Glucose-stimulated DNA Synthesis through Mammalian Target of Rapamycin (mTOR) Is Regulated by KATP Channels

EFFECTS ON CELL CYCLE PROGRESSION IN RODENT ISLETS

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The aim of this study was to define metabolic signaling pathways that mediate DNA synthesis and cell cycle progression in adult rodent islets to devise strategies to enhance survival, growth, and proliferation. Since previous studies indicated that glucose-stimulated activation of mammalian target of rapamycin (mTOR) leads to [3H]thymidine incorporation and that mTOR activation is mediated, in part, through the KATP channel and changes in cytosolic Ca2+, we determined whether glyburide, an inhibitor of KATP channels that stimulates Ca2+ influx, modulates [3H]thymidine incorporation. Glyburide (10–100 nM) at basal glucose stimulated [3H]thymidine incorporation to the same magnitude as elevated glucose and further enhanced the ability of elevated glucose to increase [3H]thymidine incorporation. Diazoxide (250 μM), an activator of KATP channels, paradoxically potentiated glucose-stimulated [3H]thymidine incorporation 2–4-fold above elevated glucose alone. Cell cycle analysis demonstrated that chronic exposure of islets to basal glucose resulted in a typical cell cycle progression pattern that is consistent with a low level of proliferation. In contrast, chronic exposure to elevated glucose or glyburide resulted in progression from G0/G1 to accumulation in S phase and a reduction in G2/M phase. Rapamycin (100 nM) resulted in an ~62% reduction of S phase accumulation. The enhanced [3H]thymidine incorporation with chronic elevated glucose or glyburide therefore appears to be associated with S phase accumulation. Since diazoxide significantly enhanced [3H]thymidine incorporation without altering S phase accumulation under chronic elevated glucose, this increase in DNA synthesis also appears to be primarily related to an arrest in S phase and not cell proliferation.

Both types 1 and 2 diabetes result from the inability of pancreatic β-cells to secrete sufficient amounts of insulin to maintain normal glucose homeostasis due to an acquired secretory defect and/or inadequate β-cell mass. Increased metabolic demands or stress responses that exert a positive effect on β-cell mass include obesity, pregnancy, partial pancreatectomy, or chronic glucose exposure. β-Cell mass is regulated by cellular mechanisms that include replication, neogenesis, hypertrophy, and apoptosis (1, 2). Recent studies have emphasized the importance of the proliferative capacity of existing adult β-cells as a major source of new β-cells during adult life that may significantly contribute to the maintenance of β-cell mass (3).

Mammalian target of rapamycin (mTOR)2 is a serine/threonine protein kinase that integrates signals derived from growth factors and nutrients to regulate cell growth and proliferation through the regulatory proteins 70-kDa ribosomal protein S6 kinase (S6K1) and the eukaryotic initiation factor 4E-binding protein-1 (4EBP1). This signaling cascade stimulates protein translation and increases the capacity of the ribosomal protein machinery necessary for the onset of DNA synthesis (4, 5). Our previous studies have demonstrated that glucose robustly activates mTOR/S6K1/4EBP1 in an amino acid-dependent manner via its metabolism in both rodent and human islets. Glucose and amino acids, especially leucine and glutamate, are the most prominent activators of mTOR in islets, possibly through ATP production mediated by mitochondrial metabolism (6–9). Insulin secreted by the β-cell and growth factors also provide input to mTOR through the insulin signaling cascade to Akt (6). Akt may directly activate mTOR but also has been shown to inhibit the tumor suppressor proteins TSC1/2. These proteins are activated by AMP-dependent protein kinase that is regulated by the ATP/AMP ratio. Rapamycin specifically inhibits mTOR activation and signaling to 4EBP1 and S6K1. Recent reports have demonstrated a negative feedback pathway from chronically stimulated mTOR to IRS2 that inhibits the insulin signaling pathway (10). Apparently, this negative feedback to IRS2 does not reduce nutrient-stimulated mTOR activation in the β-cell as S6K1 remains fully activated during a 4- or 6-day exposure to elevated glucose (7), although other IRS2-dependent pathways may be inhibited.

