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A Comparison of 17β-Hydroxysteroid Oxidoreductase Type 1 and Type 2 Activity of Cytosol and Microsomes from Human Term Placenta, Ovarian Stroma and Granulosa–Luteal Cells

Charles H. Blomquist,* Dennis G. Bealka, Hugh C. Hensleigh and George E. Tagatz

Department of Obstetrics and Gynecology, Ramsey Clinic and St Paul-Ramsey Medical Center, St Paul, MN 55101 and the Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, University of Minnesota Medical School, Minneapolis, MN 55455, U.S.A.

A large body of evidence suggests multiple forms of 17β-hydroxysteroid oxidoreductase (17-HOR) regulate estrogen and androgen levels within gonadal and peripheral tissues. Two kinetically-differing 17-HOR activities have been detected in placental homogenates. 17-HOR type 1, found mainly in the cytosol, is highly reactive with estradiol-17β (E2) and estrone (E1) but not testosterone (T) (high E2/T activity ratio). Microsomal 17-HOR type 2 is reactive with both E2 and T (low E2/T activity ratio). In this study, 17-HOR activity of cytosol and microsomes from term placenta, ovarian stroma and granulosa–luteal cells was assayed under conditions which specifically differentiate between the two forms of the enzyme. Placenta had the highest activity with either E2 or T in both cytosol and microsomes and stroma the lowest. The highest specific activity with E2 and E1 was cytosolic in all samples. The highest specific activity with T was microsomal in placenta and ovarian stroma. E2/E1 activity ratios were comparable for cytosol and microsomes while E2/T activity ratios were comparable for placenta and stroma, but markedly elevated in granulosa–luteal (G–L) cell cytosol and microsomes. The results indicate trophoblast and ovarian stroma have more 17-HOR type 2 relative to type 1. G–L cells, in contrast, are relatively enriched in 17-HOR type 1 and thus have a greater capacity for net conversion of E1 to E2 under physiologic conditions. These differences may contribute to increasing serum and follicular fluid E2/E1 ratios during development of the dominant follicle.

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INTRODUCTION

17β-Hydroxysteroid oxidoreductase (17-HOR), which catalyzes pyridine nucleotide linked oxidation and reduction at C-17 of C_{18} and C_{19} hydroxy and keto-steroids, is widespread in human tissues. A growing body of evidence demonstrating variations among and within tissues in substrate and coenzyme specificity as well as subcellular localization suggests that multiple forms of 17-HOR may be part of a family of enzymes and that the pattern of steroid metabolism within a given tissue may be regulated by the relative amounts of differing activities present [1].

Studies from various laboratories of 17-HOR kinetics and stability in term placental cytosol, microsomes and villous tissue fragments *in vitro* have yielded evidence consistent with the presence of two kinetically differing forms of the enzyme [2–8]. One, found mainly in the cytosol, is highly reactive with estradiol-17β (E2) but not testosterone (T) (high E2/T activity ratio). A second, microsomal activity is reactive with both E2 and T (low E2/T activity ratio). Current data suggest this latter activity accounts for most of T metabolism and a significant fraction of E2 metabolism in villi

*Correspondence to C. H. Blomquist, Department of Obstetrics and Gynecology, St Paul-Ramsey Medical Center, 640 Jackson Street, St Paul, MN 55101, U.S.A.
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in vitro [8]. The genes for both forms have now been cloned and cDNAs isolated and characterized [9–11]. Consistent with these latter results, we refer to the high E2/T ratio enzyme as 17-HOR type 1 and the low E2/T ratio activity as 17-HOR type 2.

For ovary, 17-HOR activity with E2, estrone (E1) and T has been detected in tissue homogenates as well as both cytosol and microsomes by direct assay [12–16]. Complementary DNA and cRNA probes for 17-HOR type 1 indicate ovary is also a rich source of this enzyme [9, 17], and this has led to the proposal that high levels of this form of 17-HOR may be characteristic of E2-producing tissues [9]. Pittaway *et al.* [12, 15] and Barbieri *et al.* [14, 16] have provided kinetic evidence of microsomal 17-HOR type 2 in ovary, as well.

