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Sulfonylurea Induced β-Cell Apoptosis in Cultured Human Islets

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Loss of β-cell mass and function raises a concern regarding the application of sulfonylureas for the treatment of type 2 diabetes because previous studies have shown that agents that cause closure of inwardly rectifying K^+ sulfonylurea receptor subtype of ATP-sensitive potassium channels, such as tolbutamide and glibenclamide, induce apoptosis in β-cell lines and rodent islets. Therefore, we investigated the effect of the new insulin secretagogues, repaglinide and nateglinide, and the sulfonylurea, glibenclamide, on β-cell apoptosis in human islets. Human islets from six organ donors were cultured onto extracellular matrix-coated plates and exposed to glibenclamide, repaglinide, or nateglinide. The doses of the three compounds were chosen according to detected maximal effects, *i.e.* efficacy. Exposure of human islets for 4 h to 0.1 and 10 μM glibenclamide induced a 2.09- and 2.46-fold increase in β-cell apoptosis, respectively, whereas repaglinide (0.01 and 1 μM) did not change the number of apoptotic β-cells. At low concentration (10 μM), nateglinide did not induce β-cell apoptosis. However, at high concentration of 1000 μM, it induced a 1.49-fold increase in the number of apoptotic β-cells. Prolonged exposure for 4 d of the islets to the secretagogues induced β-cell apoptosis. The increase was of 3.71- and 4.4-fold at 0.1 and 10 μM glibenclamide, 2.37- and 3.8-fold at 0.01 and 1 μM repaglinide, and of 3.2- and 4.6-fold at 10 and 1000 μM nateglinide, respectively. Glibenclamide at 0.1–10 nM (doses that were less efficient on insulin secretion) did not induce β-cell apoptosis after 4 h incubation as well as 0.1 nM after 4 d incubation. However, 1 and 10 nM glibenclamide for 4 d induced a 2.24- and 2.53-fold increase in β-cell apoptosis, respectively. Taken together, closure of the inwardly rectifying K^+ sulfonylurea receptor subtype of ATP-sensitive potassium channels induces β-cell apoptosis in human islets and may precipitate the decrease in β-cell mass observed in patients with type 2 diabetes. (*J Clin Endocrinol Metab* 90: 501–506, 2005)

TYPE 2 DIABETES MELLITUS is a progressive disease. At early stages diet and exercise are usually sufficient to normalize glucose homeostasis, but oral antidiabetic drugs and insulin replacement often become necessary with time due to a decrease of the functional pancreatic β-cell mass in the face of insulin resistance. Indeed, the contribution of a relative insulin deficiency to the establishment of overt diabetes is now widely accepted and is probably partly due to a decrease in β-cell mass (1–10).

Several physiological regulators of β-cell function, under certain conditions, initiate β-cell apoptosis. In a physiological environment, glucose increases the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and stimulates insulin secretion. However, chronic elevation of blood glucose concentration impairs β-cell function (11–20) and induces β-cell apoptosis (21–24). In cultured human pancreatic islets, overstimulation by high glucose leads to a rise in $[Ca^{2+}]_i$, which persists after normalization of the glucose concentration (25, 26). β-cells ATP-sensitive K^+ (K_{ATP}) channels are octamers composed of four inwardly rectifying K^+ channels and four sulfonylurea receptors. Increased intracellular glucose concentrations result in production of ATP, raising the ATP to ADP ratio, which is followed by closure of K_{ATP} channels. A decrease of K^+ conductance leads to membrane depolarization, opening of voltage-operated Ca^{2+} channels, Ca^{2+} influx, rise in $[Ca^{2+}]_i$, and subsequently induces insulin secretion (27). Sulfonylureas block β-cell K_{ATP} channels, simulating the effect of glucose in eliciting insulin release (28). Despite the widespread use of sulfonylureas, concerns emerge from studies in a β-cell line and rodent islets, demonstrating that the sulfonylureas, tolbutamide and glibenclamide, may induce, Ca^{2+} dependently, β-cell apoptosis (22, 29). However, the effect of sulfonylureas on β-cell apoptosis in human islets is unknown.

