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EPITHELIAL AND ADIPOSE CELLS ISOLATED FROM MAMMARY GLANDS OF PREGNANT AND LACTATING RATS DIFFER IN 11β-HYDROXYSTERIOD DEHYDROGENASE ACTIVITY

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Summary—Both adipose and epithelial cells isolated from the mammary glands of pregnant and lactating rats show 11β-hydroxysteroid dehydrogenase (11-HSD) activity, as measured by conversion of corticosterone to 11-dehydrocorticosterone. Activity in adipose cells from pregnant rats is 3-fold higher than in lactating rats. Epithelial cells from pregnant rats show one-twentieth of the activity of adipose cells, and activity is lower still in epithelial cells from lactating rats. Explants incubated for 48 h extensively metabolized corticosterone to 11-dehydrocorticosterone, and to a much lesser extent to a second unknown metabolite which is found in tissue extracts but not conditioned medium. Mammary gland 11-HSD may thus constitute one of the physiological mechanisms preventing premature milk production in response to glucocorticoids.

INTRODUCTION

Both differentiation and a variety of functions of the mammary gland are controlled by peptide and steroid hormones [1–4]. *In vitro* glucocorticoids increase epithelial cell synthesis of milk proteins, such as casein, α-lactalbumin and whey acidic protein, an action opposed by progestins [5–12]. These effects are altered by the stage of pregnancy or lactation; in late pregnancy and during lactation, for example, the response to both glucocorticoids and progestins at first diminishes and then disappears [8, 13].

Effector levels of steroid hormones in both pregnancy and lactation are determined in part by circulating steroid levels [14, 15] and by levels of steroid binding proteins such as transcortin [16]; in addition, metabolism of steroids within tissues can also alter effective levels [17]. Recently, there has been renewed interest in metabolism of cortisol and corticosterone by target organs [18–21]. Dehydrogenation of corticosterone/cortisol at the 11-position gives rise to products with low affinity for both mineralocorticoid (Type I) and glucocorticoid (Type II) receptors. Most tissues examined show both 11β-hydroxysteroid dehydrogenase and 11-oxo reductase activity, with the ratio varying with the tissue and even the cell type [18, 19, 22]. In terms of glucocorticoid action, the consequences of metabolism may be to increase biological activity, as for example in the reduction of cortisone (inactive) to cortisol (active) in the liver. Metabolism may also convert an active compound to an inactive one e.g. the local production of cortisone from cortisol by certain cells in the kidney [18–20]. The physiological role of these enzymes appears to be developmental in some tissues; for example, in the placenta and fetus substantial dehydrogenase activity prevents high levels of cortisol at early stages of pregnancy [18, 23, 24].

In tissues involved in salt and water balance such as the kidney, the consequence of the conversion to 11-ketosteroids has been postulated to be 2-fold [20]. First, conversion of glucocorticoids to receptor-inactive 11-ketosteroids enables Type I (mineralocorticoid) receptors in the kidney to be occupied by aldosterone, despite the much higher circulating concentrations of cortisol/corticosterone, which have equivalent affinity for Type I receptors. Secondly, such metabolism similarly lowers cortisol/corticosterone occupancy of glucocorticoid (Type II) receptors in the same tissues [20].

There are thus several potential roles for 11β-hydroxy steroid dehydrogenase. In tissues with low or absent 11-HSD, such as the hippocampus, it has been proposed that the unprotected Type I sites will be occupied by cortisol/corticosterone rather than aldosterone, reflecting their much higher free blood concentrations [20].

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The mammary gland during both pregnancy and lactation contains Type I and Type II sites [25, 26]; in the rat, glucocorticoid effects in the synthesis of casein, α-lactalbumin and whey acidic protein are mediated via Type II receptor occupancy [7, 27, 28], so that 11-HSD action in the mammary gland may serve to modulate glucocorticoid effects on milk protein synthesis.

