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Glucose-stimulated Insulin Biosynthesis Depends on Insulin-stimulated Insulin Gene Transcription*

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Glucose stimulation of pancreatic β-cells leads to insulin secretion as well as up-regulation of insulin biosynthesis. The acute elevation in pro-insulin levels is thought to be exclusively because of the activation of translation of pre-existing prepro-insulin mRNA. Glucose-stimulated insulin gene transcription is believed to be a long term effect and should therefore not contribute to the acute elevation in pro-insulin levels. We have recently shown that glucose activates insulin gene transcription within minutes and that secreted insulin is one of the key factors triggering this process in an autocrine manner. We now provide evidence that 50% of the glucose-stimulated, acute pro-insulin biosynthesis within 30 min results from up-regulated insulin gene transcription. Our data led us to propose that glucose elevates pro-insulin levels by stimulating both transcriptional and post-transcriptional/post-translational events to an equal extent. Whereas the stimulatory effect on transcription is mediated by insulin secreted in response to glucose, glucose directly stimulates the post-transcriptional/post-translational processes.

The maintenance of glucose homeostasis in mammals is of vital importance. To keep blood glucose concentrations within narrow limits, strict regulation, and fast acting mechanisms that guarantee efficient insulin secretion and biosynthesis are necessary. In these processes, glucose itself has been shown to act as the major nutrient regulator by triggering a cascade referred to as the stimulus-response coupling. With regard to insulin biosynthesis, the commonly accepted view is that glucose exerts its immediate effect at the level of translation rather than at the level of insulin gene transcription. As a consequence of this view, glucose-stimulated insulin gene transcription should be uncoupled from glucose-stimulated insulin mRNA translation. Therefore, transcription is unlikely to have an impact on the immediately triggered insulin biosynthesis. The concept or dogma of a long term effect of glucose stimulation on insulin gene transcription is challenged by Efrat *et al.* (1) and by Leibiger *et al.* (2) through nuclear run-off experiments demonstrating that insulin gene transcription is up-regulated within minutes of glucose stimulation rather than hours. By studying the mechanisms that underlie the short term regulation of insulin gene transcription by glucose, we were able to show that insulin secreted in response to glucose stimulation is a key factor in glucose-stimulated insulin gene transcription (3).

Recent reports from several groups demonstrate that insulin indeed has a *stimulatory* role in pancreatic β-cell physiology (3–8). Xu *et al.* (4) reports a positive effect of insulin on β-cell protein biosynthesis and insulin-dependent activation of PHAS-1. The involvement of insulin in the regulation of basal pro-insulin and prepro-insulin mRNA levels is shown by Xu and Rothenberg (5). A similar observation is made in mice carrying a general knockout for insulin receptor substrate-2 (6). A β-cell-restricted knockout of insulin receptors resulted in an impaired first phase insulin secretion and a decrease in insulin content (7). Aspinwall *et al.* (8) demonstrates that insulin stimulates acute insulin secretion. Finally, Leibiger *et al.* (3) demonstrate the involvement of insulin signaling in the short term glucose-stimulated control of insulin gene transcription.

In this study, we showed that secreted insulin positively influences the acute pro-insulin biosynthesis by promoting transcription and translation. Our data provided evidence, for the first time, that short term regulated insulin gene transcription contributes to the immediate up-regulation of pro-insulin biosynthesis.

MATERIALS AND METHODS

Islet Isolation and Culture—Pancreatic islets were isolated from Wistar rats (200–300 g) by collagenase digestion (9). Islets were separated by a Ficoll gradient and then hand-picked. Islet cell suspensions were prepared and washed essentially as described in Ref. 10. Islets and cells of disaggregated islets were incubated overnight 95% air, 5% CO_2 and 37 °C in RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% heat-inactivated fetal calf serum. Islets and islet cells were pretreated in RPMI 1640 medium containing 3 mM glucose, and they were supplemented as above for 90 min before the start of stimulation. Stimulation was for 15 min with either 16.7 mM glucose, 5 milliunits/ml insulin, 100 μM tolbutamide, 1 μM glibenclamide, or 50 mM KCl. Pharmacological inhibitors of voltage-gated L-type Ca^{2+} channels, such as 10 μM nifedipine (Calbiochem) or DNA-dependent RNA polymerase II, *i.e.* 5 μg/ml actinomycin D (Sigma), were added to the culture medium 30 min before stimulation and kept throughout stimulation. Anti-insulin antibodies (ICN) were added 30 min before stimulation to the culture medium (10 μg/ml) and kept throughout stimulation.

