Inhibition of GSK3 Promotes Replication and Survival of Pancreatic Beta Cells*

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Recent developments indicate that the regeneration of beta cell function and mass in patients with diabetes is possible. A regenerative approach may represent an alternative treatment option relative to current diabetes therapies that fail to provide optimal glycemic control. Here we report that the inactivation of GSK3 by small molecule inhibitors or RNA interference stimulates replication of INS-1E rat insulinoma cells. Specific and potent GSK3 inhibitors also alleviate the toxic effects of high concentrations of glucose and the saturated fatty acid palmitate on INS-1E cells. Furthermore, treatment of isolated rat islets with structurally diverse small molecule GSK3 inhibitors increases the rate beta cell replication by 2–3-fold relative to controls. We propose that GSK3 is a regulator of beta cell replication and survival. Moreover, our results suggest that specific inhibitors of GSK3 may have practical applications in beta cell regenerative therapies.

Diabetes results from an inadequate supply of the body with insulin. In type 1 diabetes, beta cells are destroyed by an autoimmune attack (1), whereas type 2 diabetes develops from a combination of peripheral insulin resistance and beta cell failure (2, 3). Despite remarkable improvements in the management of diabetic patients over the last decades, new therapies are still needed to further improve metabolic control and thereby reduce the development of diabetic complications that are associated with significant rates of morbidity and mortality (4–6). One of the most promising concepts for the treatment of diabetes is the development of therapies for the preservation and regeneration of beta cell mass and function. Ongoing formation of new beta cells in patients with type 1 diabetes has been described even in patients with long disease history. This suggests that beta cell regenerative treatments could be successful even in patients with longstanding type 1 diabetes, provided the autoimmune destruction of beta cells can be controlled (7, 8). Individuals with type 2 diabetes could also benefit from agents that preserve or expand beta cell mass, since several studies have demonstrated that the beta cell mass is significantly reduced in these patients (9–14). Beta cell mass in humans and rodents is dynamically regulated in response to changes in insulin requirements (15). A number of recent reports have demonstrated using rodent models of diabetes that pancreatic beta cell mass is responsive to pharmacologically active agents promoting beta cell expansion and/or protection. Several proteins exhibit beta cell regenerative activities in rodent models of diabetes, with long acting analogs of the incretin hormone GLP-1 being among the most promising candidates (16–20). On the contrary, effective small molecule agents or chemically tractable candidate drug targets for systematic development of small molecule therapeutics are scarce. Studies with growth factors and genetic approaches have established the significance of certain intracellular pathways, including insulin signaling, Janus kinase/signal transducer and activator of transcription signaling, and G-protein-coupled receptor signaling for the regulation of beta cell mass (21).

We have studied the role of the serine/threonine kinase GSK3 (glycogen synthase kinase 3) in beta cells. GSK3 is a well described element of the insulin signaling pathway. However, the precise role of GSK3 for beta cell growth and survival is incompletely understood. Two highly homologous GSK3 isoforms, α and β, are widely expressed (22, 23). GSK3 is a constitutively active enzyme and is inactivated by inhibitory phosphorylation in response to insulin, Wnt, or other growth factor signaling (24–26). In insulin signaling, GSK3 is inactivated by the serine/threonine kinase Akt, which in turn is regulated by phosphoinositide (PI) 3-kinase. PI 3-kinase/Akt signaling is crucial for glucose sensing and beta cell growth (27–29). For instance, the overexpression of Akt in beta cells markedly increases beta cell mass (30, 31). Previously, it has been reported that pancreatic beta cells can be protected from endoplasmic reticulum stress-induced death by RNA interference-mediated knockdown of GSK3 (32). Moreover, inactivation of GSK3 occurs in response to a number of known beta cell mitogens, such as glucose-dependent insulinotropic polypeptide (GIP), GLP-1, and insulin-like growth factor-1, mediated by the upstream PI 3-kinase/Akt signaling pathway (33–37). Based on these observations, GSK3 beta has been proposed to be a possible target for beta cell protective agents (32, 38, 39). Subsequently, it was reported that active GSK3 negatively affects beta cell function by modulating the stability and subcellular localization of the beta cell differentiation factor Pdx1 (40).

