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Differential effect of sulfonylureas on production of reactive oxygen species and apoptosis in cultured pancreatic β-cell line, MIN6

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Abstract

Sulfonylureas are considered to cause β-cell apoptosis. However, it is unclear how this occurs and whether there is a difference in such effects among various sulfonylureas. Here, we examined the effects of various sulfonylureas and a short-acting insulin secretagogue, nateglinide, on oxidative stress and apoptosis using the β-cell line MIN6. After cultured MIN6 cells were exposed to various concentrations of sulfonylureas (glibenclamide, glimepiride, and gliclazide) or nateglinide, intracellular production of reactive oxygen species (ROS) was evaluated by staining with 2′,7′-dichlorofluorescein diacetate. The effect of these agents on apoptosis was also evaluated by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick-end labeling technique. Exposure of β-cells to glibenclamide, glimepiride, and nateglinide significantly increased intracellular ROS production in a concentration-dependent manner (0.1-10 μmol/L). These effects were completely blocked by nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase inhibitors (diphenylene iodonium or apocynin) or a protein kinase C inhibitor (calphostin C). After exposure to these agents for 48 hours, the numbers of apoptotic cells were also significantly increased. These effects were significantly blocked by apocynin and antioxidant *N*-acetyl-L-cysteine. In contrast, exposure to any concentrations of gliclazide did not affect either intracellular ROS production or the numbers of apoptotic cells. Sulfonylureas (glibenclamide and glimepiride, but not gliclazide) and nateglinide stimulated ROS production via protein kinase C–dependent activation of NAD(P)H oxidase and consequently caused β-cell apoptosis in vitro. Because of the lack of such adverse effects, gliclazide may have a benefit in the preservation of functional β-cell mass.

1. Introduction

The natural history of type 2 diabetes mellitus is characterized by progression of the disease. The contribution of relative insulin deficiency to the establishment of overt diabetes or the progression of the disease is now widely accepted and is probably due to a decrease in the functional β-cell mass [1,2]. In a pathophysiological condition, persistent elevation of the glucose concentration impairs β-cell function and induces β-cell apoptosis, so-called glucose toxicity [3,4]. One potential mechanism for glucose toxicity is that of excessive formation of reactive oxygen species (ROS) in β-cells. Persistent excessive ROS formation has been reported to cause decreased insulin gene expression via a loss of the transcription factors pancreatic and duodenal homeobox protein 1 (PDX-1) and musculoaponeurotic fibrosarcoma oncogene homologue A (MafA) [5], and has also been reported to accelerate rates of β-cell apoptosis [6,7]. Accumulating evidence suggests that oxidative stress is increased in pancreatic β-cells in diabetic animal models and diabetic patients [8-11].

Sulfonylureas are commonly used in the treatment of type 2 diabetes mellitus because these drugs effectively reduce blood glucose levels in type 2 diabetes mellitus. Despite their beneficial effects, continuous use of sulfonylureas may cause β-cell dysfunction and apoptosis. Several reports have suggested that sustained enhancement of Ca^{2+} influx by sulfonylureas may be a causative mechanism for β-cell apoptotic cell death [12,13]. On the other hand, we recently

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reported that the sulfonylurea glibenclamide, as well as high glucose levels, stimulated production of ROS in the pancreatic β-cell line MIN6 [14]. Thus, this mechanism might, in part, account for β-cell dysfunction and apoptosis induced by sulfonylureas. It is therefore important to evaluate whether sulfonylureas induce β-cell apoptosis via increases in ROS production and to evaluate whether there is a difference in such adverse effects among various sulfonylureas and other insulin secretagogues. In this context, this study was undertaken to examine the dose-dependent effects of the classic sulfonylurea glibenclamide, the new-generation sulfonylureas gliclazide and glimepiride, and a short-acting insulin secretagogue, nateglinide, on ROS production and apoptosis using the pancreatic β-cell line MIN6 and mouse islets.

2. Materials and methods

2.1. Materials and chemicals

2′,7′-Dichlorofluorescein diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR). Calphostin C, diphenyleneiodonium chloride, *N*-acetyl-L-cysteine (NAC), rotenone, oxypurinol, and *N*-methyl-L-arginine were purchased from Sigma (St Louis, MO). Apocynin was purchased from Extrasynthese (Lyon, France). A guinea pig anti-insulin antibody was purchased from Dako (Carpinteria, CA), and an anti–guinea pig immunoglobulin G was purchased from Sigma. Glibenclamide and glimepiride were kindly provided by Sanofi-Aventis (Paris, France). Gliclazide was kindly provided by Dainippon Sumitomo Pharma (Osaka, Japan). Nateglinide was kindly provided by AJINOMOTO (Tokyo, Japan).