Our previous studies demonstrated that the majority of glucose-stimulated [3H]thymidine incorporation by rodent islets is inhibited by rapamycin, thus mediated through mTOR (7). Studies have further indicated that initial signaling events responsible for glucose-stimulated insulin secretion are also shared by the glucose-stimulated mTOR pathway. Thus, mTOR/S6K1 activation by glucose is mediated, in part, through modulation of the KATP channel and changes in cytosolic Ca2+ in rodent islets (7). Agents that have been used to modulate the KATP channel in β-cells include: 1) glyburide, a sulfonfonylurea type agent, which directly inhibits the KATP channel, causes depolarization, increases in Ca2+ influx and insulin secretion and 2) diazoxide, an activator of KATP channels, that causes hyperpolarization, inhibition of Ca2+ influx, and a blockage of insulin secretion.

2 The abbreviations used are: mTOR, mammalian target of rapamycin; Akt (also known as PKB), protein kinase B; eif-4e, eukaryotic initiation factor 4e; 4EBP1, eukaryotic initiation factor 4e-binding protein 1; KATP channel, ATP-sensitive potassium channel; IRS1 and -2, insulin receptor substrates 1 and 2; PI3K, phosphoinositide 3-kinase; S6K1, 70-kDa ribosomal protein S6 kinase; TSC1 (also known as hamartin) and TSC2 (also known as tuberin), respective protein products of mutated tuberous sclerosis genes TSC1 and TSC2; PI, propidium iodoide; FBS, fetal bovine serum; PBS, phosphate-buffered saline.
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At basal glucose (3 mM), glyburide-induced closure of K_{ATP} channels caused a partial phosphorylation of S6K1. Since mTOR is a regulator of DNA synthesis, these results suggested that glyburide might enhance DNA synthesis at basal glucose. In addition, diazoxide-induced activation of the K_{ATP} channel partially inhibited S6K1 phosphorylation by glucose. These results suggested that diazoxide might attenuate glucose-stimulated DNA synthesis.

Griot et al. (11) reported in 1994 that glibenclamide (glyburide) stimulated \( \beta \)-cell replication and increased \( \beta \)-cell mass in normal young mice. However, the cellular mechanisms responsible for the effects of glibenclamide were not addressed. More recent findings have indicated that the L-type Ca\(^{2+}\) channel \( \alpha 1D \) subunit is required for proper \( \beta \)-cell development in the postnatal pancreas (12). In \( \alpha 1D \) gene knock-out mice, \( \beta \)-cell proliferation was decreased, suggesting that a defect in Ca\(^{2+}\) influx may be partly responsible for a reduction in \( \beta \)-cell mass. In addition, recent findings indicated that S6K1 requires an initial Ca\(^{2+}\) dependent priming event for activation (13, 14).

Diazoxide has been used extensively in vitro to characterize the regulation of stimulus-secretion coupling mechanisms of \( \beta \)-cells that are independent of plasma membrane K\(_{ATP} \) channels. Although diazoxide has provided important mechanistic insights into the role of plasma membrane K\(_{ATP} \) channels in \( \beta \)-cells, it is also reported to exert significant effects on mitochondrial energetics (15, 16). A direct effect of K\(_{ATP} \) channel openers including diazoxide on mitochondrial function has been shown to be critical in the preservation of heart function against injury (17). The cardioprotective effects of diazoxide include decreased Ca\(^{2+}\) uptake due to depolarization of the mitochondrial membrane potential and an activated Ca\(^{2+}\) release that maintains and/or preserves mitochondrial function under conditions of increased metabolic stress. Grimmsmann and Rustenbeck (18) reported that diazoxide at concentrations used routinely to open plasma membrane K\(_{ATP} \) channels in \( \beta \)-cells also exerted direct effects on \( \beta \)-cell mitochondrial function, resulting in a decrease in mitochondrial membrane potential, an efflux of Ca\(^{2+}\) from the mitochondria, and a decrease in ATP concentration. These findings demonstrated that diazoxide exerts multiple effects on \( \beta \)-cells that are not limited to plasma membrane K\(_{ATP} \) channels. Studies by Grill and co-workers (19–21) have further demonstrated that chronic glucose exposure of pancreatic islets for 48 h under both in vitro and in vivo conditions produced nonresponsiveness to a subsequent glucose challenge. However, if the chronic exposure of islets to glucose was performed in the presence of diazoxide, the ability of \( \beta \)-cells to respond to glucose was maintained. A conclusion drawn from these studies was that the protective effect of diazoxide against glucose-induced desensitization of \( \beta \)-cells was not due to a lasting effect of diazoxide on plasma membrane K\(_{ATP} \) channels and may involve other targets possibly including the \( \beta \)-cell mitochondria.