We have recently shown that 11-HSD activity in the mammary gland decreases just after parturition [29]. In the present study, we have investigated the distribution of the enzyme between milk producing epithelial cells and adipose cells, isolated from pregnant and lactating rats, and in addition have examined the *in vivo* patterns of metabolism of corticosterone by explant systems over a 48 h incubation period.

MATERIALS AND METHODS

Materials

The reagents and equipment used were obtained as follows. Collagenase III(150–250 U/mg protein) was from Worthington, Millipore Corporation, Freehold, N.J., and Deoxyribonuclease I (from bovine pancreas, 555 U/mg protein) from Sigma Chemical Co., St Louis, Mo. Phosphate-buffered saline (PBS) and PBS minus Ca^{2+}/Mg^{2+} (PBSM) were from Gibco BRL, Preston, Vic., Australia, and Hanks' balanced salt solution 10 × conc., Dulbecco's Modified Eagle's Medium (DMEM) and HEPES from Flow Laboratories, North Ryde, N.S.W., Australia. Bovine serum albumin (BSA), Pentex Fraction V, code No 81-003-2 was from Miles Laboratories Australia, Mulgrave, Vic., Australia and Ficol 400 from Pharmacia (South Seas), North Ryde, N.S.W., Australia. Trypan Blue solution (0.5% w/v), Phenol Red (1% w/v solution) and Penicillin–Streptomycin–Fungizone (PSF), (100 × conc.: 10,000 units penicillin/ml, 10,000 μg streptomycin/ml, 25 μg fungizone/ml) were all from Commonwealth Serum Laboratories, Melbourne, Australia, and Costar 6-well plates (3.5 cm dia) Cat. No. 3416 from Phoenix Scientific Industries Ltd, Mt Waverley, Vic. Insulin (Actrapid MC, 100 U/ml) was obtained from Novo. Parramatta, Australia. Rat prolactin (rPRL) (NIH-B6) was obtained through the National Hormone and Pituitary Program from NIADDK. [1, 2, 6, 7-^{3}H]Corticosterone (sp. act. 85 Ci/mmol) TRK406, Batch 56 was from Amersham, North Ryde, N.S.W., Australia, and non-radioactive corticosterone and 11-dehydrocorticosterone from Sigma, St Louis, Mo. Thin-layer chromatography (TLC) Merck TLC plates, silica gel 60 F254 Precoated (Cat. No. 5715) were supplied by Crown Scientific, Burwood, Vic., Australia, and Whatman TLC plates LK6DF Linear K by Whatman, Singapore. All organic solvents and chemicals were analytical grade. Scintillant was Emulsifier-Safe from Canberra Packard, Mt Waverly, Vic., Australia.

Plug-mated rats were from Central Animal House, Monash University and were housed in the Prince Henry's Hospital Animal house on a 12 h light–dark regime with rat chow and water *ad libitum*.

Methods

Cell isolation. Epithelial cells were isolated as cell clusters from the mammary gland of pregnant and lactating rats as previously described [25] with the following modifications. Chopped tissue was washed prior to dissociation in a sequence of buffers: PBS with 14 mM glucose (PBSG), followed by PBSM with 14 mM glucose (PBSMG), and then 1%(w/v) bovine serum albumin (BSA) in PBSMG pH 7.4 (1%BSA–PBSMG). Dissociation medium was 1%BSA–PBSMG with Phenol Red as indicator. Ficoll gradients were made up in Hanks' medium. Following recovery of cells from the Ficoll gradient, two further washes were carried out in 1%(w/v) BSA–Hanks and two further washes with DMEM–15 mM HEPES–1%PSF pH 7.4 (DMEM–HEPES). Cells were finally resuspended in DMEM–HEPES, the buffer used in the enzyme activity determination.

