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Gliclazide protects pancreatic β-cells from damage by hydrogen peroxide

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Abstract

Oxidative stress is induced under diabetic conditions and possibly causes various forms of tissue damage in patients with diabetes. Recently, it has become aware that susceptibility of pancreatic β-cells to oxidative stress contributes to the progressive deterioration of β-cell function in type 2 diabetes. A hypoglycemic sulfonylurea, gliclazide, is known to be a general free radical scavenger and its beneficial effects on diabetic complications have been documented. In the present study, we investigated whether gliclazide could protect pancreatic β-cells from oxidative damage. One hundred and fifty μM hydrogen peroxide reduced viability of mouse MIN6 β-cells to 29.3%. Addition of 2 μM gliclazide protected MIN6 cells from the cell death induced by H_2O_2 to 55.9%. Glibenclamide, another widely used sulfonylurea, had no significant effects even at 10 μM. Nuclear chromatin staining analysis revealed that the preserved viability by gliclazide was due to inhibition of apoptosis. Hydrogen peroxide-induced expression of an anti-oxidative gene heme oxygenase-1 and stress genes A20 and $p21^{CIP1/WAF1}$, whose induction was suppressed by gliclazide. These results suggest that gliclazide reduces oxidative stress of β-cells by H_2O_2 probably due to its radical scavenging activity. Gliclazide may be effective in preventing β-cells from the toxic action of reactive oxygen species in diabetes.
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It is well known that oxidative stress impairs various cellular functions and plays important roles in the pathophysiology of many diseases. Diabetic patients are exposed to oxidative stress and complications of diabetes seem to be mediated by oxidative stress. Hyperglycemia is one of the main causes of oxidative stress in type 2 diabetes. Under hyperglycemia, the increased blood level of various reducing sugars promotes protein glycation through the Maillard reaction, which consecutively produces Schiff bases, Amadori products, and advanced glycation end products (AGEs). Reactive oxygen species (ROS) are formed in this process and trigger tissue damage. Recently, the progressive deterioration of β-cell function in type 2 diabetes has been accounted for in the oxidative stress-induced tissue damage. Due to a relatively low expression level of anti-oxidant enzymes, β-cells are implicated to be vulnerable to oxidative stress as compared with other tissues [1]. In addition, oxidative stress is reported to enhance apoptosis of β-cells and suppress insulin biosynthesis [2,3]. It has also been shown that treatment of anti-oxidant chemicals, such as cysteine or *N*-acetylcysteine (NAC), provides some protection to β-cells both in vitro and in vivo [4,5].

Gliclazide, a second-generation sulfonylurea, is used in the treatment of type 2 diabetes. It reduces blood glucose level by augmenting insulin release from

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pancreatic islets. Besides its hypoglycemic effect, gliclazide has been shown to possess anti-oxidant properties such as inhibition of LDL oxidation and reduction of platelet reactivity [6,7]. It appears that these anti-oxidant effects by gliclazide are independent of any effects of glycemic control [8]. In this study, we investigated the effects of gliclazide on β-cell death induced by H_2O_2. Glibenclamide, another hypoglycemic sulfonylurea, was used for comparative purposes. Our results show that gliclazide attenuates H_2O_2-induced oxidative stress on pancreatic β-cells and maintains a higher cellular viability.

Materials and methods

Materials. Gliclazide was obtained from Dainippon Pharmaceutical (Osaka, Japan). Glibenclamide was purchased from Sigma Biochemicals (St. Louis, USA). Hochest 33342 and propidium iodide were from Dojin (Tokyo, Japan). MIN6 cells were provided by Prof. Junichi Miyazaki (Osaka University, Osaka, Japan).

Cell culture. MIN6 cells were grown in Dulbecco's modified Eagle's medium (25 mM glucose) equilibrated with 5% CO_2 and 95% air at 37 °C as described [9].

