Characterization of the UDP-glucuronosyltransferase isoenzyme expressed in rat ovary and its regulation by gonadotropins

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Earlier studies have demonstrated that phenol UDP-glucuronosyltransferase (UGT) activity is up-regulated by pregnant mare's serum gonadotropin (PMSG) in rat ovary, but not liver. This phenomenon was investigated in more detail in the present study. Ovaries and livers of immature rats, rats synchronized with respect to their preovulatory and *corpus luteal* phases by treatment with PMSG, and mature rats hyperstimulated with PMSG were compared. Under all of these conditions, only one immunoreactive band of UGT, shown to be phenol UGT, was detected in the rat ovary. The effects of oestradiol, progesterone and/or human chorionic gonadotropin

(hCG) on the level of phenol UGT in immature rat ovary were also examined. Partial up-regulation was caused by progesterone or oestradiol, together with hCG, whereas progesterone or oestradiol alone had no up-regulating effect. Follicle-stimulating hormone also seemed to be required for the up-regulation in ovaries enriched in *corpus luteum*. The present findings demonstrate that progesterone is involved in the regulation of phenol UGT in rat ovary by gonadotropins. Regulation by both progesterone and oestradiol was dependent on induction of ovulation and steroidogenesis by luteinizing hormone.

INTRODUCTION

Microsomal UDP-glucuronosyltransferases (UGT, EC 2.4.1.17) are a family of isoenzymes which transfer glucuronic acid from UDP-glucuronic acid to endogenous and exogenous compounds and/or their metabolites, rendering these substances more polar and facilitating their excretion in the bile and urine [1,2]. Two families of UGTs have been found (designated UGT1 and UGT2) in both human and rat. At least 35 different UGT cDNAs have been cloned [3]. The family 1A gene encodes isoenzymes that primarily use phenolic compounds or bilirubin as substrate. Family 2 consists of steroid-metabolizing UGTs [4].

The phenol-metabolizing isoform UGT1A6 contains a non-coding regulatory sequence. Rat UGT1A6, also called phenol UGT, primarily uses planar phenols as substrate. This protein is up-regulated in the liver, at both the protein and mRNA levels, by 3-methylcholanthrene [5]. This enzyme is also co-induced with two forms of cytochrome *P*-450, i.e. 1A1 and 1A2, via the Ah receptor protein, which is selective for polycyclic aromatic hydrocarbons [6,7]. The UGT1 gene has been conserved in different organisms. UGT1A6 is expressed in different tissues. In addition to the liver, it has been detected in kidney, epididymis, ovary, testis [8], adrenal gland [9], lung, skin and small intestine, whereas in brain, spleen, thymus and heart, its level is very low or undetectable [2].

Ovarian cells are under the influence of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secreted by the pituitary gland [10]. The gonadotropins act together with the ovarian steroids, oestrogen and progesterone, regulating each other by feedback mechanisms during the oestrous cycle.

We have reported previously that the activity of ovarian UGT towards 1-naphthol is increased by treatment of rats with

pregnant mare's serum gonadotropin (PMSG, exhibiting equal LH and FSH activities) [11]. In the present study, we investigated whether this regulation of UGT activity reflects changes in the amount of protein and/or mRNA. At the same time, we demonstrate that only one immunoreactive band of UGT isoenzyme is expressed in the rat ovary, in contrast with rat liver. Finally, regulation of the phenol UGT by human chorionic gonadotropin (hCG/LH) and the steroids β -oestradiol and progesterone was investigated in the ovary and, in parallel, the liver.

EXPERIMENTAL

Materials

1-[¹⁴C]Naphthol (7.1 mCi/mmol), PMSG, β -oestradiol and progesterone (Sigma Chemical Co., St Louis, MO, U.S.A.), hCG (Organon, Stockholm, Sweden), the synthetic primers, *Taq* polymerase and restriction enzymes (Pharmacia & Upjohn, Stockholm, Sweden), the nick translation kit (Gibco-BRL) and [α -³²P]dCTP (3000 Ci/mmol) (Radiochemical Centre, Amersham, Bucks., U.K.) were all purchased from the sources indicated. All other chemicals were at least of reagent grade and purchased from common commercial sources.