Adipose cells were isolated by aspirating the floating adipose cell layer after the first centrifugation step following digestion of the tissue. The floating adipose cell layer was washed twice with 1%BSA–PBSMG, compacted by centrifugation at 100*g* for 1 min, with two further washes in DMEM–HEPES followed by resuspension in DMEM–HEPES. Cell numbers for epithelial and adipose cells were measured by adding 0.1 ml of resuspended cells to 0.9 ml 0.02% crystal violet (w/v) in 0.1 N citric acid [25, 30]. In this procedure, cells are disrupted and the nuclei stained and counted in a haemocytometer. This allows quantitation of cell numbers, given that epithelial cells are isolated as clusters and that adipose cells vary in size and are difficult to distinguish from lipid vesicles.

Assay of 11-HSD activity. 11-HSD activity was carried out in plastic vials in a total volume of 0.25 ml, comprising 0.1 ml of the assay buffer DMEM–HEPES, 0.1 ml of cells [range of 1.0×10^6–2.0×10^6 epithelial cells, or 0.1×10^6–

0.4×10^6 adipose cells], except when stated otherwise. The reaction was started by the addition of 0.05 ml of the substrate corticosterone to give a final concentration of 30nM of which 15 nM was unlabelled corticosterone and 15 nM [^{3}H]-corticosterone. Incubation (in duplicate and for two time points per cell preparation) was at 37°C in a rotary water bath at a setting of 1.1. With appropriate incubations times the conversion of corticosterone to 11-dehydrocorticosterone was linear with respect to cell number and time of incubation, up to about 40% conversion. Substrate blanks without cells were included in every assay. At the end of the incubation period, the vials were cooled on solid CO_2, and stored at −20°C until processed. For extraction, the samples were thawed on ice, and transferred to glass scintillation vials. PBS (2 ml) was added to rinse out the vials and the cells, media and rinse extracted with 2×5 ml ethyl acetate (extraction efficiency >85%). Separation of steroids by TLC was as previously described [29], except that in some experiments Whatman TLC plates were used. The capacity of these plates is less than that of Merck TLC plates, so that 7.5 μl of organic extract rather than 20 μl was streaked onto Whatman TLC plates.

After the plates were developed, the areas corresponding to authentic corticosterone and 11-dehydrocorticosterone were located and recovered, and the radioactivity in each area determined [29] for calculation of the conversion of corticosterone to 11-dehydrocorticosterone. No significant radioactivity was ever located in areas other than corticosterone or 11-dehydrocorticosterone. This was established in preliminary experiments where the TLC plate was divided into zones and all zones per lane were scraped, eluted and the radioactivity determined. In all experiments, at least one lane (from duplicate incubations) was likewise examined. Percentage conversion of corticosterone to 11-dehydrocorticosterone was calculated by adding the radioactivity recovered in the areas identified as corticosterone and 11-dehydrocorticosterone, and expressing the radioactive 11-dehydrocorticosterone as a percentage thereof, after subtraction of non-tissue blank values. The absolute amount of 11-dehydrocorticosterone formed was then calculated; from the number of cells added to the incubation, and time of incubation, the conversion was expressed as fmol of 11-dehydrocorticosterone formed per 10^6 cells per min. Statistical analysis was by ANOVA on log transformed data, followed by Duncan's multiple range test (SPSS statistics package, SPSS/PC$^+$, SPSS, Chicago, Ill., U.S.A.).

Metabolism of corticosterone by explants. Explants (~25 mg) from mammary glands of rats at day 13 of pregnancy of day 6 of lactation were incubated in 6-well plates on grids under conditions as previously described [7] in the presence of insulin (5 μg/ml) and rPRL (1 μg/ml). The concentrations of corticosterone used were 3–5 nM and 300 nM, of which in both cases ~2 nM was [^{3}H]corticosterone. Substrate wells (blank) without explants were included in each experiment. Medium was retained and replaced at the end of a 24 h period; medium and tissue were retained at 48 h, and stored frozen at −20°C. Extraction in ethyl acetate (2×5 ml) was as for cells, as was TLC and estimation of radioactivity. In the medium retained at 24 and 48 h there was no significant radioactivity other than corticosterone or 11-dehydrocorticosterone; in contrast in the tissue, a third peak of radioactivity was found. Accordingly, all tissue extracts were counted from the origin to the solvent front; recovery from the plates was routinely >95%. Conversion of corticosterone to 11-dehydrocorticosterone was calculated as the percentage of the radioactivity recovered in each area, with correction for blanks.

