Insulin Does Not Mediate Glucose Stimulation of Proinsulin Biosynthesis

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It has recently been suggested that insulin augments its own production by a physiologically important feed-forward autocrine loop. We studied the kinetics of glucose-regulated proinsulin gene expression and proinsulin biosynthesis in normal rat islets with emphasis on the potential role of insulin as a mediator of the glucose effect. There was a time-dependent increase in steady-state proinsulin mRNA in islets cultured at 16.7 mM glucose compared with 3.3 mM glucose; no early (1–3 h) increase in proinsulin gene expression was observed. In contrast, there was a threefold increase in proinsulin biosynthesis within 1 h of glucose stimulation that was not affected by inhibition of glucose-stimulated proinsulin gene transcription with actinomycin D. In addition, inhibition of glucose-stimulated insulin secretion with diazoxide had no effect on glucose-stimulated proinsulin mRNA or biosynthesis. Furthermore, addition of different concentrations of insulin to islets cultured in low glucose failed to affect proinsulin biosynthesis. Taken together, our data suggest that the early glucose-dependent increase in proinsulin biosynthesis is mainly regulated at the translational level, rather than by changes in proinsulin gene expression. Moreover, we could not demonstrate any effect of insulin on islet proinsulin mRNA level or rate of proinsulin biosynthesis. Thus, if insulin has any effect on the proinsulin biosynthetic apparatus, it is a minor one. We conclude that the secreted insulin is not an important mediator of insulin production in response to glucose. Diabetes 52: 998–1003, 2003

Glucose is the main physiological regulator of insulin secretion and production in pancreatic β-cells (1,2). It was reported ~30 years ago that glucose increases proinsulin gene expression and proinsulin biosynthesis (3,4). It was furthermore accepted that over short periods (2 h or less), glucose regulates proinsulin biosynthesis mainly by increasing the translation of proinsulin mRNA (5). Indeed, under resting conditions, the β-cell contains a large pool of inert proinsulin mRNA, which is recruited to membrane-bound polyribosomes in the endoplasmic reticulum in response to elevation of the glucose concentration. This translational response, which is independent of changes in proinsulin mRNA concentration, leads to a 30- to 50-fold increase in the rate of proinsulin biosynthesis (6). Over longer time intervals, glucose also increases proinsulin mRNA content by both stimulating proinsulin gene transcription and stabilizing proinsulin mRNA (7,8). We have recently shown that glucose stimulation of proinsulin gene transcription is required for maintaining augmented proinsulin biosynthetic rates under conditions of chronic secretory demand (9).

Recently, Leibiger et al. (10) suggested that proinsulin mRNA is rapidly increased in response to glucose stimulation followed by rapid degradation. Furthermore, it was claimed that the early increase in proinsulin mRNA accounts for 50% of glucose-stimulated proinsulin biosynthesis (11). This biosynthetic response to glucose was mediated by the secreted insulin, suggesting the presence of an autocrine pathway in which insulin stimulates its own production (12–15). The mechanism by which insulin exerts stimulation on its own transcription was suggested to involve phosphatidylinositol 3 (PI3) kinase, p70 S6 kinase, and the calcium-calmodulin kinase pathways (12,16). This theme was further developed by several groups, either by disrupting the insulin receptor or its signaling pathway in pancreatic β-cells of transgenic mice (14,17) or by overexpressing the insulin receptor and its downstream signaling molecules in β-cells (13,18). Specific inactivation of the insulin receptor in pancreatic β-cells results in progressive depletion of islet insulin content, selective loss of first-phase glucose-stimulated insulin secretion, and diabetes as a consequence (14). General disruption of the insulin receptor substrate 2 in mice resulted in reduced β-cell mass and diabetes (17). These findings may have wide implications in type 2 diabetes because a defect in insulin signaling could explain both the impaired β-cell function and insulin resistance.