2.2. Cell culture

Pancreatic β-cell line MIN6 cells were kindly provided by Dr S Seino (Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan) and Dr E Araki (Department of Metabolic Medicine, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan). The MIN6 cells were maintained in DMEM containing 15% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 mmol/L glucose at 37°C in an atmosphere of 95% O_2/5% CO_2.

2.3. Mouse islets isolation and culture

Pancreatic islets were isolated from 20-week–old B6 mice by collagenase digestion followed by gradient density centrifugation. Briefly, mice were killed by cervical dislocation. The pancreas was exposed and injected with Hanks balanced salt solution containing 2 mg/mL collagenase (Collagenase CLS-4; Worthington Biochemical, Lakewood, NJ) via common bile duct until distended. Digestion was performed at 37°C. Pancreas was mechanically disrupted by passing through a metal needle, and islets were purified on Histopaque (Sigma) gradients by centrifugation. Islets were handpicked and transferred into CMRL-1066 containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 5.5 mmol/L glucose at 37°C in an atmosphere of 95% O_2/5% CO_2.

Care and maintenance of all animals were in accordance with the principles of laboratory animal care and guidelines of institutional Animal Policy and Welfare Committee.

2.4. Evaluation of ROS production in MIN6 cells

2′,7′-Dichlorofluorescein diacetate was used to evaluate intracellular oxidant formation as previously reported [14]. For experiments, the cells were placed into glass-bottom culture dishes (MatTek, Ashland, MA) and cultured in DMEM containing 15% FBS and 25 mmol/L glucose. When the cells reached the loose confluent layer, the medium was replaced with DMEM containing 1% FBS and 5.5 mmol/L glucose with glibenclamide, gliclazide, glimepiride, or nateglinide for 2, 6, 12, 18, and 24 hours. All pharmacological compounds were prepared in dimethyl sulfoxide or water. The final concentration of dimethyl sulfoxide in the solution was always 0.1%. The cells were then loaded with 2 μmol/L DCF-DA, a nonfluorescent compound that freely permeates cells and interacts with intracellular oxidants to form fluorescence compound DCF. After 20 minutes, digital images of DCF fluorescence were obtained with a fluorescence microscope system (Olympus, Tokyo, Japan) at an excitation wavelength of 488 nm (argon laser) using a 515-nm long-pass emission filter. The obtained fluorescence images were converted to gray-scale images using Photoshop software (Adobe Systems, San Jose, CA), and the fluorescence intensities were quantitatively analyzed using National Institutes of Health image software (Fortner Research, Sterling, VA).

2.5. Detection and quantification of apoptosis of MIN6

Apoptosis of cells was evaluated by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay, as previously described. For experiments, MIN6 cells were placed into glass-bottom culture dishes (MatTek) and cultured in DMEM containing 15% FBS and 25 mmol/L glucose. When the cells reached the loose confluent layer, the medium was replaced with DMEM containing 1% FBS and 5.5 mmol/L glucose with glibenclamide with or without apocynin (100 μmol/L) or NAC (1 mmol/L), gliclazide, glimepiride, or nateglinide. After incubation for 48 hours, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline. The glasses were then processed for the TUNEL assay. An In Situ Apoptosis Detection Kit from TaKaRa Bio (Otsu, Japan) was used according to the manufacturer's instructions. The cells were treated with H_2O_2 and incubated with the reaction mixture containing terminal deoxynucleotidyl transferase and digoxigenin-conjugated deoxyuridine triphosphate for 1 hour at 37°C. Labeled DNA was visualized with peroxidase-conjugated anti-digoxigenin antibody using 3,3′-diaminobenzidine as the chromogen. After washing in distilled water, the cells were counterstained in 0.5% methyl green.

2.6. Detection and quantification of apoptosis of islets

Apoptosis of cells was also evaluated by TUNEL assay, as above. For experiments, cultured mouse islets were transferred to CMRL-1066 containing 1% FBS and 5.5 mmol/L glucose with glibenclamide with or without apocynin or NAC, gliclazide, glimepiride, or nateglinide. After incubation for 48 hours, the islets were dissociated with trypsin-EDTA (Sigma). After washing, cells were prepared by cytospin (Shandon, Pittsburgh, PA) on glass slide and fixed in 4% paraformaldehyde in phosphate-buffered saline. An In situ Apoptosis Detection Kit (TaKaRa) was used as above. After 3,3′-diaminobenzidine staining and washing, immunostaining for insulin was performed to distinguish β-cell. Islets were stained with a guinea pig anti-insulin antibody (Dako), followed by alkaline phosphatase conjugated anti–guinea pig immunoglobulin G (Sigma) using alkaline phosphatase substrate kit III (Vector Laboratories, Burlingame, CA) as the chromogen.

2.7. Statistical analysis

All data are expressed as means ± SEM. Statistical analysis was performed by analysis of variance followed by post hoc comparison tests.