LDH assay. MIN6 cells were cultured on 48-well microplates for two days. Hydrogen peroxide was added to the medium in the absence or presence of various concentrations of gliclazide or glibenclamide. LDH activity was measured by using CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, USA).

RNA extraction, cDNA synthesis, and semiquantitative polymerase chain reaction (PCR). Total RNA was extracted from MIN6 cells by using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction. After quantification by spectrophotometry, 2 μg RNA was reverse-transcribed into cDNA at 42 °C for 1 h using an RTG-RT-PCR Kit (Amersham Biosciences, Piscataway, USA). In 10 mM Tris–Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM primers, and 5 U/ml TaKaRa Taq DNA polymerase, PCR was carried out by cycling of 30 s at 94 °C, 30 s at the annealing temperature indicated in Table 1, and 1 min at 72 °C. PCR products were separated on 2% agarose gel in TAE buffer. Intensity of the bands was quantitated with MacWorks Analysis Software (UVP, Upland, USA).

Nuclear chromatin staining. Hochest 33342 (1.5 μg/ml) and propidium iodide (PI; 3 μg/ml) were used to detect differences between normal and apoptotic nuclei as described previously [10]. Cells were stained with a mixture of the two dyes for 10 min and washed with PBS before examination by fluorescence microscopy with excitation at 365 nm. HO342 freely passes through cell membranes and stains nuclear DNA blue when visualized under the fluorescent microscope. Intact plasma membranes are impermeable to PI, which only enters cells that have lost integrity of their cell membranes, and stains DNA red. Apoptotic cells were identified by the presence of condensed or fragmented nuclei stained either blue or pink depending on the stage of the process.

Statistical analysis. All results are presented as means ± SEM. For multiple comparisons, an analysis of variance (ANOVA) was carried out, followed by Fisher's protected least significant difference test as a post hoc test (StatView, SAS Institute, Cary, USA). A value of $P < 0.05$ was considered significant.

Table 1
Sequences of primers and PCR conditions

Gene	Forward/reverse	Primer sequence	Annealing (°C)	Product size (bp)
Catalase	F	TCTGCAGATACCTGTGAACTG	60	357
	R	TAGTCAGGGTGGACGTCAGTG		
Glutathione peroxidase	F	CTCGGTTTCCCGTGCAATCAG	65	431
	R	GTGCAGCCAGTAATCACCAAG		
SOD1	F	TTAACTGAAGGCCAGCATGGG	60	335
	R	ATCACTCCACAGGCCAAGCGG		
SOD2	F	TGCACCACAGCAAGCACCATG	55	413
	R	CTCCCACACGTCAATCCCCAG		
HO-1	F	ATGCCCCACTCTACTTCCCTG	60	309
	R	TTGGTGGGGCTGTCGATGTTCG		
Bcl-2	F	CCTGGCATCTTCTCCTTC	55	169
	R	AGAAGTCATCCCCAGCCC		
Bax	F	CAGGATCGAGCAGGGAGGATGG	55	432
	R	GATGGTCACTGTCTGCCATGTG		
P21	F	TTGTGTTTCAGCCACAGGCACCATG	60	263
	R	ACCCAGGGCTCAGGTAGACCTTG		
A20	F	TTTGAGCAATATGCGGAAAGC	60	479
	R	AGTTGTCCCATTCGTCATTCC		
Heat shock protein 70	F	ACGCAGACCTTCACCACC	60	278
	R	CGCTCGATCTCCTCCTTG		
Cyclophilin	F	AGCACTGGAGAGAAAGGATT	60	290
	R	CACAATGTTCATGCCTTCTT		

F, forward; R, reverse.