Although of long-standing interest, our understanding of the relationship between mRNA levels, patterns of steroidogenesis and the relative 17-HOR capacities of placenta and ovary is limited. Because characterizations of the kinetic properties and subcellular distribution of 17-HOR in placenta and ovary have been carried out under a variety of conditions, a direct comparison of the two organs has not been possible.

As an approach to this problem, this investigation was undertaken to quantitate specific activity with E2, E1 and T in cytosol and microsomes prepared under identical conditions from term placenta, granulosa–luteal (G–L) cells and ovarian stroma and assayed under conditions which specifically differentiate between 17-HOR types 1 and 2.

EXPERIMENTAL

Materials

Reagents and supplies were purchased from the following sources: [6,7-^{3}H]E2 (1.5 TBq/mmol, 40 Ci/mmol) and [2,4,6,7-^{3}H]E1 (4.0 TBq/mmol, 108 Ci/mmol), Amersham Corp. (Arlington Heights, IL); [1,2-^{3}H]T (119 TBq/mmol, 52.5 Ci/mmol), Dupont NEN Products (Boston, MA); Bicine (*N*,*N*-bis[2-hydroxyethyl]glycine, Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and bovine serum albumin, Sigma Chemical Co. (St Louis, MO); NAD and NADH, Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ); silical gel HL plates, Analtech, Inc. (Newark, DE) Ecolume, ICN Schwarz Mann (Cleveland, OH).

Tissue samples

All specimens were obtained with patient informed consent in accordance with the human studies policies of St Paul-Ramsey Medical Center and the University of Minnesota. Placentas were obtained from normal, term (38–40 week) vaginal deliveries. Specimens were kept on ice until taken for fractionation, usually within 30 min after the delivery. Samples of normal ovary were obtained within 30 min following surgery from patients undergoing abdominal hysterectomy and bilateral salpingoophorectomy for benign or malignant uterine disease. With the aid of a dissecting microscope fragments of stroma lacking follicles >2.0 mm in diameter were separated from the tunica albicans. G–L cells were isolated from follicular aspirates as described by Wickings *et al.* [18] following oocyte retrieval. The ovarian stimulation protocol consisted of 10 to 15 days of suppression with leuprolide acetate (Lupron; TAP Pharmaceuticals, Chicago, IL) followed by sequential stimulation with hMG (Pergonal; Serono Labs, Randolph, MA) or FSH (Metrodin, Serono) and hCG.

Tissue fractionation

All procedures were carried out at 4°C. Tissue fragments and G–L cells were washed in ice-cold Dulbecco's phosphate buffered saline, minced with scissors or homogenized by hand in a glass Dounce homogenizer in buffer containing 20% glycerol (v/v) and 1.0 mmol/l EDTA in 40 mmol/l potassium phosphate, pH 7.2 and then extracted overnight. This buffer was used because it has been shown to stabilize 17-HOR type 1 [19]. Under these conditions activity was stable for at least 48 h at 4°C. The extracts were clarified by centrifugation at 10,000 *g* for 10 min and the supernatants fractionated into microsomes and cytosol by centrifugation at 105,000 *g* for 60 min. Microsomal pellets were washed by repeat centrifugation and suspended in buffer.

17-HOR activity

17-HOR specific activity was measured as described previously [20]. Briefly, a 10 μl aliquot of cytosol or microsomes was combined with 10 μl of reaction mixture containing 1.0 mmol/l NAD and 2.0 μmol/l [^{3}H]E2 or [^{3}H]T in 0.08 mol/l Bicine, pH 9.0, or 1.0 mmol/l NADH and 2.0 μmol/l [^{3}H]E1 in 0.08 mol/l Hepes, pH 7.2. These steroid concentrations equal Michaelis constants determined previously [6, 7] and specifically differentiate between high (type 1) and low (type 2) E2/T activity ratio forms of 17-HOR in placental homogenates when E2 and T are compared as substrates. The pH values of 9.0 and 7.2 were found to be optimal for dehydrogenase and reductase activity, respectively. Assays were run at 37°C for time intervals giving linear rates of product formation.