Inactivation of the insulin receptor and/or its signaling, however, may have additional effects on β-cell survival and other key regulatory elements of β-cell physiology, such as glucokinase (19). Therefore, the relevance of these experiments for β-cell physiology and pathophysiology in unclear. To clarify this subject, we studied the kinetics of glucose-regulated proinsulin gene expression and proinsulin biosynthesis in normal rat islets with emphasis on the potential role of insulin in mediating the glucose effect on insulin production.

RESEARCH DESIGN AND METHODS

Islet isolation and culture. Male Wistar rats (140–170 g body wt) were obtained from Harlan (Jerusalem, Israel). Animals were anesthetized with...
ketamine hydrochloride (Ketalar, Park-Davis). The studies were approved by the Institutional Animal Care and Use Committee of the Hebrew University and Hadassah Medical Organization.

Islets were prepared by collagenase digestion (Collagenase P, Roche Diagnostics) as described (20). The islets were hand-picked once under the stereomicroscope, followed by purification on Histopaque 1083 density gradient (Sigma, St. Louis, MO), 10 min at 480g, and used after repeated washes with Hank’s balanced salt solution. Batches of 200–300 islets of similar size were collected and maintained in suspension in 5 ml of RPMI-1640 medium (Biological Industries, Beit-Haemek, Israel) containing 5.5 mmol/l glucose and 10% fetal bovine serum (FBS) (Biological Industries) at 37°C in 5% CO2 atmosphere for up to 24 h. Islets were then preincubated in medium containing 3.3 mmol/l for 90 min and then cultured in medium containing 3.3 or 16.7 mmol/l glucose for different periods of time. In studies in which actinomycin D was used to inhibit gene transcription, islets were first incubated with 5 μg/ml actinomycin D (Sigma) in RPMI-1640 for 1 h at 37°C, washed once in fresh RPMI-1640, then transferred to the incubation medium as indicated by the various experimental protocols. Only a 1-h exposure to actinomycin D was used to reduce potential toxicity to the islets; this was sufficient to inhibit glucose-stimulated proinsulin mRNA levels for at least 24 h (9). To study the role of secreted insulin in glucose-stimulated proinsulin gene expression and proinsulin biosynthesis, we added 10 μmol/l diazoxide to medium containing 16.7 mmol/l glucose for 24 h. In another set of experiments, different concentrations of insulin (0.5–500 μIU/ml) were added to culture medium containing 3.3 mmol/l glucose for 24 h.

Insulin release and content during culture. At the end of the incubation period, the culture medium was collected, centrifuged, and frozen at −20°C pending insulin analysis. Islets were collected and counted for reference. Islet insulin content was determined by radioimmunoassay (RIA) in extracts of batches of 25 islets subjected to repeated freeze-thaw cycles in 1.5-ml microfuge tubes containing 450 μl of 0.1% BSA in 0.1 N HCl, followed by centrifugation.

Proinsulin biosynthesis. Groups of 25 islets were collected after culture and preincubated for 1 h at 37°C in modified Krebs-Ringer bicarbonate buffer containing 20 mmol/l HEPES and 0.25% BSA (KRBB-BSA), supplemented with glucose at the concentration used during culture. After preincubation, the KRBB-BSA buffer was collected after centrifugation for determination of insulin secretion, and the islets were labeled in 50 μl of fresh KRBB-BSA buffer containing glucose as above and 25 μCi of [5,6-3H]leucine (SA, 150 Ci/mmol; Amersham, Aylesbury, U.K.). After a 15-min pulse at 37°C, leucine incorporation was terminated by the addition of 1 ml of ice-cold glucose-free KRBB-BSA buffer and rapid centrifugation. The islet pellet was suspended in 450 μl of 0.1% BSA in 0.1 N HCl and extracted by repeated freeze-thaw cycles, and the high-speed microfuge supernatant was collected and stored at −20°C for further processing by high-performance liquid chromatography, as described (21). Aliquots were used for determination of total insulin content by RIA, as well as for measurement of total protein biosynthesis by trichloroacetic acid precipitation (22). In the following, “total proinsulin biosynthesis” refers to incorporation of labeled leucine into proinsulin-like peptides, whereas “specific biosynthesis” is that after correction of glucose-induced total protein biosynthesis.