Treatment of animals

Immature (26-day-old) and mature (> 70-day-old, 240 g) female Sprague-Dawley rats (Eklunds, Sweden) were housed under conditions of constant humidity and temperature with a 12 h light/dark cycle. PMSG treatment was as follows: immature (26-

Abbreviations used: PMSG, pregnant mare's serum gonadotropin; FSH, follicle-stimulating hormone; LH, luteinizing hormone; hCG, human chorionic gonadotropin; UGT, UDP-glucuronosyltransferase.

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day-old) rats were treated with 10 IU of PMSG to synchronize the preovulatory and luteal phases. Mature (250 g) rats were hyperstimulated with 150 IU of PMSG [11].

Alternatively, the hormone treatment was as follows: immature rats were injected subcutaneously with β -oestradiol (10 μ g/rat) or progesterone (1 mg/rat) dissolved in sesame oil, once daily from 26 to 28 days of age between 08:00 and 10:00 h. Another group received a single injection of hCG (10 IU/rat) dissolved in 0.9% NaCl at 15:00 h on the 28th day of postnatal life. Control rats were injected with sesame oil or 0.9% NaCl at the same time as the treated animals [12,13]. To be certain that these hormone treatments were successful, carbonyl reductase activity in the ovary was measured with menadione as substrate [12,13] and serum progesterone level determined by RIA (Diagnostic Products Corporation, Los Angeles, CA, U.S.A.). Livers from control and treated Wistar rats were used as standards. Mature rats were treated with 0.9% NaCl, phenobarbitone (100 mg/kg) or β -naphthoflavone (100 mg/kg) for 4 days. These animals were killed on the fifth day.

The rats were killed by cervical fracture between 08:00 and 10:00 h. The ovaries and livers were immediately removed and placed in ice-cold 150 mM KCl/1 mM EDTA/20 mM Tris/HCl, pH 7.6. The ovaries were pooled, weighed and homogenized (200 rev./min) using between six and ten up-and-down strokes. Livers were handled similarly, except that they were placed in ice-cold 0.25 M sucrose and homogenized individually.

Ovaries from pigs (4–6 months old) were obtained from a local slaughterhouse (Uppsala, Sweden). They were collected in the same buffer as the rat ovaries about 20 min after death. Follicles of different sizes were prepared as described by Channing and Ledwitz-Rigby [14]. The follicles from the different groups were pooled and homogenized as described above for the rat ovary.

Subcellular fractionation of ovaries and liver

The homogenate was centrifuged at 900 g_{av} for 10 min to sediment nuclei and debris. The resulting supernatant was centrifuged at 9500 g_{av} for 10 min to yield a mitochondrial fraction. Finally, the postmitochondrial supernatant was centrifuged at 100000 g_{av} for 1 h to sediment the microsomal fraction. These microsomes were washed once and then resuspended in the same buffer supplemented with 30% glycerol. Samples were stored at -70°C until use.

Electrophoresis and immunoblot analysis

Microsomal ovarian proteins were separated by electrophoresis using a 12% (w/v)-polyacrylamide gel in the presence of 0.1% SDS [15]. The separated proteins were subsequently transferred to a nitrocellulose membrane as described by Towbin et al. [16]. This membrane was then blocked with 5% BSA in Tris-buffered saline/Tween (TBS-T) overnight. The membrane was thereafter washed in TBS-T with 0.2% BSA and incubated with sheep anti-(rat liver UGT) IgG for 1 h. This antibody (a gift from Dr. Brian Burchell, University of Dundee, Scotland, U.K.) interacts with at least the following transferase isoenzymes: 1-naphthol, bilirubin, testosterone, androsterone, oestrone and morphine [17]. After another wash, the membrane was incubated for a second hour with the secondary antibody, i.e. horseradish peroxidase-conjugated anti-sheep immunoglobulin. After the final wash, the proteins to which the antibodies bound specifically were detected by enhanced chemiluminescence (ECL).