At the end of the incubation, reaction mixtures were transferred in total to the preadsorbant layer of silica gel plates and, after the addition of unlabeled carriers, fractionated by thin layer chromatography with benzene–acetone (4:1, v/v) as the solvent. Steroids were located by a light spraying with water. The plates were air dried and steroid-containing spots scraped into 10 ml of Ecolume for liquid scintillation counting. Product formation was quantitated in terms of cpm in product as percent of total cpm in substrate and product and specific activity expressed as nmol product/mg protein · 30 min [20].

Protein quantitation

Protein was assayed by the method of Markwell *et al.* [21] with bovine serum albumin as the protein standard.

Statistical analyses

Pearson's r values were used to estimate correlations. Multiple group means were compared by analysis of variance in combination with the Newman–Keuls multiple comparisons test. When Bartlett's test indicated a non-Gaussian distribution of values, the Kruskal–Wallis non-parametric test was used. Paired means were compared by Student's t-test.

RESULTS

17-HOR specific activities of cytosol and microsomes with E2, E1 and T

Although both trophoblast and G–L cells have been shown to be rich in mRNA for the cytosolic 17-HOR type 1 [9, 17], a comparison of specific activities determined under the same assay conditions has not been made previously. Cytosolic 17-HOR activity with E2, E1 and T is shown in Table 1. Placental cytosol had the highest specific activity and stroma the lowest with each of the substrates. There was no significant difference between samples of stroma from pre- and postmenopausal patients. Although there was an overlap in the range of values, mean specific activity with E2 and E1 of placental cytosol was 2- to 3-fold greater than that of G–L cells, and that with T 10-fold greater. Cytosolic activity with E2 correlated with activity with E1 ($P < 0.03$) in each case, but was not correlated with activity with T for placenta, stroma or G–L cells.

Microsomal activities are shown in Table 2. As with cytosol, placental microsomes had the highest activity with each substrate. Here, however, the contrast between trophoblast and G–L cells was more apparent. Placental microsomal activity with E2 was 20-fold greater and activity with T approx. 100-fold greater than that of G–L cell microsomes. For placental and stromal microsomes, activity with E2 correlated with that of both E1 ($P < 0.03$) and T ($P < 0.03$). For G–L microsomes, the correlation between activity with E2 and E1 approached significance ($r = 0.68$, $P = 0.066$), but in contrast with placental and stromal microsomes, there was no correlation between activities with E2 and T ($r = 0.18$).

Table 1. Cytosolic 17-HOR activity with E2, E1, and T

Sample	17-HOR Activity		
	E2	E1	T
Placenta	224.9 ± 35.7 (6)	139.3 ± 26.2 (6)	1.63 ± 0.43 (6)
G–L cells	98.7 ± 17.5 (11)	50.0 ± 10.5 (11)	0.15 ± 0.12 (11)
Ov stroma			
Premenopausal	0.58 ± 0.07 (8)	0.33 ± 0.08 (5)	0.013 ± 0.006 (8)
Postmenopausal	0.67 ± 0.27 (4)	0.70 ± 0.57 (2)	0.008 ± 0.002 (4)

The values for 17-HOR activity (nmol/mg protein · 30 min) are the mean ± SE of (n) samples assayed in duplicate.

Table 2. Microsomal 17–HOR activity with E2, E1, and T

Sample	17-HOR Activity		
	E2	E1	T
Placenta	70.76 ± 7.20 (6)	24.42 ± 3.48 (6)	20.73 ± 5.58 (6)
G–L cells	3.50 ± 0.70 (9)	1.08 ± 0.25 (8)	0.13 ± 0.02 (8)
Ov stroma			
Premenopausal	0.12 ± 0.03 (8)	0.03 ± 0.08 (5)	0.04 ± 0.01 (8)
Postmenopausal	0.10 ± 0.03 (5)	0.02 ± 0.01 (3)	0.04 ± 0.01 (5)

The values for 17-HOR activity (nmol/mg protein · 30 min) are the mean ± SE of (n) samples assayed in duplicate.

