Glucose- and Interleukin-1β–Induced β-Cell Apoptosis Requires Ca\(^{2+}\) Influx and Extracellular Signal–Regulated Kinase (ERK) 1/2 Activation and Is Prevented by a Sulfonylurea Receptor 1/Inwardly Signal–Regulated Kinase (ERK) 1/2 Activation and Is Requires Ca\(^{2+}\) Influx and ERK Dependent and Can be Prevented by the β-cell Selective Potassium Channel Opener NN414

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Increasing evidence indicates that a progressive decrease in the functional β-cell mass is the hallmark of both type 1 and type 2 diabetes. The underlying causes, β-cell apoptosis and impaired secretory function, seem to be partly mediated by macrophage production of interleukin (IL)-1β and/or high-glucose–induced β-cell production of IL-1β. Treatment of type 1 and type 2 diabetic patients with the potassium channel opener diazoxide partially restores insulin secretion. Therefore, we studied the effect of diazoxide and of the novel potassium channel opener NN414, selective for the β-cell potassium channel SUR1/Kir6.2, on glucose- and IL-1β–induced apoptosis and impaired function in human β-cells. Exposure of human islets for 4 days to 11.1 and 33.3 mmol/l glucose, 2 ng/ml IL-1β, or 10 and 100 μmol/l of the sulfonylurea tolbutamide induced β-cell apoptosis and impaired glucose-stimulated insulin secretion. The deleterious effects of glucose and IL-1β were blocked by 200 μmol/l diazoxide as well as by 3 and 30 μmol/l NN414. By Western blotting with phosphospecific antibodies, glucose and IL-1β were shown to activate the extracellular signal–regulated kinase (ERK) 1/2, an effect that was abrogated by 3 μmol/l NN414. Similarly, 1 μmol/l of the mitogen-activated protein kinase/ERK kinase 1/2 inhibitor PD098059 or 1 μmol/l of the l-type Ca\(^{2+}\) channel blocker nimodipine prevented glucose- and IL-1β–induced ERK activation, β-cell apoptosis, and impaired function. Finally, islet release of IL-1β in response to high glucose could be abrogated by nimodipine, NN414, or PD098059. Thus, in human islets, glucose- and IL-1β–induced β-cell secretory dysfunction and apoptosis are Ca\(^{2+}\) influx and ERK dependent and can be prevented by the β-cell selective potassium channel opener NN414. Diabetes 53:1706–1713, 2004

There is now general agreement that both impaired β-cell function and decreased β-cell mass contribute to the insulin deficiency observed in patients with type 2 diabetes (1–11). Therefore, in the rationale behind new therapeutic approaches to both type 2 and type 1 diabetes maintenance or even improvement of β-cell functional mass should be considered. A key regulator of β-cell function and survival is the β-cell ATP-sensitive K\(^{+}\) (K\(_{\text{ATP}}\)) channel. It is a hetero-octamer composed of four inwardly rectifying K\(^{+}\) channels (Kir) and four sulfonylurea receptors (SUR1). Increased intracellular glucose concentrations result in the production of ATP, raising the ATP-to-ADP ratio, with subsequent closure of K\(_{\text{ATP}}\) channels. A decrease of K\(^{+}\) conductance leads to membrane depolarization, opening of voltage-operated Ca\(^{2+}\) channels, mainly of the l-type, Ca\(^{2+}\) influx, and a rise in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)), which induces insulin secretion (12). Modulation of the K\(_{\text{ATP}}\) channel also influences β-cell survival. Indeed, blocking of β-cell K\(_{\text{ATP}}\) channels with the sulfonylureas tolbutamide and glibenclamide induces Ca\(^{2+}\) influx–dependent β-cell apoptosis, as shown in a β-cell line (13) in rodent (14) and human (15) islets. In contrast, K\(_{\text{ATP}}\) channel openers may have protective effects in β-cells. In 1976, Greenwood et al. (16) were the first to report an improvement in insulin secretion after administration for 7 days of the K\(_{\text{ATP}}\) channel opener diazoxide to diabetic subjects. Preservation of residual insulin secretion by treatment with diazoxide was then shown in patients with type 1 diabetes (17) and more recently in subjects with type 2 diabetes (18). Similar protective effects were observed in the VDF Zucker rat and in a type 1 diabetic rat model treated with the recently developed β-cell–selective...
SUR1/Kir6.2 potassium channel opener NN414 (19,20). Finally, activation of K\textsubscript{ATP} channels also preserves β-cell pulsatile insulin secretion in human islets cultured at high glucose (21). Although these beneficial effects may be due to inducing β-cell "rest," they may also reflect in part an antiapoptotic effect of such drugs (14).