Enzyme assay

UGT was measured radiometrically in the microsomal fraction by the methods of Hjelle et al. and Otani et al. [18,19] as modified by Becedas and Bengtsson Ahlberg [11]: 0.5–1.0 mg of protein, a final substrate concentration of 500 μ M 1-[^{14}C]naphthol (0.04 μ Ci), and 8 mM CHAPS in 100 mM Tris/HCl, pH 7.4, were used. The reaction was started by the addition of 3 mM UDP-glucuronic acid and terminated with 0.13 M trichloroacetic acid and 0.2 M glycine, pH 2.7. Carbonyl reductase activity in the cytosol was determined spectrophotometrically by recording the change in A_{340} caused by NADPH [20]. The reaction mixture contained 0.2 mM menadione as substrate, 0.08 mM NADPH and 0.1 M sodium phosphate buffer, pH 7.0. The reaction was started by addition of the enzyme.

Protein determination

Protein was quantified using the method of Lowry and co-workers [21] with BSA as standard.

Preparation of the synthetic UGT1A6 DNA probe

Rat kidney UGT cDNA (K39) [22] coding for UGT1A6 was used to produce the specific probe by PCR. Two synthetic primers were used: 5'-CTCTGAAAGGATGGCTTGCC-3' (–10 upstream) and 5'-TGGCCGAGTCCTTCAGGAGG-3'. The PCR product, demonstrating a size of 399 bp (–10–389 nt), was purified from the agarose gel and used as a probe in Northern-blot analysis of UGT1A6 RNA in ovary and liver. This probe was labelled using a nick translation kit.

Isolation of RNA and Northern-blot analysis

mRNA was prepared from at least 10 pooled ovaries, and total RNA from about 1 g of liver. This isolation was performed on the day that the animals were killed. A kit from Promega based on the guanidinium thiocyanate method was used. These samples were stored at -70°C until use.

RNA was denatured by heating for 3 min in buffer containing 50% formamide, 2.2 M formaldehyde and 1 $\times$ MOPS buffer (20 mM Mops, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). Total RNA was electrophoresed on a 1%-agarose gel containing 2.2 M formamide. The separated RNA species were then transferred by capillary blotting to a nylon membrane (Hybond-N; Amersham Buchler) in 20 $\times$ SSC (where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and then cross-linked using UV light. The membrane was prehybridized in 10 $\times$ Denhardt's solution containing 50% deionized formamide, 6 $\times$ SSC and 1 mg of denatured sperm DNA at 44 $^{\circ}\text{C}$ for 24 h. Hybridization with the UGT1A6 probe was performed at 44 $^{\circ}\text{C}$ for 48 h. The membrane was subsequently washed twice for 20 min at 50 $^{\circ}\text{C}$ with 2 $\times$ SSC containing 0.1% SDS. Finally, the signal was detected by exposure to Kodak XAR-5 film for 14 days at -70°C [8].

RESULTS

Identification of the isoenzyme of UGT expressed in rat ovary and characterization of its regulation by PMSG

Immunoblot studies of ovarian microsomes were performed to identify and quantify the UGT isoenzyme(s) expressed in immature rats, rats synchronized with respect to their preovulatory and *corpus luteal* phases by PMSG, and mature rats hyperstimulated with PMSG. Only one immunoreactive band

Figure 1 Western blotting of UGT isoenzymes in rat ovary and liver

Top left, liver microsomes from control, phenobarbitone- and β -naphthoflavone-treated Wistar rats were used as standards for identification of the ovarian UGT isoform(s). Hepatic and ovarian microsomal protein, 1 and 23 μ g respectively, was electrophoresed on a 12% polyacrylamide gel in the presence of 0.1% SDS. The proteins were then transferred to a nitrocellulose membrane and, after blocking, incubated with the antibodies. The antibodies were then visualized by enhanced chemiluminescence. Lane 1, liver control; lane 2, liver, phenobarbitone-induced; lane 3, liver, β -naphthoflavone-induced; lane 4, ovaries from immature rats enriched in the preovulatory phase; lane 5, ovaries from immature rats enriched in the corpus luteal phase; lane 6, ovaries from mature PMSG-hyperstimulated rats. Top right, 23 μ g of ovarian microsomal protein was electrophoresed and blotted using the same procedure as for the samples on the left. The antibodies were then visualized by enhanced chemiluminescence. Lane 1, immature rats; lane 2, preovulatory ovaries; lane 3, the corpus luteal stage; lane 4, PMSG-hyperstimulated mature rats. This experiment was repeated twice with identical results. Bottom, Western blotting of UGTs in liver microsomes from mature PMSG-treated rats. Liver microsomes (1 μ g of protein) from immature and PMSG-treated rats were electrophoresed and blotted using the same procedure as for the samples in the top left gel. The antibodies were then visualized by enhanced chemiluminescence. Lane 1, immature rats; lane 2, preovulatory rats; lane 3, corpus luteal stage; lane 4, PMSG-hyperstimulated rats.