In terms of total activity with E2, 3.86 ± 1.02% ($n = 6$) and 3.40 ± 1.12% ($n = 11$) was microsomal in placental and stromal homogenates, respectively, whereas 1.91 ± 0.75% ($n = 11$) was recovered in G–L cell microsomes ($P < 0.01$ vs placenta, $P < 0.05$ vs stroma). Activity with T was distributed differently, with 64.67 ± 6.59% ($n = 6$) being microsomal in placental homogenates while 29.67 ± 4.52% ($n = 14$) and 35.64 ± 6.77% ($n = 8$) was microsomal in stromal and G–L cells homogenates, respectively. The percentage in placental microsomes was greater than in either stromal ($P < 0.01$) or G–L cell ($P < 0.05$) microsomes, which did not differ significantly.

E2/E1 and E2/T specific activity ratios

Because specific activities could vary among samples of tissue from the same organ, or between different organs because of heterogeneity in cell type or variations in protein yield in homogenates, specific activity ratios would appear to be a better indicator of enzyme heterogeneity.

E2/E1 activity ratios are shown in Fig. 1. The cytosolic ratio was low with each tissue type, characteristic of 17-HOR type 1, and did not vary significantly among tissues when placenta, G–L cells and stroma were compared. Similarly, the E2/E1 ratio for microsomes was constant among tissues. However, small but significant differences were seen when the ratios for cytosol and microsomes of each tissue type were compared. Those of placental and stromal microsomes were slightly greater than the corresponding cytosols. This is consistent with previous kinetic data showing that although the Michaelis constants for E2 are the same for the two forms of placental 17-HOR, microsomal 17-HOR type 2 has a lower affinity for E1 than does the cytosolic enzyme [6, 7, 11].

In marked contrast with the E2/E1 values, E2/T ratios varied among tissue types and between cytosol and microsomes for a given tissue. As shown in Fig. 2, the E2/T ratios of placental and stromal cytosols were comparable and significantly less than that of G–L cells. The ratios for microsomes were markedly less than the corresponding cytosol in each case. Here also the respective values for placental and stromal microsomes were comparable and less than that of G–L cell microsomes.

Although 17-HOR type 1 and type 2 are localized principally to cytosol and microsomes, respectively, they are recovered in both fractions depending on extraction and centrifugation conditions [5]. The elevated E2/T ratio for G–L cytosol appears to be due to high E2 activity relative to that with T. This probably reflects enrichment with 17-HOR type 1 but the presence of another 17-HOR type more specific for E2 or of factors in G–L cell cytosol which modify the E2/T activity ratio of the type 1 enzyme cannot be ruled out. With regard to the higher E2/T ratio for G–L cell microsomes, it has been shown for term placenta that microsomal 17-HOR activity with E2 appears to be due to both the type 2 enzyme as well as a variable amount of 17-HOR type 1 recovered in the microsomal fraction [7]. Recovery of a significant amount of 17-HOR type 1 with G–L cell microsomes, which have a low level of 17-HOR type 2, could account for the elevated E2/T activity ratio.