RESULTS

Epithelial and adipose cells isolated from the mammary glands of both pregnant and lactating rats contained 11-HSD activity. Conversion of corticosterone to 11-dehydrocorticosterone was linear with respect to both time and cell number, provided conversion was less than 40%, shown for cells from pregnant rats with respect to time (Fig. 1A, epithelial cells; Fig. 1B, adipose cells) and with respect to cell number (Fig. 1C, epithelial cells; Fig. 1D, adipose cells). Thus in later experiments, appropriate conditions of cell number and time of incubation were used for each cell type to maintain conversion below 40%. The activity of adipose cells was substantially greater than that of epithelial cells; consequently, the cell number incubated and time of incubation was routinely less for adipose cells. Similar experiments were performed with epithelial and adipose cells isolated from lactating rat to establish appropriate cell numbers and incubation times (data not shown).

When 11-HSD activity of epithelial cells and adipose cells isolated from the mammary glands of mid-pregnant and mid-lactating rats was compared, there were clear differences both between

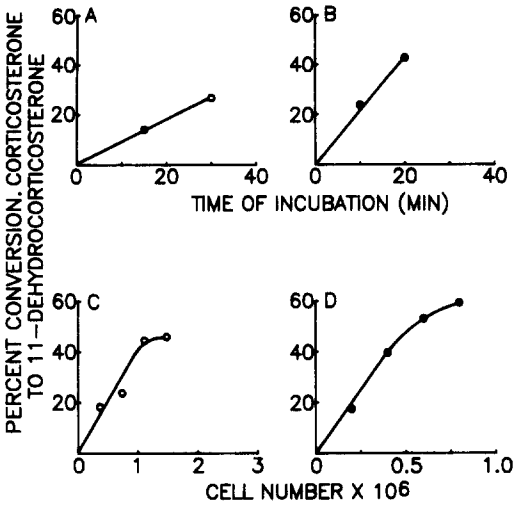

Fig. 1. 11-HSD activity as a function of time and cell number in epithelial and adipose cells. Panel A: Epithelial cells, day 15 of pregnancy (1.04×10^6 cells). B: Adipose cells, day 14 of pregnancy (0.115×10^6 cells). C: Epithelial cells, day 14 of pregnancy (incubation time 40 min). D: Adipose cells, day 16 of pregnancy (incubation time 15 min). Percentage conversion of corticosterone to 11-dehydrocorticosterone was calculated as described in Materials and Methods. Each panel shows the results of the means of duplicates from a single representative experiment from a total of at least two experiments.

