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Abstract

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HORMONAL REGULATION OF ORNITHINE DECARBOXYLASE AND POLYAMINES IN PRIMARY CULTURED RAT HEPATOCYTES — DIFFERENCES IN HORMONAL RESPONSE BETWEEN ADULT AND FETAL HEPATOCYTES —

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Abstract. Polyamines are polycationic substances which are widely distributed in living organisms and have a close relation to rapid growth phenomena. We examined ornithine decarboxylase (ODC), which is the rate limiting enzyme of polyamine biosynthesis, and polyamine induction in primary cultured rat hepatocytes by various hormones which increase during pregnancy, and revealed differences in hormonal responses between adult and fetal rat hepatocytes. Thirteen hormones, including estrone, estradiol, progesterone, teststerone, human chorionic gonadotropin (HCG), cortisol, dexamethasone, insulin, glucagon, epinephrine and epidermal growth factor (EGF), were tested. Among these hormones, only insulin, dexamethasone and EGF induced ODC activity and polyamine biosynthesis, especially that of putrescine, in both adult and fetal hepatocytes. The effects of EGF were the most significant. The combined effect of insulin and dexamethasone was additive, while that of insulin and EGF was synergistic. The rate of ODC induction was higher in adult hepatocytes than in fetal hepatocytes, however, the reaction was earlier in fetal hepatocytes. These observations suggest that ODC and polyamines in the fetal stage of development are regulated by a mechanism different from that in the adult liver.

Key words : ornithine decarboxylase, polyamine, primary culture, adult rat hepatocyte, fetal rat hepatocyte.

During intrauterine life, fetuses are in a state of almost continual anabolism and grow rapidly in a short time. It is well known that there are three enzyme clusters during differentiation, *i.e.*, "late fetal", "neonatal" and "late suckling" clusters (1). Ornithine decarboxylase (ODC), which is the rate limiting enzyme of polyamine biosynthesis, is highly active in the late fetal period (2, 3). The aliphatic polyamines, spermidine and spermine and their precursor putrescine, are formed in high concentrations in rapidly growing tissues (4, 5). Furthermore, ODC and polyamines are increased markedly by appropriate stimuli in various cells and tissues (4).

There have been few reports of polyamines in relation to fetal growth. During fetal growth, polyamines also show characteristic changes (2, 6-9). For example, polyamine, ODC and S-adenosylmethionine decarboxylase (SAMDC) concentrations

markedly increase after 16-19 days of gestation in fetal rat liver and then decreased rapidly. The concentrations of these substances are decreased in intrauterine growth retardation rat fetuses (2, 3). Additionally, polyamine concentrations in human umbilical blood are significantly higher than in maternal blood (6, 9). These data suggest that polyamines play some important role in fetal growth. However, it is difficult to explain the precise function of polyamines in fetal growth because many hormones, which increase during pregnancy, stimulate ODC and polyamine biosynthesis in some target organs (4).

To overcome this problem, we used primary cultures, so that we could investigate individually the direct action of each hormone. In this study, we examined the action of several hormones which increase during pregnancy on ODC and polyamine induction, and attempted to reveal the differences in polyamine metabolism between adults and fetuses.

MATERIALS AND METHODS

Animals and chemicals. Female Wistar rats, weighing about 200 g, and their fetuses at the twenty-first day of gestation were used in the present experiment. Animals were fed *ad libitum* on Oriental Laboratory Chow (MF) and water at constant temperature (20° to 24℃) and a 12 h light cycle.

Chemicals used in this experiment were: collagenase (Wako Pure Chemical Industries, Osaka); insulin, estrone, estradiol, teststerone, cortisol, glucagon and epinephrine (Sigma Chemical Co., St. Louis); dexamethasone (Nippon Merk-Banyu Co., Tokyo); human chorionic gonadotropin (HCG) (Mochida Pharmaceutical Co., Tokyo); epidermal growth factor (EGF) (Seragen Inc., Boston); Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co., Tokyo); Williams' medium E (WE) (Flow Laboratories, Scotland), and DL-1-^{14}C ornithine hydrochloride (56 μCi/ml) (Amersham, Buckinghamshire).