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3 The abbreviations used are: PI, phosphoinositide; GIP, glucose-dependent insulinotropic polypeptide; BrdUrd, bromodeoxyuridine; siRNA, small interfering RNA; BIO, 6-bromoindirubin-3′-oxime.
In diabetes, beta cell loss and dysfunction are most likely resulting from synergistic effects of different factors negatively affecting beta cells for prolonged periods of time. For instance, chronic or recurrent exposure of beta cells to elevated levels of glucose and lipids (glucolipotoxicity) or to proinflammatory cytokines, such as interleukin-1β, tumor necrosis factor α, and interferon-γ, interferes with beta cell function and contributes to their destruction (2, 41). Using potent and specific small molecule GSK3 inhibitors, we found that the inactivation of GSK3 protects beta cells against death induced by high concentrations of glucose and the saturated fatty acid palmitate. In addition, small molecule GSK3 inhibitors robustly stimulate cytokines, such as interleukin-1β, and glucolipotoxicity-mediated apoptosis was induced by the addition of a combination of bovine serum albumin-coupled palmitate (0.3 mM palmitate, bovine serum albumin (palmitic acid sodium salt/fatty acid-free bovine serum albumin, 6:1)) and glucose (25 mM) to the starvation medium followed by a 24-h incubation period. Test compounds were added 1 h prior to the addition of apoptosis-inducing factors. Caspase activity was quantified by an enzymatic assay (homogeneous caspase assay; Roche Applied Science catalog number 03 005 372 001). Caspase activity was measured 3 h after the addition of caspase substrate according to the guidelines of the manufacturer. The frequency of apoptosis was also monitored by the determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates using the cell death detection enzyme-linked immunosorbent assay kit (Roche Applied Science catalog number 1774425). The assay is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal anti-histone and anti-DNA peroxidase antibodies. The relative frequency of apoptosis was photometrically determined by measuring the peroxidase activity of the immunocomplexes at 405 nm. Fluorescence or photometric measurements were carried out with the FLUOstar Optima reader from BMG Labtechnologies.

**Experimental Procedures**

**Cell Culture**—INS-1E cells were maintained in culture medium (RPMI 1640 containing 11 mM glucose, 5% fetal calf serum, 10 mM HEPES, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate) and cultivated at 37 °C, 5% CO2 in a humidified atmosphere (RPMI 1640 containing 11 mM glucose, 5% fetal calf serum and 1% [v/v] penicillin/streptomycin). We found that the inactivation of GSK3 protects beta cells against death induced by high concentrations of glucose and the saturated fatty acid palmitate. In addition, small molecule GSK3 inhibitors robustly stimulate beta cell proliferation. These results suggest that GSK3 plays a key role in the regulation of beta cell mass and is a target for beta cell regenerative therapies.

**Isolation of Rat Islets**—Islets were isolated by the standard LiberaseTM digestion method from rat pancreata (LiberaseTM Cl enzyme blend BMB, catalog number 1814-435; Roche Applied Science) and cultured as described (42). In brief, 10 ml of ice-cold Liberase solution (Roche Applied Science) was injected into the pancreas via the common bile duct. After dissection, the pancreas was incubated for 40 min at 37 °C and then further dissociated by repeated pipetting using a 10-ml pipette. Islets were purified by a Ficoll density gradient centrifugation and were manually picked using a stereomicroscope. Islets were placed in bacteriological wells, and factors were administered as indicated. For quantitative reverse-phase PCR, islets were harvested in TrizolTM and immediately transferred to dry ice. RNA was isolated according to common procedures.

**Viability Assay, Cell Number Determination, Caspase Activity Assay, and DNA Fragmentation Assay**—INS-1E cells were seeded at a density of 1 × 10⁴ cells/well (6-well plate) and grown in 100 μl of culture medium/well supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin for 3 days. The metabolic activity or viability of INS-1E cells was assessed by mitochondrial reduction of the nontoxic dye Alamar Blue (Bio-source catalog number DAL1255). The dye was added to the cells 4 h before read-out according to the manufacturer’s guidelines. For the determination of relative cell numbers, INS-1E cells were maintained for 24 h in 100 μl of starvation medium (culture medium with only 5 mM glucose, 1% fetal calf serum) before the test compounds were added for an additional 4 days. Then the tissue culture plates were washed one time with 200 μl of phosphate-buffered saline and frozen at −80 °C for at least 1 h. Cell number was measured by staining of cellular DNA with CyQuant dye (CyQuant cell proliferation assay kit; Molecular Probes, Inc., catalog number C-7026), which becomes fluorescent when bound to DNA. Fluorescence was measured after 30 min of incubation using the FLUOstar Optima reader from BMG Labtechnologies. Glucolipotoxicity-mediated apoptosis was induced by the addition of a combination of bovine serum albumin-coupled palmitate and glucose to the starvation medium followed by a 24-h incubation period. Test compounds were added 1 h prior to the addition of apoptosis-inducing factors. Caspase activity was quantified by an enzymatic assay (homogeneous caspase assay; Roche Applied Science catalog number 03 005 372 001). Caspase activity was measured 3 h after the addition of caspase substrate according to the guidelines of the manufacturer. The frequency of apoptosis was also monitored by the determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates using the cell death detection enzyme-linked immunosorbent assay kit (Roche Applied Science catalog number 1774425). The assay is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal anti-histone and anti-DNA peroxidase antibodies. The relative frequency of apoptosis was photometrically determined by measuring the peroxidase activity of the immunocomplexes at 405 nm. Fluorescence or photometric measurements were carried out with the FLUOstar Optima reader from BMG Labtechnologies.