The carbamoylmethyl benzoic acid derivative repaglinide and the phenylalanine derivative nateglinide represent new classes of insulin secretagogues chemically unrelated to the sulfonylureas. Repaglinide differs from the sulfonylurea in stimulating insulin secretion solely by closure of the K_{ATP} channels, whereas nateglinide and sulfonylurea act by closure of the K_{ATP} channels and stimulation of Ca^{2+}-dependent exocytosis (30, 31). Due to distinct binding sites, the IC_{50} for nateglinide is in the low micromolar range (7 μM), whereas repaglinide resembles glibenclamide in producing a high-affinity block in the nanomolar range (IC_{50}, 0.9–7 nM). In addition to their mechanistic differences, repaglinide and nateglinide are distinguished from sulfonylureas by their rapid elimination from the body, with a plasma half-life of less than 2 h.

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Abbreviations: $[Ca^{2+}]_i$, Cytoplasmic free Ca^{2+} concentration; K_{ATP}, ATP-sensitive K^+ (channels); TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labeling.

Therefore, we investigated the short- and long-term effects of the sulfonylurea glibenclamide and the new insulin secretagogues, nateglinide and repaglinide, on β-cell apoptosis in cultured human islets.

Materials and Methods

Islet isolation and culture

Islets were isolated from pancreases of six brain-dead multiple-organ donors at the Department of Surgery, University of Geneva Medical Center, and one organ donor at the Department of Endocrinology and Diabetes, University Hospital Zurich, as described (32–34). Pancreata were procured for islet isolation according to regulations and good practice rules currently applied in Switzerland. Briefly, consent was considered obtained if the potential donor carried an official organ Swisstransplant (Swiss national organ sharing agency) donor card, on which individual reservations about procurement of specific organs or tissues are explicitly mentioned. For brain-dead potential donors not carrying an organ donor card, consent was obtained orally from the closest relatives and specifically mentioning the use of the pancreas for islet isolation. Only isolated islets not suitable for transplantation were used. Islet purity was greater than 95%, as judged by dithizone staining (if this degree of purity was not primarily achieved by routine isolation, islets were hand picked). The donors, aged 38–70 yr, were heart-beating cadaver organ donors, and none had a previous history of diabetes or metabolic disorders. Groups of 20 islets were plated on extracellular matrix-coated plates derived from bovine corneal endothelial cells, allowing the islet cells to attach to the dishes and spread (Novamed Ltd., Jerusalem, Israel), and cultured in 2 ml CMRL-1066 (5.5 mM glucose) medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Invitrogen Corp., Paisley, UK), hereafter referred to as culture medium. Two days after plating, when most islets were attached and began to flatten, the medium was changed to medium containing 5.5 or 11.1 mM glucose with or without insulin, nimodipine, nitrendipine (Sigma, Poole, UK), glibenclamide, repaglinide, or nateglinide (synthesized at Novo Nordisk A/S, Bagsvaerd, Denmark). The secretagogues were first dissolved as concentrated stock solutions in dimethylsulfoxide (Fluka, Buchs, Switzerland); the final concentration of dimethylsulfoxide was less than 0.5% (vol/vol).

Immunocytochemistry

The free 3′-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labeling (TUNEL) technique (35) according to the manufacturer's instructions (*in situ* cell death detection kit, AP; Boehringer, Mannheim, Germany) as described previously in detail (36, 37). Thereafter, islets were incubated for 30 min at 37 C with a guinea pig antiinsulin antibody (Dako, Carpinteria, CA), followed by detection using the streptavidin-biotin-peroxidase complex (Zymed Laboratories Inc., South San Francisco, CA).

Insulin release

To determine chronic insulin release, supernatants of the islets were collected and than assayed by a human insulin RIA kit (CIS Bio International, Gif-Sur-Yvette, France).

Evaluation and statistical analysis

Samples were evaluated in a randomized manner by a single investigator (K.M.) who was blinded to the treatment conditions. Care was taken to score islets of similar size. Some larger islets did not completely spread and were several cells thick. Such larger islets were excluded because a monolayer is a prerequisite for single cell evaluation (36, 37). Data were analyzed by student's *t* test or by ANOVA with a Bonferroni correction for multiple group comparisons.