Isolation of adult rat liver cells. Liver cells were isolated using the perfusion method described by Seglen (10) and Tanaka *et al.* (11). Each female adult rat was anesthetized with nembutal (5 mg/100 g body weight), and abdomen was opened. The recirculating perfusion system illustrated in Fig. 1 was formed, and Ca^{2+}-free Hanks' balanced salt pre-perfusion buffer containing 10 mM N-2-hydroxyethylpiperadine-N'-2-ethanesulfonic acid (HEPES) and 0.5 mM

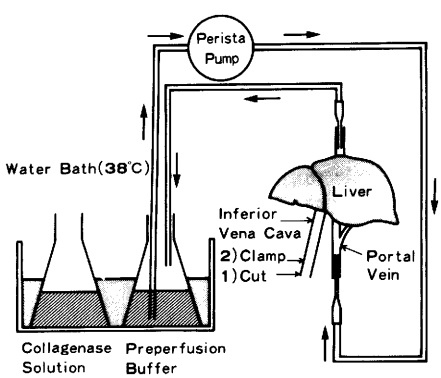

Fig. 1. Schematic illustration of perfusion system of adult rat liver, *in situ.*

ethleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA) (pH 7.2) was perfused for 5 min at a rate of 20 ml/min. Then 0.05 % collagenase solution (pH 7.5) was perfused for about 15 min. The detailed composition of these two buffers is shown in Table 1.

TABLE 1. DETAILED COMPOSITION OF THE PREPERFUSION BUFFER AND COLLAGENASE SOLUTION

	Preperfusion buffer	Collagenase solution
Nacl	8.0 g	8.0 g
KCl	0.4 g	0.4 g
$CaCl_2$	—	0.56 g
$NaH_2PO_4 \cdot 2H_2O$	0.078 g	0.078 g
$Na_2HPO_4 \cdot 12H_2O$	0.151 g	0.151 g
HEPES	2.38 g	2.38 g
Phenol Red Na	0.006 g	0.006 g
Collagenase	—	0.5 g
Trypsin inhibitor	—	0.05 g
EGTA	0.19 g	—
$NaHCO_3$	0.35 g	0.35 g
Glucose	0.9 g	—
Total	1.0 l	1.0 l
pH	7.2	7.5

The perfused liver was removed and cut into small pieces, dissociated into single cells by gentle pipetting in cold MEM, then filtrated through a 150-mesh cell filter. The collected cells were washed 3 times with cold MEM by centrifugation at 50 × g for 1 min at 4℃, and then suspended in WE containing 10 % fetal bovine serum, 1×10^{-6} M insulin, 1×10^{-5} M dexamethasone, 60 mg/l gentamycin and 0.5 mg/l fungizone.

Isolation of fetal rat liver cells. Pregnant rats were anesthetized with nembutal, and the fetuses were delivered. Collected fetal rat livers were cut into small pieces, transferred to a 100 ml Erhlenmeyer flask containing the pre-perfusion buffer and shaken twice at 100 osc./min, for 5 min in a 37℃ water bath. After that, liver tissues were shaken in 0.05 % collagenase solution for 15 min and filtrated. The undissociated tissues were shaken again and filtrated. The hepatocytes were washed and resuspended by the same method used for adult rat hepatocytes.

Culture method. The cell viability was judged from trypane blue staining. Inocula of 5×10^6 cells from adult rats and 1×10^7 cells from fetal rats were placed in 100-mm Corning plastic dishes in 10 ml WE supplemented with 10 % fetal bovine serum, 1×10^{-6} M insulin, 1×10^{-5} M dexamethasone and antibiotics. The cells were cultured in a humidified chamber at 37℃ under 5 % CO_2 and 95 % air. After 24 h, the medium was replaced with hormone free fresh medium and cultured another 16 h; then inducers were added, and cells were cultured till analysis.

Assays of ODC and polyamines. For the measurement of ODC and polyamines, dishes were washed twice with Ca^{2+}, Mg^{2+}-free Dlubecco's phosphate buffer solution (CMF-PBS), and cells were detached from dishes by incubating with CMF-PBS containing 0.1 % trypsin and 0.02 % ethlenediaminetetraacetate (EDTA). Cells collected from each dish were washed three times with CMF-PBS and resuspended in reaction mixture. The resuspended cells were dis-

rupted using a microultrasonic cell disrupter (Kontes, Vineland, N.J.). Assays were made on supernatant solutions obtained after centrifugation at 100,000 × g, for 30 min at 4℃. ODC activity was determined by the $^{14}CO_2$-release method of Ono *et al.* (12).