Our data indicate that \(^{3}H\)thymidine incorporation in adult rodent islets chronically exposed to glucose or glyburide is largely mediated through mTOR via the K\(_{ATP} \) channel and changes in intracellular Ca\(^{2+}\). Based on \(^{3}H\)thymidine incorporation and cell cycle analysis, we determined that chronic exposure of adult islets to basal glucose (3 mM) results in DNA synthesis and cell cycle progression consistent with a low level of cell proliferation. In contrast, chronic exposure to elevated glucose or glyburide results in cell cycle progression from G\(_0\)/G\(_1\) to an accumulation of newly synthesized DNA in S phase and a reduction in G\(_2\)/M. Rapamycin (100 nM) resulted in an ~62% reduction of S phase accumulation. The aim of this study was to define metabolic signaling pathways that mediate DNA synthesis and cell cycle progression in adult rodent islets to devise strategies to enhance survival, growth, and proliferation.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Collagenase type XI, Ficoll type 400-DL, diazoxide, glyburide, RNase A, and propidium iodide were obtained from Sigma. CMRL-1066 tissue culture media was from Invitrogen. Defined fetal bovine serum (FBS) was from HyClone (Logan, UT). Penicillin, streptomycin, Hanks’ balanced salt solution, and l-glutamine were obtained from the Washington University Tissue Culture Support Center. Dispase and Versene were from Roche Applied Science. Rapamycin was from Biomol (Plymouth Meeting, PA). [methyl-\(^{3}H\)]Thymidine-aqueous, 2.0 Ci/mmol, 1.0 mCi/ml was from PerkinElmer Life Sciences. Nifedipine was from Calbiochem. The primary antibody for S6K1 was from R&D Systems (Minneapolis, MN). The secondary antibody, peroxidase-conjugated donkey anti-rabbit IgG, was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). All other chemicals were from commercially available sources.

Islet Isolation—Islets were isolated from male Sprague-Dawley rats (200–250 g) by collagenase digestion as described previously (22). Briefly, pancreases were inflated with Hanks’ balanced salt solution, and the tissue was isolated, minced, and digested with 20 mg of collagenase/pancreas for 6 min at 39 °C. Islets were separated on a Ficoll step density gradient and then placed in CMRL-1066 culture medium supplemented with 10% FBS, 2 mM l-glutamine, 5.6 mM glucose, 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin (cCMRL) and selected with a stereomicroscope to exclude any contaminating tissues.

\(^{3}H\)Thymidine Incorporation—Islets (100) were counted into Falcon Petri dishes (35 × 10 mm) and were cultured for 4 days in 1 ml of CMRL (3 or 20 mM glucose, 10% FBS, 2 mM l-glutamine, 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin) containing treatment conditions as indicated in the figure legends at 37 °C, under 95% air/5% CO\(_2\). The culture media were changed everyday or after 2 days as indicated. During the final 24 h of the 96-h incubation, 10 \( \mu \)Ci of \(^{3}H\)thymidine was added to each dish. \(^{3}H\)Thymidine incorporation was determined by trichloroacetic acid extraction and scintillation counting (23).

Western Blotting—Islets were treated as described above for \(^{3}H\)thymidine incorporation. Following incubation, islets were processed for SDS-PAGE electrophoresis and Western blotting for S6K1.

Flow Cytometry—Following the 4-day treatment as described above, islets were subjected to cell cycle analysis by flow cytometry. Islets were dispersed into single cells by treatment with Versene, followed by Dispase in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hanks’ solution at 31 °C. The dispersed cells were washed once with PBS + 1% FBS and pelleted by low speed centrifugation. Cells were resuspended in 500 \( \mu \)l of PBS. Five ml of 100% ethanol was added slowly while vortexing to avoid clumping of cells. Fixed cells were stored at −20 °C until use. Cells were collected by centrifugation, washed once with PBS + 1% FBS, and resuspended in PBS + 1% FBS containing RNase A (250 \( \mu \)g/ml) and propidium iodide (10 \( \mu \)g/ml). PI-stained cells were analyzed for their DNA content using a FACSCALIBUR instrument and data analyzed with CellQuest software (Immucytometry Systems).