Measurement of Pro-insulin Biosynthesis—Groups of 10 islets or 5×10^4 cells were used for each experiment. Stimulation for 15 min with either glucose, insulin, KCl, tolbutamide, or glibenclamide was performed in 2 ml of leucine-free RPMI 1640 medium containing 100 μCi of L-[4,5-^{3}H]leucine (Amersham Pharmacia Biotech). After stimulation, the islets were washed with phosphate-buffered saline (136.9 mM NaCl, 1.5 mM KCl, 3.2 mM KH_2PO_4, 10.1 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.4) and incubated for an additional 15 min in 2 ml of leucine-free RPMI 1640 medium containing 3 mM glucose and 100 μCi of L-[4,5-^{3}H]leucine. After labeling, islets and cells were washed with ice-cold phosphate-buffered

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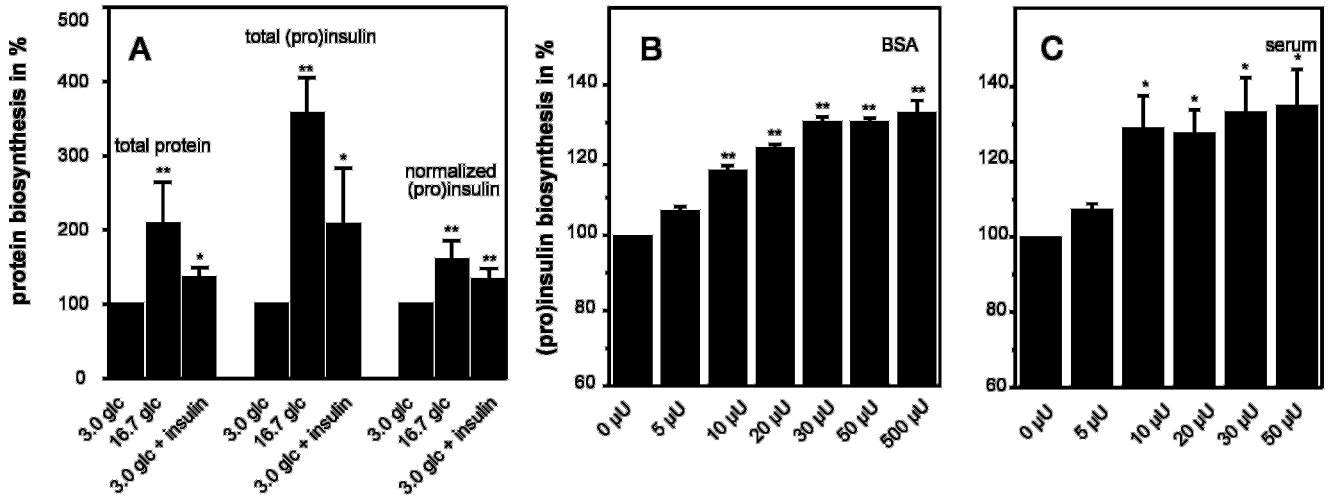