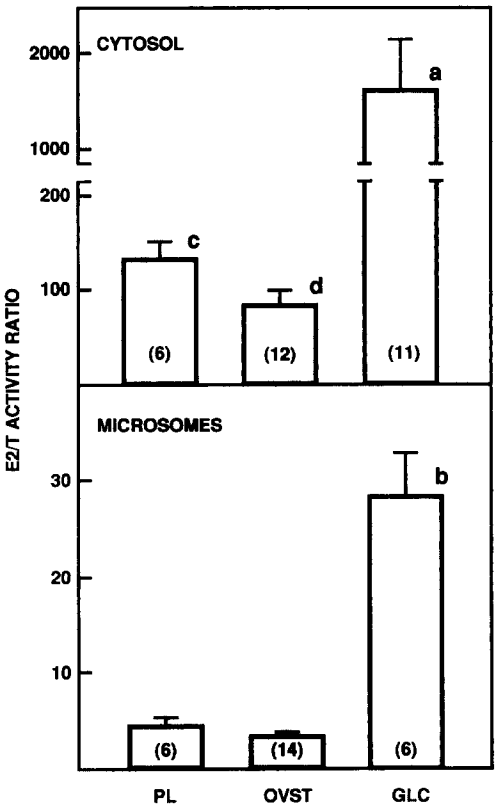

Fig. 2. E2/T activity ratios for cytosolic and microsomal 17-HOR from placenta (PL), ovarian stroma (OVST) and G–L cells. The values are the mean ± SE of (n) samples assayed in duplicate. [a] $P < 0.05$ compared to PL and OVST; [b] $P < 0.001$ compared to PL and OVST; [c] $P < 0.01$ compared to PL microsomes; [d] $P < 0.02$ compared to OVST microsomes.

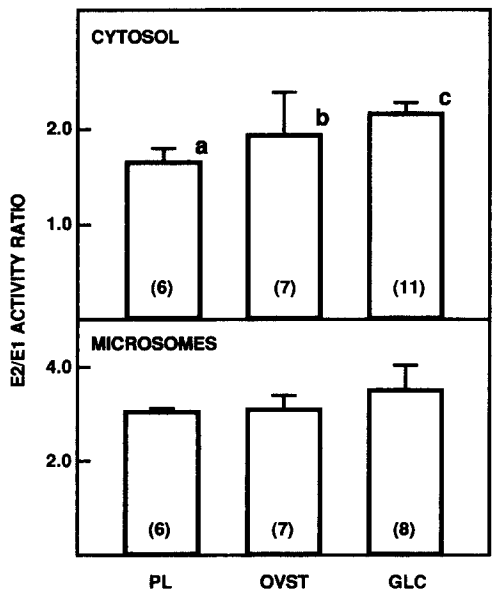

Fig. 1. E2/E1 activity ratios of cytosolic and microsomal 17-HOR from placenta (PL), ovarian stroma (OVST) and G–L cells (GLC). The values are the mean ± SE of (n) samples assayed in duplicate. [a] $P = 0.014$ compared to microsomes; [b] $P < 0.001$ compared to microsomes; [c] $P = 0.061$ compared to microsomes.

Cytosol/microsomes (C/M) specific activity ratios

C/M ratios are shown in Fig. 3. The highest specific activity with E2 or E1 was cytosolic in each case, consistent with the localization of 17-HOR type 1. With E2 as substrate the respective values for placenta and stroma were comparable and significantly less than the ratio for G–L cells. In contrast with these latter values, the C/M ratios for placental and stromal activity with T were < 1.0, reflecting the membrane association of 17-HOR type 2. The ratio for G–L cells was slightly greater, possibly due to enrichment with the type 1 enzyme which has a very low affinity for, but some activity with, T [6].

Kinetic properties of ovarian 17-HOR activity

The assay conditions used in this study were based on those used previously to characterize 17-HOR in placental cytosol and microsomes. To ensure that they were applicable to ovarian extracts the Michaelis constant of cytosolic 17-HOR of ovarian stroma with E2 was determined. When stromal cytosol was incubated at pH 9.0 with 0.5 mM NAD and 0.5, 1.0, 2.0, 4.0 or 8.0 μM steroid, the Michaelis constant for E2 was 1.0 μM, in good agreement with the value for E2 with placental 17-HOR type 1 [6, 11].

