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Research Article

Gliclazide Does Not Fully Prevent 2-Deoxy-D-Ribose-Induced Oxidative Damage Because It Does Not Restore Glutathione Content in a Pancreatic β-Cell Line

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We compared the effects of gliclazide, an antidiabetic agent with antioxidant properties, and *N*-acetyl-L-cysteine (NAC), a glutathione precursor, in protecting against 2-deoxy-D-ribose- (dRib-) induced oxidative damage in HIT-T15 cells. Using trypan blue staining and flow cytometry with annexin V/PI staining, gliclazide treatment slightly reversed dRib-induced cell death and apoptosis, and NAC treatment markedly reduced both measures. Likewise, flow cytometry using DHR 123 staining showed that the levels of dRib-induced reactive oxygen species (ROS) were partially suppressed by gliclazide and completely inhibited by NAC. Using electron spin resonance spectrometry, gliclazide and NAC scavenged hydroxyl radicals generated by Fenton reaction to a similar degree in a cell-free system. NAC, but not gliclazide, completely restored the intracellular glutathione depleted by dRib using monochlorobimane fluorescence and glutathione assays. Thus, gliclazide treatment suppressed dRib-induced oxidative damage in HIT-T15 cells less than NAC did because gliclazide did not restore the intracellular glutathione content as effectively as NAC. In addition, the elevation of intracellular glutathione rather than free radical scavenging might be an important mechanism for protecting against dRib-induced oxidative damage in a β-cell line.

1. Introduction

Pancreatic β-cell function and mass decline progressively with time in individuals with type 2 diabetes. Oxidative stress seems to be one of the causes. 2-Deoxy-D-ribose (dRib) is a powerful reducing sugar that rapidly increases oxidative stress and apoptosis in pancreatic β-cells [1, 2]. This dRib-induced oxidative damage was completely prevented by adding *N*-acetyl-L-cysteine (NAC), known as a glutathione precursor [1]. Gliclazide is an insulin secretagogue that belongs to a second-generation sulfonylurea. It also has antioxidant properties, independent of its glucose-lowering effect [3]. In an *in vitro* study, gliclazide has shown a direct free radical-scavenging effect that is not found in glibenclamide [4, 5]. Plasma oxidative stress markers, low-density lipoprotein (LDL) oxidation, and platelet aggregation have been significantly improved in gliclazide-treated patients with diabetes compared with groups treated with other sulfonylureas [6–8].

The purpose of our study was to investigate whether gliclazide could prevent dRib-induced oxidative damage in a pancreatic β-cell line. In addition, we attempted to elucidate the antioxidative mechanism of gliclazide by comparing its effects with those of NAC on dRib-induced oxidative stress and apoptosis in a β-cell line.

2. Materials and Methods

2.1. Materials. Gliclazide, NAC, dihydrorhodamine 123 (DHR 123), dimethylsulfoxide (DMSO), 5, 5-dimethyl-1-pyrroline-*N*-oxide (DMPO), $FeSO_4$, and H_2O_2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). dRib, trypan blue, and monochlorobimane (mBCl) were obtained from Amresco (Solon, OH, USA). Hydrochloric acid and ethanol were from Merck (Darmstadt, Germany). RPMI-1640, phenol red-free RPMI-1640, Dulbecco's phosphate-buffered saline (DPBS), trypsin, penicillin, and streptomycin

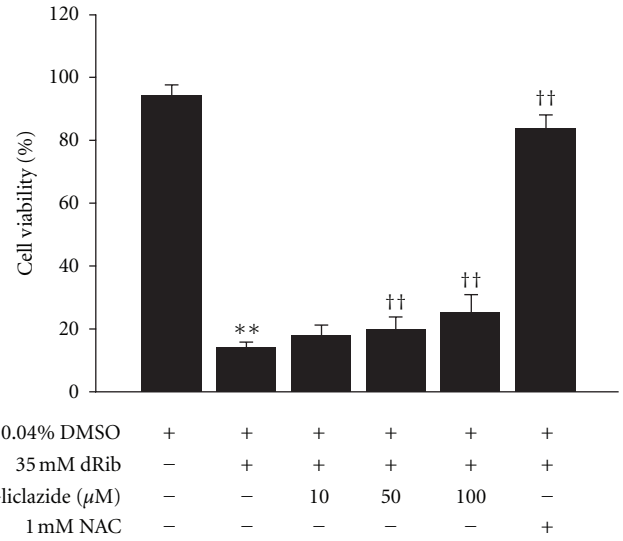

Figure 1: Effects of gliclazide and NAC on dRib-triggered cell death. HIT-T15 cells were preincubated with gliclazide or NAC for 30 min at the indicated concentrations and then cultured with 35 mM dRib for 24 h. Cell viability was determined by trypan blue exclusion assays. Data are expressed as the mean ± SD of the percentage of viable cells relative to the controls treated with vehicle (0.04% DMSO). This experiment was performed twice, in quadruplicate. $^{**}P < 0.01$ versus vehicle-treated control; $^{\dagger\dagger}P < 0.01$ versus 35 mM dRib group tested by one-way ANOVA with Duncan's *post hoc* test.

were from Gibco Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was from HyClone (Logan, UT, USA). All culture dishes were from BD Falcon (Franklin Lakes, NJ, USA).

