Glucose-stimulated DNA Replication of the Pancreatic Islets During the Development of the Rat Fetus

Effects of Nutrients, Growth Hormone, and Triiodothyronine

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SUMMARY

DNA replication and insulin release have been studied in islets isolated, using a tissue culture technique, from rat fetuses of different gestational ages. The islets were cultured for 3 days in media with high and low concentrations of glucose or amino acids. The DNA replication was determined by autoradiography and the insulin secreted into the medium was measured by radioimmunoassay. In islets of 22-day-old fetuses, DNA replication was stimulated by both glucose and amino acids. At gestational days 18 and 20, only amino acids increased DNA replication. However, both high glucose and high amino acid concentrations increased the islet insulin secretion into the culture medium at all ages studied.

In an attempt to induce glucose-sensitive DNA replication in vitro, islets obtained from 18- and 20-day-old fetal pancreata were cultured in the presence of either triiodothyronine or human growth hormone. Triiodothyronine failed to influence either DNA replication or insulin release. Growth hormone, however, increased DNA replication and insulin release in both the experimental groups but did not induce a growth response to glucose.

It is concluded that the appearance of glucose-stimulated B-cell growth is a late event in the fetal development of the rat, parallelling the late maturation of both insulin biosynthesis and release. This finding may explain the difficulties in producing islet cell hyperplasia and diabetic fetopathy previously shown in rat models of diabetes in pregnancy. DIABETES 1985; 34:803–807.

A role of glucose as a stimulus for fetal B-cell growth was suggested by Pedersen[1] in his studies of infants of diabetic mothers. In studies of diabetes in pregnancy, the rat has been widely used as an experimental model. It has, however, proved difficult to reproduce the islet hyperplasia and macrosomia of human diabetic fetopathy.[2] One reason for this could be the different developmental program of the rat B-cell as compared with the human.

In the fetal rat, pancreatic B-cells can be identified by day 12 of gestation[3] and their subsequent development has been carefully investigated by Pictet and Rutter.[4] These studies suggest that the B-cell functional capacity matures late in gestation, only a few days before term. The morphologic findings are supported by the observations that insulin release and biosynthesis mature perinatally[5,6] and respond poorly to glucose before this period. In midgestation, the B-cell does not respond to glucose with increased proliferation[7] and it is unknown when this response occurs. Such information would be of great value, since glucose-induced hyperplasia of the islets of Langerhans is probably necessary for the development of diabetic fetopathy.[1,8,9]

In the present investigation, the DNA replicatory response of the B-cell to glucose, amino acids, and hormones was investigated in vitro using islets isolated from fetal rat pancreata obtained at different gestational ages.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats weighing 200–250 g were caged overnight with males. Pregnancy was confirmed by the presence of sperm in vaginal smears on the following morning, which was designated as day 0 of pregnancy. In this strain, the duration of gestation is approximately 22.5 days and experiments were conducted on days 18, 20, and 22 of gestation.

Preparation and culture of fetal rat islets. Pregnant rats were killed by cervical dislocation between 7 and 10 a.m. and their fetuses rapidly removed. Fetal rat islets were prepared as previously described in detail[10,11] from the pancreatic glands of day 18, 20, or 22 fetuses. Briefly, the pancreatic glands were carefully dissected free of surrounding tissue, finely chopped, and digested for a short time with collagenase (Boehringer-Mannheim, Bromma, Sweden). The carefully washed digest was distributed in 50-mm culture

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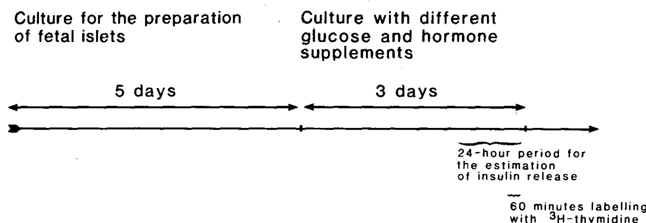

FIGURE 1. Schematic outline of the experimental procedure.

dishes, which allowed cell attachment (Nunc, Roskilde, Denmark), and cultured in 5 ml RPMI 1640 (Statens Vetrinärmedicinska Anstalt, Uppsala, Sweden) containing 11.1 mM glucose. The medium was supplemented with 20 mM N-hydroxyethyl-piperazine-N′-2-ethane sulphonic acid (Hepes), antibiotics (penicillin 100 U/ml and streptomycin 0.1 mg/ml), and 10% heat-inactivated fetal calf serum (Flow, Irvine, Scotland). The cultures were continued for 5 days at 37°C in a humidified atmosphere of 5% CO_2 in air and the culture medium was changed daily. At the end of this period, B-cell-rich islets devoid of nonendocrine tissue were harvested with the aid of a braking pipette.