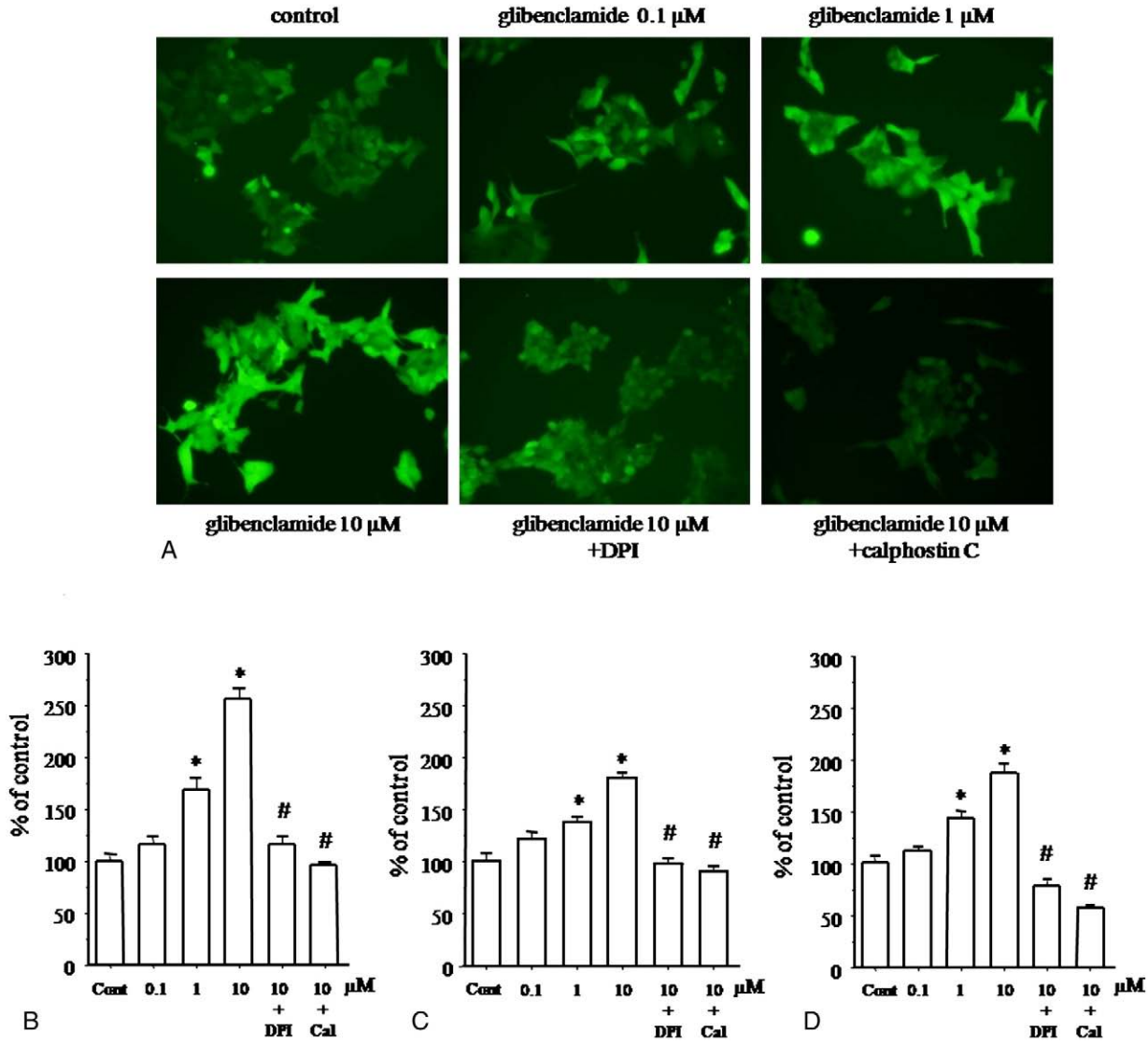

Fig. 1. Effect of glibenclamide, glimepiride, and nateglinide on production of ROS in cultured pancreatic β-cell line MIN6. The MIN6 cells were cultured in test media containing 1% FBS and 5.5 mmol/L glucose with or without various concentrations of glibenclamide, glimepiride, and nateglinide for 24 hours. Diphenyleneiodonium chloride or calphostin C was incubated simultaneously with these agents (10 μmol/L) for the last 2 hours. After 24 hours of incubation, DCF-DA was added at a concentration of 2 μmol/L. After 20 minutes, digital images of DCF fluorescence were obtained with a fluorescence microscope system. A, Representative fluorescent images of glibenclamide-treated cells. Quantitative analysis of the fluorescence intensities on the images of glibenclamide-treated (B) (n = 12), glimepiride-treated (C) (n = 12), and nateglinide-treated cells (D) (n = 10). *$P < .01$ vs nontreated cells. $^{\#}P < .01$ vs sulfonylureas- or nateglinide (10 μmol/L)-treated cells each. Data are expressed as mean percentage of control ± SEM. Cal indicates calphostin C; Cont, control.

