Corticosterone Metabolism and the Incorporation of Leucine, Uridine, and Thymidine into Fetal Mouse Brain

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Summary

The change in the pattern of biotransformation of [^{14}C]corticosterone in fetal mouse brain between gestational days 14 and 17 increased the proportion of unchanged hormone from 9-75%. A sharp decrease in the *in vitro* incorporation of [^{14}C]leucine, [^3H]uridine, and [^3H]thymidine into incubated brain coincided with this change and continued until day 19, when the incorporation of the 3 substrates had fallen to 9, 54, and 16%, respectively, of that on day 14. Injection of dexamethasone reduced values on day 14 to those normally found on days 15-18. Enzymes which metabolize

corticosteroids regulate their activity in specific tissues; these data suggest a hormonal influence on developing brain.

Speculation

Corticosteroids are known to induce metabolic processes in a number of fetal tissues; the brain can be included in these. These changes are mediated by enzymes in that tissue and do not necessarily involve the fetal pituitary-adrenal axis, nor the pattern of glucocorticoid receptors.

Introduction

Examination of the disposition of corticosteroids in the mouse fetus had led us to the concept that, since these hormones cross the placenta readily in either direction, the major factor in their regulation is the activity of enzymes in specific tissues which metabolize them (10). This communication describes the relationship between the metabolism of corticosterone in fetal mouse brain and its effects on the brain as determined by the incorporation of ^{14}C -leucine, ^3H -thymidine and ^3H -uridine.

Materials and Methods

CHEMICALS

Radioactive compounds purchased from New England Nuclear Corp. (Boston) included dexamethasone- $1,2,4\text{-}^3\text{H}$, 21.2 Ci/mmol, and corticosterone- $4\text{-}^{14}\text{C}$, 57.3 mCi/mmol. L-leucine- ^{14}C (U), 331 mCi/mmol, uridine- $5\text{-}^3\text{H}$, 30 Ci/mmol; and thymidine methyl- ^3H , 26 Ci/mmol were obtained from Amersham/Searle, Arlington Heights, IL.

The non-radioactive dexamethasone used was a gift from Merck and Co. (Rahway, NJ). The 11-epicorticosterone was kindly supplied by Dr. J.C. Babcock, The Upjohn Company, (Kalamazoo, MI).

All solvents used were distilled freshly before use.

EXPERIMENTAL ANIMALS

Non-inbred Swiss mice were raised at the Zoology Department Vivarium at this University. Matings were between 1800 and 0700 hr. If a plug was found in the morning, that day was designated gestational day 1. Mice were killed with excess CO_2 and the fetuses were removed and decapitated quickly and placed on a petri dish on ice. Tissues were minced with scissors and placed in a 25 ml erlenmeyer flask to which buffer and substrates were added. Incubations were all carried out in a water bath at 37°C shaking at 120 cycles/min. In earlier work (10), ^3H -corticosterone and ^{14}C -11-dehydrocorticosterone were incubated simultaneously to ascertain the net dehydrogenation or reduction in tissues. The ratio of reduction/dehydrogenation in head on gestational day 14, for example, was 0.12. In the present work, ^{14}C -corticosterone only was used, since it reflects, fairly well, the net conversion of corticosterone and, in addition, permits autoradiographic demonstration of other metabolites.

INCUBATIONS

Incubations for following the *in vitro* metabolism of ^{14}C -corticosterone included 5 nCi corticosterone- $4\text{-}^{14}\text{C}$ with 2 or 3 fetal heads finely minced, in 1 ml Krebs-Eggleson phosphate buffer pH 7.4 for 15 min (6). The mixture was then extracted with 6 vol n-hexane, which was discarded and then with 6 vol cold CH_2Cl_2 , which was evaporated under N_2 and spotted, either onto a silica gel TLC sheet for autoradiography, or onto Whatman no. 1 paper if quantitative recovery was required. The TLC sheets were developed in two solvent systems; (1) hexane:ethyl acetate (4:1, v:v), and (2) toluene:chloroform:methanol:water (120:60:20:1, v:v:v:v). The paper chromatography was performed using paper impregnated with formamide:methanol (1:1, v:v) and developed in benzene.