Insulin determination. Insulin was determined by RIA using anti-insulin coated tubes (ICN Pharmaceuticals, Costa Mesa, CA) and [125I]-insulin from Linco Research (St. Charles, MO). The routine intra-assay CV was 4–6%, and interassay CV was 6–10% (23). Rat insulin standards from Novo-Nordisk (Bagvaerd, Denmark) were used for determination of rat insulin-like immunoreactivity.

Quantification of proinsulin mRNA by RT-PCR. Total islet RNA was extracted with RNAzol B (Tel-Test, Friendswood, TX). For PCR analysis, total RNA was reverse transcribed using AMV reverse transcriptase (Promega, Madison, WI). The resulting cDNAs were amplified by PCR using oligonucleotides complementary to sequences in the rat proinsulin I gene: 5’-CCCTGACCGGTCTTTGTCAs-3’ and 5’-GGTTGACAGCTCTGACCAAATG-3’. Primers were designed to cross an intron and amplified fragments of 208 bp of the coding sequence of the rat proinsulin I gene. 18S rRNA (QuantumRNA kit; Ambion, Austin, TX) was used as an internal control. Polymerization reaction was performed in a 25-μl reaction volume containing 2.5 μl of cDNA (25 ng RNA equivalent), 300 μmol cold dNTPs, 2.5 μl of each primer, 100 μmol/l of appropriate oligonucleotide primers, and 1.5 units of Taq polymerase (MBI Fermentas, Amherst, NY). PCR amplification conditions and analysis were as follows: 5 min at 94°C followed by 14 cycles of 94°C, 60°C, and 72°C, 90 s each step. The amplimers were separated on a 6% polyacrylamide gel in Tris borate EDTA buffer, the gel was dried, and the incorporated [3H]CTP was measured by PhosphorImager. The number of cycles and the amplification conditions were adjusted to the exponential range phase of the amplification curve for each product (24). For quantification of proinsulin mRNA, the ratio of proinsulin/18S rRNA band intensity was determined for each reaction. In studies using actinomycin D, proinsulin mRNA/18S rRNA ratio in islets incubated at 16.7 mmol/l glucose with actinomycin D was compared with similarly treated islets at 3.3 mmol/l glucose.

Data presentation and statistical analysis. Data are expressed as mean ± SE for the indicated number of individual experiments, each done on a batch of islets pooled from six to eight animals. Groups were compared using ANOVA followed by Newman-Keuls test using the InStat statistical program from GraphPad Software (San Diego, CA). P < 0.05 was considered significant.

RESULTS

Kinetics of glucose-stimulated proinsulin gene expression and biosynthesis. We previously found that proinsulin mRNA increased in response to high glucose after at least 6 h of incubation (9). These studies were performed after a relatively short period of recovery after islet isolation, which could account for our failure to observe a rapid glucose effect on proinsulin mRNA as described by Leibiger et al. (10,11). Therefore, we allowed the islets to recover overnight in RPMI-1640 medium containing 5.5 mmol/l glucose before culturing at different glucose concentrations. Notwithstanding, exposure of isolated rat islets to 16.7 mmol/l glucose for 1 and 3 h did not increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase in proinsulin mRNA of 1.7-, 2.2-, and 2.6-fold in islets cultured at high glucose for 6, 12, and 24 h, respectively (Fig. 1). Basal proinsulin mRNA decreased over time in culture; however, the stimulatory effect of glucose on proinsulin biosynthesis was maintained. A similar decrease in basal proinsulin mRNA in cultured rat islets has been noted before (8,9,25).
Total and specific proinsulin biosynthesis rapidly increased in response to glucose stimulation, reaching maximal rate within 1 h of culture at 16.7 mmol/l glucose and maintaining this rate for up to 24 h (Fig. 2). The basal rate of proinsulin biosynthesis after different times of culture at 3.3 mmol/l showed some variation. There was a transient decrease at 3 and 6 h that returned to the initial rate at 24 h. Exposure to 16.7 mmol/l glucose resulted in a 3- to 9- and 1.5- to 2.5-fold increase in proinsulin biosynthesis, respectively, at different time points (Fig. 2).