For the measurement of polyamines, 0.1 ml of supernatant fluid was mixed with an equal volume of 10 % trichloroacetic acid (TCA) containing an internal standard, and then centrifuged at 2,000 × g, for 5 min at room temperature. Twenty μl of the supernatant was loaded on a high performance liquid chromatography (HPLC) system. The detailed composition of buffers and analytical conditions were reported previously (6-8). Putrescine, spermidine, spermine and the internal standard were clearly separated within 18 min as shown in Fig. 2. As the internal standard, we used triethylene tetramine (kindly supplied by Dr. Samejima, Josai University, Saitama) which has the molecular stracture: $NH_2(CH_2)_2NH(CH_2)_2NH(CH_2)_2NH_2 \cdot 4HCl$.

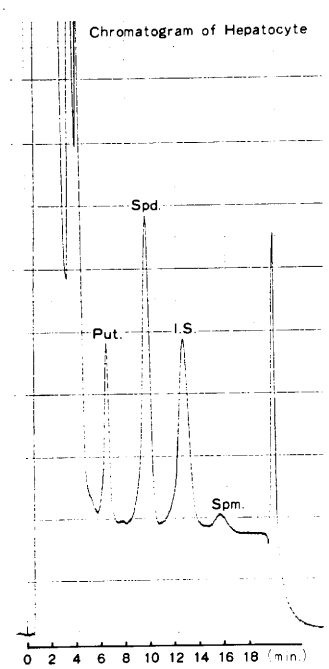

Fig. 2. Chromatogram of polyamines in primary cultured hepatocytes. Put. = putrescine, Spd. = spermidine, I.S. = internal standard, Spm. = spermine

Cellular protein was measured by the method of Lowry *et al.* (13). ODC activity was expressed as nmol CO_2/h/mg protein, and polyamines as nmol/mg protein. Coefficient variations of each polyamine were under 4 percent.

RESULTS

Viability, attachment and survival of cells. By the perfusion method, we obtained 1×10^8 cells/g liver with a viability of over 85 %, whereas the cell yield by the shaking method was one-tenth as much, but with a viability of nealy 100 %.