**In Vitro Beta Cell Proliferation Assay**—Freshly isolated islets were cultured in vitro with or without the addition of factors of interest for 48 or 72 h. Following the culture period, the islets were dispersed gently by titration in Ca²⁺- and Mg²⁺-free phosphate-buffered saline. The resulting single cell suspension was applied to adhesive slides at 3000–6000 cells/well (adhesion slides/Fa Superior Marienfeld REF 09 000 00). The adherent islet cells were fixed and stained by standard immunofluorescence techniques for C-peptide, a fragment of proinsulin, and Ki-67 a marker of proliferating cells. Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (Molecular Probes). An Olympus microscope equipped with an automatic image acquisition device (Olympus) was used for counting of C-peptide-positive beta cells. Proliferating C-peptide/Ki-67 double positive beta cells were counted manually. The fraction of proliferating beta corresponds to the percentage of Ki-67 double positive cells of all C-peptide-positive cells. Using this assay, the growth-promoting effects of a number of known beta cell mitogens, including prolactin, GIP, hepatocyte growth factor, epidermal growth factor, growth hormone, exendin-4, betacellulin, fibroblast growth factor-2, and activin on rat beta cells were detectable (data not shown). Data generated with prolactin and GIP are presented in Fig. 4.

**BrdUrd Labeling and Detection Assay**—INS-1E cells were seeded in 96-well culture plates and cultured as described above for the caspase activity assay. BrdUrd labeling and detection was carried out with a commercially available kit (cell proliferation enzyme-linked immunosorbent assay, BrdUrd (chemu- limescence) Roche Applied Science catalog number 1 669 915) according to the manufacturer’s instructions. In brief, 24 h after switching to starvation medium, test compounds were added, and cells were cultured for an additional 24 h. BrdUrd labeling solution was added to the medium for the last 4 h before the
cells were fixed using FixDenat solution and incubated with monoclonal anti-BrdUrd-POD antibodies. After substrate solution was added to each well, the light emission was measured in a microplate luminometer using the Analyst™ HT detection system from LJI Biosystems Inc.

**RNA Interference, Western Blotting, and Proliferation Assay—** For Western blotting, INS-1E cells were seeded in 12-well plates at a density of $2 \times 10^5$ cells/well and cultured overnight before transfection. Cells were transfected with 6 μl of HiPerFect Transfection Reagent (Qiagen) and mixed with 5 nm siRNA duplexes (Qiagen). For quantitative reverse transcription-PCR, cells were harvested in Qiagen lysis buffer 72 or 140 h after transfection. For Western blot analysis, cells were harvested in a standard protein lysis buffer. Total protein was separated by 12% SDS-PAGE and transferred onto nitrocellulose membrane. Blots were developed using the chemiluminescence detection system SuperSignal West Dura from Pierce (product number 34075). The following antibodies were used: mouse monoclonal anti-GSK3α and -β (Calbiochem catalog number 3686662) at a 1:1000 dilution, mouse monoclonal anti-α-tubulin (Sigma catalog number T6557) at a 1:10,000 dilution, and as a secondary antibody horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce product number 34075) at a 1:5000 dilution. For the determination of the proliferation rate of INS-1E cells, were seeded in 96-well plates at a density of $2 \times 10^4$ cells/well and cultured overnight before transfection. Following, cells were transfected with 0.7 μl of HiPerFect transfection reagent (Qiagen) mixed with 5 nm siRNA duplexes. After 24 h, incubation medium was replaced by starvation medium. 48 h later, BrdUrd labeling solution (Roche Applied Science) was added to the medium for 4 h, and the rate of proliferating cells was then determined as described above using the cell proliferation enzyme-linked immunosorbent assay (Roche Applied Science). The following siRNA duplexes were purchased from Qiagen: rat GSK3β, sense (r(CGUAUCAAGUCUAGUUA)d(TdT)) and antisense (r(UAAUCUAGACGUUGUAAUCG)d(GdT)); rat GSK3α, sense (r(GGGUAAUAAUGUGUUU)d(TdT)) and antisense (r(UAAACUAUCAUAAUUGAAAGC)d(AdA); control nonsilencing siRNA, sense (UUCUCCG-AACGUGUCAGGdTdT) and antisense (ACGUGACG-UUCGCGAGAA(ddT)).