Samples of 2 or 3 fetal heads were minced finely, and incubated in 1 ml Krebs-Eggleson bicarbonate buffer pH 7.4 (6), containing 0.14 μCi leucine- ^{14}C (U), 0.46 μCi ^3H -uridine, or 0.46 μCi ^3H -thymidine. Incubations were carried out for 1 hr at 37°C in a water bath shaking at 120 cycles/min. In some experiments the brain was removed from the outer tissues and incubated separately. The incorporation of isotopes followed a similar pattern and the metabolism of corticosterone was demonstrable in isolated brain. Because of the difficulty in separating the tissues cleanly, however, experiments were routinely carried out using whole heads. The samples were then homogenized in a Sorvall Omnimixer (Ivan Sorvall Inc., Newtown, CT). An aliquot of 0.1 ml was removed for protein determinations by the method of Lowry, Rosebrough, Farr and Randall (9). Then 0.1 vol 30% trichloroacetic acid (TCA) was added. The precipitate was washed 4 times with 3 ml 3% TCA, then solubilized in BBS-3 solubilizer (Beckman Instruments, Fullerton, CA). The sample was mixed with scintillation fluid (Econofluor, New England Nuclear, Boston, MA) and counted in a Unilux IIA liquid scintillation spectrometer (Nuclear/Chicago, Chicago, IL). Quenching was determined by the method of external standards ratios.

The assay for corticosteroid receptors in fetal mouse brain was performed in the same manner as was done for placenta (13). The 105,000 xg supernatant of brain was incubated for 30 min on ice with 8 nM (0.33 μCi) ^3H -dexamethasone and the unbound steroid was removed by addition of dextran-coated charcoal. In these experiments 2 μM 11-epicorticosterone was used to eliminate much of the non-specific binding, while not displacing the specifically bound hormone (13).

Results

The change in the pattern of biotransformation of corticosterone in fetal mouse brain incubated *in vitro* between gestational days 15 and 17 is shown in Fig. 1. Even between morning, afternoon and evening of day 15 there appeared to be differences in the relative amount of unchanged hormone which increased from 9 to 75% of the total recovered counts. Other metabolites decreased sharply on day 15.

Characterization of corticosterone and 11-dehydrocorticosterone has been described (10). The fastest moving zone was identified by co-crystallization with authentic added carrier as 11-dehydrocorticosterone acetate, and the zone directly behind it as corticosterone acetate. The slower moving derivatives have been identified tentatively as the 20 α -dihydro derivatives of 11-dehydrocorticosterone and corticosterone (the slowest zone).

Reference 20 α -dihydro compounds were prepared by incubating non-radioactive corticosterone with a dialyzed 105,000 xg supernatant of mouse liver fortified with NADPH, and separating the products chromatographically, as described by Krehbiel, Burton and Darrach (7). The radioactivity of the metabolites and their acetate derivatives coincided during chromatography with the UV absorbance of the added reference compounds and could be distinguished from their 20 β isomers.

The increase in proportion of unchanged hormone suggested that it might have a role in brain function at this time. This prompted a search for specific steroid receptors which might indicate that brain is a "target" tissue. The findings are summarized in Table 1. As in previous work with placenta (13), evidence for such a factor(s) was obtained; values of $K_d=8.33$ nM and 0.14 pmoles steroid bound per mg protein were determined on gestational day 14. The concentration of receptors did not increase thereafter, and, in fact, with continued growth of the brain the relative concentration fell. While this could reflect increased lability of the receptors, the presence of dithiothreitol did not increase binding in tissues on gestational day 19.

As seen in Table 2, the incorporation of ^{14}C -leucine, ^3H -uridine and ^3H -thymidine began to fall at the time the change in corticosterone conversion occurred. This decrease continued until day 19, when the values for these 3 substrates had fallen to 9, 54 and 16%, respectively, of those on day 14. In order to determine whether or not this was attributable directly to the steroid, mothers were injected on day 13.5 with 200 μg dexamethasone. As indicated in Table 2, the synthetic steroid decreased values on gestational day 14 for all substrates to those normally observed between days 15 and 18.