FIG. 1. **Glucose and insulin stimulate acute pro-insulin biosynthesis.** *A,* effect of 15-min stimulation with either 16.7 mM glucose (*glc*) or 5 milliunits of insulin/ml at 3 mM glucose (*glc + insulin*) on acute total protein biosynthesis, acute total pro-insulin biosynthesis, and acute specific pro-insulin biosynthesis, *i.e.* normalized to protein biosynthesis within 30 min in islets. Biosynthesis values are given in percentages relative to those obtained at 3 mM glucose, which were set to 100%. Data are given as means ± S.E. All data are from at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$. *B,* dose-response curve of various exogenous insulins on pro-insulin biosynthesis in cells cultured in medium with bovine serum albumin (*BSA*). *µU,* microunits. *C,* dose-response curve of various exogenous insulins on pro-insulin biosynthesis in cells cultured in fully supplemented medium. Biosynthesis values are given in percentages relative to those obtained at 3 mM glucose, which are set to 100%. Data are given as means ± S.E. All data are from at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

saline, harvested, resuspended in 100 µl of ice-cold immunoprecipitation buffer (25 mM $Na_2B_4O_7$, pH 9.0, 3% (w/v) bovine serum albumin, 1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *n*-[*N*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucine]agmatine, 1 mM EDTA, 20 µM leupeptin, and 0.01% NaN_3) and sonicated two times for 20 s. Immunoprecipitation with guinea pig anti-porcine insulin antibody (ICN) was performed according to Hutton *et al.* (11) using protein A-Sepharose. The washed immunoprecipitates were counted for radioactivity by liquid scintillation counting after dispersion in 1 M acetic acid containing 0.25% bovine serum albumin. Immunoprecipitation of radiolabeled pro-insulin was verified by discontinuous Tricine[1] SDS-polyacrylamide gel electrophoresis (16.5% separating gel, 10% spacer gel, 4% stacking gel) as described in Ref. 12 and by phosphorimaging of the dried gel. Total protein synthesis was measured in aliquots of the samples obtained for pro-insulin biosynthesis. Total protein was precipitated by the addition of 1 volume of 10% trichloroacetic acid. After centrifugation, the pellet was resuspended in 0.15 M NaOH and counted for radioactivity by liquid scintillation counting.

Quantification of Prepro-insulin mRNA Amounts—Levels of prepro-insulin mRNA were analyzed by comparative reverse transcription-PCR as described previously (2, 3) using primers 5′-TGCCCAGGCTTT-TGTCAAAC-3′ and 5′-CTCCAGTGCCAAGGTCTGAA-3′ for prepro-insulin mRNA in rat pancreatic islets. Levels of β-actin mRNA were analyzed by using primers 5′-AACTGGAACGGTGAAGGCGA-3′ and 5′-AACGGTCTCACGTCAGTGTA-3′. Total RNA obtained from 10 pancreatic islets was reverse-transcribed using Moloney murine leukemia virus revertase. Aliquots of the generated cDNA were used for PCR-mediated amplification using the reverse transcription-PCR kit (Stratagene) and α-[^{32}P]dCTP. PCR conditions were chosen that guaranteed the amplification of insulin and actin fragments within the linear range (Fig. 1*C* in Ref. 2). PCR was performed in an AutogeneII-thermocycler (Grant Instruments, Cambridge, U.K.) using a linked program (1 cycle of 5 min at 94 °C, 5 min at 54 °C, and 2 min at 72 °C and 23–27 cycles of 1 min at 94 °C, 2 min at 54 °C, and 2 min at 72 °C). Labeled PCR products were separated on a 6% polyacrylamide sequencing gel and analyzed through phosphorimaging. Quantification was performed with TINA software 2.07d (Raytest) using co-amplified reverse transcription-PCR products for β-actin as the internal standard.

Data Analysis—Data were analyzed by Student's *t* test using the software package Microcal Origin, version 5 (Microcal Software, Inc., Northampton, MA).

RESULTS AND DISCUSSION

The immediate effect of elevated glucose concentration on prepro-insulin mRNA translation is well accepted (reviewed in Ref. 13). Recently, we have shown that glucose stimulation for only 15 min resulted in a transient rise in insulin gene transcription and in a transient elevation of steady-state prepro-insulin mRNA levels (2). We were able to demonstrate that insulin, secreted in response to glucose stimulation, is a key factor involved in the up-regulation of insulin gene transcription (3). Because insulin has been demonstrated as a positive regulator of protein biosynthesis in several tissues (reviewed in Ref. 14) and in pancreatic β-cells (4, 5), we wanted to know whether secreted insulin also contributes to the stimulus-dependent elevation in pro-insulin biosynthesis.