Results

Effects of glibenclamide on β-cell apoptosis in human islets

Human islets were cultured on extracellular matrix-coated dishes and exposed to increasing concentrations of glibenclamide for up to 4 d. Short-term exposure of 4 h to 0.1–10 nM glibenclamide did not change the number of nuclei displaying DNA fragmentation (TUNEL-positive nuclei), compared with solvent-treated controls, but higher concentrations of 0.1 and 10 μM induced a 2.09- and 2.46-fold increase in TUNEL-positive β-cells, compared with solvent-treated islets, respectively (Figs. 1, 2A, and 3A). Long-term incubation with glibenclamide for 4 d significantly increased the number of TUNEL-positive β-cells already at concentration of 1 nM and was maximal at 10 μM (2.24-, 2.53-, 3.71-, and 4.4-fold increase at 0.001, 0.01, 0.1, and 10 μM, respectively, compared with solvent) (Figs. 2A and 3A). In parallel to the dose-dependent induction of β-cell apoptosis, 4 h exposure to glibenclamide increased insulin secretion 1.32-, 1.82-, 1.87-, 3.27-, and 3.4-fold at 0.0001, 0.001, 0.01, 0.1, and 10 μM, respectively, compared with solvent (Figs. 2B and 3B). Long-term incubation for 4 d blurred the acute glibenclamide-induced increase in insulin secretion; only 0.01, 0.1, and 10 μM glibenclamide significantly increased chronic insulin secretion, by 1.67-, 2.0-, and 2.1-fold, respectively, compared with control.

To investigate whether glibenclamide induced β-cell apoptosis requires Ca^{2+} influx, islets were coincubated with the L-type Ca^{2+} channel blockers, nitrendipine and nimodipine. At a concentration of 1 μM, both drugs inhibited the deleterious effects of a 4-d exposure to 0.1 μM glibenclamide; however, this protective effect failed to reach statistical significance after 4 h (Fig. 4).

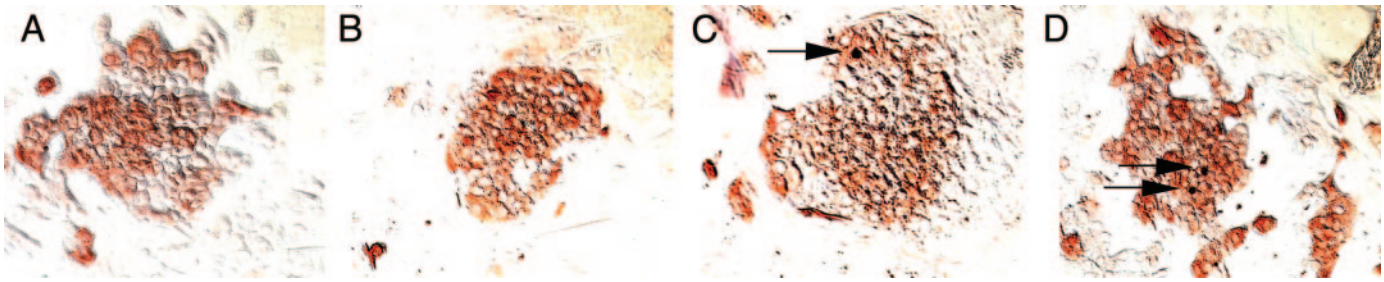

FIG. 1. Characterization of the effect of repaglinide, nateglinide, and glibenclamide on β-cell DNA fragmentation by double staining with the TUNEL assay (alkaline phosphatase) and antiinsulin antibody (peroxidase). Human islets were cultured on extracellular matrix-coated dishes for 4 h at 5.5 mM glucose and solvent alone (control) (A) or in the presence of 0.01 μM repaglinide (B), 10 μM nateglinide (C), or 0.1 μM glibenclamide (D). The *arrows* indicate nuclei stained positive for the TUNEL reaction (magnification, ×250)