was observed in the ovarian microsomes from all of these animals cross-reacted with sheep anti-(rat liver UGT) antibodies. Hepatic microsomes from male Wistar rats treated with phenobarbitone and β -naphthoflavone were used as standards in these Western blots to identify the ovarian UGT isoenzyme (Figure 1, top left). The ovarian band was the same apparent size as phenol UGT in the liver standard, i.e. 53 kDa (Figure 1, top right). The highest amounts of immunoreactive protein was present in the ovary of hyperstimulated rats, the next highest amount in rats synchronized for their preovulatory and corpus luteal phases, and the lowest in immature rats.

Microsomes from the liver of PMSG-hyperstimulated rats were also analysed by immunoblotting. No differences compared with liver microsomes from untreated mature female rats were seen, in agreement with activity measurements [11] (Figure 1, bottom). Immunochemical analysis of microsomes prepared from porcine ovarian follicles of different sizes revealed the presence of only phenol UGT in this organ. No differences in the level of this protein in different follicles were observed (not shown).

Comparison of UGT activity and levels of the corresponding protein in rat ovarian microsomes

Table 1 presents a comparison of rat ovarian microsomal UGT activities towards 1-naphthol and the corresponding band densities obtained by immunoblotting. There was a similar relationship between these two parameters in the immature group, the animals in the preovulatory and corpus luteal phases

Table 1 Comparison between the activity of ovarian microsomal phenol UGT towards 1-naphthol and the level of the corresponding protein, with and without PMSG pretreatment

The activities presented are from our earlier study [11]. The bands on the exposed film were analysed with a densitometer and their densities compared with that obtained for immature rats. The activity and density for immature rats were defined as 100% (100% activity = 0.23 pmol/min per mg; data from ref. [11]).

Hormonal state	Activity (%)	Immunoblot density (%)
Immature	100	100
Preovulatory	567	754
Corpus luteum	936	1618
PMSG-hyperstimulated	712	2648
Mature	341	265

and for mature rats. However, the activity in PMSG-hyperstimulated animals was almost the same as that seen for the corpus luteal stage, whereas the amount of protein was 60% higher in the former case.

Serum progesterone concentrations and ovarian carbonyl reductase activities in rats in different hormonal states

These measurements were performed in order to be certain that the hormone treatments had been effective. As expected, serum progesterone concentration was higher in animals treated with progesterone (Table 2). The progesterone-treated group injected with hCG demonstrated the highest concentration. Carbonyl reductase activity was increased 2-fold in the animals treated with oestradiol and 1.8-fold increased in the animals treated with oestradiol and 10 IU of hCG, in comparison with control animals, in agreement with the report by Inazu et al. [12].

Immunoblot analysis of the regulation of rat ovarian UGT by hCG, oestradiol and progesterone

Rats were treated with oestradiol, progesterone and hCG separately or together. The level of the phenol UGT protein increased in ovarian microsomes from animals pretreated with progester-

Table 2 Carbonyl reductase activities and progesterone concentrations in the serum of rats in different hormonal states

The carbonyl reductase activities and concentrations of progesterone (ng/ml serum) were measured by RIA to verify the effectiveness of the hormone treatments. For further details, see the Experimental section. —, Not measured.