Chronic elevation of the blood glucose concentration impairs β-cell function (22–31) and induces β-cell apoptosis (14,32–34). Furthermore, in cultured human pancreatic islets, overstimulation by high glucose leads to a rise in [Ca\textsuperscript{2+}], which persists after normalization of the glucose concentration (35,36). Therefore, we hypothesized that parts of these toxic effects of glucose are mediated via a reduction in K\textsubscript{ATP} channel activity and elevated [Ca\textsuperscript{2+}], and thus may be counteracted by potassium channel openers and/or calcium channel antagonists.

Interleukin (IL)-1β is thought to be a key mediator of both impaired function and destruction of pancreatic β-cells during the development of autoimmune type 1 diabetes (37). Furthermore, IL-1β seems to contribute to β-cell glucotoxicity in the pathogenesis of type 2 diabetes. Indeed, exposure of cultured human islets to elevated glucose levels leads to β-cell production and release of IL-1β (38). In turn, IL-1β feeds back on the β-cell to induce impaired function and apoptosis. Among several signaling pathways activated by IL-1β in the β-cell is the activation of extracellular signal–regulated kinase (ERK) 1/2, a mitogen-activated protein kinase (MAPK). This has been shown in β-cell lines, rat islets, and purified primary rat β-cells (39–41). ERK activation has been demonstrated to be required both for cytokine-induced expression of inducible nitric oxide (NO) synthase (iNOS) (39) and β-cell apoptosis (40). Glucose also stimulates activation of ERK1/2 in β-cells (42–47), and in macrophages, ERK1/2 activation was recently shown (48) to be required for double-stranded RNA- and virus-induced IL-1β expression. However, whether glucose-induced β-cell production of IL-1β as well as IL-1β–induced β-cell toxicity in human islets require ERK1/2 is unknown. Therefore, we investigated the effect of diazoxide and NN414 on glucose- and IL-1β–induced impaired function and apoptosis in human β-cells and to what extent their mechanisms of action involved modulation of [Ca\textsuperscript{2+}], and ERK1/2 activity.

**RESULTS**

K\textsubscript{ATP} channel openers protect against glucose-induced apoptosis and impaired function, whereas tolbutamide augments the deleterious effect of glucose in human β-cells. Human islets were cultured on extracellular matrix–coated dishes and exposed for 4 days to increasing concentrations of glucose in the presence or absence of NN414, diazoxide, or tolbutamide. Analysis of β-cell nuclei for DNA fragmentation (TUNEL positive) revealed a 1.9- and 3.9-fold increase in islets cultured at
medium glucose concentration of 11.1 and 33.3 mmol/l, respectively, relative to islets at 5.5 mmol/l glucose (Fig. 1A). The $\text{K}_\text{ATP}$ channel openers NN414 and diazoxide did not significantly change baseline apoptosis at 5.5 mmol/l glucose. However, 3, 30, and 100 $\mu$mol/l NN414 partially protected the $\beta$-cells from apoptosis induced by 11.1 mmol/l glucose, whereas only 3 and 30 $\mu$mol/l NN414 were protective at 33.3 mmol/l glucose. Diazoxide had no significa