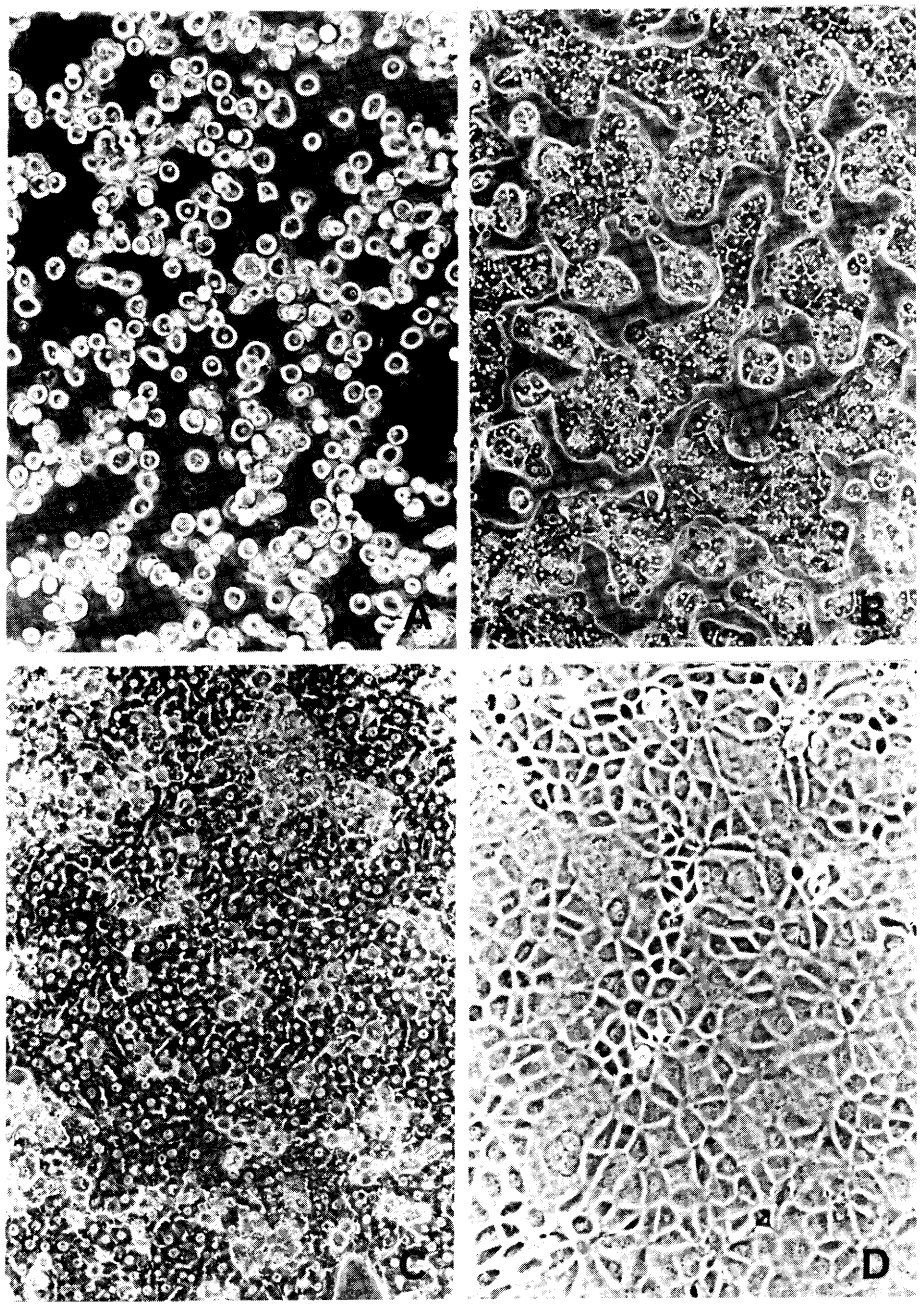

Fig. 3. Phase-contrast microscopy of cultured cells from adult rat liver. A-D were cells cultured for 2 h, 12 h, 40 h and 14 days, respectively. × 366

Freshly isolated spherical hepatocytes of adult adhered to the surface of dishes and began to flatten within 6 h (Fig. 3-A). After 24 h of culture, they formed a subconfluent monolayer (Fig. 3-C) and remained so for about 7 days. After 10 days, however, the number of these polygonal cells with granular cytoplasm decreased, and cells with clear cytoplasm appeared (Fig. 3-D). This change in cell proliferation has been reported by others (11, 14).

Hepatocytes of fetal rats, which were smaller than those of adults, also formed a subconfluent monolayer after 24 h culture (Fig. 4), but the fibroblast-like cell population increased after 5 days under the conditions of our study. For this reason, biochemical investigation using primary cultured fetal rat hepatocytes were completed within 5 days.

Changes in the number of adult hepatocytes in primary culture are shown in Fig. 5. Insulin and dexamethasone were indispensable for the maintenance of hepatocytes, as repoted previously (11). After seeding 1×10^6 cells/ml of fetal hepatocytes, they grow as adult rat hepatocytes for 5 days, after which the number of fibroblast-like cells increased.

Induction of ODC activity and polyamine synthesis by various hormones in primary cultured adult rat hepatocytes. It is well known that many enzyme activities including ODC activity are induced markedly in hepatocytes cultured for 40 h (15). The reason for this phenomenon is thought to be that the function of cell membrane is impared in freshly isolated hepatocytes, but are restored by culture of cells for 1 day (16-18). Therefore, studies were carried out using cells cultured for 40 h.

