Polyamines in pancreatic islets of obese-hyperglycemic (ob/ob) mice of different ages

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Sjöholm, Åke, Per Arkhammar, Per-Olof Berggren, and Arne Andersson. Polyamines in pancreatic islets of obese-hyperglycemic (ob/ob) mice of different ages. Am J Physiol Cell Physiol 280: C317–C323, 2001.—To further evaluate the role of polyamines in insulin production and cell replication in diabetic pancreatic islets, we have studied hyperplastic islets of obese-hyperglycemic mice of different ages and normal islets of the same strain. The aims of the study were to investigate the impact of the diabetic state and aging on polyamine contents and requirements in these islets. Cultured islets from lean and obese animals contained significantly less polyamines than freshly isolated islets. Spermine-to-spermidine ratio was elevated in freshly isolated islets from young obese mice compared with those from lean mice. In islets from old obese animals, spermidine content was decreased, whereas the content of spermine was not different from that of young obese mice. The physiological significance of polyamines was investigated by exposing islets in tissue culture to inhibitors of polyamine synthesis. This treatment caused a partial polyamine depletion in whole islets but failed to affect polyamine content of cell nuclei. Insulin content was not affected in polyamine-deficient islets of obese mice, irrespective of age, in contrast to decreased islet insulin content in polyamine-depleted young lean animals. Polyamine depletion depressed DNA synthesis rate in obese mouse islets; in lean mice it actually stimulated DNA synthesis. We concluded that important qualitative and quantitative differences exist between islets from obese-hyperglycemic and normal mice with respect to polyamine content and requirements of polyamines for regulation of insulin content and cell proliferation. The results suggest that spermine may be involved in mediating the rapid islet cell proliferation noted early in obese-hyperglycemic syndrome, but changes in spermine concentration do not seem to account for the decline in islet cell DNA synthesis in aged normoglycemic animals.

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The recessively inherited obese-hyperglycemic syndrome in mice (gene symbol ob) is characterized by hyperphagia, insulin resistance, nonketotic hyperglycemia, marked β-cell hyperplasia, and hypersecretion of insulin (12, 42). It has frequently been used as an animal model of diabetes mellitus, resembling human type 2 diabetes. Studies of the development of the syndrome suggest that an early peripheral insulin resistance leads to hyperglycemia and an increased demand for insulin, which induces excessive replication of the pancreatic β-cells (42). Despite this expansion of the β-cell mass and elevated insulin secretion, the mice develop hyperglycemia. However, this is a transient state, and late in the syndrome the animals become normoglycemic again and islet cell replication is markedly decreased (3, 42).

The obese-hyperglycemic syndrome has recently engendered renewed interest because of the discovery of the leptin system. Leptin, the protein encoded by the ob gene, is secreted from white adipose tissue and regulates satiety and energy expenditure through hypothalamic receptors (15, 22, 25). Interestingly, ob/ob mice have a mutation in the leptin gene and are thus deficient in leptin (22, 25). Islets from obese-hyperglycemic mice show hyperplastic changes (3, 13, 40, 42) and abnormal insulin secretory behavior (4–6, 8–11, 14, 21, 42).

We previously implicated polyamines as stimulatory or permissive factors for DNA synthesis and insulin production in pancreatic islets isolated from normoglycemic mice and fetal rats (32–34, 41). In an attempt to further elucidate the role of polyamines in diabetic pancreatic islets, isolated islets from obese-hyperglycemic mice at different stages of the syndrome have now been studied with specific attention to the relation between the polyamine content, on one hand, and the proliferative activity and insulin content of the islet cells, on the other. Thus the two aims of this study were to investigate the impact of the diabetic state and aging on polyamine contents and requirements in these islets.