Hormonal treatment	Carbonyl reductase activity (nmol/min per mg)	Progesterone concentration (ng/ml)
None	5.9 $\pm$ 0.65	8.8
Oestradiol	11.6 $\pm$ 2.2	1.8
Progesterone	—	33
hCG	7.15 $\pm$ 1.4	14
hCG + oestradiol	10.6 $\pm$ 1.2	10
hCG + progesterone	—	55

Figure 2 Immunoblot of UGT in ovarian microsomes from rats treated with hCG, β -oestradiol and/or progesterone

Ovarian microsomal protein (20 μ g) or hepatic microsomal protein (1 μ g) was electrophoresed on a 12%-polyacrylamide gel in the presence of 0.1% SDS. The proteins were then transferred to a nitrocellulose membrane and, after blocking, incubated with the antibodies. The antibodies were then visualized by enhanced chemiluminescence. The microsomal samples applied were as follows: lane 1, ovarian microsomes from control rats; lane 2, ovarian microsomes from rats receiving oestradiol; lane 3, ovarian microsomes from rats treated with progesterone; lane 4, ovarian microsomes from rats treated with hCG; lane 5, ovarian microsomes from rats treated with oestradiol + hCG; lane 6, ovarian microsomes from rats treated with progesterone + hCG; lanes 7 and 8, ovarian and liver microsomes respectively from PMSG-hyperstimulated rats.

one and hCG (Figure 2). The highest increase, i.e. 7-fold (as determined by densitometry), was found after treatment with hCG together with progesterone. A smaller increase with oestradiol and hCG together, i.e. 3–4-fold (as determined by densitometry), was also seen. For comparison, the density of the UGT band in ovaries from PMSG-treated immature rats was 12–13-fold greater than that of the corresponding band for untreated immature rats.

Figure 3 Northern-blot analysis of the level of the mRNA species coding for UGT1A6 in the ovaries and livers of rats in different hormonal states

Top, ovarian polyadenylated RNA (14 μ g) and hepatic total RNA (20 μ g) were separated, blotted on to a membrane and hybridized with a UGT1A6-specific probe (see Experimental section for further details). Lane 1, immature rat liver; lane 2, liver from rats in the preovulatory stage; lane 3, liver from rats in the *corpus luteal* stage; lane 4, PMSG-hyperstimulated liver; lane 5, PMSG-hyperstimulated ovary; lane 6, ovary from immature rats. Bottom, Total hepatic RNA (20 μ g) was electrophoresed and blotted as above and then hybridized with a common UGT probe (see Experimental section for more details). Lane 1, immature rat liver; lane 2, liver from rats in the preovulatory stage; lane 3, liver from rats in the *corpus luteal* stage; lane 4, PMSG-hyperstimulated liver.

Immunochemical analysis demonstrating the lack of regulation of rat liver UGT isoenzymes by hCG, oestradiol and/or progesterone

Female rats were treated with oestradiol, progesterone and hCG separately and together and immunoblot analysis was subsequently performed. The levels of the hepatic UGT isoenzymes were apparently unaffected by these hormones (not shown).

Northern-blot analysis of mRNA from the ovary and of total RNA from the liver of rats treated with PMSG

mRNA from pooled ovaries of immature and PMSG-hyperstimulated rats and total RNA from the liver of rats in the preovulatory and *corpus luteal* stages were compared by Northern blotting, employing hybridization with a probe specific for UGT1A6. The mRNA levels of interest in the ovaries are very low and difficult to detect. However, it can be seen that the level of UGT1A6 mRNA in the ovaries of PMSG-hyperstimulated rats is the same as or higher than that in immature rats (Figure 3, top).

In the liver, we saw a regulation of the level of mRNA coding for UGT1A6. The highest level was observed in the immature rats, and PMSG hyperstimulation caused a decrease. This same regulation was seen with a UGT1A6-specific probe (Figure 3, top) and with a probe hybridizing to the C-terminal portion of the cDNA for UGT1A6, which is highly conserved in different isoenzymes (Figure 3, bottom). The level of mRNA in PMSG-hyperstimulated animals, in comparison with the immature rat, appeared to be higher using the common UGT probe.