Results

In order to assess the protective effect of gliclazide on pancreatic β-cells against the oxidative stress, LDH assay of MIN6 cells was conducted (Fig. 1). Exposure to 150 μM H_2O_2 for 6 h reduced the viability of MIN6 cells to 29.3%. Addition of 2 μM gliclazide protected MIN6 cells from the cell death induced by H_2O_2 to 55.9% (A). Protective effect of gliclazide was significant at 0.5–10 μM. Glibenclamide had no significant effect even at 10 μM. Enhanced viability by gliclazide was significant from 6 to 18 h after H_2O_2 addition (B). One to five μM gliclazide showed a protective effect against damage caused by higher concentrations (150 and 200 μM) of H_2O_2 (C).

DNA binding dyes, PI and HO342, were used for nuclear double staining (Fig. 2). In control MIN6 cells, no chromatin condensation or nuclear fragmentation was evident (A). In contrast, the nucleus of MIN6 cells exposed to 150 μM H_2O_2 exhibited condensed chromatin (B), which is characteristic of apoptosis [11]. In the presence of 2 μM gliclazide, the number of apoptotic cells showing condensed, bright chromatin was decreased (C), suggesting that gliclazide confers resistance to β-cells by inhibiting apoptosis. Again, there was no significant effect observed with glibenclamide (D).

To evaluate the molecular mechanism involved in the β-cell protection by gliclazide against oxidative stress, mRNA levels of anti-oxidative enzymes were compared (Fig. 3). At 3 h after H_2O_2 addition, the expression levels of glutathione peroxidase, not catalase, were markedly reduced. Neither gliclazide nor glibenclamide showed any effect on the reduction of glutathione peroxidase expression. On the other hand, both gliclazide and glibenclamide induced the expression of catalase in the presence of H_2O_2 as compared with the basal levels. No significant change of expression levels of SOD1 or SOD2 was evident by H_2O_2. Only in the presence of gliclazide, SOD1 mRNA was upregulated by the addition of H_2O_2. Meanwhile, glibenclamide alone induced the expression of SOD2 in the presence of H_2O_2. Hydrogen peroxide markedly induced the expression of heme oxygenase (HO)-1. This remarkable induction of HO-1 expression was also observed in the presence of glibenclamide; however, it was nearly suppressed by gliclazide.

Next, the possible effects of gliclazide on the expression of apoptosis-related genes were examined (Fig. 4). No significant effect by the sulfonylureas on the expression of Bcl-2 and Bax was observed. The expression levels of the anti-apoptotic gene A20 and the cell cycle-related protein $p21^{CIP1/WAF1}$ were significantly increased by adding H_2O_2, while the induction was suppressed by gliclazide. No such suppression in $p21^{CIP1/WAF1}$ or A20 mRNA was observed by the addition of glibenclamide. The expression of heat shock protein (Hsp) 70 was increased by H_2O_2. This induction was significantly enhanced by the addition of glibenclamide. Although the