MATERIALS AND METHODS

Materials. Difluoromethylornithine (DFMO) and methyl-acetylenic putrescine (MAP) were generously provided by Dr.
Peter P. McCann (Merrell Dow Research Center, Cincinnati, OH), and ethyglyoxal bis(guanylhydrazone) (EGBG) was provided by Dr. Juhani Jänne (University of Helsinki). RPMI 1640 culture medium, L-glutamine, penicillin, streptomycin, and donor calf serum were obtained from Flow Laboratories (Irvine, CA); cyanogen bromide-activated Sepharose 4B was from Pharmacia Fine Chemicals (Uppsala, Sweden); antibovine insulin serum was from Miles-Yeda (Rehovot, Israel); crystalline mouse insulin and 125I-insulin were from Novo; and spermidine, spermine, L-proline, dansyl chloride, and HEPES were from Sigma Chemical (St. Louis, MO). Tissue culture dishes were supplied by Heger (Stockholm, Sweden). Collagenase (type CLS, EC 3.4.24.3) was obtained from Boehringer-Mannheim (Mannheim, Germany); Hanks’ balanced salt solution was from Statens Bakteriologiska Laboratorium (Stockholm, Sweden); and [methyl-3H]thymidine (5 Ci/mmol) and 1-[4,5-3H]leucine (40 Ci/mmol) were from Amersham (Amersham, UK). Unisolve was supplied by New England Nuclear (Boston, MA), and Soluene was purchased from Packard Instruments (Downers Grove, IL). All other chemicals of analytical grade were obtained from E. Merck (Darmstadt, Germany).

Preparation and culture of isolated islets. Genetically obese mice (genotype ob/ob) of both genders and lean mice of the same strain (+/?) were obtained from the inbred Uppsala colony, which originated from a breeding couple obtained from Jackson Laboratories (Bar Harbor, ME) (12). Obese mice were used at 2 and 8 mo of age and lean animals, at 2 mo of age only. Islets from animals starved overnight were isolated by a collagenase digestion technique (19) and subsequently picked free of exocrine tissue by means of a braking pipette. They were used immediately or cultured freely floating (1) for 2 days at 37°C in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10% calf serum, and the polyamine synthesis inhibitors as indicated.

Use of polyamine synthesis inhibitors. We have in this study used inhibitors of key enzymes in polyamine biosynthesis, i.e., DFMO, MAP, and EGBG. DFMO and MAP are specific and irreversible inhibitors of ornithine decarboxylase, the enzyme regulating putrescine formation (24, 26). EGBG is a highly selective and potent, albeit not completely specific, inhibitor of S-adenosylmethionine decarboxylase, which controls synthesis of spermidine and spermine (31). The rationale for using both MAP and DFMO is, as we previously reported, that MAP alone is not very effective in inhibiting ornithine decarboxylase (41). Throughout our islet studies, we found that partial putrescine and spermidine depletion by DFMO/MAP has little impact on islet hormone production and does not at all affect islet cell replication (32–34, 41). In contrast, when EGBG is included, pronounced effects are seen on both of these parameters. It does not seem to matter, however, whether EGBG is used alone or in combination with the ornithine decarboxylase inhibitors, because both alternatives produce virtually identical results. For these reasons, we used EGBG in combination with the ornithine decarboxylase inhibitors.

Whole cell polyamine content. The total islet polyamine content was determined by one-dimensional TLC (30, 34, 41). For this purpose, the islets were washed in PBS and homogenized by sonication in 20 μl of 0.3 M perchloric acid at 4°C. After centrifugation (5 min, 12,000 g), the supernatant was alkalinized by addition of 10 μl of 1 M Na2CO3 followed by 75 μl of 10 mg/ml dansyl chloride in acetone. After incubation at room temperature overnight in the dark, excess reagent was reacted with 5 μl of L-proline (250 mg/ml) and then sonicated for 2 min. Dansyl polyamines were extracted in 100 μl of toluene. The toluene was evaporated, and the residue was redissolved in 5 μl of toluene for application on TLC plates (high-performance TLC Pertlplatten, Kieselgel 60 F254, E. Merck). The dansylated polyamines were separated by one run with ethyl acetate-cyclohexane (1:1, vol/vol) followed by two runs with diethyl ether-cyclohexane (2:3, vol/vol). The spots were scraped off the plates, and the fluorescence intensities of the supernatants were measured in a luminescence spectrometer (model LS5, Perkin-Elmer) connected to a plate reader at an excitation wavelength of 360 nm and an emission wavelength of 510 nm. This method has, in our hands, an intra-assay variability (SE/mean) of 6%, an interassay variability of 3%, and a sensitivity of ~15 pmol (i.e., an amount of polyamine resulting in a fluorescence intensity 2 SD above the blank reading). Standard curves were linear up to ≥1,000 pmol and showed correlation coefficients of ≥0.99.