Expression of Data and Statistics—Data are presented as mean ± S.E. Statistically significant differences between groups were analyzed using unpaired one-tailed t tests, where \( p < 0.05 \) was considered significant.

RESULTS

In our experimental design, isolated primary rat islets were chronically exposed to either basal (3 mM) or elevated glucose (20 mM) for 4 days to quantitate the incorporation of \(^{3}H\)thymidine that was added to the culture media on day 3 as an index of DNA synthesis. Time course
studies established that a 4-day incubation period was optimal for [³H]thymidine incorporation under these conditions. Separate studies also demonstrated, based on S6K1 phosphorylation, that mTOR remained fully activated in response to elevated glucose or glyburide and was not down-regulated over this time period. This same 4-day treatment period was used to measure cell cycle progression by flow cytometry. Both methods measure DNA synthesis, although in the case of [³H]thymidine incorporation only the last 24 h of incubation are measured, whereas with the flow cytometry method, total DNA is intercalated with PI.

**Glyburide Dose Response**—Our initial approach was to determine whether glyburide, an inhibitor of K\textsubscript{ATP} channels that enhances Ca\textsuperscript{2+} influx, modulates DNA synthesis through mTOR. As shown in Fig. 1, chronic exposure of rat islets to elevated glucose for 4 days resulted in an increase in DNA synthesis as assessed by [³H]thymidine incorporation compared with islets exposed to basal glucose (3 mM, lane 2 versus lane 1). Glyburide dose-dependently (1–100 nM) increased DNA synthesis at basal glucose (lanes 4 and 5). These concentrations of glyburide are within the physiological range typically used to modulate K\textsubscript{ATP} channels.

**Role for Ca\textsuperscript{2+} Influx**—Studies were next performed to determine whether glyburide-mediated increases in DNA synthesis were due to cytosolic Ca\textsuperscript{2+} derived from the extracellular media. In Fig. 2, an increase in glucose concentration enhanced DNA synthesis above basal glucose (lane 2 versus lane 1) and glyburide (10 nM) at basal glucose (lane 4) stimulated DNA synthesis to the same level attained by elevated glucose alone (lane 2). Nifedipine (10 μM), an inhibitor of the L-type Ca\textsuperscript{2+} channel, blocked glyburide-stimulated DNA synthesis (lane 5) to a level comparable with basal glucose (lane 1), and all of these effects were inhibited by rapamycin (25 nM) as shown in lane 6. The level of DNA synthesis in the presence of rapamycin (lane 6) was substantially lower than basal DNA synthesis (lane 1), supporting our previous findings that mTOR mediates both basal and nutrient stimulated DNA synthesis (7). These results support the conclusion that the ability of glyburide to mediate [³H]thymidine incorporation is due, in part, to an increase in Ca\textsuperscript{2+} influx through the nifedipine-sensitive L-type Ca\textsuperscript{2+} channel.

These results also correlated with S6K1 phosphorylation based on gel shift mobility assays (upper inset) as an indicator of mTOR activation. Elevated glucose robustly enhanced S6K1 phosphorylation as indicated by the intense and more slowly migrating upper band (lane 2) compared with basal glucose (lane 1). An effective concentration of glyburide (10 nM) resulted in a partial activation of S6K1 phosphorylation at basal glucose (lane 4) as indicated by the doublet band in comparison to basal glucose (lane 1) that is sensitive to nifedipine (lane 5), and all of these effects are blocked by rapamycin (lane 6). These studies, however, suggest that the level of S6K1 activation is not linearly related with [³H]thymidine incorporation, since different levels of S6K1 activation (lane 2 versus lane 4) resulted in comparable levels of DNA synthesis. We propose that S6K1 is an excellent indicator of mTOR activation but other mTOR-mediated signals in addition to S6K1 activation are important for the regulation of DNA synthesis.