FIG. 1. $\text{K}_\text{ATP}$ channel openers protect from glucose-induced $\beta$-cell apoptosis and impaired secretory function in human islets. Human islets were cultured on extracellular matrix–coated dishes for 4 days in 5.5, 11.1, and 33.3 mmol/l glucose alone or with 3, 30, and 100 $\mu$mol/l NN414; 200 $\mu$mol/l diazoxide; or 10 and 100 $\mu$mol/l tolbutamide. A: Results are means ± SE of the percentage of TUNEL-positive $\beta$-cells per islet normalized to control incubations at 5.5 mmol/l glucose alone (100%; in absolute value, 0.32 ± 0.03 TUNEL-positive $\beta$-cells per islet). Islets were isolated from five organ donors. The mean number of islets scored from each donor was 28 (range 21–45) for each treatment condition. B: Chronic insulin release into the culture medium during the 4-day incubation period. Results are means ± SE of the insulin secreted per islet relative to control incubations at 5.5 mmol/l glucose alone (100%; in absolute value, 3.45 ± 0.59 pmol · islet$^{-1}$ · day$^{-1}$). C: Ratio of stimulated to basal insulin secretion during successive 1-h incubation at 3.3 (basal) and 16.7 (stimulated) mmol/l glucose after the 4-day culture period. Results are means ± SE. Islets were isolated from three organ donors. In each experiment, the data were collected from three plates per treatment. $*P < 0.05$ vs. control islets at 5.5 mmol/l glucose alone; $**P < 0.05$ vs. islets at 11.1 mmol/l glucose alone; $#P < 0.05$ vs. islets at 33.3 mmol/l glucose alone. 

The effect of closure of $\text{K}_\text{ATP}$ channels by the sulfonylurea tolbutamide was also tested. At 5.5 mmol/l glucose, exposure to 10 or 100 $\mu$mol/l tolbutamide induced a 1.7-fold increase in the number of TUNEL-positive $\beta$-cells, relative to islets at 5.5 mmol/l glucose alone. At 11.1 mmol/l glucose, 10 $\mu$mol/l tolbutamide had no additive effect on $\beta$-cell apoptosis induced by the closure of $\text{K}_\text{ATP}$ channels by glucose itself, whereas 100 $\mu$mol/l tolbutamide induced a similar deleterious effect to that of 33.3 mmol/l glucose alone. Tolbutamide had no additive effect to 33.3 mmol/l glucose–induced DNA fragmentation.

Exposure of cultured human islets to elevated glucose concentrations for 4 days decreased the number of proliferating (Ki-67$^+$) $\beta$-cells. Proliferation was reduced by 34 and 61% in medium that contained 11.1 and 33.3 mmol/l glucose, respectively, relative to islets at 5.5 mmol/l glucose alone (100%; in absolute value: 0.89 ± 0.2% Ki-67$^+$).
β-cells; P < 0.01). Tolbutamide, diazoxide, and NN414 had no significant influence on β-cell proliferation at those glucose concentrations (data not shown).

Accumulated insulin release into the culture medium was measured after the 4-day culture period. Glucose (11.1 and 33.3 mmol/l) as well as 10 μmol/l tolbutamide increased accumulated insulin release, which was decreased by the K<sub>ATP</sub> channel openers NN414 and diazoxide (Fig. 1B).

Exposure of human islets to 11.1 or 33.3 mmol/l glucose for 4 days completely abolished acute glucose-stimulated insulin release (Fig. 1C). Coincubation with 3 and 30 μmol/l NN414 or 200 μmol/l diazoxide partially restored glucose responsiveness. Similar to elevated glucose concentrations, chronic exposure to tolbutamide also impaired β-cell secretory function.