The islets were subsequently transferred in batches of about 150 to nonattachment culture dishes (Hegar, Stallarholmen, Sweden) and the cultures continued in 5 ml RPMI 1640 supplemented with Hepes, antibiotics, and serum as above, and with a glucose concentration of either 2.7 or 16.7 mM (subsequently denoted as 2.7 G and 16.7 G, respectively). A third experimental group consisted of islets cultured in the low glucose concentration with a supplement of 250 μl per dish of an amino acid concentrate (100× basal Eagle's medium amino acid solution, Flow; denoted as 2.7 GAA). The pH was readjusted to 7.4 with NaOH and the final amino acid concentration was approximately five times greater than that found in the standard RPMI 1640 medium. Some cultures maintained at each glucose and amino acid concentration were supplemented with 1 μg/ml human growth hormone (GH; Crescormone, generously supplied by Kabi, Stockholm, Sweden) or 1 μg/ml triiodothyronine (T_3; Sigma, St. Louis, Missouri). The cultures were continued for 3 days with a medium change after 2 days. During the culture period, there was no significant change in the medium in concentration of either of the test hormones. A schematic outline of the experimental procedure is shown in Figure 1.

Estimation of insulin release. To estimate the insulin accumulation in the culture medium, groups of about 20 fetal islets were removed from the larger batches and cultured in 2 ml medium for the last 24 h (see Figure 1). Aliquots of media were then removed and stored frozen until assayed for insulin.[12] The islets were sonicated in distilled water and assayed for DNA content.[13,14] The results were finally expressed as ng insulin released/μg of islet DNA/24 h.

Estimation of islet cell DNA replication. To estimate the DNA replication at the end of the 3-day culture period, ^{3}H-thymidine (The Radiochemical Centre, Amersham, United Kingdom; specific activity 5 Ci/mmol) was added to the culture medium to give a final activity of 10 μCi/ml. After 60 min, the incubation was terminated by rinsing the islets briefly in Hanks' balanced salt solution (HBSS; Statens Vetrinärmedicinska Anstalt) and fixing them in Bouin's solution. The islets were then dehydrated, embedded in paraffin, and sectioned at 7 μm. After removal of the paraffin, the slides were dipped in Kodak NTB-2 emulsion and exposed at 4°C for 1 wk. They were then developed in Kodak D-19 for 5 min and fixed for 10 min in Kodak F-24. The slides were dried overnight, counterstained with hematoxylin, and mounted. To determine the labeling index (LI), radiolabeled islet cell nuclei were counted using an oil immersion lens (final magnification 1250×) and expressed as a percentage of the total number of nuclei scored. In each experiment at least 1000 nuclei in at least six islet sections were counted.

Statistical analyses. Differences in insulin release and DNA replication between experimental groups of the same gestational age were evaluated using Student's two-tailed *t*-test for independent observations. Differences between the groups of different gestational ages were evaluated by analysis of variance.

TABLE 1
Insulin secretion into the culture medium of islets isolated from rat fetuses at day 18 of gestation

Hormone supplement	Glucose concentration 2.7 mM	2.7 mM + amino acids	16.7 mM
0	65 ± 8 (10)	211 ± 43† (8)	333 ± 42‡ (10)
Growth hormone	115 ± 22 (11)	405 ± 53‡ (10)	544 ± 50‡ (11)
Triiodothyronine	63 ± 8 (9)	155 ± 35* (8)	337 ± 51‡ (9)

The islets were cultured for 3 days in media with different glucose and amino acid concentrations as indicated above. Cultures were also supplemented with 1.0 μg/ml triiodothyronine or 1.0 μg/ml human growth hormone. After 2 days of culture, the media were changed and at the end of the culture period the insulin concentration in the media and the DNA content of the islets were determined. The insulin secretion into the medium was finally expressed as ng immunoreactive insulin/μg islet DNA · 24 h. The values are given as means ± SEM for the number of observations in parentheses. Significance of difference between values from islets cultured in low glucose medium and those cultured in either high glucose or high amino acid medium but with the same hormone supplement: *$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$.