**Diazoxide Potentiates Glucose-stimulated DNA Synthesis**—Our next approach was to determine whether diazoxide, an opener of K\textsubscript{ATP} channels, would decrease glucose-stimulated DNA synthesis due to its ability to attenuate Ca\textsuperscript{2+} influx derived from the extracellular media. As shown in Fig. 3, diazoxide (250 μM) paradoxically enhanced the ability of elevated glucose to stimulate DNA synthesis (lane 3 versus lane 2). This enhanced DNA synthesis also occurred in the absence of diazoxide treatment during the final 24 h of the total 96-h incubation period as shown in the washout protocol (lane 4). Rapamycin (25 nM) blocked the ability of diazoxide to significantly enhance glucose-stimulated DNA synthesis (lane 5). These unexpected results suggest that diazoxide might exert multiple effects to mediate the potentiation of glucose-stimulated DNA synthesis in addition to the opening of plasma membrane K\textsubscript{ATP} channels. The observation that diazoxide did not have to be present for the total 4 day incubation period to potentiate glucose-stimulated DNA synthesis was consistent with this conclusion, since the ability of diazoxide to activate plasma membrane K\textsubscript{ATP} channels is rapidly reversible following its removal.

**Multiple Sites of Action for Diazoxide**—Studies were next performed to determine whether the ability of diazoxide to attenuate Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels is responsible for the significant potentiation of glucose-stimulated DNA synthesis. If this were the case, an inhibitor of the L-type Ca\textsuperscript{2+} channel such as nifedipine should mimic...
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the potentiating effects produced by diazoxide on glucose-stimulated DNA synthesis. As shown in Fig. 4, the ability of elevated glucose alone to increase DNA synthesis (lane 2) was significantly enhanced by diazoxide exposure (lane 3). However, nifedipine (0.1–10 μM) failed to mimic the potentiating effect of diazoxide on glucose-stimulated DNA synthesis (lanes 4–6). These results suggest that diazoxide stimulates DNA synthesis by mechanisms other than solely attenuating Ca\(^{2+}\) influx.

Evidence that the potentiating effect of diazoxide on glucose-stimulated DNA synthesis is associated with a nifedipine-sensitive component of Ca\(^{2+}\) influx is shown in Fig. 5. As shown in lane 3, diazoxide enhanced the ability of glucose (20 mM) to stimulate DNA synthesis compared with glucose alone (lane 2). In lane 4, nifedipine (10 μM) in the presence of diazoxide (250 μM) significantly inhibited glucose-stimulated DNA synthesis to the level produced by glucose alone (lane 2). These results indicate that the significant enhancement of glucose-stimulated DNA synthesis by diazoxide (lane 3) is dependent on a low level of nifedipine-sensitive Ca\(^{2+}\) influx into β-cells. In contrast, chronic exposure of islets to elevated glucose alone as shown in Fig. 4 (lane 2) resulted in a smaller increase in DNA synthesis that is independent of Ca\(^{2+}\) influx due to the lack of inhibition by nifedipine (lanes 4–6).

The conclusion that the stimulation of Ca\(^{2+}\) influx, under conditions in which primary rat islets are chronically exposed to elevated glucose, will enhance DNA synthesis is further supported by the findings shown in Fig. 6. In this experimental protocol, glyburide due to its ability to directly inhibit K\(_{ATP}\) channels was used in combination with elevated glucose to enhance Ca\(^{2+}\) influx. Chronic exposure of islets to glucose (20 mM) in combination with glyburide (10 mM) significantly potentiated DNA synthesis compared with an elevated glucose concentration alone (lane 3 versus lane 2) in a nifedipine-sensitive manner (lane 4).

Rapamycin-mediated Inhibition of Glucose-stimulated DNA Synthesis Is Irreversible in Vitro—An important question raised in these in vitro studies is whether the ability of rapamycin to inhibit basal and glucose-stimulated DNA synthesis in primary rodent islets is reversible. This concern is also relevant to the current use of rapamycin as an immunosuppressant in islet transplantation protocols in type 1 diabetes (24). As shown in Fig. 7, the inhibitory effects of rapamycin (1 nM) present for 4 days on glucose-stimulated DNA synthesis (lane 3 versus lane 2) is nearly identical to the inhibition observed when rapamycin is present for only 1 day and absent during the remaining 3 days of the incubation period (lane 6). As shown in the inset, S6K1 phosphorylation, based on gel shift analysis, correlates well with the effects of rapamycin on DNA synthesis. These results indicate that inhibition of DNA synthesis by rapamycin (1 nM) is essentially irreversible during this 3-day exposure period under these in vitro conditions with primary adult islets. Furthermore, these findings suggest that the synthesis of new mTOR protein may be required to restore mTOR activity following the removal of the inhibitor, rapamycin.