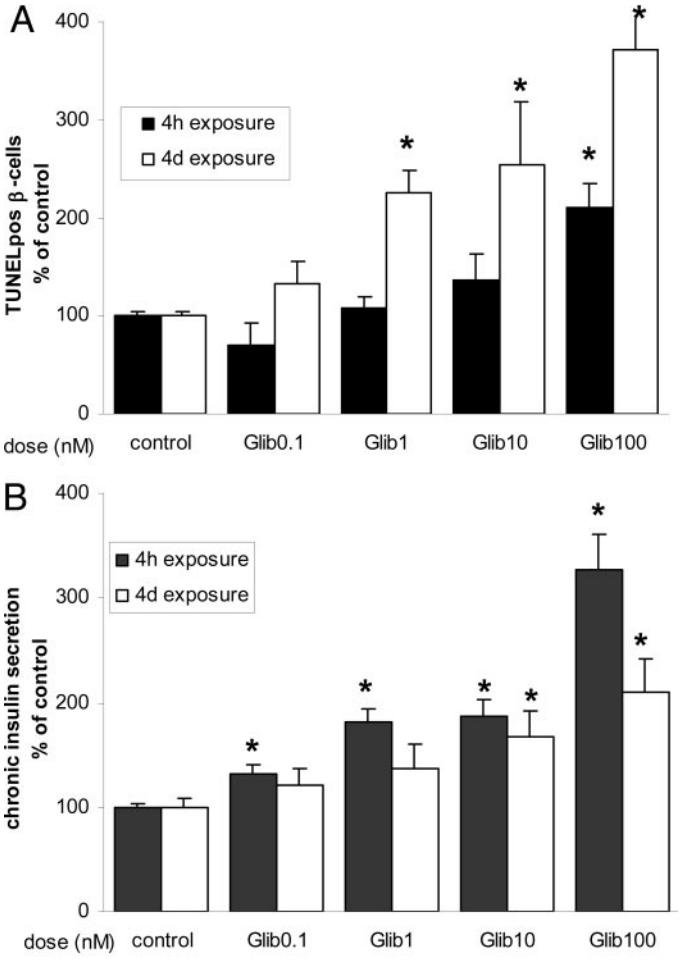

FIG. 2. Glibenclamide induces β-cell apoptosis in human islets. Human islets were cultured on extracellular matrix-coated dishes for 4 h and 4 d in 5.5 mM glucose and solvent alone or in the presence of 0.1, 1, 10, or 100 nM glibenclamide (Glib). A, Results are means ± SE of percentage of TUNEL-positive β-cells relative to control incubations (100%; in absolute value: 0.36 ± 0.03% TUNEL-positive β-cells). The mean number of islets scored from each donor was 42 (range 20–61) for each treatment condition. Islets were isolated from five organ donors. B, Chronic insulin release into the culture medium during the 4-h and 4-d incubation period. Results are means ± SE of the insulin secreted per islet relative to control incubations (100%, in absolute values: 0.62 ± 0.05 and 3.09 ± 0.30 pmol/islet after 4 h and 4 d, respectively). Islets were isolated from three organ donors. *, $P < 0.01$ relative to controls.

Effects of repaglinide, nateglinide, and insulin, compared with glibenclamide, on β-cell apoptosis

The effects of glibenclamide on β-cell apoptosis were compared with those of repaglinide and nateglinide. We chose the lowest concentration of glibenclamide inducing β-cell apoptosis after 4 h exposure (0.1 μM, see above) and compared its effect with repaglinide and nateglinide at doses of similar efficacy in stimulating β-cell insulin secretion. After 4 h of exposure, 0.01 and 1 μM repaglinide as well as 10 μM of nateglinide did not induce β-cell apoptosis (Figs. 1 and 3A). Suprisingly,1 μM repaglinide even decreased baseline apoptosis by 30%. Only a higher concentration of nateglinide (1 mM) significantly increased β-cell apoptosis 1.5-fold, an increase that was much lower than the dose-corresponding glibenclamide-induced β-cell apoptosis (Fig. 3A). After 24 h of exposure, 0.01 μM repaglinide did not significantly change β-cell apoptosis, but higher doses of 1 μM repaglinide and 10–1000 μM nateglinide increased β-cell apoptosis 1.9-, 1.6-, and 2.6-fold, respectively. In comparison, exposure to 0.1 and 10 μM glibenclamide for the same period of 24 h increased the number of apoptotic β-cells 2.5- and 2.7-fold. After 4 d of exposure, all added insulin secretagogues increased β-cell apoptosis, compared with solvent-treated controls (Fig. 3A). The increase was 2.37- and 3.8-fold at 0.01 and 1 μM repaglinide and 3.2- and 4.6-fold at 10 and 1000 μM nateglinide, respectively. Assaying chronic insulin secretion revealed no differences in efficacy to increase secretion among the three secretagogues for exposures of 4–24 h, whereas prolonged exposure for 4 d maintained a significant increase only in glibenclamide-treated islets (Fig. 3B).