Thirteen hormones were tested for their ability to induce ODC activity and

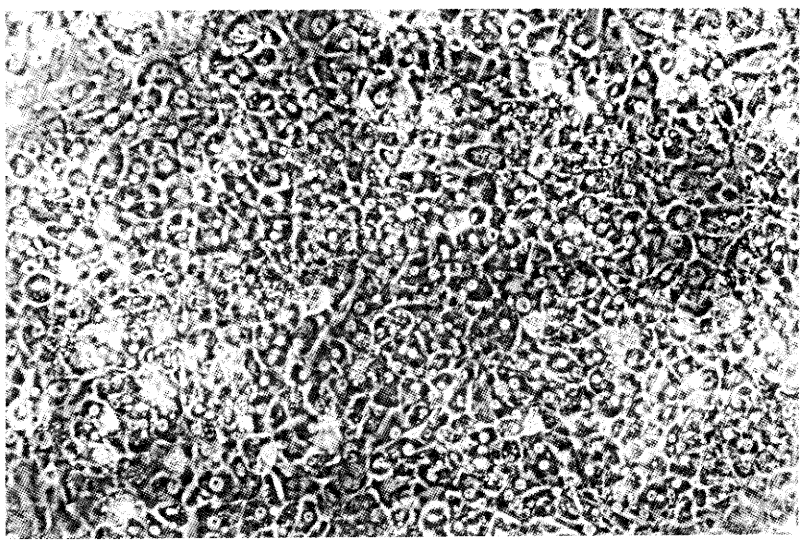

Fig. 4. Phase-contrast microscopy of fetal rat hepatocytes cultured for 40 h. × 580

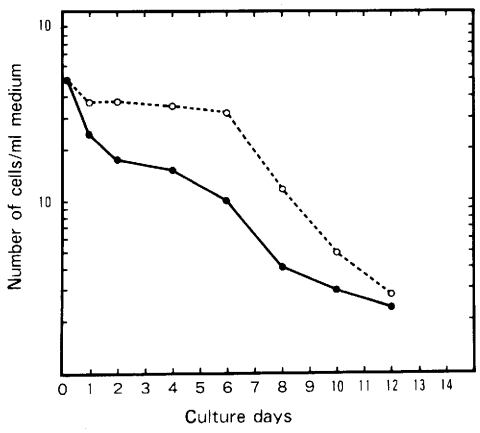

Fig. 5. Maintenance of isolated adult rat hepatocytes in primary culture. ○WE, 10% fetal bovine serum, 1×10^{-6}M insulin and 1×10^{-5}M dexamethasone. ●WE and 10% fetal bovine serum.

TABLE 2. INDUCTION OF ODC AND POLYAMINES BY VARIOUS HORMONES IN PRIMARY CULTURED ADULT RAT HEPATOCYTES

Hormones	Conc.	n	ODC	Putrescine	Spermidine	Spermine
Estrone	2×10^{-5}M	8	82.6±16.2	104.6±22.0	105.8±20.5	100.7±14.6
Estradiol	7×10^{-5}M	5	90.4± 8.3	109.9± 9.4	107.2±15.2	102.6± 9.0
Progesterone	6×10^{-4}M	8	84.6±13.2	87.3±13.2	98.2±15.0	84.6±12.1
Teststerone	5×10^{-5}M	5	43.2± 4.5	69.0± 5.0	78.8±10.0	82.0± 4.0
HCG	60 IU/ml	5	90.7±12.9	114.2±17.2	101.2±14.3	100.7± 6.2
Dexamethasone	10^{-6}M	8	162.6±17.5	101.1±11.0	95.3±11.5	100.6± 7.5
Cortisol	10^{-6}M	5	100.8± 7.5	81.7± 5.8	108.3± 9.2	100.8± 7.5
Insulin	10^{-6}M	8	293.0± 8.3	136.5± 7.3	101.1± 6.2	79.5±10.6
Glucagon	10^{-6}M	5	123.9±21.4	120.0±18.3	84.5±17.3	91.8±16.2
Epinephrine	10^{-6}M	5	119.8±18.3	119.7±20.3	92.1±15.4	102.1±12.8
EGF	0.1 μg/ml	5	480.3±32.5	136.6±10.5	94.0± 3.9	107.6±13.4
Insulin + Dexamethasone	10^{-6}M 10^{-6}M	8	314.6±12.6	138.9± 8.8	94.5± 3.5	95.6± 9.6
Insulin + EGF	10^{-6}M 0.1 μg/ml	5	1180.4±54.3	356.7±18.9	115.9± 5.9	100.0±19.8