Cell Cycle Analysis—To determine to what extent increases in [\(^{3}H\)]thymidine incorporation translate into changes in cell proliferation, cell cycle progression was analyzed by flow cytometry with PI-stained...
islet cells. As shown in Fig. 8 and Table 1, under basal glucose conditions, quantitation of cell cycle progression indicates that 7.3 ± 0.5% of gated cells were in G2/M phase, a result that is consistent with a low level of cell proliferation normally observed in adult islet cells. In contrast, chronic exposure to elevated glucose resulted in a marked accumulation of total gated cells in S phase (50.7 ± 5.5%) and a reduction in G2/M to 2.5 ± 0.3%. Furthermore, chronic exposure of islets to basal glucose in combination with glyburide (10 nM) also resulted in an accumulation in S phase (39.6 ± 9.9%) and reduction in G2/M to 3.4 ± 0.8%. In separate experiments, rapamycin at 100 nM maximally inhibited S phase accumulation by 61.9 ± 2.6% (n = 4), whereas rapamycin at 25 nM resulted in 32.7% (n = 2) inhibition in response to chronic elevated glucose. Although diazoxide significantly enhanced [3H]thymidine incorporation above elevated glucose alone, it did not alter S phase accumulation (data not shown).

**DISCUSSION**

We have attempted to define the pathways and mediators responsible for modulating glucose-stimulated DNA synthesis through mTOR and to determine the extent that this relates to cell cycle progression and proliferation in adult rodent islets. Since glucose, through its metabolism, activates mTOR continuously for at least 6 days, we utilized an *in vitro* model of chronic exposure of rat islets to glucose (20 mM) under conditions shown previously to enhance DNA synthesis 1.5–2-fold compared with basal glucose (3 mM) (7). Our studies have shown that appropriately regulating cytosolic Ca2+ levels via the modulation of KATP channels is critical for mTOR-mediated [3H]thymidine incorporation.

In rodents, chronic exposure of islets to elevated glucose has been shown to increase hypertrophy, proliferation, and/or the neogenesis of β-cells. Steil et al. (25) have shown that glucose infusions in rats for 1–2 days resulted in an increase in β-cell mass that paralleled increased rates of β-cell proliferation. Paris et al. (26) also reported that chronic exposure of rodent islets to glucose *in vivo* for 48 h provoked a rapid compensatory increase in β-cell mass that was independent of plasma insulin levels. Lipsett et al. (27) reported that β-cell neogenesis contributed...
significantly to glucose-stimulated expansion of \( \beta \)-cell mass during pro-
longed hyperglycemia in rats infused with elevated glucose. In in vitro
models, the mitogenic effects produced by elevated glucose were shown
to be long lasting in rat islets and more transitory in human islets (28). In
addition, prolonged exposure of rodent islets to elevated glucose
resulted in impaired insulin secretion and increased \( \beta \)-cell apoptosis
that is attributed, in part, to glucose toxicity.