**Quantitative Reverse Transcription-PCR—** Total RNA from $8 \times 10^4$ INS-1E cells growing on 4-cm² surface area of a tissue culture dish was extracted using Qiagen RNAeasy kit according to the instructions of the manufacturer (Qiagen), and 2 μg was converted into cDNA. Primers for the analyzed genes were designed using the Primer Express 1.5 Software from Applied Biosystems, and sequences are available upon request. Quantitative real time PCR was performed using Applied Biosystems SDS 7000 detection system. Amplifications for a gene were performed in duplicate, and mean values were normalized to the mean value of the reference RNA, 18 S RNA. Mean gene expression values in cycle thresholds ± S.D. were determined from three wells of untreated rat islets kept for 24 h in culture medium (see Fig. 4E). The values for the indicated genes are as follows: bcl-xl, 23.6 ± 0.3; c-myc, 26.6 ± 0.4; id2, 22.7 ± 0.3; pax4, 32.2 ± 0.28; CDK4, 22.3 ± 0.2; neuroD, 19.35 ± 0.3; pdx1, 23.3 ± 0.2; cypB, 22.7 ± 0.3. A corresponding analysis was carried out with untreated INS-1E (see Fig. 3A). Mean cycle thresholds ± S.D. for GSK3α or -β and cyclophilin B were 21.5 ± 0.09, 25 ± 0.12, and 21.5 ± 0.1, respectively.

**GSK3 Inhibitors—** The Chiron inhibitor CT99021 (6-[2-[4-(2,4-dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)-pyrimidin-2-ylamino]ethyl-amino]nicotinonitrile) was synthesized by Asinex Inc. for DeveloGen AG. The compound was >98% pure by NMR and analytical high pressure liquid chromatography. All other GSK3 inhibitors used in this study were obtained from Calbiochem.

**RESULTS**

**Small Molecule Inhibitors of GSK3 Alleviate Glucolipotoxicity in INS-1E Beta Cells—** INS-1E, a rat insulinoma cell line (42), shares many features with primary beta cells and is therefore widely used in the field to study beta cell function. We have studied the effects of well known and specific small molecule inhibitors of GSK3 on INS-1E cells exposed to toxic concentrations of glucose and the unsaturated fatty acid palmitate. INS-1E cells were treated for 24 h with a combination of high glucose (25 mM) and high palmitate (0.3 mM) concentrations. High glucose/high palmitate treatment was shown previously to induce cell death in INS-1-derived cell lines (44, 45) and induces endoplasmic reticulum stress in INS-1 cells (32, 46). In fact, microscopic examination of INS-1E cells exposed to the mixture revealed typical features of apoptotic cells, such as condensed nuclei and membrane blebbing (data not shown). In order to quantitatively evaluate these apoptotic processes in the presence or absence of GSK3 inhibitors, two biological assays were established, enabling us to determine DNA fragmentation in the cytoplasm (Fig. 1, B, E, and H) and the enzymatic activity of caspases in cell lysates (Fig. 1, C, F, and I). Low levels of these apoptotic processes were detectable in untreated INS-1E cells (Fig. 1, Co). The treatment with a combination of high glucose and palmitate stimulated these processes severalfold, confirming microscopic observations. For our studies, three structurally diverse, small molecule kinase inhibitors were selected: CHIR99021 (47), 6-bromoindirubin-3’-oxime (BIO) (48), and 1-azakenpaullone (49), since they are cell-permeable and among the most potent and selective GSK3 inhibitors currently available. In particular, CHIR99021 has been considered an early clinical candidate and has previously been used to study the potential of GSK3 inhibitors for the treatment of type 2 diabetes (50) and hematopoietic stem cell repopulation (51) in the respective animal disease models. Treatment of INS-1E cells with 1-azakenpaullone or CHIR99021 alone did not compromise the viability of INS-1E cells even at high compound concentrations (Fig. 1, A and D, respectively). On the contrary, the GSK3 inhibitor BIO significantly reduced INS-1E viability at concentrations above 2 μM (Fig. 1G). In order to avoid potential off target effects, BIO was therefore used in the following studies at concentrations below 2 μM. Interestingly, all three GSK3 inhibitors counteract cell death induced by high glucose and high palmitate in INS-1E cells in a concentration-dependent manner as demonstrated by reduced DNA fragmentation (Fig. 1, B, E, and H) and caspase activity (Fig. 1, C, F, and I), indicating a GSK3-mediated mechanism. On the other hand, GSK3 inhibitors did not completely abolish apoptotic processes, pointing...
at the involvement of GSK3-independent signaling events. Taken together, these results are consistent with previous reports demonstrating that inactivation of GSK3β protects beta cells against endoplasmic reticulum stress-induced apoptosis (32, 46).

