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Maternal adrenocortical hormones maintain the early development of pancreatic B cells in the fetal rat

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ABSTRACT

To investigate the effect of maternal adrenocortical hormones on the development of fetal pancreatic islet cells, pregnant rats were adrenalectomised on d 6 of gestation. On d 12–16 the growth patterns of fetal insulin-producing B cells, glucagon-producing A cells, and somatostatin-producing D cells were observed histometrically. Maternal adrenalectomy resulted in growth retardation of fetal B cells on d 12–15. Maternal corticosterone therapy prevented this retardation. Maternal adrenalectomy, however, did not affect the developmental patterns of A and D cells. By Western blotting and immunohistochemistry, glucocorticoid receptors were demonstrated to be present in the islet cells from d 12 to d 15. These results suggest that maternal adrenocortical hormones, glucocorticoids in particular, maintain the early development of fetal pancreatic B cells through their specific intracellular glucocorticoid receptor.

Key words: Adrenalectomy; fetal pancreatic islets; glucocorticoid receptors.

INTRODUCTION

It is well known that adrenocortical hormones may participate in the development of many organs during the prenatal period. Corticosterone, the principal adrenocortical hormone in rats, promotes the differentiation of the exocrine fetal rat pancreas in organ culture (Rutter et al. 1974; McEvoy & Hegre, 1976). In cultured 19 d rat fetal pancreas, the concentration of corticosterone (0.1 μg/ml) at which the acinar cells are not fully preserved can stimulate the growth of the islet cells (McEvoy et al. 1976). The same concentration of corticosterone (0.1 μg/ml) inhibits the release of glucagon into the medium in cultured 17 d rat fetal pancreas (McEvoy, 1980), but in contrast, similar concentration (0.1 μg/ml) stimulates the release of somatostatin in cultured 21 d rat fetal pancreas (McEvoy et al. 1981). It is unknown, however, whether or not these in vitro results hold in vivo as well.

In general, glucocorticoids act through their specific intracellular receptors (Yamamoto, 1985). The tissue-specific biopotency of a steroid hormone depends both upon its concentration and the amount of its specific receptor in its target tissue (Danielsen & Stallcup, 1984). It is as yet unknown whether glucocorticoid receptors exist in the rat fetal pancreas, as they do in the adult (Matthes et al. 1994) and the neonatal pancreas (Lu et al. 1987).

It has been well documented that adrenocortical hormones can cross the placenta from the mother to the fetus and vice versa (Kamoun, 1970; Zarrow et al. 1970; Dupouy et al. 1975). The rat fetal adrenal cortex may not secrete any trace of its own hormones until d 16, since plasma corticosterone is first detected on d 17 (Cohen, 1973). In the present experiment, therefore, pregnant rats were adrenalectomised on d 6 of gestation so that their fetuses developed in the absence of transplacental maternal adrenocortical hormones. With this manipulation, by the time that pancreatic islets first appear, maternal hormones would no longer be present in the fetal circulation in utero. The objectives of this work were, first, to determine whether maternal adrenocortical hormones

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affect the early development of fetal pancreatic A, B, and D cells in vivo, and second, to determine the time when glucocorticoid receptors first appear and the sites where they are located in the rat fetal pancreas.

MATERIALS AND METHODS

Animals and experimental groups

Wistar rats (CLEA, Tokyo, Japan) were given a commercial diet (Labo MR Breeder, Nihon Nosan Kogyo K.K., Japan) and water ad libitum. Females were placed with males overnight and the day on which sperm were detected in the vaginal smear was designated as d 0 of gestation.

Pregnant rats were divided into 3 groups, non-operated (Control group), adrenalectomised (Adx group), and adrenalectomised and corticosterone-treated group (Adx + CS group). Maternal adrenalectomy was performed on d 6 of gestation. Corticosterone (Sigma Chemicals, St Louis, MO) was injected subcutaneously at a dose of 40 mg/kg body weight once a day at 10:00 from the day of operation to the day of autopsy. Rats in both the Adx and Adx + CS groups were given 0.9% saline solution as drinking water. Maternal autopsy was conducted at 13:00 on each day from d 12 to d 16; under ether anaesthesia, each pregnant rat was killed by cardiac puncture to collect a blood sample. At the same time, her fetuses were quickly removed from the uterus.