Our current studies showed that glyburide in the nanomolar range at
basal glucose stimulated DNA synthesis to the same magnitude as ele-
vated glucose, and these effects were blocked by rapamycin. Nifedipine
prevented glyburide-mediated DNA synthesis indicating that the ability
of glyburide to stimulate DNA synthesis was due to an increase in Ca\(^{2+}\)
influx from the extracellular media. Maedler et al. (29) reported with
isolated human islets that 0.1 and 10 nM glyburide in the presence of
basal glucose after 4 days of exposure produced small increases (2.2- and
2.5-fold, respectively) in \( \beta \)-cell apoptosis. In the \( \beta \)-cell line, INS-1, Bri-
aud et al. (30) reported that glyburide (5 m\( \mu \)M) increased DNA synthesis
at basal glucose (3 mM) but was ineffective at an elevated glucose
concentration. Glyburide also failed to stimulate phosphorylation of S6K1
in this \( \beta \)-cell line. Their results indicated that glyburide and/or glucose
exposure stimulated increases in cytosolic Ca\(^{2+}\) that were primarily
associated with Erk1/2 phosphorylation, and DNA synthesis likely
occurred through a protein kinase A-dependent pathway. The effects
of rapamycin on glucose- or glyburide-mediated DNA synthesis were not
evaluated in this study. A plausible explanation for the lack of agreement
of the effects of glyburide on S6K1 phosphorylation with our findings is
that these studies were performed in a rapid proliferating cell line,
INS-1, as opposed to primary islets.

In our previous studies, diazoxide treatment resulted in a partial inhibi-
tion of S6K1 phosphorylation suggesting that diazoxide might reduce
glucose-stimulated DNA synthesis (7). Paradoxically, diazoxide (250
m\( \mu \)M) in the presence of glucose (20 mM) resulted in a marked 2–4-fold
enhancement of DNA synthesis above that of elevated glucose alone
and occurred in a rapamycin-sensitive manner. This potentiating effect
also occurred under conditions when diazoxide was absent from the
incubation media during the final 24 h of the 96 h total incubation
period. These effects of diazoxide were also compared with those of
pinacidil, another K\(_{\text{ATP}}\) channel opener (18). Pinacidil mimicked the
effects produced by diazoxide on glucose-stimulated DNA synthesis but
at a significantly lesser degree possibly due to its lower potency in opening
K\(_{\text{ATP}}\) channels in comparison with diazoxide in pancreatic \( \beta \)-cells
(data not shown). The ability of diazoxide to attenuate nutrient over-
stimulation of mTOR (7) might also be anticipated to down-regulate the
negative feedback pathway from mTOR/S6K1 to IRS2. It is possible that
attenuating this negative feedback pathway with diazoxide may increase
\( \beta \)-cell mass by reducing apoptosis through increased growth factor acti-
vation of IRS2-, PI3K-, and Akt-regulated pathways other than mTOR
in vivo. Functional IRS2/PI3K/Akt signaling is considered essential for
\( \beta \)-cell survival (1).

Studies were next performed with nifedipine, an inhibitor of \( \beta \)-type
Ca\(^{2+}\) channels, to determine whether the ability of diazoxide to attenu-
ate Ca\(^{2+}\) influx was responsible for the marked potentiation of gluo-
ses-stimulated DNA synthesis. Nifedipine failed to mimic the stimula-
tory effects produced by diazoxide (Fig. 4) even though nifedipine
completely blocked insulin secretion in response to glucose (data not
shown). Overall, these results suggested that diazoxide might be exert-
ing effects independent of the activation of plasma membrane K\(_{\text{ATP}}\)
channels that are responsible for the potentiation of glucose-stimulated
DNA synthesis. Findings by Kullin et al. (31) also reported that K\(_{\text{ATP}}\)
channel openers including diazoxide decreased the vulnerability of rat
islets to free radicals induced by alloxan, nitroprusside, or IL-1\( \beta \). It was
proposed that K\(_{\text{ATP}}\) channel openers by their ability to decrease Ca\(^{2+}\)
influx due to \( \beta \)-cell membrane hyperpolarization in combination with
their direct effect to decrease the mitochondrial membrane potential
reduced ATP production necessary for the activation of apoptotic path-
ways. In cardiac mitochondria, Holmuhamedov et al. (16) have also
proposed that K\(_{\text{ATP}}\) channel openers modulate Ca\(^{2+}\) homeostasis
through dissipation of the mitochondrial membrane potential that leads
to reduced mitochondrial Ca\(^{2+}\) influx and provides protection against
ischemic injury.