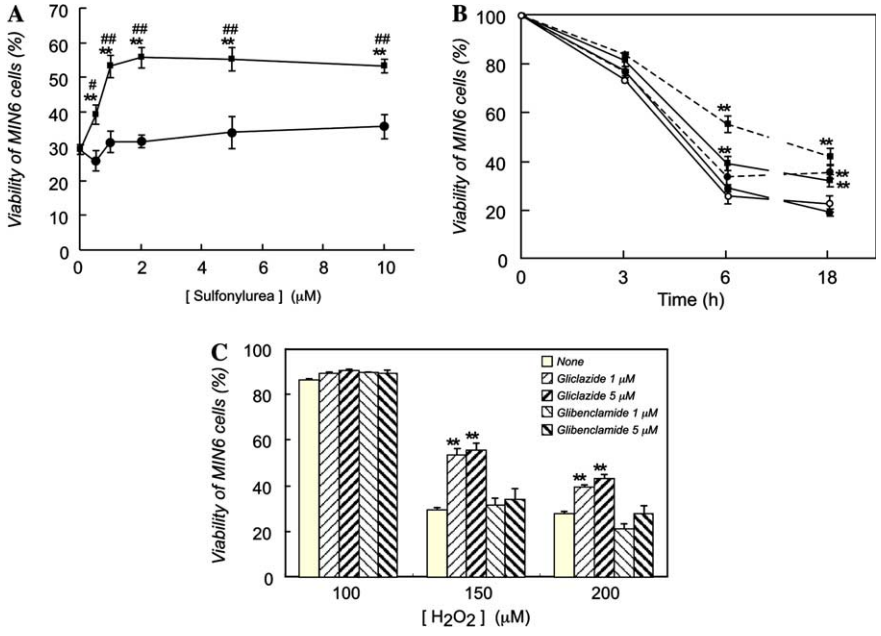

Fig. 1. MIN6 cell viability quantitated by LDH assay. (A) MIN6 cells were treated with 150 μM H_2O_2 for 6 h in the presence of either gliclazide (square) or glibenclamide (circle). Results are presented as means ± SEM ($n = 5$). $^{**}P < 0.01$ vs. vehicle treatment, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ vs. glibenclamide treatment. (B) MIN6 cells were treated with 150 μM H_2O_2 in the absence or presence of gliclazide (square) or glibenclamide (circle) (0.5 [solid line] or 5 [dotted line] μM). Results are presented as means ± SEM ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle treatment. (C) MIN6 cells were treated with H_2O_2 for 6 h in the absence or presence of gliclazide or glibenclamide. Results are means ± SEM ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle treatment.

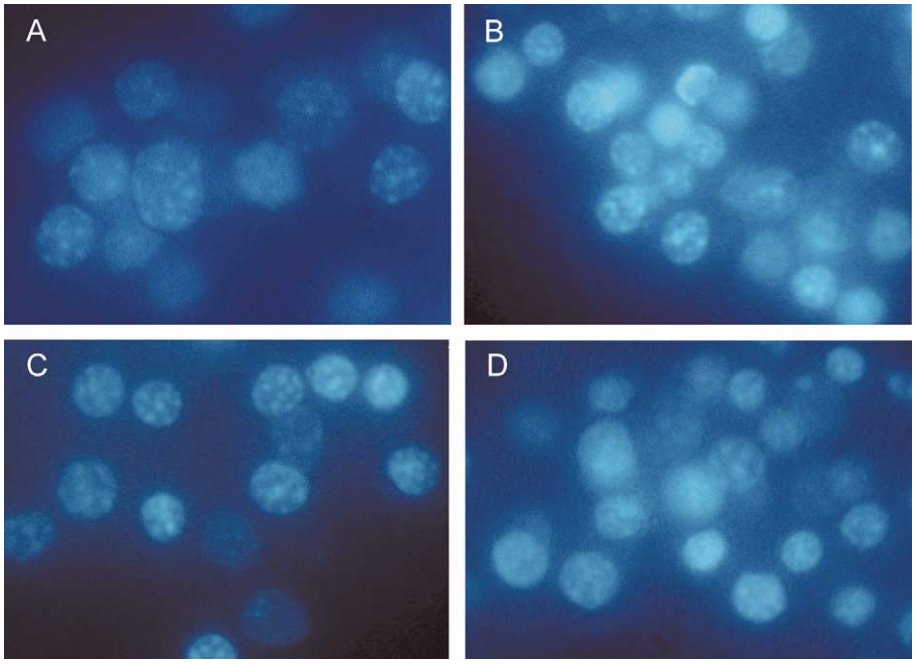

Fig. 2. Effect of gliclazide on apoptotic cell death of MIN6 cells induced by H_2O_2. Cells were treated with 150 μM H_2O_2 for 1 h in the presence (C) or absence (B) of 2 μM gliclazide. Cells were stained with HO342 and propidium iodide and visualized using a fluorescent microscope. Untreated cells (A) and H_2O_2-treated cells in the presence of 2 μM glibenclamide (D) are shown as a comparison.