Previous studies from this laboratory have shown that for placenta, 17-HOR type 1 activity with E2 is insensitive to inhibition by 5α-dihydrotestosterone (5α-DHT) while microsomal 17-HOR type 2 activity with T is inhibited [6, 7]. Similar results were obtained when cytosolic and microsomal 17-HOR from G–L cells or ovarian stroma were tested. In Fig. 4(A) representative data obtained with cytosol and microsomes from a sample of G–L cells are shown. Cytosolic activity with E2 was not affected by 5α-DHT in 100-fold excess of substrate while microsomal activity with T was reduced by approx. 80% under the same conditions. In separate experiments with 5α-DHT at 50 μM, mean cytosolic activity (±SE) with E2 was 99.3 ± 4.6% ($n = 6$) while microsomal activity with T was 41.8 ± 5.6% ($n = 4$) of that of control assays lacking 5α-DHT [Fig. 4(B)].

E2/E1 activity ratio of G–L cell cytosol at pH 9.0 and 7.2

The growth of the dominant follicle is associated not only with an increase in E2 and E1 levels but also with a marked increase in the E2/E1 ratio in follicular fluid and serum [22–25]. In preliminary experiments we established that dehydrogenase activity with either E2 or T was optimal at pH 9.0 and reductase activity at pH 7.2. On that basis dehydrogenase assays were run routinely at pH 9.0 while reductase activity with E1 was assayed at pH 7.2, with the result that E2/E1 activity ratios were >1 in all cases (Fig. 1). However when G–L cytosol was assayed with either E2 or E1 at pH 7.2, the E2/E1 ratio was <1. Thus in terms of enzymatic capacity within intact trophoblast and G–L cells, the reductase reaction yielding E2 would appear to be favored at physiologic pH, in agreement with results obtained with unfractionated placental and ovarian homogenates [17] and placental cytosol [12] and microsomes [12, 26].

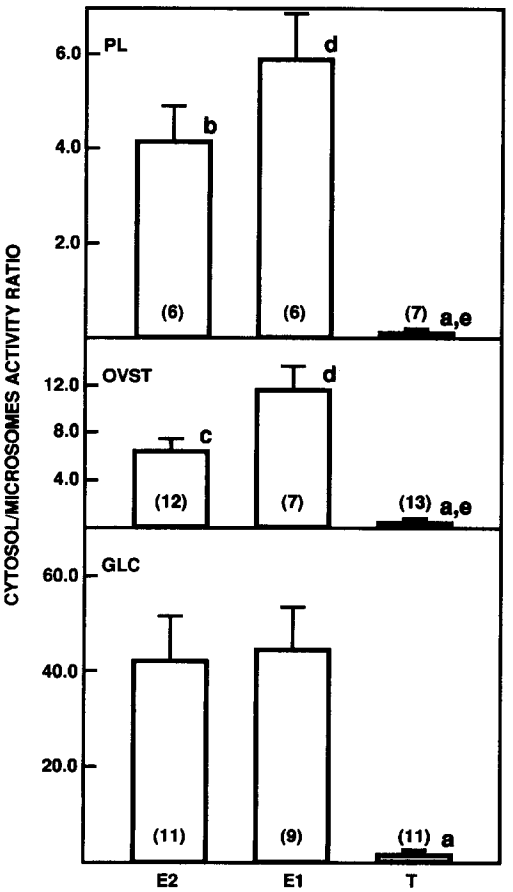

Fig. 3. Cytosol/microsomes 17-HOR activity ratios for placenta (PL), ovarian stroma (OVST) and G–L cells (GLC). The values are the mean ± SE of (n) samples assayed in duplicate. [a]$P < 0.01$ compared to E2 and E1; [b]$P < 0.01$ compared to GLC; [c]$P < 0.05$ compared to GLC; [d]$P < 0.01$ compared to GLC; [e]$P < 0.01$ compared to GLC.