Regarding putrescine, by far the least abundant amine in islets, we have encountered some problems in obtaining reliable measurements of this diamine in the minute amounts of islet tissue available. We thus do not consider it very meaningful to measure putrescine in this study, particularly because we have excluded a role for putrescine itself by lack of influence of DFMO or MAP on β-cell function (conversely, a stimulatory role for putrescine was also excluded, because islet cell replication and insulin synthesis are depressed by EGBG, a treatment that leads to a large accumulation of putrescine (41)).

Polyamine content in cell nuclei. Cultured islets in groups of 300 were washed in ice-cold PBS and mechanically homogenized in 100 μl of a buffer containing 250 mM sucrose, 2% Triton X-100, 2 mM EDTA, and 20 mM Tris (pH 7.5). After sedimentation of nuclei (10 s, 12,000 g), the pellet was suspended once in the homogenization buffer and recentrifuged. Twenty microliters of 0.3 M perchloric acid were subsequently added, and the polyamine content of this nuclear fraction was analyzed as described above. All steps of the fractionation procedure were carried out at 0–4°C to prevent possible redistribution of polyamines between cellular compartments. Because previous studies have shown that polyamines are found mainly in the insulin secretory granules (18), it was important to ascertain the purity of the nuclear fraction. For this purpose, we determined the insulin content in acid-ethanol extracts of the pellets obtained after perchloric acid extraction. The results revealed that the nuclear fraction contained ~1% of total cellular insulin as determined by RIA (16). This means that the nuclear fraction was essentially devoid of secretory granule contamination.

Islet DNA synthesis and contents of DNA. Islets in groups of 50–150 were cultured for 2 days as described above. During the last 5 h of culture, 1 μCi/ml [methyl-3H]thymidine was present in the culture medium. At the end of the labeling period, islets were washed in PBS and homogenized in 0.3 M perchloric acid, and the acid-insoluble material was pelleted by centrifugation, solubilized in redistilled water, sonicated, and precipitated in ice-cold 10% TCA. The precipitate was washed twice in TCA and dissolved in 50 μl of Soluene. The radioactivity incorporated was determined by scintillation counting after addition of 1 ml of Unisolve. Duplicate samples of the aqueous homogenates were analyzed fluorometrically for DNA (17, 20).

For determination of labeling indexes, batches of 25–30 islets cultured as described above in the presence of 1 μCi/ml [3H]thymidine for the final 5 h were used. After careful rinsing, islets were fixed in Bouin’s solution and subsequently processed for autoradiography as previously described (3, 36). A minimum of 2,000 cells (in some cases 4,000
cells) were counted, and a labeled cell was defined as carrying 
≥15 grains over the nucleus.

**Insulin production and secretion.** Cultured islets in duplicate
groups of 20 were incubated at 37°C and pH 7.4 for 2 h
in a bicarbonate buffer (41) containing 10 mM HEPES, 2
mg/ml BSA, 16.7 mM glucose, and 50 μCi/ml L-[4,5-3H]
leucine. After incubation, the islets were washed in PBS and
ultrasonically homogenized in redistilled water. Rates of
(proinsulin biosynthesis were measured using an immuno-
precipitation technique (41).

The islet insulin content (extracted from sonicates over
night at 4°C in 70% ethanol plus 0.18 M HCl) was measured
by RIA (16).

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night at 4°C in 70% ethanol plus 0.18 M HCl) was measured
by RIA (16).

For short-term insulin release experiments, duplicate
batches of 10 islets were selected and preincubated at 37°C
for 45 min in a bicarbonate buffer (41) supplemented with 2
mg/ml BSA, 3.3 mM glucose, and 10 mM HEPES (pH 7.4).