Each hormone was added in dish after 40 h culture as descrived in MATERIALS AND METHODS, and ODC activity and polyamine concentrations were measured 6 h later, respectively. All values are mean ± S. D. (% of control)

polyamine synthesis (Table 2). Only dexamethasone, insulin and EGF induced ODC activity. The maximal effective concentration of insulin and dexamethasone was about 1×10^{-6}M, and that of EFG was 0.1 μg/ml. The combined effect of insulin and dexamethasone was additive, whereas that of insulin and EFG was

synergistic. Estrone, estradiol, progesterone, teststerone, HCG and cortisol show marked changes during pregnancy, but they did not induce ODC activity in adult rat hepatocytes. Glucagon and epinephrine tended to increase ODC activity, but not significant.

Concerning polyamine synthesis, only putrescine synthesis was incresed by insulin and EGF. Spermidine and spermine synthesis, however, was not increased by any hormones.

Induction of ODC and polyamines by various hormones in primary cultured fetal rat hepatocytes. In fetal rat hepatocytes, dexamethasone, insulin and EGF also induced ODC activity (Table 3). The combined effects of insulin, dexamethasone and EGF were not as marked as in adult rat hepatocytes. Putrescine and spermidine biosynthesis was slightly stimulated by insulin, dexamethasone and EGF, but other hormones showed no remarkable effects on polyamine biosynthesis.

Time course of changes in ODC activity and polyamine synthesis stimulated by insulin and EGF. ODC activity and polyamine synthesis were stimulated most by the combination of insulin and EGF in both adult and fetal rat hepatocytes (Tables 2, 3). Therefore, the time course of changes in ODC activity and polyamine synthesis produced by insulin and EGF stimulation was examined. In adult rat hepatocytes, ODC activity which increased rapidly after stimulation was about 18-fold the control level 9 h later, and then decreased rapidly.

The concentration of putrescine increased gradually and reached the maximal

TABLE 3. INDUCTION OF ODC AND POLYAMINES BY VARIOUS HORMONES IN PRIMARY CULTURED FETAL RAT HEPATOCYTES

Hormones	Conc.	n	ODC	Putrescine	Spermidine	Spermine
Estrone	2×10^{-5} M	6	78.4±12.3	104.0± 9.8	109.2±10.8	97.5± 9.2
Estradiol	7×10^{-5} M	4	71.4± 4.5	77.2± 2.7	99.7± 9.8	99.3±13.9
Progesterone	6×10^{-4} M	6	108.8±10.5	78.2± 5.6	87.9±10.3	82.5± 7.5
Teststerone	5×10^{-5} M	6	102.2±14.7	77.5± 9.8	96.9± 6.3	90.7± 5.8
HCG	60 IU/ml	10	115.3±13.5	110.8±10.2	117.3±11.4	105.8±11.1
Dexamethasone	10^{-6} M	8	142.5± 6.7	126.0± 6.0	124.1± 7.9	95.6± 3.7
Cortisol	10^{-6} M	4	68.8± 5.2	68.2± 5.0	83.2± 4.8	84.1±11.1
Insulin	10^{-6} M	10	196.8±19.3	120.6±15.7	121.3±14.8	100.3± 9.3
Glucagon	10^{-6} M	4	97.2± 5.7	73.5± 3.1	84.1± 3.7	91.7± 6.4
Epinephrine	10^{-6} M	4	57.1±10.3	70.0± 5.9	79.7± 3.3	77.9± 8.4
EGF	0.1 μg/ml	5	260.7±18.5	131.4±13.4	126.3± 8.9	105.4±10.3
Insulin + Dexamethasone	10^{-6} M 10^{-6} M	5	209.7±19.3	128.6±12.7	86.7± 4.5	79.5± 5.3
Insulin + EGF	10^{-6} M 0.1 μg/ml	5	363.1±22.8	148.8±13.3	128.5±11.2	113.7± 8.9

Each hormone was added in dish after 40 h culture as descrived in MATERIALS AND METHODS, and ODC activity and polyamine concentrations were measured 6 h later, respectively. All values are mean ± S. D. (% of control)