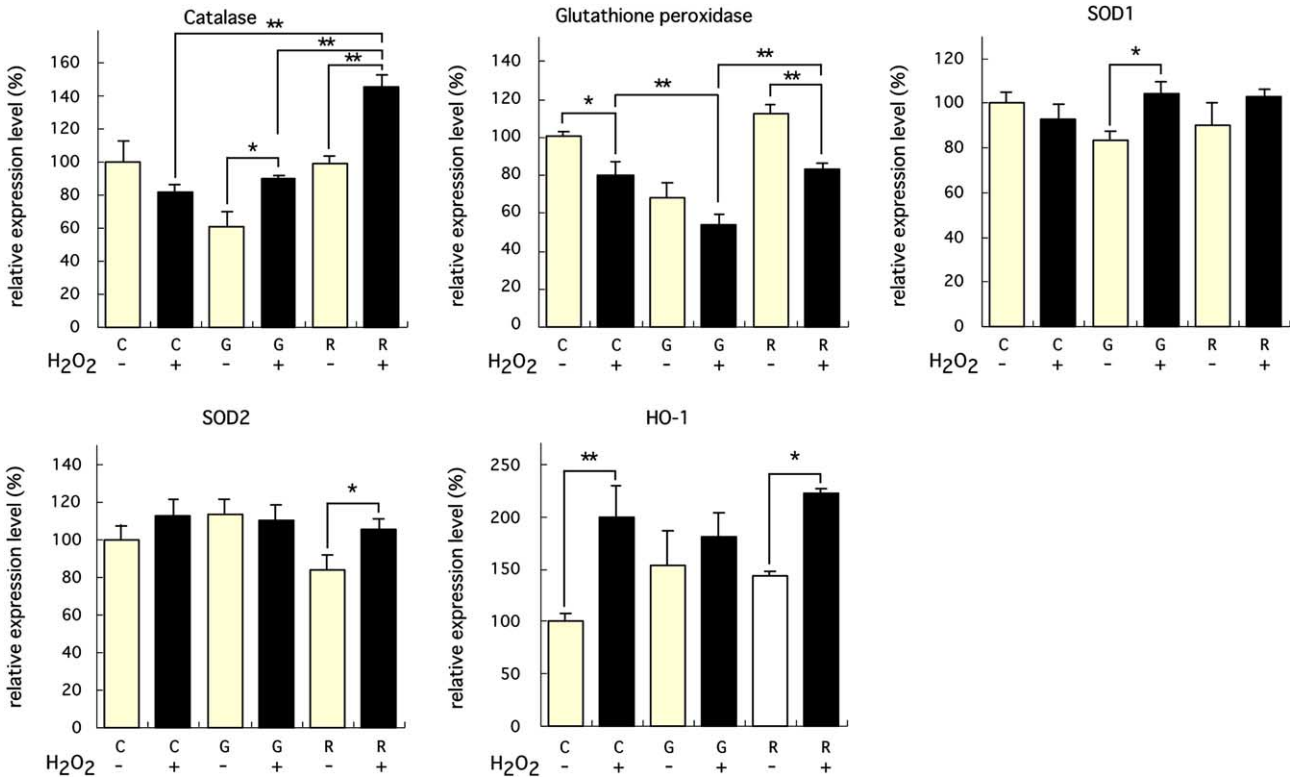

Fig. 3. Changes in anti-oxidative enzyme gene expression levels in MIN6 cells treated with H_2O_2. Cells were treated with 150 μM H_2O_2 for 3 h in the presence or absence of 2 μM sulfonylureas before isolation of total RNA. mRNA levels were compared by semiquantitative RT-PCR. mRNA levels after normalization of the specific gene to cyclophilin are expressed as a percent of that of untreated cells. Values are means ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$. C, untreated; G, gliclazide; R, glibenclamide.