Next the effect of exogenous insulin and increased glucose concentration were studied. At 100 mg/dl (5.5 mM) glucose, exposure to 20 nM exogenous insulin had no significant effect on β-cell apoptosis (Fig. 3C). However, insulin partially protected the β-cells from apoptosis induced by a 4-d exposure to 200 mg/dl (11.1 mM) glucose. In contrast, closure of the K_{ATP} channels with nateglinide, repaglinide, and glibenclamide tended to increase glucose-induced β-cell apoptosis, an effect that reached statistical significance only for 10 μM glibenclamide (Fig. 3C).

Finally, to mimic the intermittent effects of the *in vivo* treatment, cultured human islets were exposed to repaglinide and nateglinide every 6 h for a period of 2 h over a period of 3 d and compared with islets with the same number of medium changes. Analysis of β-cell apoptosis revealed no significant increase of β-cell apoptosis after the intermittent exposure of repaglinide and nateglinide (0.13 ± 0.03% TUNEL-positive β-cells at 5.5 mM glucose alone *vs.* 0.19 ± 0.08 and 0.28 ± 0.09 at 0.01 and 1 μM repaglinide and 0.19 ± 0.08 and 0.28 ± 0.12 at 10 and 1000 μM nateglinide, respectively).

Discussion

Recent studies convincingly highlight the shortcoming of β-cell mass in type 2 diabetes mass (2, 7, 8, 10). It follows that drugs increasing the rate of β-cell apoptosis may have deleterious effects in the long term, precipitating insulin requirement. In the present study, we show that the sulfonylurea glibenclamide induces β-cell apoptosis in human islets. This effect was observed *in vitro*, but it may, nevertheless, be of potential clinical importance because the concentrations that produced effects in our experiments are within the range encountered in plasma of treated patients. Indeed, peak plasma concentrations for glibenclamide, in doses of 2.5 mg, are 72 ng/ml (0.15 μM) (38), and exposure of isolated human islets to 0.1 μM glibenclamide for 4 h significantly induced β-cell apoptosis with prolonged exposure for 4 d being even more deleterious. When one considers that treatment with glibenclamide is often maintained over several years, there emerges a need to study the relevance of this effect *in vivo*. Butler *et al.* (2) quantified relative β-cell volume in type 2 diabetic patients treated with diet, sulfonylureas, or insulin. Whereas they did observe a decrease in β-cell volume in