Our failure to detect a small increase in glucose-induced proinsulin mRNA level could be related to potential limited sensitivity of the assay used in this study. Such an increase could still be important for an adequate biosynthetic response to glucose. To answer this question, the transcription inhibitor actinomycin D was added to the culture medium. We have previously shown that actinomycin D efficiently inhibits glucose-stimulated proinsulin gene expression, resulting in a 50% decrease of proinsulin biosynthetic response after 24 h of incubation (9). Exposure of islets to actinomycin D abolished the glucose-dependent increase of proinsulin mRNA (Fig. 3A). Nevertheless, inhibition of glucose-stimulated proinsulin gene transcription did not prevent the rapid increase in proinsulin biosynthesis in response to short-term stimulation (1 h) by 16.7 mmol/l glucose (Fig. 3B and C). Thus, our data show that the proinsulin mRNA response to high glucose is time-dependent and that an early increase in proinsulin mRNA is not required for the rapid stimulation of proinsulin biosynthesis.

**Role of insulin in glucose-stimulated proinsulin gene expression and biosynthesis.** To test the hypothesis that the secreted insulin is an important mediator of glucose-stimulated proinsulin gene expression and proinsulin biosynthesis, we added diazoxide to the culture medium to inhibit insulin release. Indeed, there was nearly 90% inhibition of insulin release in islets cultured at 16.7 mmol/l glucose in the presence of diazoxide (Fig. 4A) to levels similar to those observed at basal glucose (3.3 mmol/l). The inhibition of insulin release was associated with an 70% increase in islet insulin content (Fig. 4B). Diazoxide did not inhibit glucose-stimulated proinsulin gene expression (Fig. 4C) or proinsulin biosynthetic response to glucose (Fig. 4D and E), indicating that glucose-stimulated insulin secretion is not required for glucose-regulated insulin production.

Exposure of rat islets cultured at low glucose to 500 mmol/l insulin, a concentration similar to that observed in the medium after 24 h of incubation at 16.7 mmol/l
glucose, had only a minimal effect on proinsulin mRNA (Fig. 5), with no effect on proinsulin biosynthesis (Fig. 6). Lower concentrations of insulin did not affect either proinsulin mRNA or biosynthesis (not shown). The failure to detect an insulin effect on proinsulin biosynthesis could be related to loss of insulin responsiveness over time in culture. In an attempt to address this question, we studied insulin effect on proinsulin biosynthesis also in freshly isolated islets without prolonged (24 h) recovery in the culture medium (Fig. 6). Under these conditions, 16.7 mmol/l glucose stimulated 10- to 15-fold the total proinsulin biosynthesis and ~3-fold the specific proinsulin biosynthesis; none of these parameters was influenced by insulin.

FIG. 4. Effect of diazoxide on glucose-stimulated insulin secretion, insulin content, proinsulin mRNA, and proinsulin biosynthesis. Isolated rat islets were cultured for 24 h in the presence of 3.3 and 16.7 mmol/l glucose, or 16.7 mmol/l glucose with 10 μmol/l diazoxide. A: Insulin secretion. B: Insulin content. C: Proinsulin mRNA. D: Proinsulin biosynthesis per islet. E: Specific proinsulin biosynthesis normalized to total protein biosynthesis. The insert in C shows a representative quantitative PCR experiment; the upper band is 18S rRNA, and the lower band is proinsulin mRNA (the order from left to right is as indicated on the x-axis). Results are mean ± SE of four individual experiments, each using islets pooled from six to eight rats. *P < 0.01 for the difference in insulin secretion (A) between 16.7 mmol/l glucose and either 3.3 mmol/l glucose or 16.7 mmol/l glucose + diazoxide, and for the difference in biosynthesis (D and E) relative to 3.3 mmol/l glucose. **P < 0.05 for the difference in insulin content (B) between 16.7 mmol/l with diazoxide and 16.7 mmol/l glucose alone, and for the difference in proinsulin mRNA (C) between 16.7 mmol/l glucose with and without diazoxide and 3.3 mmol/l glucose.