Based on cell cycle analysis, our studies suggest that the ability of
chronic elevated glucose or glyburide to enhance DNA synthesis in
adult islets is linked to an accumulation of cells in S phase. Conditions of
chronic hyperglycemia or pregnancy have been shown to cause hyper-
trophy of islet cells; however, the possibility that hypertrophy is respon-
sible for S phase accumulation of DNA is unlikely, since the synergy of
cell growth and proliferation is a G1 phenomenon regulated by retino-
blastoma protein and not by S phase (32). Although the mechanism
responsible for this defect in cell cycle progression is unknown, it may be
related to excess free radical production which predisposes the mito-
chondria to Ca\(^{2+}\) overload that mediates DNA damage. It has been
shown under chronic hyperglycemic conditions that mRNA levels of
some stress-related genes are increased in islets cells such as, heme
oxygenase-1, glutathione peroxidase, A20, Fas, INOS, and c-Myc (33,
34). Based on flow cytometry, S phase accumulation represents new
DNA synthesis. The arrest of islet cells in S phase may be due to the lack
of protein expression, i.e. cyclins, necessary to complete cell cycle pro-
gression or possibly a checkpoint in cell cycle progression due to the
over expression of stress-related proteins that cause a response to DNA
damage.

The ability of diazoxide to significantly enhance \[^{3}H\]thymidine
incorporation above that of elevated glucose alone might be related to the
protective effect that diazoxide exerts on the \( \beta \)-cell mitochondria.
Since the ability of diazoxide to enhance DNA synthesis occurs without
any significant alterations in S phase accumulation, this increase in
DNA synthesis appears to be linked to an arrest in S phase cell cycle
progression and not translated to cell proliferation. In contrast, chronic
exposure of islets to basal glucose that only partially activates mTOR
results in a typical cell cycle progression pattern that is consistent with a
low level of proliferation. Santiago-Walker et al. (35) have recently
reported that the ability of PKC\( \theta \) to stimulate apoptosis of primary rat
thyroid cells is linked to its ability to stimulate G1 phase cell cycle pro-
gression and S phase arrest. Evidence that apoptosis was a direct conse-
quence of replication stress was established by using the PI3K inhibitor,
LY294002, that prevented S phase entry and apoptosis. Based on cell
cycle analysis by flow cytometry, we have no evidence to indicate that a
significant degree of apoptosis occurs under our in vitro conditions with
primary rat islets (data not shown). Interestingly, Belanger et al. (36)
reported that the anti-apoptotic protein Bcl-2 delays cell proliferation
and promotes accumulation of cells in S phase without affecting the rate
of apoptosis by human ovarian carcinoma cells. Bcl-2 as a regulator of
apoptosis is believed to alter mitochondrial permeability transition
through its ion channel activity and inhibits the release of both cyto-
ochrome c and apoptosis-inducing factor from the mitochondria,
thereby promoting cell survival. Importantly, it has been reported that
damage to DNA results in activation of damage response pathways in
human epidermal keratinocytes (37). The signal derived from DNA
damage to the activation of these response pathways is mediated by
mTOR. A similar mechanism may be responsible for enhanced \[^{3}H\]thym-
idine incorporation that occurs in a rapamycin-sensitive manner as a
consequence of chronic exposure of adult rodent islets to elevated glucose. The ability of rapamycin to partially prevent in a dose-dependent manner the accumulation of DNA in S phase may reflect the necessity of inhibiting mTOR more completely at the higher concentration of 100 nM that maximally inhibits cell cycle progression from G0/G1 to S phase. The reason for the differences in sensitivity to rapamycin of [3H]thymidine incorporation versus cell cycle progression are unknown but may be due to different mTOR-dependent pathways involving DNA damage responses and/or cell cycle checkpoints.

Overall, in our model of chronic glucose exposure we observed an accumulation of DNA in S phase that does not translate to an increase in cell proliferation. This is consistent with the well established finding that only a few adult islet cells are progressing through the cell cycle at any one time. Thus, it appears that the enhanced [3H]thymidine incorporation observed under conditions of prolonged elevated glucose alone or in combination with diazoxide or glyburide may be related to a response to DNA damage that is mediated by mTOR. Although an increase in DNA synthesis based on either [3H]thymidine or bromodeoxyuridine incorporation is required for cell division, this essential prerequisite does not always result in completion of the cell cycle. Our studies suggest that the appropriate regulation of mTOR by nutrients and pharmacologic agents that mediate DNA synthesis and cell cycle progression may be a component of an effective strategy to enhance the survival, growth, and proliferative capacity of adult rodent islets.

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