The preincubation media were discarded, incubations were
continued for another 60 min in fresh buffer, and media were
frozen for subsequent analysis of their insulin concentration
(16). Fresh media, now containing 16.7 mM glucose, were
added to the same islets, and incubations were continued for
another 60 min; media were then frozen for subsequent
analysis of their insulin concentration (16). Polyamine syn-
thesis inhibitors were not present during these short-term
experiments.

**Statistical analysis.** Means ± SE were calculated, and
values were compared using Student's t-test for
unpaired data. In case of multiple comparisons, data were
compared by multivariate factorial ANOVA in combination with
Bonferroni's modified t-statistics by use of a StatView 512+
(version 1.0) software package from Abacus Concepts and
BrainPower (Calabasas, CA).

### RESULTS

#### Whole cell and nuclear polyamine content.**
The spermine-to-spermidine ratio was markedly increased
in islets from 2-mo-old obese animals compared with lean
mice of the same age (Table 1). In fresh islets isolated
from aged (8-mo-old) obese mice, the spermidine content
was significantly reduced compared with that in
2-mo-old obese animals (Table 1). The content of
spermine was, however, not altered in the islets of the
older mice. Also, the spermine-to-spermidine ratio was
further elevated in the islets from the 8-mo-old obese
mice compared with the young animals. In all groups,
the islet content of spermidine decreased markedly
during the 2-day culture period compared with freshly
isolated islets (Table 1). In contrast, the spermine content
was decreased only in cultured islets of 8-mo-
old obese mice. We are not quite sure why polyamines
fall during culture, but we have repeatedly observed it
in previous islet experiments. It could of course be an
artifact of culture, meaning that the tone of control of
polyamine formation normally maintained in vivo may
be lost in vitro. The islet contents of spermidine and
spermine were significantly reduced in all groups cul-
tured for 2 days in the presence of DFMO (5 mM) +
EGBG (100 μM).

There were no effects of the polyamine synthesis
inhibitors on the polyamine content of islet cell nuclei
from lean or obese animals (Table 2). There was also no
difference in nuclear polyamines when correcting for
differences in islet size (as inferred from DNA con-
tenents). It was not feasible to directly measure the DNA
content of the nuclear fraction, because the fraction-
ation procedure seemingly interfered with the DNA
assay.
Table 3. Influence of culture, obesity, age, and polyamine synthesis inhibitors on islet DNA synthesis rate, labeling index, and insulin content

<table>
<thead>
<tr>
<th>Cultured Islets</th>
<th>DNA Synthesis, cpm/μg DNA</th>
<th>Labeling Index, %</th>
<th>Insulin Content, ng/μg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean 2 mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Inhibitors</td>
<td>1,046 ± 140</td>
<td>0.30 ± 0.04</td>
<td>1,647 ± 133</td>
</tr>
<tr>
<td>+ Inhibitors</td>
<td>2,364 ± 521</td>
<td>0.80 ± 0.03</td>
<td>1,138 ± 125</td>
</tr>
<tr>
<td>Obese 2 mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Inhibitors</td>
<td>550 ± 90</td>
<td>0.21 ± 0.07</td>
<td>2,036 ± 278</td>
</tr>
<tr>
<td>+ Inhibitors</td>
<td>253 ± 33</td>
<td>0.07 ± 0.01</td>
<td>1,636 ± 194</td>
</tr>
<tr>
<td>8 mo Inhibitors</td>
<td>380 ± 99</td>
<td>0.17 ± 0.05</td>
<td>2,323 ± 207</td>
</tr>
<tr>
<td>+ Inhibitors</td>
<td>135 ± 31</td>
<td>0.05 ± 0.002</td>
<td>2,577 ± 321</td>
</tr>
</tbody>
</table>

Values are means ± SE for 8–9 (DNA synthesis), 3–4 (labeling index), and 8–10 (insulin content) observations. Islets were cultured after 2 days of culture in RPMI 1640 medium + 10% calf serum with or without inhibitors of polyamine synthesis (5 mM DFMO, 200 μM MAP, or 100 μM EGBG). Statistical analysis of differences between groups was performed by multiway factorial ANOVA in conjunction with Bonferroni’s modified t-statistics. *P < 0.01, **P < 0.001 vs. islets from lean animals cultured with polyamine synthesis inhibitors. *P < 0.05, *P < 0.01, *P < 0.001 vs. islets cultured without polyamine synthesis inhibitors. **P < 0.001 vs. islets from lean animals cultured without polyamine synthesis inhibitors.