RESULTS

Insulin release. The insulin accumulation in the culture medium of islets from day 18 (Table 1) and day 20 rat fetuses

TABLE 2
Insulin secretion into the culture medium of islets isolated from rat fetuses at day 20 of gestation

Hormone supplement	Glucose concentration 2.7 mM	2.7 mM + amino acids	16.7 mM
0	59 ± 8 (13)	269 ± 50‡ (11)	412 ± 42‡ (13)
Growth hormone	97 ± 14 (14)	313 ± 58† (13)	666 ± 72‡ (15)
Triiodothyronine	61 ± 11 (13)	168 ± 45* (11)	355 ± 40‡ (13)

For details see legend to Table 1.
*$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$.

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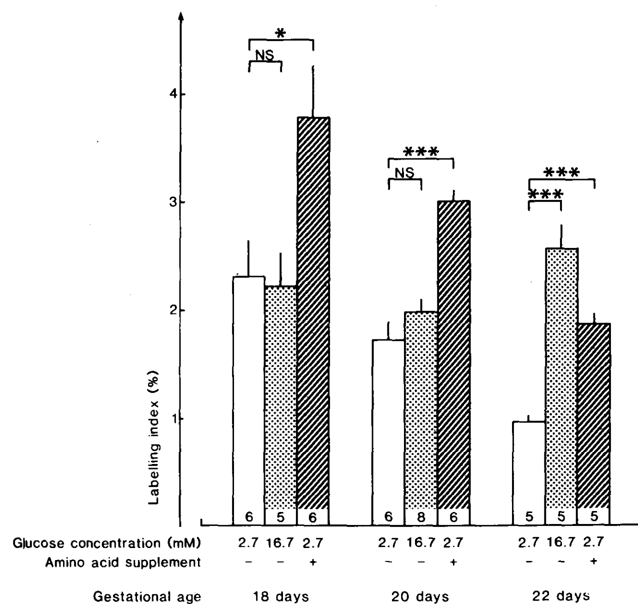

FIGURE 2. DNA synthesis in cultured fetal rat islets. Islets isolated from rat fetuses of different gestational ages were cultured for 3 days in media with different glucose and amino acid concentrations as indicated in the figure. At the end of the culture period, the islets were labeled with ^{3}H-thymidine and processed for autoradiography. For details of the procedure for isolation, culture, and incubation see the MATERIALS AND METHODS section. The DNA synthesis is expressed as the labeling index, i.e., the percentage of the total islet cell population that was radiolabeled. The values are given as mean ± SEM for the number of observations given at the bottom of each bar. Significance of difference between values from islets cultured in low glucose medium and those cultured either in high glucose or high amino acid medium: *P < 0.05, ***P < 0.001, NS = not significant.

(Table 2) was stimulated by both 16.7 G and 2.7 GAA when compared with 2.7 G cultures. The high glucose concentration caused a five- to sevenfold increase of the medium insulin content, whereas 2.7 GAA increased the insulin accumulation three- to fourfold. At both fetal ages, the addition of GH to the culture medium increased the insulin secretion of the 16.7 G cultures ($P < 0.01$). In islets of the 18-day-old fetuses, GH stimulated insulin secretion in the 2.7 GAA cultures ($P < 0.05$) and in the islets of the 20-day-old fetuses, GH stimulated insulin secretion also in the 2.7 G cultures ($P < 0.05$). Supplementation of the medium with T_3 did not increase the insulin accumulation in the medium of any of the experimental groups. The stimulation of insulin secretion caused by 16.7 G and 2.7 GAA appeared similar in hormone-supplemented and control cultures, 16.7 G always being more efficient than 2.7 GAA as an insulin secretagogue. No differences in insulin secretion between day 18 and day 20 fetal islets could be demonstrated under any of the culture conditions used.