3. Results

3.1. Effects of sulfonylureas and nateglinide on intracellular ROS production in MIN6 cells

Exposure of the MIN6 cells to glibenclamide, glimepiride, and nateglinide for 24 hours induced a significant increase in intracellular ROS production in a concentration-dependent manner (0.1-10 μmol/L) (Fig. 1A-D, respectively). These stimulatory effects were completely blocked by the incubation with a nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase inhibitor, diphenyleneiodonium chloride (1 μmol/L), or a protein kinase C (PKC) inhibitor, calphostin C (500 nmol/L). Other flavoprotein inhibitors including rotenone (100 μmol/L), oxypurinol (100 μmol/L), or *N*-methyl-L-arginine (10 μmol/L) did not significantly affect ROS production induced by these agents (data not shown). In contrast, exposure of the cells to gliclazide did not induce a significant increase in ROS production at any concentration (0.1-10 μmol/L) (Fig. 2A, B). As shown in Fig. 3, when the intensity of the stimulatory effect was compared among these agents at the concentration of 10 μmol/L, the stimulatory effect of glibenclamide was significantly greater than that of glimepiride and nateglinide. These stimulatory effects were observed at 2 hours after the start of incubation and lasted for up to 24 hours, but no stimulatory effect of gliclazide was found at any incubation time (Fig. 4).

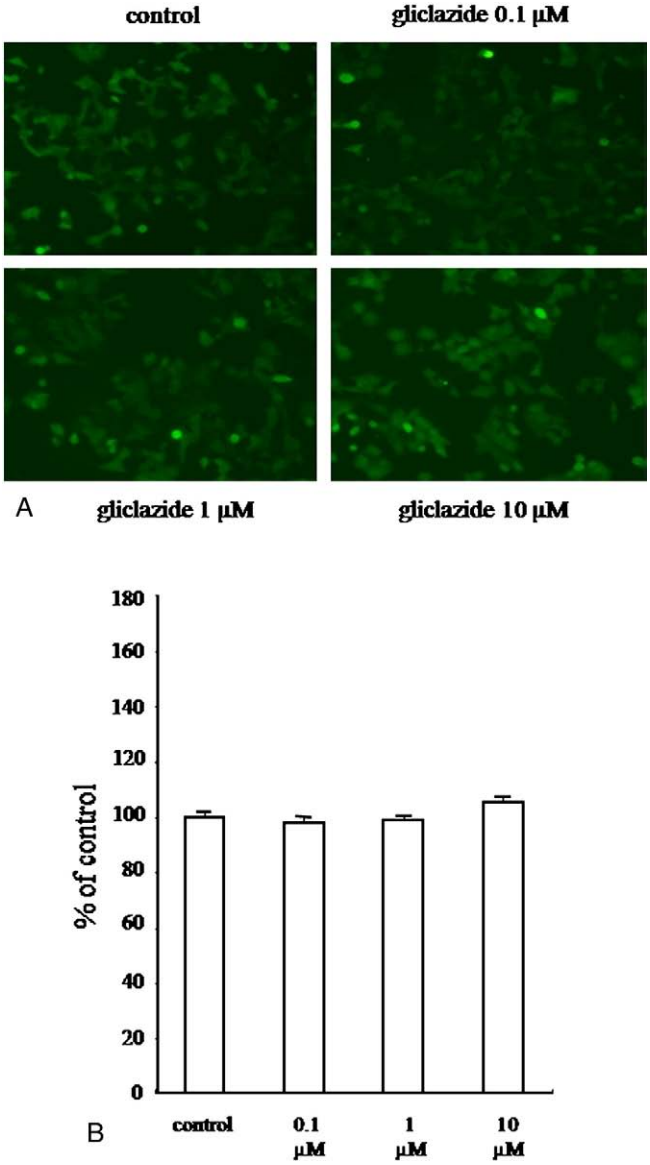

Fig. 2. Effect of gliclazide on ROS production in cultured pancreatic β-cell line MIN6. A, Representative fluorescence images of gliclazide-treated cells. B, Quantitative analysis of the fluorescence intensities on the images of gliclazide-treated cells. Data are expressed as mean percentage of control ± SEM (n = 10).

3.2. Effects of sulfonylureas and nateglinide on β-cell apoptosis

As shown in Fig. 5A, B, exposure of the cells to glibenclamide, glimepiride, and nateglinide at the concentration of 10 μmol/L for 48 hours induced a significant increase in the number of brown-colored TUNEL-positive cells compared with control. In parallel with the results of ROS production, the stimulatory effects of glibenclamide on apoptosis were significantly greater than those of glimepiride and nateglinide. In contrast, exposure of the cells to gliclazide did not induce a significant increase in the number of apoptotic cells (Fig. 5A, B). Simultaneous addition of both the NAD(P)H oxidase apocynin (100 μmol/L) and the antioxidant NAC (1 mmol/L) significantly inhibited glibenclamide-induced apoptosis (Fig. 6).