Discussion

Of the metabolites of corticosterone found in fetal mouse brain (Fig. 1), the C-20 dihydro derivatives are known to be biologically inactive, whereas acetate conjugates and C-11 ketone derivatives can be converted to corticosterone in tissues which contain the appropriate enzymes (4). The formation of acetate esters is not a common finding, but has been reported in the brain of two other species (5). The most striking change observed was the sharp increase in the relative amount of the biologically active component, corticosterone. Since the concentration of steroid receptors did not increase (Table 1), it seems most likely that it is the increased hormone concentration in the tissue which mediates changes which are induced by corticosteroid at this time.

The sharp decrease in the incorporation of labelled substrates into the acid-insoluble components of fetal brain (Table 2) coincided with the decrease in the rate of corticosterone conversion, and continued for several days. That this event is in fact attributable to a change in steroid level is indicated by the fact that injection of dexamethasone on gestational day 13.5 resulted in values on day 14 which were normally found between days 15 and 18 (Table 2). The synthetic steroid was used here because it largely escapes the normal regulatory mechanisms which protect the fetus from maternal corticosteroids, with the result that a more effective dose reaches the fetus (14).

A relationship similar to that described here has been reported in fetal rabbit lung, where induction of phospholipid synthesis by cortisol was related to reductase activity which converts cortisone to cortisol (11). Inhibition of this reaction by 11-ketoprogesterone was shown to inhibit lung maturation in fetal rats (12). In a similar manner, inhibition of the dehydrogenase reaction by 11-epicorticosterone enhanced the action of corticosterone as measured by glycogen deposition in neonatal mice (11). These experiments provide direct evidence that enzymes in a tissue which metabolize corticosteroids influence their biological activity in the same and probably also in other tissues. It should be noted that these events can occur in specific fetal tissues independently, without necessarily involving the fetal pituitary and/or adrenal.

Morphological studies have not revealed any significant differences evident by light microscopy, at least within the 16 hours following dexamethasone treatment; further examination is in progress (15). Corticosteroid-induced maturation of rat and rabbit lung was accompanied by decreased growth and cell division in lung tissue (3,12). Such effects are suggested by the parameters which were measured here as a reflection of growth.

The introduction of corticosteroid therapy administered prenatally to accelerate maturation of the lung in premature infants does not appear to have caused other deleterious effects (8). This effect on the lung is a rather late event; experimental results such as those described herein implicate corticosteroids in several critical roles but at a much earlier stage in fetal development. However, the presence of the 11 β -reductase in human fetal liver as early as 20 weeks (2), raises the likelihood that similar events occur in human fetal development, especially during the last trimester.

References

1. Burton, A.F., Greenall, R.M. and Turnell, R.W.: Corticosteroid metabolism and liver glycogen in fetal and newborn mice. *Can. J. Biochem.* 48: 178 (1970).
2. Burton, A.F., McClellan, D.J., Drummond, M.R., Mah, T.G., Thomson, M.J., Wong, W. and Turnell, R.W.: Corticosteroids in premature cord blood determined fluorometrically by enzymatic reduction of cortisone. *J. Clin. Endocrinol. Metab.* 39: 950 (1974).
3. Carson, S.H., Taesch, H.W. and Avery, M.E.: Inhibition of lung cell division after hydrocortisone injection into fetal rabbits. *J. Appl. Physiol.* 34: 660 (1973).
4. Fieser, L.F. and Fieser, M.: Steroids, Reinhold Publishing Corp., New York (1959) p. 600.

