1 week [16], FMO activity was determined on days 7, 10 and 14 of culture. In Fig. 1, the effects of testosterone, 5α-DHT, 17β-estradiol and hGH on FMO activity in co-cultured rat hepatocytes is shown as a function of culture time.

Neither testosterone nor 5α-DHT significantly changed FMO activity. The addition of 17β-estradiol, however, resulted in a significant inhibition of FMO activity for all time points examined. After 14 days, the FMO activity was only 44 ± 14% of the control value. To determine whether the decrease in FMO activity in response to 17β-estradiol could have been due to a decrease in microsomal protein content, a Western blot experiment was carried out. The amount of immunoreactive FMO was lower in microsomal membranes isolated from 17β-estradiol cells compared to that present in untreated co-cultures (Fig. 2). The decrease ranged from 1.5 to 3-fold in the three culture preparations analysed. The above results are in line with earlier *in vivo* observations made by Dannan and coworkers [4]. They found that continuous administration of 17β-estradiol to neonatal and postpubertal male rats, gonadectomized at birth, inhibited FMO activity by *ca.* 50%. Administration of male sex hormones to these animals had no effect on FMO activity. It is believed that gender-dependent metabolic differences in rats are primarily determined by events that occur during the neonatal period [25]. At this time, secretion of testicular androgens imprints a latent masculine potential onto an otherwise feminine pattern [4]. Gonadectomy of male animals at birth thus prevents the effects of neonatal imprinting by androgens.

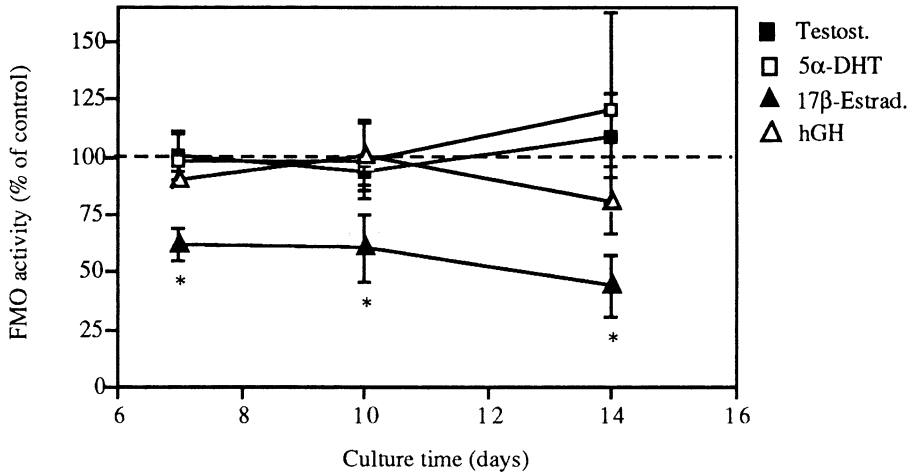

FIG. 1. Effects of testosterone, 5α-DHT, 17β-estradiol, and hGH on FMO activity in co-cultures of male rat hepatocytes as a function of culture time. The absolute value for freshly isolated hepatocytes is 12.8 ± 3.0 nmol methimazole/min/mg of microsomal protein. The absolute value for the control co-cultures at day 7 is 4.7 ± 0.9 nmol methimazole/min/mg of microsomal protein (no significant differences for control samples with or without 0.1% ethanol). The results are expressed as a percentage of the control condition, which is set at 100%. Each value represents the mean (expressed as a percentage of the nontreated control value, i.e. dashed line) ±SD. The mean is an average of three separate isolation and culture experiments. *Significantly different from the control value according to a paired Student's *t*-test ($P < 0.05$).

Using an *in vitro* approach of co-cultures of hepatocytes from nongonadectomised adult male rats, we have shown that in the absence of androgens the female sex hormone 17β-estradiol inhibits FMO activity. Effects of androgens on hepatic xenobiotic-metabolizing enzymes can be regulated, either directly or indirectly, by other androgen-sensitive organs such as the pituitary [26], of which GH is a major regulator. A combined effect of sex hormones and hGH could therefore account, at least in part, for the previously observed *in vivo* results. The *in vitro* methodology used for the study of the regulation of FMO activity, is straightforward, omits elaborate and for the animals painful procedures such as castration, ovariectomy and hypophysectomy, and permits the effect of a single hormone to be monitored.

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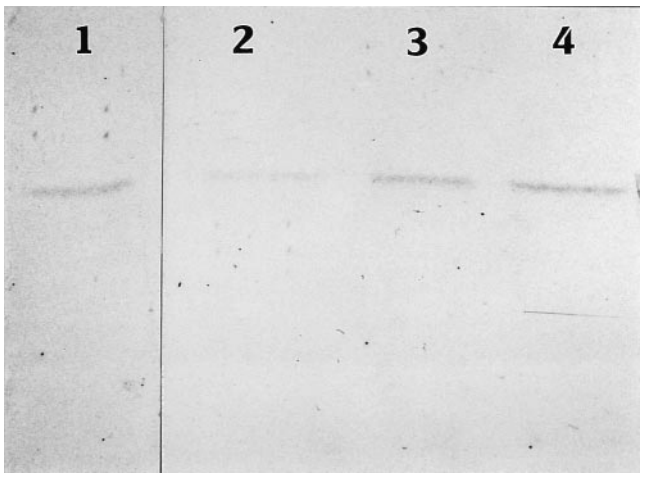

FIG. 2. Western blot analysis of FMO in microsomal membranes isolated from co-cultured rat hepatocytes. Cells were treated with either 0.1% ethanol (lane 1), 10 μM 17β-estradiol (lane 2), 10 μM 5α-DHT (lane 3), or 10 μM testosterone (lane 4). Each track contained 30 μg of protein. For this particular blot, values after scanning were 33%, 88% and 91% for lanes 2, 3 and 4, respectively, when the band in lane 1 was set at 100%.

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