Maternal corticosterone assay

The blood samples were centrifuged at 4 °C, and the sera stored at −80 °C until assayed. The concentration of maternal serum corticosterone was measured by a radioimmunoassay using the rat corticosterone [^{125}I] assay system (Amersham, Little Chalfont, Bucks, UK) which can measure in the range 0.78–200 ng/ml.

Immunohistochemistry for insulin, glucagon and somatostatin

On d 12 and 13 of gestation, fetuses were fixed in Bouin's fluid in toto. On d 14, 15 and 16, the pancreas of each fetus was removed together with the stomach and duodenum and was fixed in the same fluid. The fixed materials were embedded in Paraplast (Sherwood Medical, St Louis, MO), and sectioned serially at 5 μm. The sections were immunostained with anti-insulin serum (Incstar, Stillwater, MN), antiglucagon serum (Incstar, Stillwater, MN) or antisomatostatin serum (Zymed Laboratories, San Francisco, CA). The outline of every insulin-positive B cell or glucagon-positive A cell in each section of the series was measured with an image analysis system (Image Command 5098, Olympus, Tokyo, Japan). The total volume of the positive cells in each pancreas was determined by the following formula:

$$\text{total volume} = \begin{pmatrix}\text{sum of}\\ \text{outline areas}\end{pmatrix} \times \begin{pmatrix}\text{thickness}\\ \text{of section}\end{pmatrix}$$

The total number of somatostatin-positive D cells in each pancreas was counted. The counts were made on all the serial sections for each pancreas. Care was taken to avoid repeated counts of profiles of the same cell in adjoining sections.

Statistical analysis

The statistical analyses were performed with Duncan's new multiple range test (Duncan, 1975). A P value < 0.05 was considered significant.

Western blotting for the glucocorticoid receptor

On d 15 of gestation, maternal and fetal pancreases were quickly removed, frozen and stored at −80 °C until analysis. Frozen tissues were homogenised and then centrifuged at 2 °C for 45 min at 105000 *g* in an ultracentrifuge (Optima TLX, Beckman, Fullerton, CA). Supernatants were collected and used for the protein determination and Western blotting. Proteins were measured by the method of Lowry et al. (1951).

Western blotting was carried out according to the protocol of Laemmli (1970). Samples (100 μg protein/lane) were separated electrophoretically on 7.5% polyacrylamide gels (Bio-Rad, Richmond, CA). Proteins were then transferred electrophoretically according to the method of Towbin et al. (1979) onto nitrocellulose membranes (Bio-Rad, Richmond, CA). The membranes were immunoblotted with the rat glucocorticoid receptor monoclonal antibody, BuGR2 (Affinity Bioreagents, Golden, CO). It has been reported that the specificity of this monoclonal antibody can be demonstrated well by Western blotting (Gametchu & Harrison, 1984; Jaskoll et al. 1994; O'Donnell et al. 1995).

Immunohistochemistry for the glucocorticoid receptor

Fetuses on d 12–15 were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h

at 4 °C, embedded in Paraplast, and sectioned serially at 5 μm. The sections were immunostained with the glucocorticoid receptor polyclonal antibody no. 57 (Affinity Bioreagents, Golden, CO). It has been reported that the specific reaction to this polyclonal antibody appears well in the cell nuclei (LaFond et al. 1988; ten Cate et al. 1993).

RESULTS

Maternal serum corticosterone concentrations

From d 12 to d 15 of gestation, no serum corticosterone concentrations were detectable in the adrenalectomised mothers (Table 1).

Effect of maternal adrenalectomy on the development of pancreatic B cells

Maternal adrenalectomy caused no decrease in the fetal body weights (Table 1).

In the Control group, insulin-positive B cells were observed in the pancreatic anlage on d 12 of gestation, and their total volume was gradually increased until d 15, followed by a marked increase on d 16 (Table 2). In contrast, the maternal adrenalectomy caused a significant decrease in the total volume of these cells on d 12–15. In the Adx + CS group, however, the decrease was prevented (Table 2). On d 16, the total volume of B cells was not significantly different between the 3 groups.