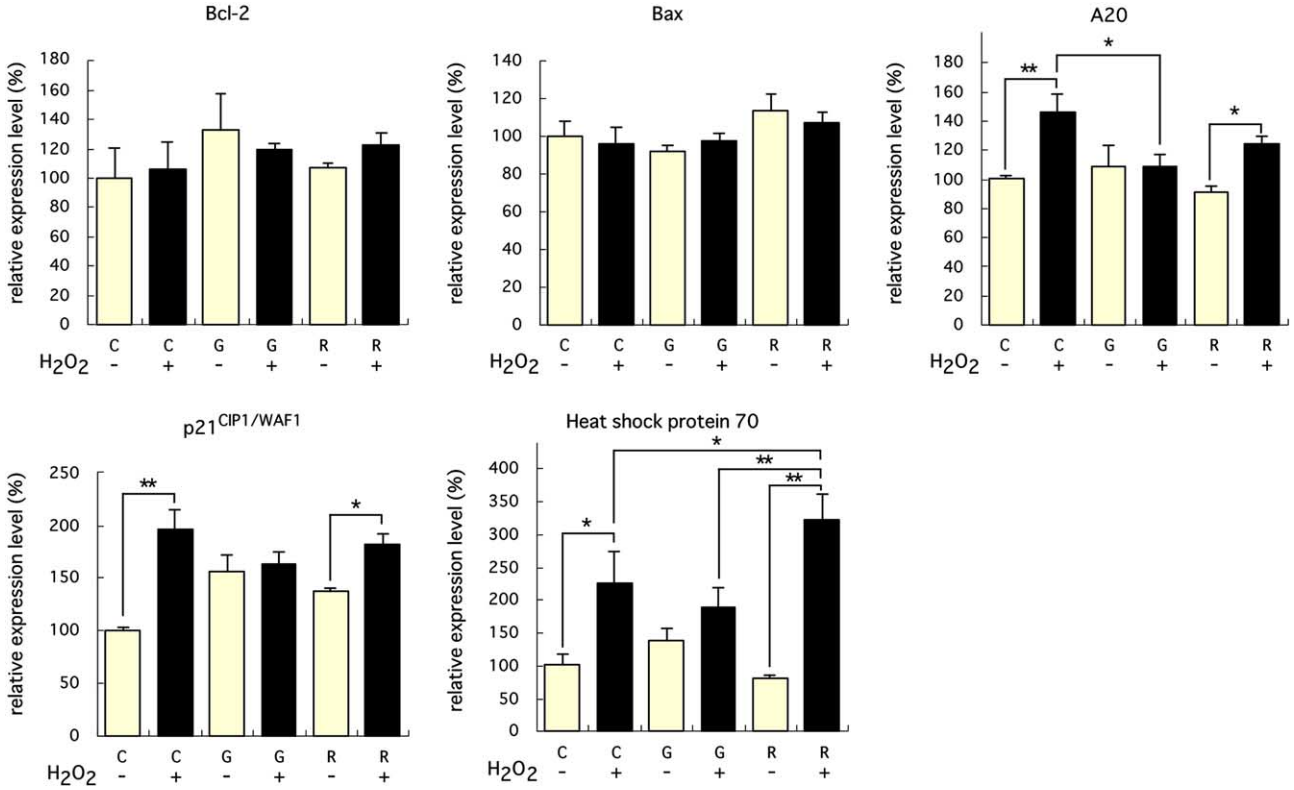

Fig. 4. Changes in stress gene mRNA levels in MIN6 cells treated with H_2O_2. Cells were treated with 150 μM H_2O_2 for 3 h in the presence or absence of 2 μM sulfonylureas before isolation of total RNA. mRNA levels were compared by semiquantitative RT-PCR. mRNA levels after normalization of the specific gene to cyclophilin are expressed as a percent of that of untreated cells. Values are means ± SEM ($n = 3$). $^*P < 0.05$,$^{**}P < 0.01$. C, untreated; G, gliclazide; R, glibenclamide.

difference was not significant between "untreated" and "gliclazide-treated," the induced expression level of Hsp70 in the presence of gliclazide was the lowest.