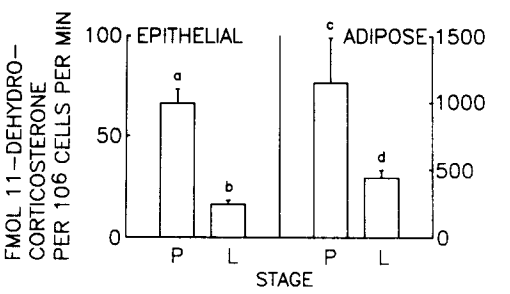

Fig. 2. The conversion of corticosterone to 11-dehydrocorticosterone by epithelial and adipose cells isolated form mammary glands of pregnant (P) (day 13–16) and lactating (L) (day 6–8) rats. 11-HSD activity was measured as described in Materials and Methods, under conditions of linear conversion in which corticosterone to 11-dehydrocorticosterone conversion was less than 40%. Number of cells per incubation for epithelial cells ranged from 1×10^6–2×10^6 cells, with incubation times of 15–30 min for cells from pregnant rats, and 30–60 min for cells from lactating rats. Number of cells per incubation for adipose cells ranged from 0.1×10^6–0.4×10^6 cells, with incubation times of 10–20 min for cells from pregnant rats, and 15–30 min for cells from lactating rats. In each experiment duplicate incubations were carried out at least two time points and duplicate values differed by less than 10%. Values are expressed as fmol 11-dehydrocorticosterone produced per 10^6 cells per min and are the means ± SEM; the number of independent experiments was for epithelial cells from pregnant rats, $n = 6$; from lactating rats, $n = 4$; for adipose cells, $n = 3$ for both pregnant and lactating rats. Means with different letters are different from each other ($P < 0.01$) on statistical analysis of log transformed data by ANOVA followed by Duncan's multiple-range test.

cell types and between pregnancy and lactation (Fig. 2). In both epithelial and adipose cells, enzyme activity was 3–4-fold greater in pregnant rats than lactating rats. In both pregnancy and lactation, activity in adipose cells was markedly (~20-fold) higher than in epithelial cells. In a single experiment on epithelial cells from day 21 of pregnancy, 11-HSD activity was 82 fmol 11-dehydrocorticosterone produced per 10^6 cells per min, similar to that earlier in pregnancy; in two experiments on adipose cells from the same stage, the activity was 146 and 374 fmol 11-dehydrocorticosterone produced per 10^6 cells per min, much lower than earlier in pregnancy.

When the conversion of corticosterone to 11-dehydrocorticosterone was examined in explants taken from mid-pregnant rats, approximately 50% conversion occurred over the first 24 h incubation period, with slightly lower values over the second 24 h period, at both 3 and 300 nM corticosterone (Table 1). Explants from lactating rats showed lower rates of conversion, about 15–25% during the first 24 h time period, and with a fall to < 10% over the second 24-h period at the higher concentration. Thus under conditions used to examine the effects of corticosterone on milk protein production in explants [27], considerable metabolism of the active steroid occurs over the period of incubation. In the medium, no significant radioactivity was found at positions other than those of authentic corticosterone or 11-dehydrocorticosterone.

Table 1. Conversion of corticosterone to 11-dehydrocorticosterone in medium from explants

Stage	Corticosterone added	% Conversion to 11-dehydrocorticosterone in the medium 1st 24 h	2nd 24 h
Pregnant ($n = 3$)	3–5 nM	51 ± 1.4	46 ± 2.6
Lactating (n = 2)		27 ± 0.5	23 ± 1.1
Pregnant ($n = 3$)	300 nM	52 ± 2.5	33 ± 1.5
Lactating ($n = 2$)		16 ± 0.2	8.4 ± 1.1

Explants were incubated for 48 h, with a medium change at 24 h; extraction of medium and analysis by TLC was as described in Materials and Methods. The number of experiments was 3 (pregnant) and 2 (lactating); within each experiment, triplicate incubations were made for dose in each experiment.

Table 2. Conversion of corticosterone in explants

Stage	Corticosterone added	Tissue % recovered as: B	A	Y
Pregnant ($n = 3$)	3–5 nM	32 ± 1.9	33 ± 0.3	19 ± 3.2
Lactating ($n = 2$)		42 ± 0.2	19 ± 0.5	17 ± 0.8
Pregnant ($n = 3$)	300 nM	38 ± 2.0	22 ± 2.7	24 ± 3.5
Lactating ($n = 2$)		45 ± 0.4	7.9 ± 0.2	21 ± 2.6

Explants were retained from the experiments described in Table 1. Extraction of tissue and analysis by TLC was as described in Materials and Methods. Values for corticosterone (B), 11-dehydrocorticosterone (A), and unknown metabolite Y are the means ± SEM of the percentage of steroid recovered from the TLC plate. The number of experiments was 3 for pregnant and 2 for lactating rats; triplicate incubations were carried out for each dose in each experiment.