Insulin content of islets cultured for 4 days in high glucose or in the presence of tolbutamide was decreased. Insulin content was reduced by 12.9, 47.9, and 55.8% in medium that contained 11.1 mmol/l glucose, 33.3 mmol/l glucose, and 100 μmol/l tolbutamide, respectively, relative to islets at 5.5 mmol/l glucose alone (P < 0.01). Coincubation with 3 μmol/l NN414 prevented the decrease in insulin content by 30.2% (P < 0.05) and 111.2% (P < 0.01) compared with 11.1 and 33.3 mmol/l glucose alone, respectively, whereas 200 μmol/l diazoxide prevented the decrease by 33.9% at 33.3 mmol/l (P < 0.05) but had no significant effect at 11.1 mmol/l glucose.

**Glucose-induced β-cell apoptosis and impaired function require l-type Ca<sup>2+</sup> influx and activation of ERK MAPK.** To investigate whether ERK1/2 is activated by glucose and IL-1β, we performed Western blotting with phosphospecific antibodies that recognize only Thr<sup>202</sup>/Tyr<sup>204</sup>-phosphorylated ERK1/2 (39,54). As seen in Fig. 2, exposure of cultured human islets to 11.1 and 33.3 mmol/l glucose or 2 ng/ml IL-1β for 30 min enhanced the phosphorylation of ERK1/2 (P-ERK1/2), whereas total ERK1/2 remained unchanged. In contrast, baseline as well as IL-1β- and glucose-induced ERK1/2 phosphorylation were decreased by preincubation for 1 h with 1 μmol/l of the inhibitor of MAPK/ERK1/2 PD098059. Similarly, ERK1/2 phosphorylation was also prevented by preincubation for 1 h with 1 μmol/l of the l-type Ca<sup>2+</sup> channel blocker nimodipine. It is interesting and in contrast to PD098059 and nimodipine that preincubation for 1 h with 3 μmol/l of the β-cell–selective potassium channel opener NN414 had no influence on baseline ERK1/2 phosphorylation but decreased glucose- and IL-1β–induced ERK activation.

We next studied the functional role of glucose-induced, calcium-mediated, ERK activation in human β-cells; specifically, we investigated the ability of nimodipine and PD098059 to protect β-cells from glucose-induced apoptosis and impaired function. Exposure of human islets to 11.1 and 33.3 mmol/l glucose in the presence of 1 μmol/l nimodipine as well as 1 μmol/l PD098059 protected the β-cells from 11.1 and 33.3 mmol/l glucose–induced apoptosis and restored acute glucose-stimulated insulin secretion (Fig. 3). Furthermore, 1 μmol/l nimodipine and 1 μmol/l PD098059 induced a 2.4- and 1.9-fold increase of insulin content, respectively, in islets cultured at 33.3 mmol/l glucose (P < 0.05). A total of 1 μmol/l of each compound had no significant effect at 5.5 mmol/l glucose (Fig. 3), whereas higher concentrations of nimodipine (10 μmol/l) and PD098059 (10 μmol/l) by themselves induced β-cell apoptosis at 5.5 mmol/l glucose (data not shown). To rule out possible nonspecific actions, we confirmed the protective effects of PD098059 and nimodipine using an additional MAPK inhibitor (UO126) and Ca<sup>2+</sup> channel blocker (nitrendipine) (Fig. 3D). Glucose-induced IL-1β release and IL-1β–induced β-cell apoptosis are also Ca<sup>2+</sup> influx and ERK dependent and can be prevented by potassium channel openers.

The ability of nimodipine, PD098059, diazoxide, and NN414 to protect from the deleterious effects of IL-1β was tested. Exposure of human islets cultured on extracellular matrix–coated dishes to 2 ng/ml IL-1β for 4 days resulted in a 4.8-fold increase in β-cell apoptosis compared with control at 5.5 mmol/l (Fig. 4A). This was strongly reduced by coincubation with 1 μmol/l nimodipine, 1 μmol/l PD098059, 200 μmol/l diazoxide, or 3 μmol/l NN414. Similarly, these compounds restored glucose-stimulated insulin secretion (Fig. 4B and C). Furthermore, IL-1β decreased insulin content by 26.0% compared with islets at 5.5 mmol/l glucose alone, a decrease that was prevented by 61.6, 79.4, 62.1, and 70.0% by 200 μmol/l diazoxide, 3