Islet cell DNA replication. In islets from 18-day-old and 20-day-old fetuses, DNA replication was stimulated by 2.7 GAA but not by 16.7 G, as evidenced by increased autoradiolabeling indices (Figure 2). In islets from 22-day-old fetuses, however, both 16.7 G and 2.7 GAA stimulated DNA replication, with glucose being the more powerful stimulus. Analyses of variance revealed that islets cultured in 16.7 G maintained a similar rate of DNA replication in the different gestational age groups, while those islets cultured in a low glucose concentration or a low glucose plus high amino acid concentration had a rate of DNA replication that decreased with gestational age ($P < 0.01$, see Figure 2). Addition of GH to the culture medium increased the rate of DNA replication in all age groups irrespective of the glucose and amino acid concentrations (Tables 3 and 4: day 18, $P < 0.05$; day 20, $P < 0.01$). T_3 stimulated DNA replication only in the islets of the 20-day-old fetuses when they were cultured in 2.7 GAA (Table 4; $P < 0.001$). The inability of islets from the two younger age groups to respond to 16.7 G with an increased rate of DNA replication was also observed in cultures to which either GH or T_3 had been added. The rate of DNA replication in the hormone-supplemented cultures was similar in islets from 18-day-old and 20-day-old fetuses.

TABLE 3
Islet cell replication in islets isolated from rat fetuses at day 18 of gestation

Hormone supplement	Glucose concentration		
	2.7 mM	2.7 mM + amino acids	16.7 mM
0	2.31 ± 0.35 (6)	3.79 ± 0.48* (6)	2.21 ± 0.30 (5)
Growth hormone	3.27 ± 0.21 (6)	5.45 ± 0.47† (7)	3.84 ± 0.38 (6)
Triiodothyronine	2.93 ± 0.18 (6)	4.46 ± 0.30† (6)	3.14 ± 0.29 (6)

The islets were cultured for 3 days in media with different glucose and amino acid concentrations as indicated above. Cultures were also supplemented with either 1 μg/ml triiodothyronine or 1.0 μg/ml human growth hormone. At the end of the culture period, the islets were labeled with ^{3}H-thymidine and processed for autoradiography. For details of the procedure see the MATERIALS AND METHODS section. The DNA synthesis is expressed as the labeling index, i.e., the percentage of radiolabeled cells of the total islet cell population. The values are given as means ± SEM for the number of observations in parentheses. Significance of difference between values from islets cultured in low glucose medium and those cultured in either high glucose or high amino acid medium but with the same hormone supplement: *P < 0.05 and †P < 0.01.

DISCUSSION

The fetal rat islet preparation technique used in the present study is a simple method that yields a large number of islets and has previously been employed in several investigations of islet cell proliferation.[11,15–22] Similarly, the use of ^{3}H-thymidine incorporation as an index of islet cell proliferation is well

TABLE 4
Islet cell replication in islets isolated from rat fetuses at day 20 of gestation

Hormone supplement	Glucose concentration		
	2.7 mM	2.7 mM + amino acids	16.7 mM
0	1.72 ± 0.15 (6)	2.99 ± 0.11‡ (6)	1.97 ± 0.11 (8)
Growth hormone	3.33 ± 0.20 (5)	4.65 ± 0.21† (5)	3.79 ± 0.24 (6)
Triiodothyronine	2.36 ± 0.37 (5)	4.07 ± 0.21* (5)	2.73 ± 0.54 (5)

For details see legend to Table 3.
*P < 0.05, †P < 0.01, and ‡P < 0.001.

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documented.[11,20,23] The fetal islets isolated using this technique are rich in B-cells (>90%)[16] and it can therefore be assumed that the results obtained reflect B-cell function. During the culture period of 5 days needed to isolate the islets, they undergo a functional development toward a mature, adult-like capacity for insulin secretion.[10,22,24] This development depends on the culture conditions, but the developmental stage of the fetuses yielding the islets must also be taken into account when the results are interpreted.