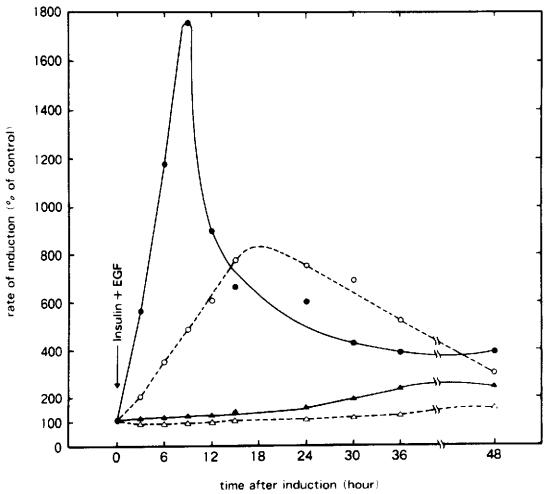

Fig. 6. Induction of ODC and polyamines by insulin and EGF in primary cultured adult rat hepatocytes. 1×10^{-6} M insulin and 0.1 μg/ml EGF were added after 40 h of culture. ● ODC, ○ putrescine, ▲ spermidine and △ spermine. Each point shows the mean value of 5 experiments.

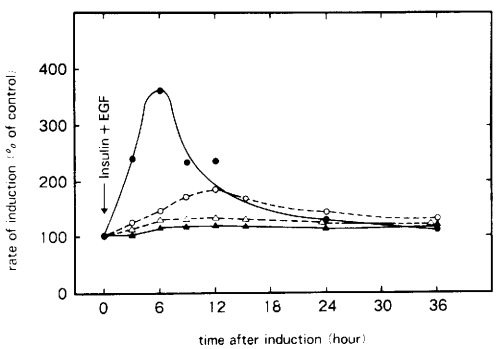

Fig. 7. Induction of ODC and polyamines by insulin and EFG in primary cultured fetal rat hepatocytes. 1×10^{-6} M insulin and 0.1 μg/ml EGF were added after 40 h of culture. ● ODC, ○ putrescine, ▲ spermidine and △ spermine. Each point shows the mean value of 4 experiments.

level 15 h after stimulation, whereas the concentrations of spermidine and spermine increased slightly from 30-36 h after stimulation (Fig. 6).

On the other hand, in fetal rat hepatocytes, ODC activity reached the maximal level 6 h after stimulation, 3 h earlier than in adult rat hepatocytes. The maximal rate of induction was about 3.6-fold that of the control. The concentration of putrescine gradually increased after stimulation, reaching a peak 6 h later than the peak of ODC. The concentration of spermidine and spermine also increased slightly (Fig. 7).

DISCUSSION

Liver is the tissue in which the largest number of enzymes have been quantified as a function of age. To study the developmental changes of this complicated tissue, primary culture, in which hepatocytes regain their functions after one day of culture, is very useful (17-19). We examined the action of thirteen hormones on polyamine metabolism using primary cultured hepatocytes. Among the hormones tested, insulin and EGF showed marked effects on ODC and polyamine induction. Our study revealed that insulin and EGF caused greater induction of ODC and polyamines in adult rat hepatocytes than in fetal rat hepatocytes, but an earlier response in fetal hepatocytes than in adult hepatocytes. It has been established that hepatic membranes from fetal and early neonatal rats bind more insulin than adult tissues, because of more hepatic insulin receptors in the former (19). It also is known that serum insulin concentrations in fetuses are higher than in adults (19).

Our data indicate, however, that the sensitivity of fetal rat hepatocytes to insulin and EGF is lower than that of adult rat hepatocytes. Namely, the receptors of adult rat hepatocytes appeared to be more functionally differentiated than those of fetal hepatocytes. These observations could be explained by the presence of immunologically detectable but biologically inactive species of fetal insulin (20) or the inability of developing fetal hepatocytes to down-regulate insulin production in responce to high serum insulin concentrations. In support of the latter explanation, Greeberg *et al.* (24) have noted that 24 h exposure to insulin results in only limited depletion of insulin receptors in hepatic membrane from fetal rats. Furthermore, in variety of other developing tissues, excess circulating insulin is either not associated with receptor depletion (22-24) or may actually result in stimulation of receptor formation (25).