**FIG. 2. Glucose and IL-1β activate ERK1/2 in human islets.** Human islets were cultured in suspension for 30 min in 5.5 mmol/l glucose alone or in the presence of 2 ng/ml IL-1β or in 11.1 or 33.3 mmol/l glucose with or without preincubation for 1 h with 1 μmol/l PD098059, 1 μmol/l nimodipine, or 3 μmol/l NN414. Islet extracts were analyzed by Western blotting for Thr<sup>202</sup>/Tyr<sup>204</sup>-phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (ERK1/2) or actin. One of three experiments with all conditions, except for the presence of PD098059 and 11.1 mmol/l glucose (two experiments), from six organ donors is shown. All experiments gave similar results.
FIG. 3. Glucose-induced β-cell apoptosis and impaired function require L-type Ca\(^{2+}\) influx and ERK1/2 activation. Human islets were cultured on extracellular matrix-coated dishes for 4 days in 5.5, 11.1, and 33.3 mmol/l glucose alone or with 1 μmol/l nimodipine, 1 μmol/l nitrendipine, 1 μmol/l PD0898059, or 1 μmol/l UO126. A: Results are means ± SE of the percentage of TUNEL-positive β-cells per islet normalized to control incubations at 5.5 mmol/l glucose alone (100%; in absolute value, 0.32 ± 0.03 TUNEL-positive β-cells per islet). The mean number of islets scored from each donor was 32 (range 25–43) for each treatment condition. B and C: Basal and stimulated insulin secretion during successive 1-h incubation at 3.3 (basal) and 16.7 (stimulated) mmol/l glucose after the 4-day culture period. Results are means ± SE of the insulin secreted per islet relative to control incubations at 5.5 mmol/l glucose alone (100%; in absolute value, 0.79 ± 0.08 pmol/islet for basal insulin). D: Results are means ± SE of the percentage of TUNEL-positive β-cells per islet normalized to control incubations at 5.5 mmol/l glucose alone. Islets were isolated from three (A–C) or one (D) organ donor. In each experiment, the data were collected from three plates per treatment. *P < 0.05 vs. control islets at 5.5 mmol/l glucose alone; **P < 0.05 vs. islets at 11.1 mmol/l glucose alone; #P < 0.05 vs. islets at 33.3 mmol/l glucose alone.

**DISCUSSION**

The central role of the K\(_{ATP}\) channel in mediating acute glucose-stimulated insulin release is well established. In the present study, we described an additional role of this channel as a key mediator of β-cell survival/apoptosis. Indeed, long-term (4 days) exposure to either glucose or IL-1β led not only to impaired β-cell secretory function but also to β-cell apoptosis. These effects were mediated by closure of K\(_{ATP}\) channels and therefore could be prevented by the potassium channel openers diazoxide and NN414. Furthermore, our data demonstrated that both glucose and IL-1β effects were mediated by Ca\(^{2+}\) influx and activation of ERK1/2.

The protective mechanisms of potassium channel openers have been associated with β-cell “rest.” However, defective insulin secretion may be secondary to decreased β-cell mass (23,55–57). Therefore, the beneficial effect of potassium channel openers could be due to the antia apoptotic effects of these compounds described in the present study. After a 4-day exposure to high glucose or IL-1β, ~1% of the β-cells undergo apoptosis. Therefore, it is unlikely that this apoptotic process has a significant impact on β-cell function in vitro during this time period, whereas the direct toxic effects of chronic exposure to high glucose and to IL-1β on β-cell function can clearly be reversed by potassium channel openers. Nevertheless, the potent antiapoptotic effect of these compounds may be of potential importance in vivo, when one considers that a decrease in β-cell mass is the hallmark of both type 1 and type 2 diabetes and the long-term consequence of even a small daily turnover of apoptotic β-cells.