3.3. Effects of sulfonylureas and nateglinide on islet cell apoptosis

As shown in Fig. 7A, exposure of the islet cells to glibenclamide, glimepiride, and nateglinide at the concentration of 10 μmol/L for 48 hours also induced a significant increase in the number of TUNEL-positive cells compared with controls. Again, exposure of the islet cells to gliclazide did not induce a significant increase in the number of apoptotic cells. Simultaneous addition of both the apocynin and NAC significantly inhibited glibenclamide-induced islet cell apoptosis (Fig. 7B).

4. Discussion

Although sulfonylureas are widely used for the treatment of type 2 diabetes mellitus, it is of concern that continuous use of sulfonylureas may cause β-cell dysfunction and apoptosis. In a prospective study comparing insulin and glibenclamide treatment of type 2 diabetes mellitus, it was shown that treatment with insulin preserved β-cell function more effectively than glibenclamide [15]. Thus, given the possible deleterious effects of sulfonylureas, it is important to understand how sulfonylureas may cause β-cell dysfunction and apoptosis, and whether there is a difference in such effects among various sulfonylureas.

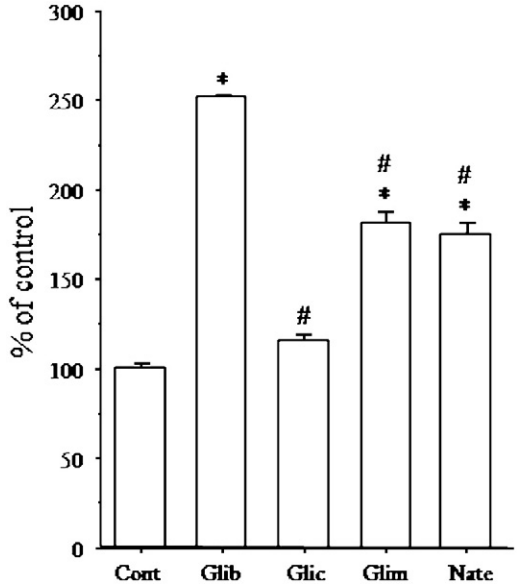

Fig. 3. Comparison of the stimulatory effects on ROS production with sulfonylureas and nateglinide. Data are expressed as mean percentage of control ± SEM (n = 10). *$P < .01$ vs nontreated cells (control). #$P < .01$ vs glibenclamide-treated cells. Glib indicates glibenclamide; Glic, gliclazide; Glim, glimepiride; Nate, nateglinide.

Sulfonylureas block β-cell adenosine triphosphate–sensitive K^+ channels, which leads to membrane depolarization, opening of voltage-dependent Ca^{2+} channels, Ca^{2+} influx, and elevated cytosolic Ca^{2+} concentration, and consequently induces insulin secretion. Several reports have suggested that sustained enhancement of Ca^{2+} influx caused by glibenclamide or tolbutamide induces apoptotic cell death in a β-cell line or islets [12,13]. In contrast, increased oxidative stress has been implicated as a potential mechanism for glucose toxicity on β-cells. Persistent exposure of β-cells to elevated glucose and ROS levels was reported to cause loss of transcription factors PDX-1 and MafA, which are essential to maintain normal levels of insulin promoter activity. Thus, the loss of PDX-1 and MafA leads to diminished insulin secretion [5]. Furthermore, several reports have shown that persistent exposure of β-cells to elevated glucose induces β-cell apoptosis, which was also mediated by excessive ROS production [6,7]. In addition, treatment with several antioxidants or antioxidant enzyme overexpression by gene transfer method was reported to protect from β-cell damage in animal models of type 2 diabetes mellitus [8,9,16]. Together, these findings suggest that oxidative stress may play an important role in the progressive deterioration of β-cell function and apoptosis. We recently reported that glibenclamide stimulated ROS production in the pancreatic β-cell cell line MIN6 [14], suggesting that increased ROS production may be a causative mechanism for glibenclamide-induced β-cell damage. In the present study, we further revealed that another sulfonylurea, glimepiride, and a new insulin secretagogue, nateglinide, also stimulated ROS production in MIN6 in a concentration-dependent manner from 0.1 to 10 μmol/L. The clinical relevance of these findings seems to be applicable because the concentrations used in the present study is within the therapeutic range. As for the source of ROS production, it has largely been established that the mitochondrial respiratory chain is an important site of ROS production within cells, including β-cells [17]. In contrast, NAD(P)H oxidase has received increasing attention as one of the most important source of