Islet DNA synthesis. After 2 days of culture, there was a significant decrease in [3H]thymidine incorporation in islets from obese mice compared with lean mice (Table 3). In islets from lean 2-mo-old mice, treatment with DFMO + MAP + EGBG evoked a significant increase in [3H]thymidine incorporation and labeling index (Table 3). In contrast, [3H]thymidine incorporation rates were markedly depressed by the polyamine synthesis inhibitors in islets from the obese animals at 2 and 8 mo of age. Labeling indexes in the obese islets decreased as well, but the difference did not attain statistical significance for the islets of the 8-mo-old mice. In a separate set of experiments conducted with islets from 8-mo-old obese mice only, DFMO (5 mM) alone failed to affect the [3H]thymidine incorporation rate, whereas EGBG (100 μM) alone was not as effective as DFMO + EGBG in this respect, evoking a 65–70% decrease in DNA synthesis (Fig. 1).

Insulin production and secretion. The insulin content in islets isolated from 2-mo-old lean, but not obese, mice was significantly reduced by treatment with DFMO + MAP + EGBG (Table 3). These drugs also failed to influence the content of insulin in islets isolated from 8-mo-old obese animals. In a separate set of experiments conducted with islets from 8-mo-old obese mice only, DFMO (5 mM) alone failed to affect insulin production, whereas EGBG (100 μM) alone was as effective as DFMO + EGBG in this respect, significantly impairing (pro)insulin biosynthesis (Fig. 2). When islets from this latter series were incubated for 60 min in low or high glucose concentrations, glucose-sensitive, but not basal, insulin release was significantly impaired in islets cultured in the presence of EGBG but not in islets treated with DFMO alone (Fig. 3).

DISCUSSION

We have 1) analyzed the polyamine content of fresh islets isolated from diabetic obese-hyperglycemic and lean mice of different ages, assumed to reflect the in vivo situation, and 2) compared differences in requirement of polyamines for proliferation and hormone con-
tent between young and senescent islets of obese-hyperglycemic mice, on one hand, and lean and obese mouse islets, on the other. This was done in an attempt to delineate any differences in polyamine synthesis and requirements between diabetic mice and healthy animals and whether these parameters are altered in aged islets. The obese-hyperglycemic syndrome in these mice in many respects resembles type 2 diabetes mellitus and has frequently been used as an animal model for human diabetes (12, 13).

Direct addition of spermidine and spermine to cell cultures is not always feasible because of toxic effects due to polyamine-catabolizing enzymes present in serum and also interactions with cell membranes (29, 37). A way of obviating this problem is to use selective inhibitors of rate-limiting enzymes in polyamine biosynthesis. The results show that freshly isolated islets from 2-mo-old obese-hyperglycemic mice showed an increased spermine-to-spermidine ratio compared with those of lean animals. Considering that the islet cell replicatory activity in the obese mice is maximally enhanced at this age (3), and because spermine is the polyamine essential for DNA replication (31, 33, 41), the elevated ratio likely represents a preferential spermine synthesis related to the enhanced rate of proliferation. The crucial issue of whether this event is the cause or the consequence of the increased \( \beta \)-cell replication was addressed by the use of polyamine synthesis inhibitors. This treatment elicited not only the expected decrease in polyamine content but also resulted in a marked decrease in DNA synthesis. These findings suggest that spermine indeed may be involved in mediating the rapid \( \beta \)-cell replication associated with the obese-hyperglycemic syndrome. Nevertheless, because the specificity of EGBG is not absolute (31), it cannot be excluded that part of the actions of EGBG observed here may not be attributable solely to spermine depletion.