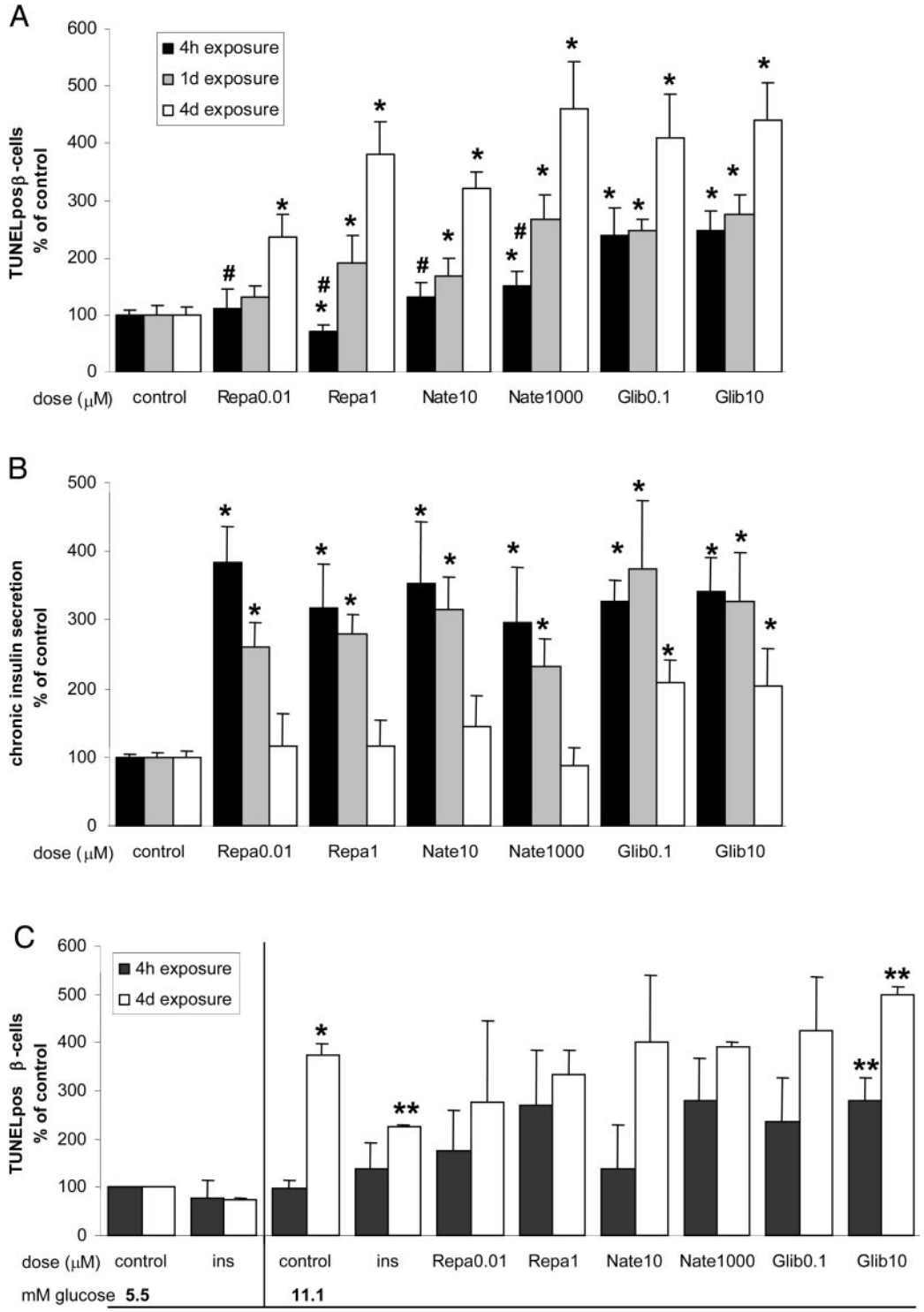

FIG. 3. Distinct effects of repaglinide, nateglinide, and glibenclamide on β-cell apoptosis. Human islets were cultured on extracellular matrix-coated dishes for 4 h and 1 and 4 d in 5.5 or 11.1 mM glucose and solvent alone or in the presence of and 0.01 or 1 μM repaglinide (Repa), 10 or 1000 μM nateglinide (Nate), 0.1 or 10 μM glibenclamide (Glib), or 20 nM insulin (ins). A, Results are means ± SE of percentage of TUNEL-positive β-cells relative to control incubations (100%; in absolute value: 0.36 ± 0.03% TUNEL-positive β-cells). The mean number of islets scored from each donor was 45 (range 25–72) for each treatment condition. Islets were isolated from five organ donors. B, Chronic insulin release into the culture medium during the 4-h and 1- and 4-d incubation period. Results are means ± SE of the insulin secreted per islet relative to control incubations (100%, in absolute values: 0.62 ± 0.05, 1.06 ± 0.07, and 3.09 ± 0.30 pmol/islet after 4 h and 1 and 4 d, respectively). Islets were isolated from three organ donors. *, $P < 0.01$ relative to controls; #, $P < 0.05$ relative to islets exposed to 0.1 and 10 μM glibenclamide. C, Results are means ± SE of percentage of TUNEL-positive β-cells relative to control incubations (100%; in absolute value: 0.17 ± 0.01% TUNEL-positive β-cells). The mean number of islets scored was 35 (range 20–49) for each treatment condition. Islets were isolated from one organ donor. *, $P < 0.01$ relative to control at 5.5 mM glucose; **, $P < 0.01$ relative to control at 11.1 mM glucose.