To address the question of whether secreted insulin contributes to pro-insulin biosynthesis, we first compared the effects of short term glucose stimulation (15 min) with that of a short term insulin stimulation at substimulatory glucose concentrations (15 min) on acute overall protein biosynthesis and acute prepro-insulin biosynthesis in isolated pancreatic islets, i.e. within a 30-min period. Therefore, we performed protein labeling with [^{3}H]leucine in response to the stimulation of pancreatic islets for 15 min with either 16.7 mM glucose or 5 milliunits of insulin/ml at 3 mM glucose. We then harvested the islets after culturing for another 15 min at 3 mM glucose (Fig. 1). Both the cultures and stimulations were performed in RPMI 1640 medium supplemented with 10% fetal calf serum. To show that the obtained effect on pro-insulin biosynthesis was a result of stimulation by either glucose or insulin rather than a result of recovery from a lack of glucose, growth factors, and amino acids, we deliberately used culture conditions where the medium was not depleted of serum, glucose, or insulin.

As shown in Fig. 1*A*, total protein biosynthesis was increased by the glucose stimulus up to 3-fold (average 2.08 ± 0.17, $n = 11$, $p < 0.01$) and by the insulin stimulus up to 2-fold (average 1.35 ± 0.13, $n = 9$, $p < 0.05$) within 30 min. Absolute pro-insulin biosynthesis was elevated by glucose up to 6-fold (average 3.56 ± 0.48, $n = 9$, $p < 0.01$) and by insulin up to 3-fold (average 2.07 ± 0.34, $n = 5$, $p < 0.05$), respectively. The effect of glucose and insulin stimulation on specific pro-insulin biosynthesis, *i.e.* after normalization to total protein biosynthesis, was up to 2-fold (average 1.6 ± 0.08, $n = 10$, $p < 0.01$) and up to 1.5-fold (average 1.32 ± 0.05, $n = 9$, $p < 0.01$), respectively (Figs. 1–3).

[1] The abbreviations used are: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PCR, polymerase chain reaction.