The present investigation shows that a high glucose concentration is unable to stimulate DNA replication in fetal rat islets obtained as late as day 20 of gestation. This finding corresponds to the late maturation of the glucose regulation of other B-cell functions such as insulin biosynthesis, which, although present at midgestation,[25] acquires its adult-like glucose sensitivity close to birth.[6] Likewise, the insulin secretory response to glucose is low during the last days of gestation but matures during the first postnatal days.[26,27] In the present investigation, DNA replication was, however, strongly maintained by medium with a high amino acid content. This finding forms an interesting parallel with both the rapid insulin accumulation in pancreatic rudiments cultured in such medium[7] and the considerable insulin secretory response to amino acids observed in late pregnancy in the rat.[27] Indeed, the amino acid-enriched medium resembles the physiologic condition late in gestation in the fetal rat in which the serum level of glucose is low[28] but that of amino acids high.[29] It may be that during gestation, when the fetal blood sugar level is low, an ample supply of amino acids is necessary both as an energy source and as building blocks for peptides. Between days 20 and 22 of gestation, immediately before term, a glucose-sensitive DNA replication develops. The DNA replication now resembles that of adult islets with respect to the potency of glucose stimulation.[11,21] The fetal islets, however, have a larger proportion of cells capable of proliferation and consequently a greater total capacity for islet growth.[19,21]

Whether there is a hormonal control of the transition of the B-cell from fetal glucose independency to a more mature state in which glucose is the major modulator of function is not yet known. The serum levels of both thyroid hormones[30] and growth hormone[31-33] increase markedly during the perinatal period and may influence B-cell development. Of the two, thyroid hormones influence the perinatal induction of glucokinase in the liver.[34] A similar enzyme is also present in the islets of Langerhans[35] and may play a key role in the B-cell glucose metabolism and insulin secretory response.[36] However, in the present investigation, triiodothyronine, in pharmacologic doses, did not affect DNA replication or insulin release. This finding and a previous study in vivo[37] suggest that thyroid hormones are not important factors for the perinatal B-cell development. They may, however, be important permissive factors for the maintenance of optimal B-cell function.[38]

The present observed stimulation of DNA replication by GH is similar to the effects reported on neonatal rat and mouse B-cells maintained in tissue culture.[39-41] The increase in insulin production during culture in the presence of GH also confirms previous data obtained with postnatal islets.[39,42,43] There was, however, no indication that the hormone is able to induce a glucose-sensitive DNA replication. The present results may therefore reflect only the growth promotion and increased protein biosynthesis generally caused by GH.[44]

In this context, it should be noted that glucose, at a level of 11.1 mM during the first 5 days of culture, was unable to induce a glucose-sensitive DNA replication. Thus, islets cultured in 2.7 G or 16.7 G after this initial culture period had similar rates of DNA replication. This finding contrasts the effects on insulin release and other parameters associated with the secretory process, which in this system tend to mature and acquire an adult-like pattern during high glucose culture.[10,22,24] This effect of the culture conditions is presently indicated by the increased insulin accumulation in the high glucose media. It may be that the development of a glucose-sensitive B-cell growth is not a preprogrammed event that proceeds according to a fixed schedule, but rather, is induced by factor(s) which may be lacking or present in insufficient concentrations in the present tissue culture system. The different effects of culture on DNA replication and insulin secretion are nevertheless not surprising, since it has been previously demonstrated that factors affecting both insulin biosynthesis and secretion do not necessarily influence islet growth.[11,45]

The present findings may offer an explanation as to why islet hyperplasia and diabetic fetopathy have been difficult to produce in experimental models of diabetes in pregnancy. In man, maternal hyperglycemia causes islet hyperplasia and overproduction of insulin in the fetus. These changes are not only part of the fetopathy syndrome but are also necessary to produce both the increased deposition of body fat and the excessive body weight typical of infants of diabetic mothers. Indeed, there is a direct correlation between the B-cell mass and the body weight of the infant.[8,46,47] The increased body weight and the other symptoms of fetopathy can be observed from about the 28th wk of human pregnancy, suggesting that the fetal B-cell responds to glucose from about this time point.[9] By contrast, in the rat the B-cell DNA replication becomes glucose-sensitive only during the last 2 days of pregnancy. This would explain the observation that in mildly diabetic pregnant rats, the fetuses failed to exhibit an increased pancreatic insulin content before day 20 of gestation.[48] In pregnant rats with severe diabetes, fetal islet hyperplasia is not observed,[49] but the development of the fetus as a whole is retarded.[2] Thus in these fetuses, we would not have expected a glucose-sensitive B-cell DNA replication to have developed.

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