5. Grosser, B.I. and Axelrod, L.R.: Acetylation of cortisol by neonatal rat brain in vitro. *Steroids* 9: 229 (1967).
6. Krebs, H.A. and Eggleston, L.V.: The oxidation of pyruvate in pigeon breast muscle. *Biochem. J.* 34: 442 (1940).
7. Krehbiel, R.E., Burton, A.F. and Darraeh, M.: Studies on corticosterone C-20 reduction in the mouse. *Can. J. Biochem.* 40: 1797 (1962).
8. Liggins, G.C. and Howie, R.N.: A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. *Pediatrics* 50: 515 (1972).
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.R.: Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* 193: 265 (1951).
10. Michaud, N.J. and Burton, A.F.: Maternal-fetal relationships in corticosteroid metabolism. *Biol. Neonate* 32: 132 (1977).
11. Smith, B.T., Torday, J.S. and Giroud, C.J.P.: Evidence for different gestation-dependent effects of cortisol on cultured fetal lung cells. *J. Clin. Invest.* 53: 1518 (1974).
12. Smith, B.T.: The role of pulmonary corticosteroid 11-reductase activity in lung maturation in the fetal rat. *Pediat. Res.* 12: 12 (1978).
13. Wong, M.D. and Burton, A.F.: Studies on corticosterone-receptor complexes from mouse placenta. *Can. J. Biochem.* 52: 190 (1974).
14. Wong, M.D., Thomson, M.J. and Burton, A.F.: Metabolism of natural and synthetic corticosteroids in relation to their effects on mouse fetuses. *Biol. Neonate* 28: 12 (1976).
15. The authors are grateful to Dr. Mary E. Todd, Dept. of Anatomy, for microscopic examination of tissue samples.
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Table 1. Properties of corticosteroid receptor(s) in fetal mouse brain.

K_d	8.33 nM
Total steroid bound	0.14 pmoles/mg protein (day 14)
Relative binding (dpm/mg protein)	
on gestational day 14:	6433 ± 1109 (6)
on gestational day 17:	1551 ± 345 (5)

The 105,000 xg supernatant of fetal mouse brain was incubated on ice with 8 nM, 0.33 μ Ci 3 H-dexamethasone for 30 min. The unbound steroid was removed by addition of dextran-treated charcoal which was centrifuged down, and the supernatant was assayed for radioactivity. Values shown are Mean $\pm$ SEM of number of samples shown in parentheses.

Table 2. Incorporation of substrates into fetal mouse brain.

Gestational Day	Incorporation, dpm/mg protein		
	Leucine	Uridine	Thymidine
14	9900 ± 451	1668 ± 81	19283 ± 557
15	5856 ± 473		
16	4295 ± 170	921 ± 146	11376 ± 1021
18	2085 ± 352		
19	862 ± 163	889 ± 35	3131 ± 526
14 + DEX*	5083 ± 559	1213 ± 107	16009 ± 511

* Dexamethasone, 200 μ g, was injected subcutaneously 16 hr earlier

Two or three fetal heads were pooled and incubated in 1 ml buffer containing 2 mg glucose at 37° for 60 min, with either 0.14 μ Ci leucine- 14 C, or 0.46 μ Ci uridine- 3 H or thymidine- 3 H. Each sample was then homogenized and an aliquot removed for protein determination, and 0.1 vol 30% TCA was added. The precipitates were washed 4x with 3% TCA, then solubilized and counted. Each value is a mean $\pm$ SEM of 4 or more values from several litters. Day 14 vs Day 16: P < 0.01 in all cases; Day 14 vs Day 14 + DEX: P < 0.01 in all cases.

Fig. 1. Autoradiograms of chromatographed extracts of tissues incubated with 14 C-corticosterone. CH_2Cl_2 extracts were spotted onto silica gel TLC sheets and developed in first, hexane:ethyl acetate (4:1) and second, toluene:chloroform:methanol:water (120:60:20:1). 1 & 2: Gestational day 14; 3 & 4: day 15; 5 & 6: day 17. The zones and the average counts recovered in each on day 14 are:

AAC: 11-dehydrocorticosterone acetate	364 dpm
BAC: corticosterone acetate	443
A: 11-dehydrocorticosterone	3631
B: corticosterone	535
20aA: 20 α -dihydro-11-dehydrocorticosterone	666
20aB: 20 α -dihydrocorticosterone	344

Note the increase in size of the B zone and decrease in size of the A zone in 3 to 6, as compared with 1 and 2. The B zone increased from 9% on day 14 to 75% of the total counts on day 17.