Inhibition of GSK3 Activity Stimulates Growth of INS-1E Beta Cells—As previously reported by others, a functional PI 3-kinase/AKT pathway is instrumental for the regulation of beta cell mass. To study the role of the AKT target GSK3 in beta cell replication, INS-1E cells were incubated with the above described potent and specific GSK3 inhibitors. The treatment with any of the three GSK3 inhibitors increased the rate of proliferation of INS-1E cells in a dose-dependent manner as assessed by BrdUrd incorporation (Fig. 2, A, C, and E). The rate of proliferation reached a maximum in the presence of 2.5–10 μM CHIR99021 and 1-azakenpaullone, whereas BIO maximally stimulated proliferation between 0.5 and 1 μM. Above 1 μM, the proproliferative effects of BIO gradually disappeared, probably due to the off-target effects already observed in Fig. 1G. GSK3 inhibitors promote survival and replication of INS-1E cells, implying that GSK3 inhibitor treatment may support net growth of INS-1E cells. In fact, INS-1E cell cultures treated with GSK3 inhibitors grew faster than vehicle-treated INS-E cell cultures, since cultures subjected for 4 days to GSK3 inhibitors contained more cells (Fig. 2, A, C, and E) and the measurement of caspase activity (C, F, and I) and the measurement of caspase activity (C, F, and I). The kinase inhibitors 1-azakenpaullone (1-AKP) and CHIR99021 (CHIR) are well tolerated by INS-1E cells over a broad concentration range as indicated by the results of the metabolic activity or viability assay (A and D). The kinase inhibitor BIO was used at concentrations below 2 μM, because it compromised INS-1E cell viability at higher concentrations (G). The figure shows representative experiments of at least three independently performed studies. The given values are means of measured data of at least eight wells. Values show fold change relative to control ± S.D. The asterisks indicate statistically significant differences (Student’s t test). *, p < 0.05 vs INS-1E cells not treated with the indicated inhibitor.

FIGURE 1. Small molecule inhibitors of GSK3 kinase protect INS-1E cells from glucolipotoxicity. Cell death was induced by the treatment of INS-1E cells with a mixture (Gluc/Pal) of high concentrations of palmitate (0.3 mM palmitate coupled to bovine serum albumin) and glucose (25 mM) for 24 h. Apoptosis was quantitatively monitored by the determination of cytosolic DNA fragments (DNA-fragment), (B, E, and H) and the measurement of caspase activity (C, F, and I). The kinase inhibitors 1-azakenpaullone (1-AKP) and CHIR99021 (CHIR) are well tolerated by INS-1E cells over a broad concentration range as indicated by the results of the metabolic activity or viability assay (A and D). The kinase inhibitor BIO was used at concentrations below 2 μM, because it compromised INS-1E cell viability at higher concentrations (G). The figure shows representative experiments of at least three independently performed studies. The given values are means of measured data of at least eight wells. Values show fold change relative to control ± S.D. The asterisks indicate statistically significant differences (Student’s t test). *, p < 0.05 versus INS-1E cells treated with a mixture of high palmitate and glucose in the absence of the indicated inhibitor.