Our present findings indicate that the labeling index of islets from diabetic obese mice was not different from that of lean mice after culture. This means that the enhanced DNA synthesis known to occur in vivo in islets of obese mice (3) is normalized by 2 days of tissue culture. Unfortunately, it is not possible for technical reasons to perform reliable DNA synthesis measurements in vitro on freshly isolated islets, because the collagenase isolation procedure seems to severely interfere with thymidine uptake and metabolism (36). The use of labeling index measurements is a well-established method for studies of islet cell DNA synthesis (technique reviewed in Ref. 36). While it is clear that changes in this index do not necessarily imply that mitotic cell division is altered, a net change in cell number would be impossible to detect after 2 days in culture, because only \(-2\% \) of adult islet cells traverse the cell cycle. In all groups, there was a decrease in polyamines, particularly spermidine, during culture. This phenomenon was reported by us previously (41), but it is not known why it is occurring.

The \( \beta \)-cell replicatory capacity decreases during senescence (3, 35), and so does the expression of polyamine biosynthetic enzymes in other cell systems (7), suggesting a possible interrelationship between these two phenomena. In the presently studied fresh islets isolated from aged obese mice, there was a clear decrease in the content of spermidine, whereas the spermine content did not differ from that of young obese controls, suggesting that the lowered proliferative activity of the aged cells was not due to a change in spermine content. Further characterization of the subcellular polyamine distribution revealed that the nuclear polyamine content was not altered by the polyamine synthesis inhibitors in any group. Thus it appears that the \( \beta \)-cell is equipped with a mechanism translocating spermidine and spermine into the nucleus, when there is a depletion of cytoplasmic polyamines. This may serve as a means by which the \( \beta \)-cell in islets of the lean mice can amplify its rate of DNA synthesis, despite a decrease in the cytosolic polyamine content. Such reversible fluxes of polyamines between nuclei and cytosol are also likely to take place in
normal conditions. The elevated DNA synthesis in lean mouse islets, occurring despite a decreased cytosolic polyamine content, may be conveyed also by other mechanisms that transduce the mitogenic message into the nucleus. One such mechanism could be the polyamine-dependent protein kinases, which make them more susceptible to polyamine synthesis inhibitors than β-cells from normal mice. In agreement with this, previous reports have indicated a number of functional and secretory abnormalities in islets from the diabetic ob/ob mice (4–6, 8–11, 14, 21, 40, 43).

Another area in which polyamines in general, and spermine in particular, have been implicated is insulin production and secretion (18, 32–34, 41). These studies were conducted in normal mouse or rat islets and demonstrated a glucose-regulated spermine content, suggesting a stimulatory or permissive role of spermine at multiple sites of insulin production and glucose-sensitive insulin release, results reproduced in the present study. Such a role is also in conformity with the present observations of an increased spermine-to-spermidine ratio in conjunction with a previously reported enhanced (pro)insulin biosynthesis (2) and hypersensitive secretory response to glucose (5, 11, 21, 40) in islets of the obese-hyperglycemic mouse.

Because pancreatic islets contain a mixed population of hormone-secreting cells, it cannot completely be excluded that the effects reported here to a minor extent may result from non-β-cells. However, because in the islets of obese-hyperglycemic mice there is a marked enrichment in insulin-producing β-cells (40, 42), this possibility seems remote. Likewise, the proportion of β-cells within the islet changes with time and also between lean and obese mice. Although the differences are not great, they should be taken into consideration when viewing the results.

It is concluded from the present data that islets from obese-hyperglycemic mice exhibit important qualitative and quantitative differences in their polyamine content and requirement of polyamines for cell proliferation compared with lean mice of the same strain. The results furthermore conform to the view that spermine may be involved in mediating the rapid islet cell proliferation noted early in the obese-hyperglycemic syndrome, but changes in spermine do not seem to account for the decline in DNA synthesis in senescent β-cells. Whether these differences in the formation and requirements of polyamines between islets from lean and obese-hyperglycemic mice also exist in islets from diabetic patients remains to be elucidated.

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