Discussion

There is increasing evidence to suggest that apoptosis is the main mode of β-cell death leading to type 1 diabetes and that the destruction of β-cells is caused by the infiltration of lymphocytes into the pancreatic islets [12]. On the other hand, it is also well documented that β-cells undergo apoptosis in type 2 diabetes [13,14]. In general, the apoptosis cascade is triggered by various kinds of stimuli such as DNA damage, cell cycle perturbation, metabolic imbalance, withdrawal of growth factors, cytokines as well as oxidative stress. Possible sources of oxidative stress in diabetes include an increased production of ROS, especially from enhanced glycation, and decreased enzymatic or non-enzymatic anti-oxidant defense systems. ROS participate in the toxic actions that lead to necrosis or apoptosis of the insulin-producing cells. Chronic hyperglycemia induces protein glycation and oxidative stress is increased in type 2 diabetes. The level of 8-hydroxy-2′-deoxyguanosine was increased in mononuclear cells from the whole blood and urine of diabetic patients, suggesting that oxidative stress is induced in diabetic patients [11,15]. It has also been reported that pancreatic β-cells are oxidatively stressed in diabetic GK rats [16]. Progressive impairment of β-cell function in type 2 diabetes has been demonstrated to be attributed to chronic hyperglycemia [17–19].

For the purpose of glycemic control of patients with type 2 diabetes, hypoglycemic sulfonylureas have been widely used. Besides its metabolic effects, gliclazide shows some non-metabolic effects that are specifically related to vascular diseases in diabetes. More specifically, it is reported that gliclazide decreases the oxidation of LDL cholesterol and the adhesion of monocytes to endothelial cells induced by oxidatively modified LDL [7]. In addition, a clinical study documented a decline in the vasoconstrictor prostanoid TXA2 together with a decline in lipid peroxidation [20]. Since gliclazide has been shown to possess free radical scavenging activity both in vitro and in vivo, these beneficial effects have been claimed to be due to its anti-oxidant properties rather than to its hypoglycemic activity [21,22]. However, little is known about whether gliclazide has a protective effect on β-cells against oxidative stress.

In the present study, we have demonstrated that β-cell death induced by H_2O_2 is partially suppressed by the addition of gliclazide using MIN6 cells. Instead of β-cell-specific toxins such as alloxan or streptozotocin, hydrogen peroxide was used as a trigger of oxidative stress since it is known to act physiologically during most oxidative processes. Moreover, the destruction of β-cells in the present study occurred in a relatively short time period. Fluorescent microscopic analysis suggested that the induced viability of MIN6 cells in the presence of gliclazide is due, in part, to inhibition of apoptosis.

Cellular anti-oxidant defense systems are composed of several different levels. Among them, the primary enzymatic defenses are conducted by SOD, which catalyzes the conversion of superoxide radicals into H_2O_2, and by catalase and glutathione peroxidase, both of which eliminate H_2O_2 [1]. In pancreatic β-cells, these protective genes are expressed at low levels. The importance of the anti-oxidant enzymes in protection against the toxicity of ROS was confirmed by overexpressing these genes in insulin-producing cells [23]. HO-1, the inducible form of HO, is also suggested to play a pivotal role in anti-oxidant defense in pancreatic β-cells [24]. When oxidative stress was added to the cell, these anti-oxidative enzymes were induced as an adaptive response [23]. On the other hand, Tiedge et al. [25] reported that the induction of cellular stress by high glucose treatment did not affect anti-oxidant enzyme expression at 48 h in rat pancreatic islets and RINm5F cells. In the present study, expression of HO-1 was markedly induced at 3 h after H_2O_2 addition, whereas glutathione peroxidase was reduced. There was no change induced by oxidative stress in expression of catalase, SOD1, or SOD2. Such variations in response of anti-oxidant enzymes to oxidative stress may be due to the differences in the time-course and the applied stimuli. Gliclazide suppressed the changes in the expression of both glutathione peroxidase and HO-1, suggesting that the compound decreased the oxidative stress toward MIN6 cells. A protective effect was observed at the μM level. Scott et al. [26] reported that gliclazide shows a free radical scavenging activity in vitro at a comparative concentration.