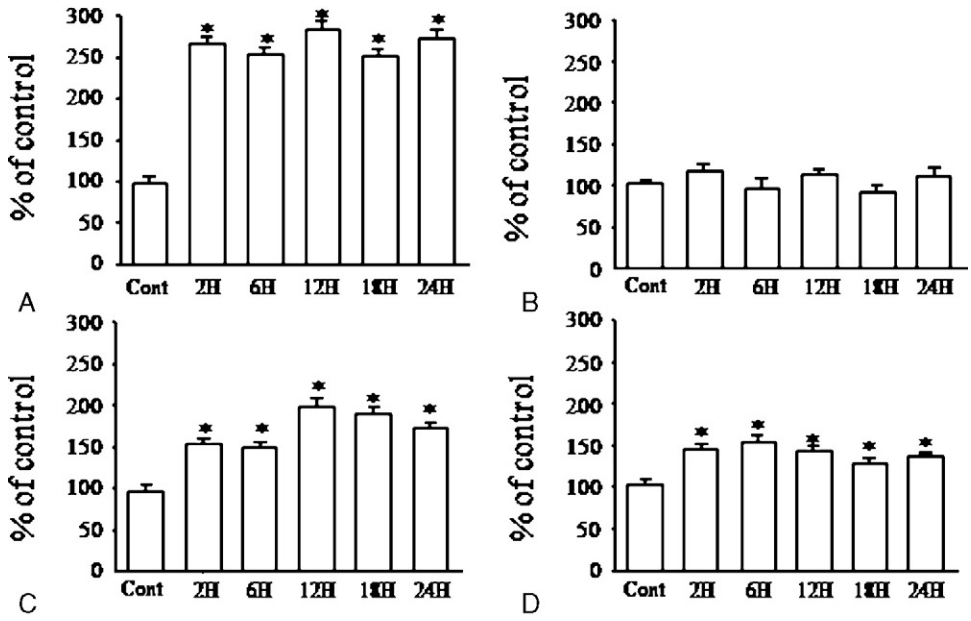

Fig. 4. Time course of ROS production by sulfonylureas and nateglinide. After incubation with sulfonylureas or nateglinide at a concentration of 10 μmol/L for 2, 6, 12, 18, or 24 hours, production of ROS was evaluated by DCF-DA method. Glibenclamide (n = 12) (A), gliclazide (n = 10) (B), glimepiride (n = 12) (C), and nateglinide (n = 10) (D). Data are expressed as mean percentage of control ± SEM. *$P < .01$ vs nontreated cells each (control).