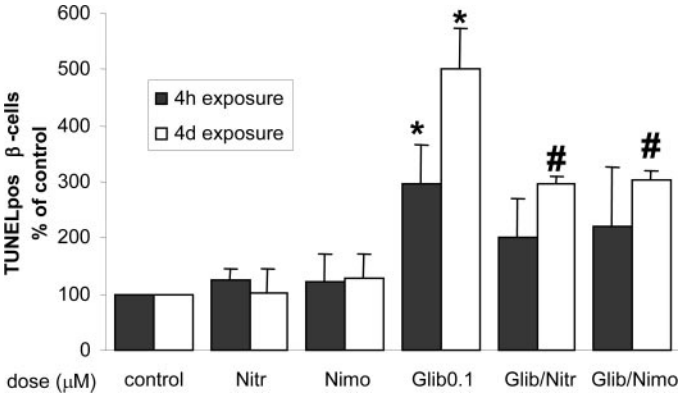

FIG. 4. Glibenclamide-induced β-cell apoptosis requires L-type Ca^{2+} influx. Human islets were cultured on extracellular matrix-coated dishes for 4 h and 4 d in 5.5 mM glucose and solvent alone or in the presence of 0.1 μM glibenclamide (Glib) with or without 1 μM nitrendipine (Nitr) or 1 μM nimodipine (Nimo). Results are means ± SE of percentage of TUNEL-positive β-cells relative to control incubations at 5.5 mM glucose alone (100%; in absolute value: 0.17 ± 0.01% TUNEL-positive β-cells). The mean number of islets scored was 35 (range 20–49) for each treatment condition. Islets were isolated from one organ donor. *, $P < 0.01$ relative to controls; #, $P < 0.05$ relative to islets exposed to 0.1 μM glibenclamide.

sulfonylurea-treated patients, compared with diet only, the β-cell volume did not differ from that of insulin-treated patients. However, it was not stated whether the insulin-treated patient group previously received sulfonylureas, but based on current treatment strategies, it is most probable. In an important recent prospective study comparing insulin and sulfonylurea treatment of type 2 diabetes, it was shown that treatment with insulin preserved β-cell function more effectively than glibenclamide (39). It remains to be established whether it is the beneficial effects *per se* of insulin or possible β-cell toxicity of glibenclamide that accounts for this observation. Whereas a deterioration of insulin secretion was seen in patients treated with sulfonylurea in the U.K. Prospective Diabetes Study, those treated with insulin were not evaluated in this regard (40).

Given the observed possible deleterious effects of glibenclamide, alternative insulin secretagogues may have to be considered. Peak plasma concentrations for nateglinide, in doses of 120 mg, are 19.1 μM (41), whereas *in vitro* exposure of human islets for 4 h to 10 μM did not and 1000 μM only slightly increase the β-cell apoptosis rate. Peak plasma concentrations for 2 mg repaglinide are 0.08 μM (42), and even a 1 μM repaglinide exposure of cultured islets for 4 h had no deleterious effects on β-cell survival. Thus, although both drugs have effects similar to glibenclamide in terms of stimulating insulin secretion, it appears that nateglinide and repaglinide are less toxic for β-cells. This is in line with previous findings demonstrating other distinct effects among sulfonylurea, repaglinide, and nateglinide (31, 43–45). The reason for these differences may be related to different binding characteristics to the K_{ATP} channels (30, 31, 46). Indeed, repaglinide differs from the sulfonylurea in stimulating insulin secretion solely by closure of the K_{ATP} channels, whereas nateglinide and sulfonylurea act by closure of the K_{ATP} channels and stimulation of Ca^{2+}-dependent exocytosis. Because Ca^{2+} is a typical mediator of apoptosis, differences in Ca^{2+} handling may translate in different sensitivity to apoptotic signals.

In summary, for the duration of their respective circulating half-lives, glibenclamide but not repaglinide and nateglinide induces β-cell apoptosis in human islets. Thus, and all other things being equal, short-acting insulin secretagogues may be preferred to long-acting ones due to the fact that patients will be less exposed to proapoptotic stimuli during protracted treatment with short-acting agents. Clearly, the present *in vitro* study of human islets needs *in vivo* confirmation because several additional factors including β-cell proliferation and regeneration may compensate for glibenclamide-induced apoptosis. However, if these findings translate to patients with type 2 diabetes, sulfonylureas may have adverse effects on β-cell mass.

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