DISCUSSION

It is known that xenobiotic-metabolizing enzymes can be induced by endogenous compounds, such as hormones, including gonadotropic hormones. Earlier studies by our group revealed that UGT and sulphotransferase activities in rat ovary are regulated during the oestrous cycle and by the gonadotropins FSH and LH. Another xenobiotic-conjugating enzyme that has its level of expression regulated by hormones is glutathione transferase. This enzyme is up-regulated in the liver of PMSG-hyperstimulated female rats [11], as well as during sexual maturation of female rats [23]. It is regulated by other hormones as well, including adrenocorticotrophic hormone in the adrenal gland [24] and growth and thyroid hormones and testosterone in the liver (L. Staffas, C. Yanog and J. W. De Pierre, unpublished work).

In the present study we have demonstrated the following. (1) UGT1A6 is apparently the only isoenzyme of UGT expressed in rat ovary. Steroids play an important role in ovarian function and it is possible that other isoenzymes of UGT, e.g. steroid UGTs, are also present, but at considerably lower levels not detectable by the methodology used here. Indeed, two UGT bands have been detected in human ovary (C. Brierley and B. Burchell, personal communication). (2) Changes in the activity of this enzyme on treatment with PMSG reflect up-regulation of the amount of protein and, apparently, of the corresponding mRNA. (3) Regulation of these changes involves LH/hCG and progesterone.

A similar correlation between activity and the amount of protein was found in the ovaries of immature rats, animals in the preovulatory and *corpus luteal* stages and mature animals. However, the activity of PMSG-hyperstimulated rats did not increase as much as the amount of protein, in comparison with the other four groups. This may indicate the existence of another

ovarian UGT form in addition to the phenol form. If this is the case, the other isoenzyme should have a molecular mass very similar to that of the phenol form.

UGT is an integral membrane protein, with its active site oriented towards the lumen of the endoplasmic reticulum. It is very important to have the correct lipid environment for maximal specific activity of this enzyme [25]. In the case of the PMSG-hyperstimulated rats, the high rate of steroidogenesis in ovarian cells may influence the lipid composition of the endoplasmic reticulum, which may account for the relatively low specific activity in this case. Another possible explanation for the lower specific activity in PMSG-hyperstimulated rats is post-translational modification of the UGT protein.

It is known that hepatic bilirubin metabolism during pregnancy and lactation is altered by hormones, probably β -oestradiol and progesterone [26]. hCG and progesterone appear to be important factors in the up-regulation of ovarian UGT. Up-regulation of this protein by progesterone is potentiated after the ovulation induced by hCG/LH. However, hCG alone does not bring about such up-regulation. This agrees with the finding that the highest activity and amount of ovarian microsomal phenol UGT is seen in the *corpus luteal* phase induced by PMSG, i.e. the stage at which the highest serum progesterone levels are found.

These results suggest an involvement of the LH receptor in regulation of ovarian phenol UGT. However, the up-regulation by hCG/LH and progesterone was not as pronounced as that observed in the *corpus luteal* phase. Thus we can also speculate that FSH is involved in the regulation of UGT in the ovary.

In liver, treatment with the gonadotropins and/or steroids has no effect on the level of the phenol UGT protein. It is known that rat liver does not express the LH receptor [27], which may explain these findings.

The distribution of UGT in the different ovarian cell types is not yet known. Unpublished results from our laboratory demonstrate that the quantitatively most important cells for ovarian metabolism of 1-naphthol (a substrate for phenol UGT) are the theca and/or stroma cells, which also have the highest rate of progesterone production and are the only ovarian cells that express the LH receptor in immature animals.

In contrast with the lack of regulation of hepatic UGT protein by the hormones of interest here, up-regulation of the hepatic mRNA level in rats treated with PMSG (to synchronize their preovulatory and *corpus luteal* stages) was observed. This mRNA level is decreased by gonadotropin treatment and its level thus does not correlate with enzyme activity. This may mean that the mRNA detected does not code for, or is not the only mRNA species coding for, UGT1A6 in rat liver. Splicing of the species detected may be required to obtain the mature mRNA. Regulation of the level of mRNA coding for UGT1A6 in the liver is not the same as in the ovary, which may mean that two different mechanisms are involved.

UGT plays an important role in the conjugation of xenobiotics in the ovary and liver. It is not yet known whether the phenol UGT isoenzyme also plays an endogenous role during ovulation and/or the luteinizing phase in rat ovary.

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