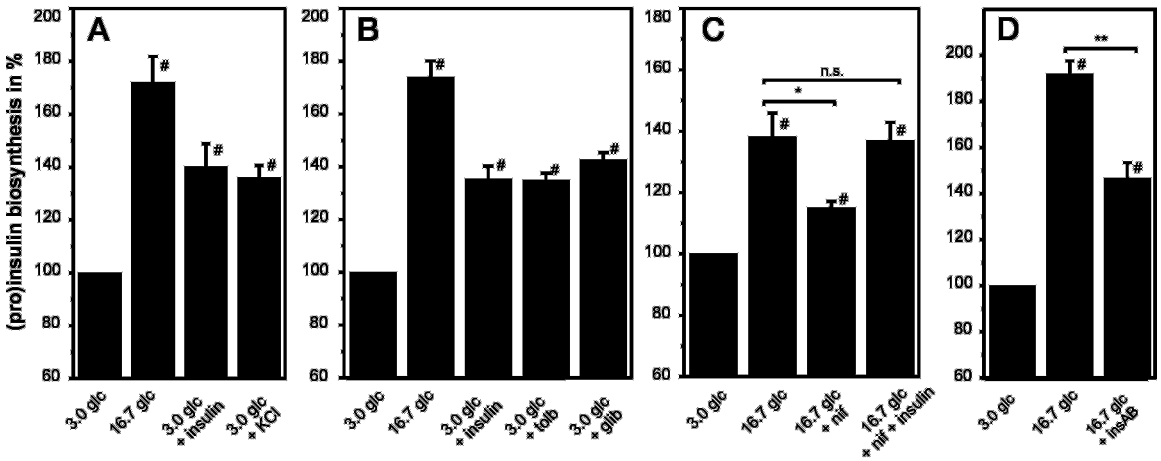

FIG. 2. **Secreted insulin stimulates pro-insulin biosynthesis.** *A*, effect of 15-min stimulation with either 16.7 mM glucose (*glc*) or 50 mM KCl (*3.0 glc + KCl*) on the increase in specific pro-insulin biosynthesis within 30 min in islets. *B*, effect of stimulation with 100 μM tolbutamide (*tolb*) or 1 μM glibenclamide (*glib*) on the increase in specific pro-insulin biosynthesis within 30 min in islets. *C*, effect of the voltage-gated L-type Ca^{2+} channel blocker, 10 μM nifedipine (*nif*), on glucose- or insulin-stimulated increase in specific pro-insulin biosynthesis in islet cells. *D*, effect of absorption of stimulus-secreted insulin by anti-insulin antibodies (*insAB*) on pro-insulin biosynthesis in islet cells. Biosynthesis values are given in percentages relative to those obtained at 3 mM glucose (*glc*), which were set to 100%. Data are given as means ± S.E. All data are from at least three independent experiments. #, statistical significance in relation to the control (*3.0 glc*), *t* test, one population ($p < 0.05$); * and **, statistical significance between compared groups, *t* test, two populations (*, $p < 0.05$; **, $p < 0.01$); *n.s.*, not statistically significant.

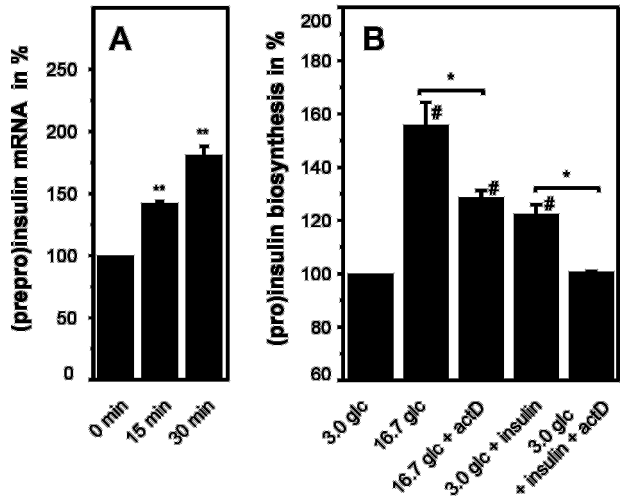

FIG. 3. **Insulin gene transcription contributes to acute pro-insulin biosynthesis.** *A*, effect of a 15-min glucose stimulus at 16.7 mM on prepro-insulin mRNA steady state levels after 0, 15, and 30 min of incubation. Data are given in percentages relative to those obtained at 3 mM glucose (*3.0 glc*), which are set to 100%. Data are given as means ± S.E. All data are from at least three independent experiments. **, statistical significance in relation to the control (*3.0 glc*), $p < 0.01$. *B*, effect of inhibition of transcription by actinomycin D (*actD*) on glucose (*16.7 glc + actD*) or insulin-stimulated (*3.0 glc + insulin + actD*) increase in specific pro-insulin biosynthesis in islet cells. Biosynthesis values are given in percentages relative to those obtained at 3 mM glucose (*3.0 glc*), which are set to 100%. Data are given as means ± S.E. All data are from at least three independent experiments. #, statistical significance in relation to the control (*3.0 glc*), *t* test, one population ($p < 0.05$); *, statistical significance between compared groups, *t* test, two populations ($p < 0.05$).

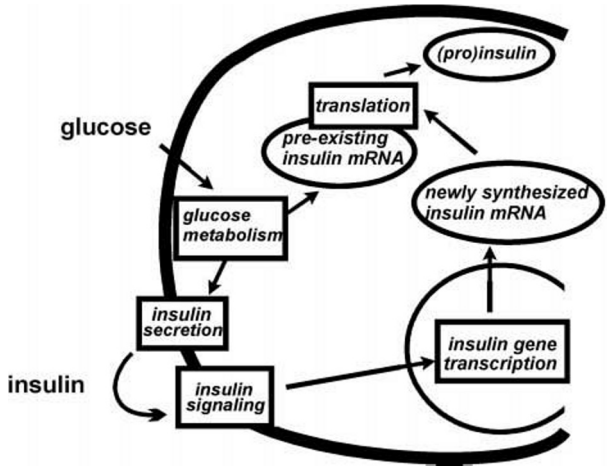

FIG. 4. **Schematic illustration of glucose- and insulin-dependent events in glucose-stimulated specific pro-insulin biosynthesis.**