FIGURE 2. Stimulation of INS-1E beta cell growth by structurally diverse small molecule inhibitors of GSK3 kinase. INS-1E cells were treated with the test compounds 1-azakenpaullone (1-AKP), CHIR99021 (CHIR), or BIO at the indicated concentrations for 24 h. Cell replication was determined by BrdUrd incorporation. A, C, and E illustrate the relative increase in incorporated BrdUrd in INS-1E cells (Fold increase BrdU). Mitogenic effects were confirmed by cell counts (B, D, and F). The relative cell number of INS-1E cells was determined after treatment of INS-1E cells for 4 days with any of the three kinase inhibitors using the CyQuant cell proliferation assay. Results are presented as -fold change relative to control ± S.D. The figure shows representative experiments of two independently performed studies. Given values are means of measured data of at least eight wells. The asterisks indicate statistically significant differences (Student’s t test). *, p < 0.05 versus INS-1E cells not treated with the indicated inhibitor.
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TABLE 1
Summary of effects of small molecule GSK3 inhibitors on INS-1E cell functions

| GSK3 inhibitors | Assays in INS-1E cells | Resistance to induced cell death (24 h) | Replication (24 h) | Growth (after 4 days of treatment) |
|-----------------|------------------------|-----------------------------------------|-------------------|-----------------------------------|
| 1-AKP           |                        | +                                       | +                 | +                                 |
| CHIR99021       |                        | +                                       | +                 | +                                 |
| BIO (toxic >2 μM) |                       | +                                       | +                 | +                                 |
| SB216763        |                        | +                                       | +                 | ND                                |
| AR-A014418      |                        |                                         |                   |                                    |
| (toxic >2.5 μM) |                        |                                         |                   |                                    |
| Lithium (toxic >3 mM) |                  | ND                                     |                   |                                    |

much less is known about this molecule because it is comparatively new and has not yet been widely used in experimental biology. SB216763 is another relatively potent and selective GSK3 inhibitor that has been shown to efficiently inhibit GSK3 activity in cells (57, 58). The effects of this compound on INS-1E cells were very similar to those of 1-azakenpaullone, CHIR99021, and BIO (Table 1), further supporting our conclusion that GSK3 is a regulator of INS-1E cell replication and survival.

Simultaneous Inhibition of GSK3α and -β Expression Stimulates Replication of INS-1E Beta Cells—In order to support the notion that GSK3 is a key regulator of INS-1E replication, gene silencing experiments targeting both GSK3 isoforms via small double-stranded RNA interference (siRNA) were carried out in INS-1E cells. siRNA duplexes specific for GSK3α or -β were transfected into INS-1E cells, leading to a drastic decrease of GSK3α and -β protein expression (Fig. 3B) as well as a reduction of GSK3α and -β RNA by about 75% (Fig. 3A). Transfection of isoform-specific siRNAs did not affect the expression levels of the respective other isoform or of unrelated genes like cyclophilin B or α-tubulin, indicating a high selectivity of the used siRNA oligonucleotides (Fig. 3, A and B). Transfection of GSK3α or -β siRNA alone specifically reduced the expression of the respective RNA or protein; however, it did not stimulate the rate of INS-1E proliferation. Interestingly, siRNA oligonucleotides targeting both GSK3 isoforms had to be transfected simultaneously to stimulate cell replication. This indicates redundancy between the two GSK3 isoforms in the regulation of beta cell replication (Fig. 3C). In summary, the outcomes of the siRNA experiments are consistent with the above presented data obtained with small molecule GSK3 inhibitors, confirming that GSK3 inactivation has mitogenic effects on INS-1E beta cells.