Effect of maternal adrenalectomy on the development of pancreatic A and D cells

In the Control group on d 12, the total volume of glucagon-positive A cells was approximately 6.5 times greater than that of the B cells (cf Tables 2, 3). The total volume of A cells slowly increased until d 14, followed by an abrupt increase on d 15, the volume

Table 2. *Total volumes of insulin-positive B cells* (*mean* ± *S.E.M.*)

Age (d)	Group	Number of samples (litters)	Volume of insulin-positive cells ($\times 1000\ \mu m^3$)
12	Control	11 (4)	7.8 ± 0.9
	Adx	13 (3)	3.3 ± 0.8*
	Adx + CS	13 (4)	7.0 ± 0.8
13	Control	13 (5)	12.2 ± 1.3
	Adx	14 (5)	6.6 ± 1.3*
	Adx + CS	10 (3)	10.1 ± 0.6
14	Control	13 (3)	26.9 ± 2.9
	Adx	11 (3)	17.3 ± 2.0*
	Adx + CS	10 (3)	25.4 ± 1.2
15	Control	13 (4)	46.8 ± 5.0
	Adx	9 (3)	29.9 ± 4.3**
	Adx + CS	13 (4)	41.6 ± 3.6
16	Control	10 (3)	352.6 ± 62.1
	Adx	9 (3)	308.5 ± 41.3 ns
	Adx + CS	8 (3)	325.4 ± 28.6

* Significantly different only from the Control group ($P < 0.05$).
** Significantly different from both the Control and Adx + CS groups ($P < 0.05$).
ns, not significantly different either from the Control or the Adx + CS groups.

Table 1. *Maternal serum corticosterone concentrations and fetal body weights* (*mean* ± *S.E.M.*)

Gestational (d)	Group	Number of samples	Concentration (ng/ml)	Number of fetal samples	Body weight (mg)
12	Control	5	336 ± 116	—	—
	Adx	5	Undetectable	—	—
	Adx + CS	5	1032 ± 189*	—	—
13	Control	5	470 ± 152	—	—
	Adx	5	Undetectable	—	—
	Adx + CS	5	842 ± 116*	—	—
14	Control	5	562 ± 65	33	153 ± 2
	Adx	5	Undetectable	30	149 ± 2
	Adx + CS	5	697 ± 58	27	151 ± 3
15	Control	5	639 ± 173	32	271 ± 3
	Adx	5	Undetectable	28	275 ± 5
	Adx + CS	5	1136 ± 147*	31	279 ± 5
16	Control	—	—	18	445 ± 7
	Adx	—	—	18	425 ± 9
	Adx + CS	—	—	17	428 ± 7

* Significantly different from the Control group ($P < 0.05$).

Table 3. *Total volumes of glucagon-positive A cells* (*mean* ± *S.E.M.*)

Age (d)	Group	Number of samples (litters)	Volume of glucagon-positive cells (× 10000 μm³)
12	Control	10 (4)	5.0 ± 0.4
	Adx	10 (3)	5.5 ± 0.3
	Adx + CS	9 (3)	5.7 ± 0.4
13	Control	11 (3)	8.4 ± 0.7
	Adx	10 (3)	8.7 ± 0.5
	Adx + CS	11 (2)	7.5 ± 0.6
14	Control	11 (4)	9.1 ± 1.0
	Adx	11 (3)	13.8 ± 0.8*
	Adx + CS	9 (3)	8.9 ± 1.0
15	Control	11 (4)	20.2 ± 2.4
	Adx	11 (4)	20.5 ± 1.8
	Adx + CS	11 (3)	21.4 ± 0.8

* Significantly different both from the Control and the Adx + CS groups ($P < 0.05$).

Table 4. *Total number of somatostatin-positive D cells* (*mean* ± *S.E.M.*)

Age (d)	Group	Number of samples (litters)	Total number of D cells
12	Control	7 (3)	2.4 ± 0.3
	Adx	9 (3)	2.4 ± 0.4
	Adx + CS	9 (3)	2.6 ± 0.4
13	Control	8 (3)	5.4 ± 0.6
	Adx	9 (3)	4.2 ± 0.6
	Adx + CS	9 (3)	4.7 ± 0.6
14	Control	9 (3)	8.3 ± 0.9
	Adx	8 (3)	8.9 ± 0.7
	Adx + CS	8 (3)	7.5 ± 0.5
15	Control	8 (3)	18.0 ± 1.7
	Adx	8 (3)	21.0 ± 2.1
	Adx + CS	7 (3)	15.7 ± 1.8
16	Control	8 (3)	26.4 ± 2.2
	Adx	8 (3)	29.5 ± 3.8
	Adx + CS	7 (3)	26.0 ± 2.9

being about 2-fold of that on d 14 (Table 3). Maternal adrenalectomy resulted in a significant increase in the volume only on d 14.

Somatostatin-positive D cells first appeared in the pancreatic anlage on d 12, and their number gradually increased until d 16 (Table 4). During the period observed, the number of D cells was approximately equal among the 3 groups.