Both glucose- and IL-1β–induced apoptosis and impaired function were dependent on Ca\(^{2+}\) influx and ERK1/2 activation and could be counteracted by the action of K\(_{ATP}\) channel openers. The similarity of the effects induced by the nutrient glucose and the cytokine IL-1β is explained by the ability of glucose to induce β-cell production of IL-1β (38). Surprisingly, the K\(_{ATP}\) channel opener NN414, the L-type Ca\(^{2+}\) channel blocker nimodipine, and the MAPK/ERK1/2 inhibitor PD0898059 also prevented glucose-induced IL-1β release. It follows that the pathway that leads to islet production of IL-1β is also used by IL-1β itself to induce its toxic effects. That glucose-induced β-cell release
of IL-1β is dependent on ERK1/2 is in line with a recent report (48) showing that ERK activation is required for virus-induced IL-1 β expression in macrophages.

In the present study, we observed phosphorylation of ERK1/2 by glucose and IL-1β (already) after a 30-min incubation, whereas apoptosis and impaired function were investigated after 4-day exposures. Detection of ERK1/2 activation was investigated after such a short incubation time because it is shown to be rapidly activated. However, activation was investigated after such a short incubation, whereas apoptosis and impaired function were detected after 24 h (39). Moreover, PD098059 inhibited ERK1/2 phosphorylation not only in the short-time experiment, but also apoptosis and impaired function in the 4-day incubations. Therefore, ERK1/2 is also a mediator of the long-term effects of glucose and IL-1β.

IL-1β activation of ERK1/2 has previously been observed in rat islets cells (39–41). Moreover, in rat islets, IL-1β–induced expression of iNOS requires ERK1/2 activation (39). Although IL-1β also induces ERK1/2 activation in human islets, IL-1β alone does not induce iNOS, as has been shown repeatedly (38,58,59). Because of this difference between human and rat islets, it was important to demonstrate that IL-1β indeed activates ERK1/2 in human islets. Furthermore, we show that ERK1/2 mediates the deleterious effects of IL-1β on β-cell function and is required for IL-1β–induced apoptosis.

The TUNEL assay detects DNA fragmentation associated with both apoptotic and necrotic cell death. Therefore, the observation of glucose-induced TUNEL positivity and its antagonism by NN414, nimodipine, and PD098059 could reflect a modulation of necrosis and not apoptosis. Nevertheless, in several previous studies, we tested, in parallel to the TUNEL reaction, the DNA-binding dye propidium iodide, triple immunostaining for DNA fragmentation, insulin, and cleaved caspase 3 and were able to demonstrate solely an apoptotic process in the presence of high glucose (34,38,53).

The beneficial effect of NN414 against glucotoxic effects was maximal at a concentration of 3 µmol/l, whereas 100 µmol/l was much less effective. Accordingly, it was shown recently (60) in HEK293 cells stably expressing the pancreatic β-cell type KATP channel that the EC50 of NN414 is 0.45 µmol/l and that very high concentrations may be toxic.

β-Cell mass depends not only on β-cell apoptosis but also on the rate of neogenesis or replication. In the present study, tolbutamide, diazoxide, and NN414 had no significant influence on β-cell proliferation. However, our in vitro system does not allow for detection of neogenesis. Nevertheless, in type 2 diabetes, increased apoptosis seems of greater importance in the reduced β-cell mass than impaired neogenesis/proliferation (2).

In conclusion, this study suggests that in human islets, glucose- and IL-1β–induced β-cell secretory dysfunction and apoptosis are KATP channel, Ca2+ influx, and ERK dependent. Glucotoxicity and IL-1β are suggested to be major driving forces for β-cell death and impaired β-cell function in both type 1 and type 2 diabetes. Therefore, the use of orally active KATP channel openers may be a promising novel therapeutic strategy to preserve β-cell mass and function in type 1 and 2 diabetes.

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