GSK3 Inhibitors Promote Replication of Beta Cells in Islets—The effects of GSK3 inhibitors on cell replication were further assessed in primary rat beta cells. Replicating beta cells were identified by double immunocytochemical staining using antibodies against C-peptide, a proteolytic fragment of proinsulin, and the nuclear cell division marker Ki-67 (Fig. 4A). 1-Azakenpaullone and CHIR99021 were found to promote beta cell replication in isolated rat islets at concentrations as low as 1 μM after 72 h of incubation (Fig. 4, B and C, respectively). Two to three times more replicating beta cells were found in islets treated with 5 μM 1-azakenpaullone or CHIR99021, respectively, compared with vehicle-treated controls. This result is consistent with observations made in INS-1E cells (Fig. 2, A and C), although the relative increase in the proliferation rate was lower in INS-1E cells. In our hands, GSK3 inhibitors revealed stronger mitogenic activities on primary beta cells than prolac-
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This study indicates that GSK3 is not only a regulator of beta cell survival but also of beta cell replication. We report that selective and potent small molecule inhibitors of GSK3 stimulate the replication of both insulinoma cells and primary beta cells in isolated islets. Interestingly, under the specific conditions used in our proliferation assay, the mitogenic effects of GSK3 inhibitors were found to be stronger than those elicited by prolactin and in the same range as those elicited by GIP. To the best of our knowledge, GSK3 inhibitors are currently the only small molecule compounds with protective as well as replication-stimulating effects on beta cells. In contrast to other reported beta cell growth factors, which need to be administered by injection, GSK3 inhibitors are orally available, adding to their potential clinical significance. Pharmacological inhibitors of the extracellular enzyme dipeptidyl peptidase IV have also been shown to improve beta cell function under prediabetic as well as diabetic conditions (62–64). However, these molecules only indirectly support beta cells by increasing the blood levels of growth factors, such as endogenous GLP-1 and GIP.

GSK3 is a substrate of the PI 3-kinase/Akt pathway (65), which has previously been shown to be a central regulator of beta cell growth. However, PI 3-kinase and Akt have numerous and diverse intracellular targets, and the relative contribution of GSK3 to PI 3-kinase/Akt-mediated growth-promoting and antiapoptotic effects in beta cells were unknown. Here we demonstrate that the inactivation of GSK3 is sufficient to induce proliferative and antiapoptotic effects in beta cells. This implies that GSK3 is an important mediator linking PI 3-kinase/Akt signaling with downstream signaling elements in both antiapoptotic and proliferation pathways. For example, GSK3 may relay Akt signals to cell cycle regulators, such as c-Myc, D-type cyclins, p21\(^{CDK1}\), or Pdx1 in beta cells.

mitogenic effects of 1-azakenpaullone on rat beta cells were found to be comparable with those of the incretin hormone GIP (Fig. 4D). Taken together, these findings illustrate that GSK3 is a regulator of beta cell replication.

To further elicit the effects of GSK3 inhibitors on primary beta cells, the RNA expression levels of some genes involved in the regulation of beta cell function were analyzed by quantitative reverse transcription-PCR (Fig. 4E). \(g_{\text{ax}}\), \(\text{pax4}\), \(\text{bel-xl}\), \(c\text{-myc}\), and \(id2\) were selected, because an increase of their mRNA expression level is associated with an increase in beta cell replication (43). The expression of \(\text{CDK4}\), a specific regulator of the G\(_1\) checkpoint, was analyzed, because \(\text{CDK4}\) overexpression in beta cells stimulates their proliferation (60). NeuroD and Pdx1 are key regulators of insulin transcription, beta cell development, and function (61). Except for \(id2\), the treatment with 1-azakenpaullone did not significantly modulate the expression of these genes on the RNA level, leaving the GSK3 downstream events unclear. On the other hand, the result indicates that GSK3 inhibitors do not compromise the transcription of important regulators of beta cell function, survival, and proliferation. In conclusion, further research is required to dissect signaling events following the inhibition of GSK3 in beta cells.

FIGURE 4. Small molecule inhibitors of GSK3 kinase stimulate replication of primary beta cells in isolated rat islets. Replicating beta cells in isolated rat islets were identified by double immunofluorescence staining (A) using antibodies against C-peptide (green) and antibodies recognizing the nuclear proliferation marker Ki-67 (red). Nuclear DNA was stained with 4\',6-diamidino-2-phenylindole (blue). After incubation with 1-azakenpaullone (1-AKP), CHIR99021 (CHIR), prolactin (PRL), or glucose-dependent insulinotropic polypeptide (GIP) for 72 h, islets were disaggregated, and the resultant cell suspension was spotted on microscope slides before staining with antibodies and analysis. Islets processed in parallel to the above described samples but not treated with any factor (Co.) were included as controls. B–D show the percentage of Ki-67-expressing cells of all C-peptide-positive cells (%Ki-67*/C-peptide* cells), which corresponds to the fraction of replicating beta cells. B and C illustrate representative experiments of at least three independent studies, and each data point includes about 30,000 C-peptide-positive cells. GIP seems to further enhance the growth-stimulating effect of the GSK3 inhibitor 1-azakenpaullone. D shows a representative experiment of two independent experiments where isolated islets were treated with GIP or 1-azakenpaullone alone or in combination. The RNA expression levels of selected genes important for beta cell functions were analyzed in rat islets treated for 24 h with 1-AKP (E). Data are presented as relative expression levels in percent compared with the untreated control, which was defined as 100%. Data of representative experiments are shown in which at least three independently treated and processed culture wells were analyzed per data point, and the calculated average and the S.D. are indicated. Error bars represent ± S.D. Asterisks, statistically significant differences (Student’s t test), \(*p<0.05; **p<0.01\) versus control and in B for the values indicated by brackets also versus prolactin.