To evaluate whether the amount of insulin, which is necessary to stimulate pro-insulin biosynthesis, is within the physiological range, we generated a dose-response curve of exogenous insulin on pro-insulin biosynthesis. With regard to insulin gene transcription, we found a stimulatory effect when the endogenous insulin release was higher than 10 microunits/ml per 10 islets per 15 min (3). The range for endogenous release upon stimulation with 16.7 mM glucose was quite broad and could reach levels of up to 100 microunits/ml per 10 islets per 15 min. Therefore, we analyzed the necessary amounts of exogenous insulin to stimulate pro-insulin biosynthesis when culturing islets in the medium without serum, *i.e.* medium supplemented with bovine serum albumin. As shown in Fig. 1*B*, the addition of 10 microunits of insulin/ml of medium (*i.e.* 60 pmol) was necessary to significantly increase pro-insulin biosynthesis within a 30-min period. The addition of 30 microunits/ml (*i.e.* 180 pmol) triggered the maximal response. Next we wanted to know the necessary amount of exogenous insulin to stimulate pro-insulin biosynthesis when culturing islets in fully supplemented medium. As illustrated in Fig. 1*C*, here also the addition of 10 microunits of insulin/ml of medium was necessary to significantly increase pro-insulin biosynthesis within a 30-min period. Addition of exogenous insulin above the physiological range did not lead to a further increase in pro-insulin biosynthesis.

To test whether endogenous insulin that secreted upon stimulation had a similar effect as exogenous insulin, we next stimulated the isolated pancreatic islets for 15 min with 50 mM KCl at 3 mM glucose. This allowed insulin secretion without the additional effects of glucose metabolism. Indeed, KCl-stimulated insulin secretion resulted in a similar 55% elevation of specific pro-insulin biosynthesis within 30 min (Fig. 2*A*). To

exclude an unspecific effect of KCl and to further demonstrate that insulin secreted by the β-cell promotes pro-insulin biosynthesis, we tested the effects of classical insulin secretagogues, *i.e.* the sulfonylurea compounds, tolbutamide and glibenclamide, on acute pro-insulin biosynthesis. Indeed, stimulation with either 100 μM tolbutamide or 1 μM glibenclamide at 3 mM glucose led to a similar amount of increase in pro-insulin biosynthesis as obtained by the addition of exogenous insulin (Fig. 2*B*) or stimulation with 50 mM KCl (Fig. 2*A*).

To test the effects of glucose metabolism without the additional effects of secreted insulin, we stimulated islet cells for 15 min with 16.7 mM glucose but blocked the depolarization-mediated influx of Ca^{2+} via voltage-gated L-type Ca^{2+} channels and the subsequent insulin secretion by treatment with 10 μM nifedipine. This protocol has been shown to abolish the Ca^{2+} influx via L-type Ca^{2+} channels and to completely block insulin secretion (15). As shown in Fig. 2*C*, nifedipine treatment decreased glucose-stimulated, pro-insulin biosynthesis by roughly 50%.

So far our data suggest that neither secreted insulin nor glucose metabolism *per se* can account for the total effect on acute glucose-stimulated, pro-insulin biosynthesis, but each of the stimuli had an impact of approximately 50%. If both effects were independent, they should be additive. To test this hypothesis, we stimulated pancreatic islets with 16.7 mM glucose and blocked secretion of endogenous insulin with 10 μM nifedipine but substituted the insulin effect by administering 5 milliunits/ml exogenous insulin together with the sugar. As shown in Fig. 2*C* (*16.7 glc + nif + insulin*), exogenous insulin could restore glucose-induced, pro-insulin biosynthesis in the presence of nifedipine, indeed demonstrating that the effects of glucose metabolism and insulin feedback were additive.