At the end of the second 24 h incubation period we measured the radioactivity retained in the tissue (Table 2). In explants from pregnant rats, at 3 nM corticosterone approximately 4–6% of the label was retained; at 300 nM, 6–7% was retained by the tissue. In addition to the considerable metabolism of corticosterone to 11-dehydrocorticosterone, another less polar metabolite was formed, which is not identified to date. The levels of this metabolite (Y in Table 2) varied, but in all incubations it was greater than 15% of the recovered radioactivity, regardless of dose, in both pregnancy and lactation.

DISCUSSION

Many tissues such as liver, kidney, adipose, hippocampus, uterus, fetus, and lung (reviewed in Ref. 19), parotid [20] and mammary gland [29] show 11-HSD activity. In the present study, we have shown that activity is about 20-fold higher in adipose than in epithelial cells in the mammary gland; for both cell types, activity decreased to about one-fourth between mid-pregnancy and established lactation. It is highly unlikely that epithelial levels of enzyme activity represent adipose cell contamination, since the percentage of glandular tissue at day 16 of pregnancy is about 40% of the total tissue, while at day 6–8 of lactation the percentage of glandular tissue rises to 80% [31]. Given the clear preponderance of epithelial tissue during lactation, it is unlikely that the isolation procedure in both instances would afford a constant 5% contamination.

We have also shown that explants are capable of extensive metabolism of corticosterone to 11-dehydrocorticosterone. Our results for corticosterone are at variance with the results of Bolander *et al.*[32], who reported that in their incubation system 99% of added cortisol retained by explants was recovered as such. Given the evidence from the present studies of very similar extent of conversion of 3 and 300 nM corticosterone, it is possible (but unlikely) that the meagre metabolism reported by Bolander *et al.*[32] reflects the very high doses of cortisol added (14 μM). A more likely explanation is a difference in conversion rate by 11-HSD for these two 11-hydroxylated steroids, only one of which (corticosterone) is seen under physiological circumstances in rats and mice.

In the medium from explants there was no evidence for any metabolite other than 11-dehydrocorticosterone. In the tissue itself, however, we found a third peak, production of which (unlike 11-dehydrocorticosterone) was equivalent in lactating and in pregnant rats. Hampel *et al.* [33] have previously reported the existence of a 21-acylated derivative of corticosterone in freshly prepared alveoli incubated with corticosterone. Their experimental conditions differed from ours most markedly in that they extracted only cells rather than cells and media together. In our explant experiments, the overall proportion of metabolite Y was very small, in that it was not found in medium and only approximately 5% of the added corticosterone was found in explants at the end of 48 h incubation. Though the production is relatively minor, this does nor *per se* exclude a physiological role for metabolite Y.

The location of 11-HSD activity in both adipose and epithelial cells suggests a general mechanism in the tissue to lower corticosterone levels, not one confined to a particular cell type. The physiological role of 11-HSD in the mammary gland during pregnancy may be to decrease local concentrations of corticosterone by the formation of the inactive metabolite, 11-dehydrocorticosterone and thus prevent premature milk production. The question of whether the 11-HSD activity in adipose and epithelial cells is due to the same enzyme species remains unknown; recent findings suggest there is a family of 11-HSD enzymes [34, 35] one member of which has been purified from rat liver [36], and the cDNA sequence established [34]. Northern blot analysis has detected this species in liver, lung, kidney and testis [34] but not parotid [35] and the enzyme was demonstrated by immunocytochemistry to be present in proximal convoluted tubes of the kidney [22] but not cortical collecting tubules [22, 35]. High levels of this species was also demonstrated by immunofluorescence in the testis, where a developmental role involving modulation of glucocorticoid receptor occupancy has been suggested [37]. Whether the role of the enzyme present in the mammary gland is to modulate glucocorticoid occupancy of either or both Type I and Type II receptors, both of which are present in the mammary gland [25, 26] remains to be established. The lower 11-HSD activity during lactation is consistent with *in vitro* studies which suggest that glucocorticoids have negligible direct effects on α-lactalbumin and whey acidic protein production during established lactation [8, 13] despite the continued presence of glucocorticoid receptors.

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