On the other hand, it is possible that fetal rat hepatocytes reflect contamination by red blood cells and immature hematopoietic cells. In fact, fetal red blood cells contain a significantly higher concentrations of polyamines than adult red blood cells (8). However, control levels of polyamines in fetal hepatocytes are similar to those in adult hepatocytes (2, 3), and in this study, red blood cells were removed almost perfectly during the isolation of hepatocyres. Some hematopoietic cells remain at 21 days of pregnancy, but levels of ODC and polyamine in fetal rat liver cells at 21 days of pregnancy are similar to those in adult hepatocytes, and most importantly, ^{125}I-iodoinsulin binding has recently been demonstrated to occur mainly with hepatocytes, and not with hematopoietic elements in fetal rat liver (23, 26). For this reason, our data seem to indicate a difference in polyamine metabolism between fetal and adult rat hepatocytes.

The epidermal growth factor (EGF), which also stimulates significantly ODC and polyamine production, is a small single polypeptide (molecular weight 6,000) which belongs to a new class of cytomodulatory factors that are hormone-like in their biological action (27). It is known that EGF stimulates the proliferation of

epidermis and several internal epithelial tissues *in vivo* (27, 28). An *in vivo* role for the EGF receptor system in embryonic and organ development is suggested by various studies on EGF binding and EGF action in embyonic, amniotic, placental and other developing tissues (29-32).

It has also been reported that EGF stimulates DNA synthesis in hepatocytes (15,33). Only Tomita *et al.*(15) have reported that EGF stimulates ODC induction in primary cultured adult rat hepatocytes. There have been no reports of EGF concerning the metabolism of polyamines in fetal rat hepatocytes, and the precise function of EGF during fetal growth is poorly understood. Our observations revealed that the induction of ODC and polyamines by EGF is greater than that by insulin in both adult and fetal hepatocytes. However, the rate of ODC induction in fetal hepatocytes is less than in adult hepatocytes with either polypeptide.

EGF has been shown to stimulate the phosphorylation of proteins, including the EGF receptor, in membrane from a epidermoid carcinoma cell line (34). ODC and polyamines might be induced by this mechanism, but the precise function of EGF, including the action on polyamine metabolism, must be investigated further.

The effect of insulin and dexamethasone on ODC induction was additive, while the effect of insulin and EGF was synergistic, suggesting different mechanism of induction. Besides insulin and EGF, dexamethasone also showed induction of ODC and polyamines in both type of hepatocytes. Epinephrine and glucagon tended to induce ODC in adult hepatocytes and HCG in fetal hepatocytes, but the changes were not significant.

It is generally accepted that the growth hormone and steroid hormones most likely exert their metabolic effects through a mechanism independent of the stimulation of cyclic AMP production, whereas the action of other peptide hormones and amino acid derivatives may involve a transient increase in the concentration of intracellular cyclic AMP (4). It has also been reported that the induction of ODC would result from a stimulation of protein kinase by cyclic AMP (35). However, glucagon, a potent elevator of cyclic AMP level in the liver *in vivo* (36), was ineffective in inducing ODC and polyamines in fetal hepatocytes. Similary, epinephrine, which can also raise intracellular liver cyclic AMP level *in vivo*, although to a lesser extent than glucagon (36), showed no effect on ODC and polyamine induction in fetal hepatocytes. The unresponsiveness of fetal hepatocytes to glucagon may be a reflection of the low number of glucagon receptors in the liver at the fetal stage of development (37), so that the elevation of intracellular cyclic AMP observed in fetal hepatocytes following glucagon treatment (38) is insufficient to mediate a detectable increase in ODC activity.

Recently, ODC antizyme and acetyl-polyamine have been reported, so that polyamine metabolism has become complicated. The result of our study, however, suggest that ODC and polyamines in the liver at the fetal stage of development are regulated by a different mechanism than in the adult liver. The detailed mechanism of ODC induction in fetal tissues is still obscure, but these precise

investigations may resolve the pathogenesis of intrauterine growth retardation fetus. Our studies are ongoing to reveal the precise functions of insulin, EGF and other growth factors such as somatomedin in fetal growth.

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