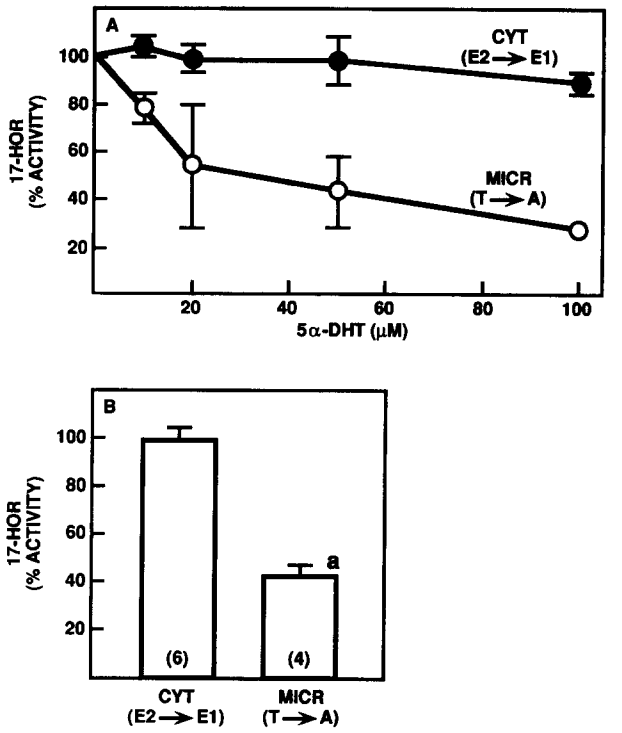

Fig. 4. Effect of 5α-DHT on cytosolic (CYT) and microsomal (MICR) 17-HOR of G–L cells and ovarian stroma. (A) Duplicate assays of G–L cell cytosol and microsomes were run at pH 9.0 as described under Experimental with 0 to 100 μM 5α-DHT. (B) Effect of 5α-DHT on cytosol and microsomes from ovarian stroma. The values are the mean ± SE of (n) samples assayed in duplicate. [a]$P < 0.001$ compared to CYT.

DISCUSSION

Results from a number of laboratories suggest 17-HOR type 1 may be of particular importance in E2-producing organs such as the ovary and placenta [9, 17]. Our objective in this investigation was to quantitate cytosolic and microsomal 17-HOR of ovary and placenta under conditions which would allow for a direct comparison of the two organs and further our understanding of the possible regulatory roles of the differing forms of 17-HOR. Extraction conditions

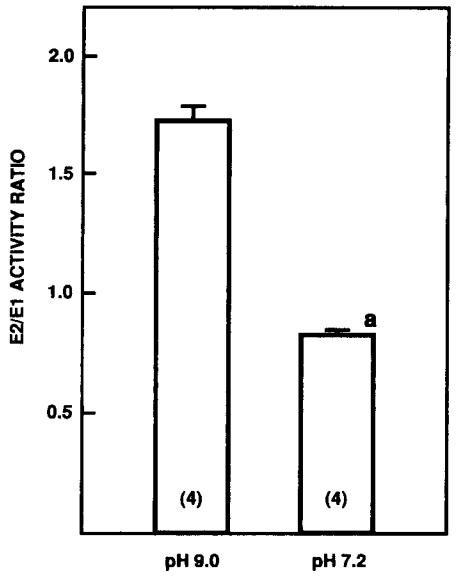

Fig. 5. E2/E1 activity ratio for cytosolic 17-HOR from ovarian stroma and G–L cells at pH 7.2 and 9.0. The values are the mean ± SE of combined values from four separate experiments in which cytosol from either ovarian stroma ($n = 2$) or G–L cells ($n = 2$) was assayed in duplicate. $^aP = 0.002$ compared to pH 9.0.

which stabilize activity were chosen, and we used assay conditions shown previously to specifically differentiate in placental homogenates between the type 1 and type 2 enzymes.

The data from this investigation show that 17-HOR type 1 specific activity of G–L cells is comparable to that in term placenta in agreement with estimates of mRNA [9, 17]. The Michaelis constant for E2, pH optimum and inhibition pattern of the ovarian enzyme in both stroma and G–L cells are identical to that of the placental activity, as well, confirming that not only is the mRNA for 17-HOR type 1 present in both tissues but also that the expressed protein has the same kinetic properties *in vitro* as that of term placenta. Thus at physiologic pH, both trophoblast and G–L cells have a large capacity for net E2 formation.