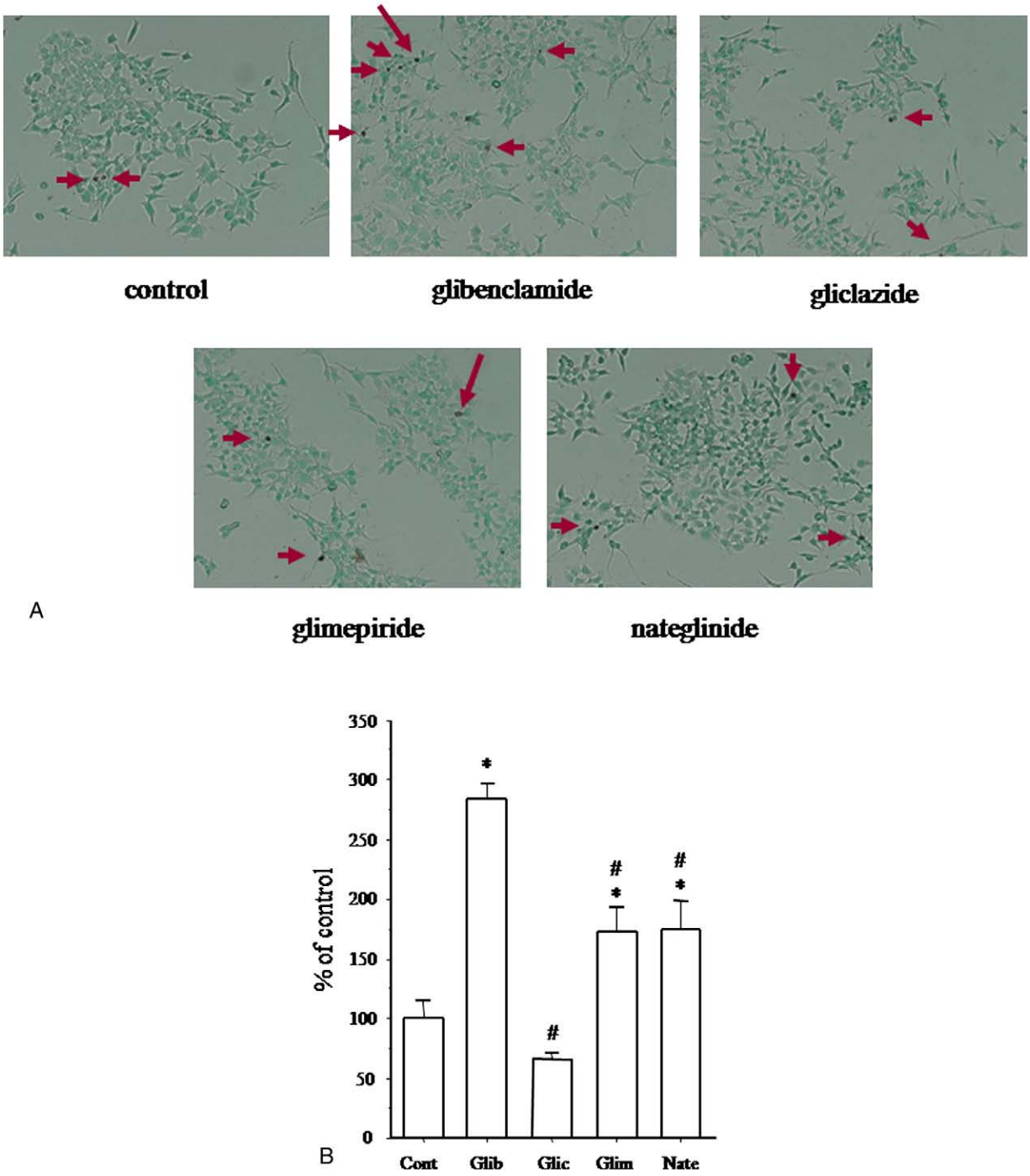

Fig. 5. Effect of sulfonylureas and nateglinide on apoptosis in MIN6 cells. The MIN6 cells were incubated with test media containing 1% FBS and 5.5 mmol/L glucose with glibenclamide, gliclazide, glimepiride, and nateglinide at a concentration of 10 μmol/L. After 48 hours of incubation, apoptosis was evaluated by TUNEL assay. A, Representative images of apoptotic cells. Arrows indicate brown-colored TUNEL-positive cells. B, Quantitative analysis of the rates of apoptotic cells induced by sulfonylureas and nateglinide. Data are expressed as mean percentage of control ± SEM (n = 4). *$P < .01$ vs control. $^{\#}P < .01$ vs glibenclamide-treated MIN6 cells.

ROS production in vascular cells [18]. Previously, we and other investigators have shown that elevated glucose levels stimulate ROS production via PKC-dependent activation of NAD(P)H oxidase [19-21]. Recently, several reports have shown the presence of NAD(P)H oxidase in pancreatic β-cell [22,23]. In the present results, the stimulatory effects on ROS production induced by sulfonylureas and nateglinide were completely blocked by treatment with a PKC inhibitor and NAD(P)H oxidase inhibitors, but not significantly affected by treatment with other inhibitors of flavoproteins such as mitochondria electron transport chain (rotenone), xanthine oxidase (oxypurinol), or nitric oxide synthase (*N*-methyl-L-

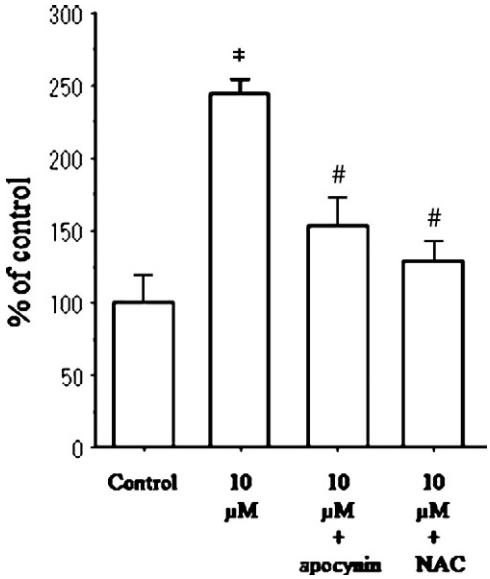

Fig. 6. Effect of apocynin and NAC on the rates of apoptotic cells induced by glibenclamide. The MIN6 cells were exposed to glibenclamide with or without apocynin or NAC. Data are expressed as mean percentage of control ± SEM (n = 6). *$P < .01$ vs control. #$P < .01$ vs glibenclamide-treated MIN6 cells.

arginine). Thus, these findings suggest that the stimulatory effects on ROS production induced by sulfonylureas and nateglinide may be mediated by a PKC-dependent activation of NAD(P)H oxidase. The present study also revealed that these agents further increased the numbers of apoptotic β-cells, but this was significantly blocked by the treatment with NAD(P)H oxidase inhibitors or antioxidant NAC. These results suggest that these agents might induce β-cell apoptosis via NAD(P)H oxidase–dependent ROS production.

