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 mmol/l compared with 3.3 mmol/l 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
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 proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1). However, a longer period of incubation resulted in an average increase proinsulin mRNA levels (Fig. 1).

**FIG. 1.** Time course of the effect of glucose on proinsulin mRNA in isolated rat islets. Islets were cultured at 3.3 and 16.7 mmol/l glucose for 1, 3, 6, and 24 h. Relative quantitative PCR for proinsulin mRNA and 18S rRNA was performed on cDNA prepared from total RNA extracted from 200–300 islets from each group. Proinsulin gene expression was first normalized to 18S rRNA (internal control) and then expressed as fold of values obtained in islets at 3.3 mmol/l glucose. The upper panel shows a representative time course, and the lower panel shows quantification of the results relative to islets at 3.3 mmol/l glucose. Results are mean ± SE. The number of individual experiments, each performed on islets pooled from six to eight animals, is indicated in parentheses. *P* < 0.001, 24-h cultured islets vs. islets cultured for 1 and 3 h; $P$* < 0.05, 6-h cultured islets vs. 24-h cultured islets and 12-h cultured islets vs. 1-h cultured islets.

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.

**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'-CCTGC CCAGGCTTTGCTCA-3' and 5'-GGTGCAGACTGTCATCCACAATA TG-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 (QuantumRNAakit; 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 μCi of α-32PdCTP, 100 μmol/l of appropriate oligonucleotide primers, and 1.5 units of Taq polymerase (MBI Fermentas, Anherst, 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, 30 s each step. The amplifiers were separated on a 6% polyacrylamide gel in Tris borate EDTA buffer, the gel was dried, and the incorporated α-32PdCTP was measured in a phosphorimager. The number of cycles and the final reaction conditions were adjusted to the exponential range phase of the amplification curve for each product (24). For quantification of proinsulin mRNA, the ratio...
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 biosynthetic rate by 12 and 24 h of culture, suggesting that the culture conditions were not toxic to the islets. Exposure to 16.7 mmol/l glucose resulted in a 3- to 9- and 1.5- to 2.5-fold 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 nmol/l insulin, a concentration similar to that observed in the medium after 24 h of incubation at 16.7 mmol/l glucose.
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 < 0.05 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 with diazoxide, and for the difference in biosynthesis (D and E) relative to 3.3 mmol/l glucose. **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. 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.

FIG. 5. Effect of exogenously added insulin on proinsulin mRNA. Rat islets were incubated for 24 h in 3.3 and 16.7 mmol/l glucose, as well as in 3.3 mmol/l glucose to which insulin (500 nmol/l) was added during the last 6 h or throughout the 24-h incubation. Relative quantitative PCR for proinsulin mRNA and 18S rRNA was studied as described in the legend to Fig. 1. The insert 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 < 0.05 for the difference between islets cultured at 16.7 vs. 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 several-fold increase in 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, nonsignificant, increase in proinsulin mRNA with no effect on proinsulin biosynthesis. Moreover, inhibition of endogenous insulin secretion with diazoxide did not affect glucose-stimulated proinsulin gene expression and biosynthesis. Similarly, Alarcon et al. (29) recently observed that inhibition of insulin secretion by somatostatin or calcium depletion did not affect glucose-stimulated proinsulin biosynthesis. However, inhibition of endogenous insulin secretion with diazoxide did not affect glucose-stimulated proinsulin gene expression and biosynthesis. In addition, augmentation of insulin secretion by depolarization of the plasma membrane of pancreatic β-cells with sulfonylurea analogues (30) or nateglinide (N. Kaiser, unpublished observation) had no effect on proinsulin biosynthesis. Our data are also in accordance with Gasa et al. (31), who found that prevention of insulin secretion with diazoxide had no effect on 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, 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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