In addition to anti-oxidant genes, induction of stress gene expression is part of the adaptation of β-cells to oxidative stress, and the destruction of β-cells is mediated by an altered expression level of anti-apoptotic or pro-apoptotic genes. In many cell types including pancreatic cells, the onset of the apoptosis process is regulated by the relative abundance of Bcl-2 and Bax [27,28]. Cycline-dependent kinase inhibitor $p21^{CIP1/WAF1}$, which is responsive to oxidative stress in β-cells, is shown to act as an inhibitor of apoptosis [5,29,30]. The zinc finger protein A20 is also reported to inhibit apoptosis of islets, probably by blocking the nuclear factor-κB activation [31,32]. At 3 h after H_2O_2 stimulation, expression of neither Bcl-2 nor Bax was modified in MIN6 cells. Both p21 and A20, however, were significantly induced by oxidative stress. Again, this induction was completely suppressed by adding gliclazide, thereby suggesting that gliclazide attenuates oxidative stress to β-cells and thus protects them from apoptotic cell death.

Since the involvement of oxidative stress in β-cell dysfunction and also complications in type 2 diabetes have been well recognized, the effect of supplementation with anti-oxidants on pancreatic β-cells has been investigated in vitro and in vivo. Rasilainen et al. [33] reported the protective effect of extracellular cysteine on H_2O_2-induced oxidative stress in insulinoma RINm5F cells. In their system, the damage at higher concentrations of H_2O_2 was not completely abolished by cysteine, which is consistent with our results. NAC and aminoguanidine prevented defective insulin gene expression induced by glucose toxicity in HIT-T15 cells. In Zucker diabetic fatty rats, these anti-oxidants ameliorate the progression of diabetes mellitus [4]. In combination with Vitamin C plus E, NAC exerts beneficial effects in diabetic C57BL/KsJ-db/db mice [5]. Increased β-cell mass in anti-oxidant-treated diabetic mice may prove the validity of the hypothesis that apoptosis induced by oxidative stress in chronic hyperglycemia causes reduction of β-cell mass in type 2 diabetes. Since improved glycemic control also seems to be a beneficial factor to decrease oxidative stress in diabetic patients, a hypoglycemic sulfonylurea gliclazide with possible anti-oxidant activities may have an enhanced therapeutic role.

It is possible that suppression of apoptotic β-cell death induced by H_2O_2 is mediated through some other effects of gliclazide that are not correlated with its radical scavenging activity. Hypoglycemic sulfonylureas elicit insulin secretion by binding to the high affinity sulfonylurea receptor (SUR-1) that is part of the ATP-sensitive K^+ channel, with a resultant β-cell depolarization. Recently, there have been several reports which indicate that the agents augment Ca^{2+}-dependent insulin secretion via other mechanisms [34–36]. In addition, Efanova et al. [37] reported that another sulfonylurea, tolbutamide, induces apoptosis in pancreatic β-cells at high glucose concentrations, indicating that sulfonylurea may have additional effects directly or indirectly connected to the programmed cell death pathways. Further studies will be needed to elucidate the molecular mechanisms involved in the β-cell protection by gliclazide.

The present study provides the first evidence for a protective effect of gliclazide on pancreatic β-cells damaged by oxidative stress, but the underlying mechanism remains to be clarified. Our data favor the view that the protective effects of gliclazide are derived from its anti-oxidative activity.

Acknowledgments

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