Previous findings from this laboratory demonstrated the presence in term placenta of microsomal 17-HOR type 2 which is distinctly different in steroid specificity and physical properties from 17-HOR type 1 [6, 7]. Wu *et al.* [11] have reported that it also differs from the type 1 enzyme in amino acid composition and sequence, as well as molecular weight. In addition to differing in steroid specificity, 17-HOR type 1 and type 2 differ in that 17-HOR type 1 is equally reactive with both E2 and E1 while 17-HOR type 2 has a relatively lower affinity for A and E1 and thus favors reaction in the oxidative direction yielding less biologically-active, 17-ketosteroid products [6, 7, 11]. Pittaway and coworkers [12, 15] and Barbieri [14, 16] have presented evidence of a similar activity in human ovary. The data in Tables 1 and 2 indicate that although placental microsomes are enriched in 17-HOR type 2 activity with T relative to that with E2 and E1, G–L cells, by comparison, have relatively less activity with T in both cytosol and microsomes. As a result, G–L cells have an elevated E2/T activity ratio in both subcellular fractions. This is consistent with their being the major site for the interconversion of E2 and E1 within the ovary. It is noteworthy that placental and stromal cytosol and microsomes have comparable E2/T activity ratios. Thus microsomal 17-HOR type 2 may play a dominant role in stromal interconversion of T and A as has been shown for villous tissue fragments *in vitro* [7, 8].

However, the relationship between mRNA levels, estimates of enzyme capacity based on *in vitro* activity measurements, as in this investigation, and factors such as intracellular pH, NAD(P)/NAD(P)H ratios, substrate availability and, for microsomal enzymes, membrane lipid and protein composition remains to be clarified for both placenta and ovary. There is evidence of metabolic regulation in trophoblast and of intrafollicular regulation in ovary, as well.

For example, even though the apparent capacity of trophoblast for net E2 formation is high, E2 formation from E1 by fragments of villous tissue *in vitro* is very low [8]. With E1 at 1.0 μM, E2 formation was observed to be near zero in the absence of NAD(P)H-generating 17β- or 20α-hydroxysteroids, suggesting that the rate of net E2 formation by 17-HOR type 1 may be regulated by concomitant metabolism of hydroxysteroids by 17-HOR type 2. Inhibition data [8] also suggest that in villous tissue fragments 17-HOR type 2 can account for up to 50% of the conversion of E2 to E1 and essentially all of the conversion of T to A. Thus although 96–97% of the E2 to E1 activity is cytosolic (17-HOR type 1) in placental homogenates, the microsomal enzyme (17-HOR type 2) appears to play a major role in intact trophoblast.

With regard to ovary, McNatty *et al.* [25] observed that cultures of stromal, thecal or granulosa cells supplemented with fetal calf serum differed in their biosynthetic capacities but that A was the predominant C_{19} steroid formed, with an A/T ratio of approx. 10 for each cell type. In contrast, the E2/E1 ratio was 10 or greater for each cell type. Thus although stromal tissue and G–L cells differ in their relative contents of 17-HOR types 1 and 2, the patterns of steroidogenesis by intact cells *in vitro* are similar. In an important additional study in which intact follicles and antral fluid were characterized [27], McNatty and coworkers found that although the maximum number of granulosa cells per follicle increased with increasing follicle diameter, follicles with a full complement of granulosa cells, regardless of follicle diameter, contained the highest levels of estrogens and the highest E2/E1 ratio. This latter ratio increased 10-fold as the complement of granulosa cells increased from 25 to 95% of total. A/T ratios, in contrast, were constant. And while the total amount (ng/ml) of antral fluid steroids did not change with the percentage of granulosa cells, the percentage

as estrogens and the E2/E1 ratio did. This suggests that intrafollicular regulation of both aromatase and 17-HOR underlies the increase in estrogen levels and the marked change in the E2/E1 ratio. In conclusion, this investigation has yielded kinetic evidence confirming that 17-HOR types 1 and 2 are present in ovary as well as placenta. Whether there are still other forms of 17-HOR in these organs, a possibility suggested by results obtained with both normal and malignant breast tissue [28], remains to be clarified.

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