Of great interest, in contrast with other sulfonylureas and nateglinide, gliclazide did not significantly stimulate ROS production. These findings may be consistent with one previous report showing that gliclazide attenuated apoptotic β-cell death induced by hydroxyl peroxide treatment [24]. Gliclazide may also possess radical scavenging properties including inhibition of low-density lipoprotein oxidation [25], inhibition of *o*-dianisidine photooxidation [26], and reduction of lipid peroxides [27]. Furthermore, a recent report has confirmed its scavenging activity against hydroxyl and superoxide radicals using an electron spin resonance method [28]. Therefore, it is likely that gliclazide might stimulate ROS production in β-cells via the same mechanism as other sulfonylureas, but at the same time diminishes oxidative stress via its radical scavenging effect. In parallel with the results of ROS production, gliclazide did not significantly induce the increased rates of β-cell apoptosis. Thus, gliclazide may be more protective against β-cell damage than other sulfonylureas or nateglinide. In support of this concept, a recent retrospective analysis showed that patients treated with gliclazide needed to administer insulin less frequently than glibenclamide, suggesting that gliclazide is less likely to induce β-cell failure compared with glibenclamide [29]. However, there are very few clinical studies showing the reduced frequencies of β-cell failure of gliclazide as compared with those clinical studies related to glimepiride or nateglinide. The short-acting insulin secretagogue nateglinide has a distinct action from sulfonylureas in vivo, with rapid elimination from the body and a plasma half-life of less than 2 hours. Therefore, it is predictable that the stimulatory effect of nateglinide on ROS produc-

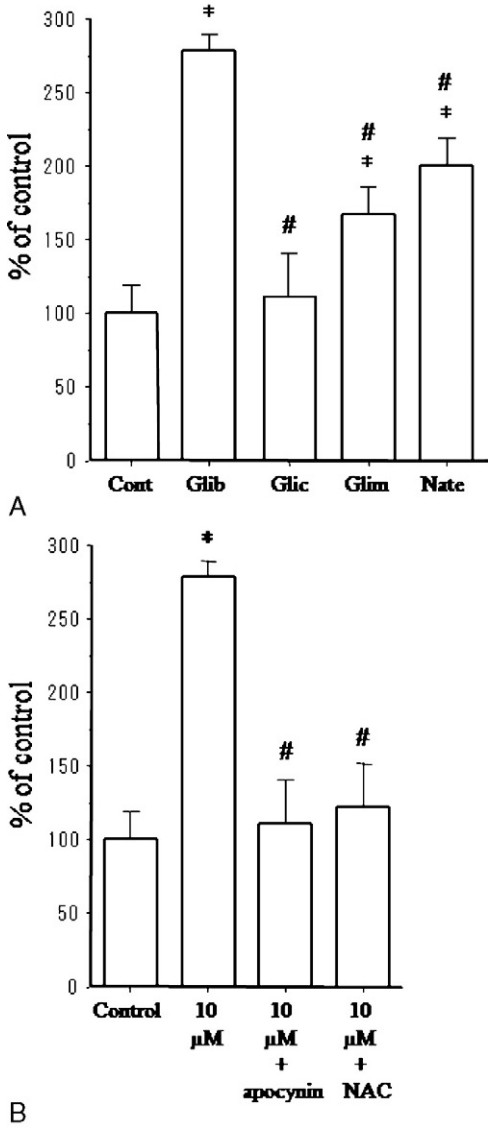

Fig. 7. Effect of sulfonylureas and nateglinide on apoptosis and effect of apocynin and NAC on the rates of apoptotic cells induced by glibenclamide in pancreatic islets. Islet cells were incubated with glibenclamide, gliclazide, glimepiride, and nateglinide at a concentration of 10 μmol/L. After 48 hours of incubation, apoptosis was evaluated by TUNEL assay. A, Quantitative analysis of the rates of apoptotic cells induced by sulfonylureas and nateglinide. B, Effect of apocynin and NAC for the rates of apoptotic cells induced by glibenclamide. The islet cells were exposed to glibenclamide with or without apocynin or NAC. Data are expressed as mean percentage of control < .01 vs control. #$P < .01$ vs glibenclamide-treated islet cells.

tion may be much lower in vivo than that shown in vitro. Glimepiride has also been demonstrated to offer therapeutic advantages over other sulfonylureas in terms of its glucose level–dependent insulinotropic action and insulin-sparing effects due to the improvement of insulin resistance. It is also possible that its stimulatory effect on ROS production may be minimized in vivo. Further studies are needed to evaluate in vivo ROS production induced by nateglinide and glimepiride.

In summary, the present study showed for the first time that sulfonylureas (glibenclamide, glimepiride) and nateglinide induced ROS production via a PKC-dependent activation of NAD(P)H oxidase in β-cells and consequently caused β-cell apoptosis in vitro. This may, at least in part, account for the mechanism underlying sulfonylurea secondary failure. Because of the lack of such adverse effects, gliclazide may have a benefit in the preservation of functional β-cell mass.

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