Western blotting for the glucocorticoid receptor

On d 15 of gestation, Western blotting showed specific staining for immunoreactive glucocorticoid receptor protein at approximately 97 kDa in the fetal pancreatic extraction (Fig. 1, lane 1). The glucocorticoid receptor level of the fetal pancreas was less than that of the maternal pancreas (Fig. 1, lane 2).

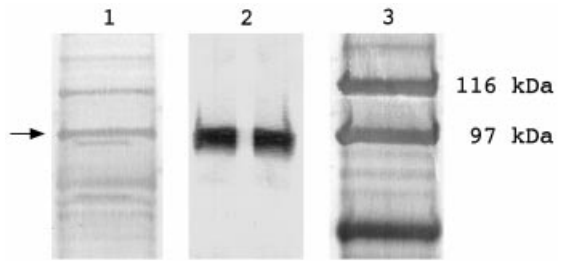

Fig. 1. Representative Western blot of glucocorticoid receptor protein in fetal and maternal pancreases. Lane 1, 15 d fetal rat pancreas. Lane 2, 15 d maternal rat pancreas. Lane 3, marker proteins. Both fetal and maternal tissue extractions show the glucocorticoid receptor protein with a molecular size of 97 kDa (arrow).

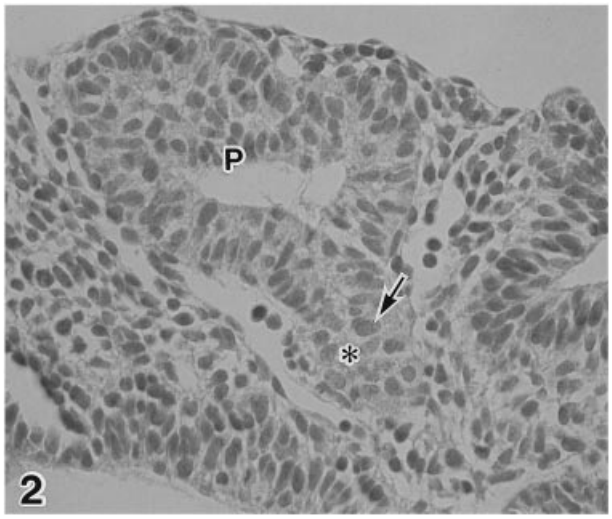

Fig. 2. Photomicrograph of a 12 d fetal pancreas. Glucocorticoid receptors are present in the cell nuclei (arrow) of the cell masses presumably forming a primitive islet (asterisk). Pancreatic anlage (P) shows tubular structure. × 470.

Ontogeny of the pancreatic glucocorticoid receptor

On d 12, the fetal pancreatic anlage showed glucocorticoid receptors. A positive immunoreaction for the receptor antibody was detected in the cell nuclei of cell masses presumably forming primitive islets (Fig. 2, arrow). On d 13, the positive reaction was observed mainly in the nuclei of the islet cells. On d 14, the degree of reactions was greater than that on the foregoing day. Most of the islet cells showed positive immunoreactivity in their nuclei. Some endocrine cells

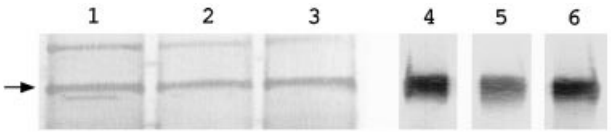

Fig. 3. Western blot analysis of the glucocorticoid receptor. Lanes 1–3, 15 d fetal pancreas. Lanes 4–6, 15 d maternal pancreas. Lanes 1 and 4, Control. Lanes 2 and 5, Adx. Lanes 3 and 6, Adx + CS.

derived from ductal cells had glucocorticoid receptors as well.

Effect of maternal adrenalectomy on the glucocorticoid receptor levels and localisation in the fetal pancreas

On d 15, there was no significant difference in the glucocorticoid receptor levels between the 3 fetal groups (Control, Adx and Adx + CS) (Fig. 3, lanes 1–3), although the maternal Adx caused a decreased content of the immunoreactive proteins in the maternal pancreas (Fig. 3, lane 5; cf. lane 4). A specific reaction to the receptor was found mainly in the nuclei of the islet cells, with both the localisation and the intensity of immunoreaction being almost the same in the Control and Adx groups.