To finally prove that insulin *secreted* by the pancreatic β-cell is promoting pro-insulin biosynthesis, we stimulated insulin secretion by 16.7 mM glucose but absorbed the secreted insulin with anti-insulin antibodies. The antibodies were added 30 min before stimulation and were kept throughout stimulation in the culture medium (10 μg/ml). This approach resulted in approximately a 50% reduction in pro-insulin biosynthesis (Fig. 2*D*).

Next we wanted to test the impact of the short term glucose/insulin-stimulated insulin gene transcription on the immediate glucose/insulin-stimulated pro-insulin biosynthesis. As shown in Fig. 3*A*, already 15 min after glucose stimulation, prepro-insulin mRNA steady state levels were elevated by 40%, increasing further with time. By performing a nuclear run-off assay on islets, we found a 3.5-fold elevation in insulin gene transcription initiation 45 min after the start of stimulation (data not shown). To evaluate the importance of insulin gene transcription, we combined glucose/insulin stimulation with actinomycin D treatment. This approach allowed stimulus-dependent translation but blocked renewed synthesis of mRNA. Indeed, blocking transcription through actinomycin D treatment led to a decrease in pro-insulin biosynthesis by approximately 50% (Fig. 3*B*, compare *16.7 glc versus 16.7 glc + actD*). Moreover, actinomycin D in combination with exogenous insulin at substimulatory glucose concentrations almost abolished an increase in pro-insulin biosynthesis (Fig. 3*B,* compare *3.0 glc + insulin versus 3.0 glc + insulin + actD*).

This observation led us to suggest that part of the glucose effect on specific pro-insulin biosynthesis is independent of acute insulin gene transcription and reflects glucose-stimulated translation of pre-existing prepro-insulin mRNA. On the other hand, the insulin effect seems to be directly dependent on acute transcription. That the newly transcribed prepro-insulin mRNA is accessible for translation within a 30-min period is in agreement with the data presented here (Fig. 3*A*) as well as with earlier findings, which show that an elevation in cytoplasmic prepro-insulin mRNA steady state levels can be observed as early as 10 min after the start of glucose stimulation (1, 2).

Glucose stimulation of insulin-producing cells has been shown to result in an increase in the biosynthesis rate of more than 50 proteins including insulin (16). To what extent this effect is due to a "glucose"effect, "insulin" effect, or effect on transcription or translation (or both) remains to be clarified for each individual protein. Both glucose and insulin have been shown to stimulate transcription and translation in several tissues (reviewed in Refs. 17 and 14, respectively). In this study we show that insulin, secreted in response to glucose stimulation, positively affects acute pro-insulin biosynthesis in an autocrine manner. Both glucose and insulin have a stimulatory effect on total protein biosynthesis as well as on absolute pro-insulin biosynthesis (the effect of glucose being more pronounced than that of insulin).

As illustrated in Fig. 4, our data led us to propose that glucose elevates specific pro-insulin levels by stimulating both the transcriptional and post-transcriptional/post-translational events to an almost equal extent. The glucose dependence of the post-transcriptional/post-translational processes, such as the translocation of prepro-insulin, mRNA-bound ribosomes to the endoplasmic reticulum, the initiation, and the elongation in prepro-insulin mRNA translation, is in agreement with earlier reports (reviewed in Ref. 13). Although we cannot totally exclude a direct effect of glucose/glucose metabolism on insulin gene transcription, the activation of glucose metabolism is mediated via glucose-stimulated exocytosis of insulin and the positive paracrine feedback of insulin as described previously (3).

Our data demonstrate for the first time that glucose-stimulated insulin gene transcription directly contributes to the immediately triggered pro-insulin biosynthesis. The involvement of insulin signaling in stimulation-dependent pro-insulin biosynthesis supports the new evolving concept that the pancreatic β-cell itself is a target for insulin and that the positive autocrine feedback loop contributes to β-cell physiology.

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