mitogen (Fig. 4B), a factor that drives beta cell expansion during pregnancy. However, the situation may be different when cells are treated for longer periods of time, since prolactin requires several days of incubation to reach maximal activity (59). The
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(35). Further research is required to understand the signaling events downstream of GSK3 in beta cells.

GSK3 also transmits signals of the canonical Wnt pathway by regulating the levels of β-catenin, whose temporal and spatial expression during development is instrumental for pancreas organogenesis. Signaling via this pathway, however, is probably not essential for beta cell growth in adults (66–69). Taken together, there is accumulating evidence for a key role of GSK3 in the regulation of beta cell mass, and additional pathway analysis using pharmacological inhibitors of GSK3 in animals or transgenic mice expressing kinase dead mutants in beta cells is now required to substantiate these findings.

The loss of beta cell mass under diabetic conditions is most likely due to an increase in beta cell death and insufficient compensatory regeneration (8, 9). Human beta cells are responsive to regenerative signals, as demonstrated by the effect of overexpression of regulatory factors, such as Pax4 (43) or CDK4/cyclin D (60), and the exposure to growth factors in vitro and in vivo (70–73). GSK3 inhibitors combine protective as well as proliferative effects and thus might be expected to significantly expand the beta cell mass in patients with diabetes. In our experiments, the co-treatment of islets with GIP and GSK3 inhibitors has an additive effect on beta cell proliferation, which indicates that beta cells remain sensitive to signals from other factors, such as endogenous incretins released after meals or injected incretin analogs. This also indicates that a combination therapy approach could be beneficial.

GSK3 is already considered as a potential drug target for type 2 diabetes, because GSK3 inhibitors mimic some actions of insulin and improve insulin sensitivity. They enhance glucose disposal and have previously been shown to exert antidiabetic effects in animal models of type 2 diabetes by addressing key tissues malfunctioning in type 2 diabetes, such as liver and muscle (47, 50, 74–76). Thus, the combination of the effects on glucose metabolism together with the beneficial effects on beta cells, as described herein, warrant further investigation of the therapeutic potential of GSK3 inhibitors for type 2 diabetes.

The protective and beta cell proproliferative activities of GSK3 inhibitors also make them suitable for the treatment of type 1 diabetes, especially immediately after disease onset. Ideally, GSK3 inhibitors would be combined with an immunomodulatory agent suppressing the autoimmune attack on the beta cells (19, 20, 77). Finally, GSK3 inhibitors may find applications in islet transplantation procedures. Isolated islets could be treated with GSK3 inhibitors before transplantation to expand and preserve beta cells. Alternatively, transplant recipients could be treated after islet transplantation to improve graft survival and function.

The search for GSK3 inhibitors is a very active field, because GSK3 has been identified as an interesting drug target for several diseases (26). They are primarily being developed for the treatment of Alzheimer disease (78). The main concern about GSK3 as a drug target has to do with its regulatory role in a variety of cellular processes, including behavior, bone turnover, immunity, or the circadian clock (24, 79–83) and the possibility that its long term inhibition may cause serious adverse effects. The inhibition of GSK3 may activate pathways involved in tumor development and progression, such as the Wnt signaling pathway. Here, inactivation of GSK3 leads to a stabilization of β-catenin, which in turn can drive tumorigenesis in the gut epithelium and other tissues. It appears, however, that specific thresholds for GSK3 inhibition are associated with specific cellular processes. It may thus be possible to enhance beta cell proliferation and survival as well as insulin sensitivity without provoking consequences of enhanced β-catenin stability (51, 76). With the recent availability of potent small molecule GSK3 inhibitors, these safety issues as well as therapeutic efficacy can now be addressed in a systematic fashion. In the future, more selective and possibly non-ATP-competitive inhibitors (84) or the development of targeting technologies that direct inhibitors to certain cell types (85) may also lower the potential risks. An intensive benefit-risk analysis may eventually resolve, whether patients with diabetes will profit from therapeutics inactivating GSK3.

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