DISCUSSION

The main messages of this article are that 1) there is no rapid increase in steady-state proinsulin mRNA levels in response to glucose, 2) the early increase in proinsulin biosynthesis is regulated at the translational level, and 3) insulin has minimal effect, if any, on proinsulin mRNA levels and proinsulin biosynthesis. Thus, the insulin secreted during glucose stimulation of the islet does not act as an autocrine mediator of insulin production.

These data argue against the presence of a physiologically important autoregulatory loop in insulin production and are in marked contradiction with those of Leibiger et al. (10,11), who reported increased proinsulin biosynthesis paralleled by a fivefold increase in steady-state proinsulin mRNA levels within <1 h of exposure of rat islets to high glucose. Fifty percent of this early increase in proinsulin biosynthesis was attributed to the rapid regulation of proinsulin gene transcription by glucose. Transfection and nuclear run-off experiments showed only an approximate twofold stimulation of proinsulin gene transcription (10). Furthermore, the stimulatory effect of glucose was short-lived; transcriptional activity was maximal at 30 min but markedly decreased thereafter. Under resting conditions, the β-cell contains a large pool of cytoplasmic proinsulin mRNA, amounting to 10–15% of the total cellular mRNA (26). Furthermore, the half-life of existing proinsulin mRNA is relatively long (29 and 77 h in rat islets cultured at 3.3 and 17 mmol/l glucose, respectively) (8). Therefore, in quantitative terms, it is difficult to explain the severalfold increase of the steady-state proinsulin mRNA after short-term glucose stimulation, which induced a relatively modest and short-lived transcriptional response. We found a glucose-stimulated increase in proinsulin mRNA level after 6 h in culture. Activation of transcription by glucose can start within minutes (27); however, the accumulation of proinsulin mRNA against the high background of steady-state mRNA is time-dependent and progressive. It is unlikely that the contradiction between our data and those of Leibiger et al. is explained by the fact that the islets used in our study are less responsive to glucose; we obtained a two- to threefold increase in proinsulin mRNA levels after exposure to high glucose, and there was a
three- to ninefold increase in proinsulin biosynthetic activity in response to glucose, similar to that observed by Leibiger et al. (11).

In line with our observation that proinsulin mRNA is not increased rapidly after glucose stimulation, inhibition of glucose-stimulated proinsulin gene transcription with actinomycin D did not prevent the early increase in proinsulin biosynthesis. Taken together, these data suggest that the early biosynthetic response to glucose is regulated at the level of translation rather than transcription; this paradigm is supported by others (6,22,26,28).

The recently published studies (11,12) suggesting an important physiological role for the secreted insulin in glucose-stimulated insulin production are also puzzling. In these studies, islets exposed briefly to low concentrations of insulin showed a marked increase in proinsulin mRNA and proinsulin biosynthesis. Surprisingly, the response to insulin stimulation was higher than that to glucose (12). In contrast, we found that addition of exogenous insulin equivalent to the amount secreted after glucose stimulation (500 nmol/l/25 islets per 24 h) induced only a modest, equivalent to the amount secreted after glucose stimulation. Surprisingly, the response to insulin showed a marked increase in proinsulin mRNA levels. Others suggested that insulin might have a negative feedback effect on its own expression (32).

Transfection experiments suggested that insulin could increase insulin promoter activity, with conflicting data as to the mechanisms involved (13,18). However, artificial systems with overexpression of genes involved in insulin signaling may result in overestimation of the physiological role of this pathway in insulin production. For example, it was recently shown that expression of the insulin receptor and its downstream signaling molecules increase insulin promoter activity; however, exposure of islets to insulin in the presence of a physiological glucose concentration had no effect, whereas glucose increased the production of insulin as expected (18).

It is our firm belief that the physiological role of pathways involved in insulin production are best studied in a physiological β-cell preparation (islets), examining the interactions between steady-state proinsulin mRNA levels, proinsulin biosynthesis, insulin content, and secretion. The present study suggests that secreted insulin has no major physiological role in mediating the glucose effect on insulin production.

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