DISCUSSION

In the present study, pregnant rats were adrenalectomised early on d 6 of gestation, and consequently no serum corticosterone was detected during the period from d 12 to d 15 when fetal islet cells appeared and developed in different ways. In the condition of maternal adrenal deficiency, the B cells were clearly retarded in development, and the retardation was prevented by maternal corticosterone therapy. This retardation was not caused by a delay in fetal body weight gain. The maternal adrenal deficiency caused a significant increase in the total volume of A cells only on d 14.

From d 12 to d 15, the total volume of B cells was less than that of A cells; the volume of B cells reached only 15.6% of that of A cells on d 12 and 23.4% on d 15. On d 16, however, the B cells were markedly increased in volume, a result which is in good agreement with previous reports (Clark & Rutter, 1972; Rall et al. 1973). It has more recently been shown that the situation is eventually reversed; B cells become predominantly greater in volume than A cells on d 18 (McEvoy & Madson, 1980; Kaung, 1994). The pancreatic insulin content has been reported to be constant until d 15 (Clark & Rutter, 1972), followed by a marked and abrupt increase in the volume of B cells (Rall et al. 1973; McEvoy & Madson, 1980). In the present study, maternal adrenalectomy caused a retarded growth of the B cells from d 12 to d 15, but the retarded growth appeared to catch up to the normal level on d 16. Evidently, a maternal adrenal deficiency influences the growth of B cells in early development. The catching-up of retarded growth to the normal level on d 16 could be explained in terms of the marked increase of the B cell volume at that time.

Insulin acts competitively against glucagon. Insulin from B cells first reaches A and D cells via the islet microcirculation (Bonner-Weir & Orci, 1982). When B cells secrete a high level of insulin, A cells react rapidly to it and stop releasing glucagon. A cells on d 14 responded to Adx by a significant increase in their volume, coincident with a significant decrease in the volume of B cells. Several investigators have measured the insulin content of the pancreas of 17 d or 18 d fetuses (Golob et al. 1970; Kervran et al. 1978; Perrier-Barta, 1984). Nevertheless, it was technically very difficult to measure circulating insulin levels in 14 d fetuses which were too small to collect blood samples. However, the decline of the total volume of B cells in the present Adx group indicates a reduced quantity of released insulin. Consequently, it may be possible that on d 14, A cells reacted to the reduced insulin concentration to release glucagon with a significant increase in A cell volume. On d 15, because the volume of A cells in the Control group abruptly increased about 2-fold of that on d 14, the A cell volume in the Adx group which may respond to the effect of maternal adrenal deficiency showed no significant increase. Regarding D cells, any indication that slight but not significant differences in the total number of D cells may reflect the influence of maternal adrenal deficiency or the result of corticosterone therapy can neither be affirmed nor supported by the present observations, the total number of the cells being too small to estimate the total cell volume.

Our immunohistochemical observations thus revealed that the glucocorticoid receptor exists in the rat fetal pancreas from d 12. On d 15, Western blotting showed that the content of the fetal pancreatic glucocorticoid receptor remained almost the same in the Control and Adx groups, although the content of maternal receptors was reduced in the Adx group. In general, hormone receptors are regulated both by their own ligands and by other regulatory molecules. Glucocorticoids are involved in the autoregulation of the glucocorticoid receptors (Okret et al. 1986). When 10-d-old rat pups are adrenalectomised, the binding

capacity of the pancreatic glucocorticoid receptor is increased 3 d later by an autoregulative reaction of glucocorticoids (Yoon & Lee, 1992). Dexamethasone administration induces changes in the level of tissue-specific glucocorticoid receptor mRNA in the liver of 7-d-old rats and in the brain of 14-d-old rats (Kalinyak et al. 1989). It has been suggested that glucocorticoid receptors bind to dexamethasone in the rat fetal brain (Meaney et al. 1985) and liver cytosol (Feldman, 1974). Thus the time at which the glucocorticoid receptor starts binding to its ligands in the fetal tissues seems to depend on the type of tissue and seems not to be the same as the time when glucocorticoid begins to regulate its receptor levels. The present findings showed that the glucocorticoid receptors in the fetal pancreas on d 12–15 did not seem to be autoregulated by their own ligands, since the receptor levels were changed neither by maternal adrenal deficiency nor corticosterone therapy.

In conclusion, maternal adrenocortical hormones, glucocorticoids in particular, maintain the early development of rat pancreatic B cells. The glucocorticoid receptor exists in the islet cells as early as on d 12 of gestation, the time of which coincides with that of the first appearance of the pancreatic anlage.

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