2.2. Cell Culture. Insulin-secreting HIT-T15 cells were provided by the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 mU/mL penicillin, and 100 mg/mL streptomycin. The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere and subcultured by trypsinization with 0.05% trypsin—0.02% EDTA in Ca^{2+}- and Mg^{2+}-free DPBS when they reached about 70% confluence. Two days after subculture, the culture medium was replaced with fresh RPMI-1640 containing 10% FBS, and 35 or 50 mM dRib was added to the medium after pretreatment with various concentrations of gliclazide or 1 mM NAC for 30 min. The cultures were then incubated for 6 or 24 h.

2.3. Assessment of Cell Viability. Cells were cultured in 24-well plates at a density of 1×10^5 per well. They were incubated with 35 mM dRib for 24 h, with or without gliclazide or NAC. Then, cells were harvested and stained vitally with 0.4% trypan blue for 5 min. In the sample transferred to a hemocytometer, dead cells that did not exclude the dye and viable cells that excluded it were counted. The results were expressed as the percentage of viable cells in the whole population.

2.4. Flow Cytometry for Measuring Apoptosis. A flow cytometric analysis with annexin V and propidium iodide (PI) double staining was performed as described [2]. HIT-T15 cells were cultured in 6-well plates at a density of 5×10^5 per well. The cells were stimulated with 50 mM dRib and gliclazide or NAC for 24 h. Results were calculated as the mean fluorescence intensity and expressed as the fold difference from the control group treated with vehicle alone (0.04% DMSO).

2.5. Assessment of Intracellular Reactive Oxygen Species (ROS) Levels. Intracellular ROS levels were assessed using the fluorescein-labeled dye DHR 123 as described [2]. HIT-T15 cells were plated in 6-well culture plates at a density of 5×10^5 per well. The cells were stimulated with 50 mM dRib and gliclazide or NAC for only 6 h because extensive cell death could interfere with ROS measurement.

2.6. Assessment of Hydroxyl Radical-Scavenging Activity. Electron spin resonance (ESR) spectroscopy was used for assessing hydroxyl radical-scavenging activity *in vitro*. Hydroxyl radicals were generated by the iron-catalyzed Fenton reaction and reacted quickly with the spin trap DMPO. Levels of the resultant DMPO-hydroxyl radical adducts were measured using an ESR spectrometer (JEOL, Tokyo, Japan). Gliclazide or NAC was first dissolved in ethanol and then diluted with DPBS (pH 7.4). The reaction mixtures for ESR analysis consisted of 0.2 mL of 0.3 M DMPO, 0.2 mL of 10 mM $FeSO_4$, 0.2 mL of 10 mM H_2O_2, and 0.2 mL of control vehicle (ethanol) or samples (gliclazide or NAC dissolved in ethanol) incubated at room temperature for 2.5 min. The spectrometer conditions were magnetic field 336.5 mT, power 1.00 mW, frequency 9.4380 GHz, modulation amplitude 0.2 mT, gain 200, scan time 0.5 min, scan width 10 mT, sampling time constant 0.03 s, and temperature 25°C. The ESR signal intensities of gliclazide and NAC groups were estimated by comparison with an ethanol-treated control group.

2.7. Measurement of Intracellular Glutathione Levels. The content of intracellular reduced glutathione (GSH) was assessed using a fluorescent dye, mBCl. GSH binds specifically to mBCl to form a fluorescent GSH-mBCl adduct in a reaction mediated by intracellular glutathione S-transferases [9]. HIT-T15 cells were plated in 96-well culture plates at a density of 1×10^4 per well. The cells were preincubated with gliclazide or NAC for 30 min and then cultured with 35 mM dRib for only 6 h because cell death could interfere with GSH measurements. The stock solution of 100 mM mBCl was prepared in DMSO, which was further dissolved in phenol red-free RPMI-1640 medium at a final concentration of 5 mM for use in experiments. Then, 2 μL of 5 mM mBCl solution was added to each well containing 100 μL of the medium 20 min before measurement. The cells were washed twice with cold DPBS and the resulting fluorescence was measured using a DTX 880 multimode detector (Beckman Coulter, Fullerton, CA, USA) using an excitation wavelength of 360 nm and an emission wavelength of 465 nm. The

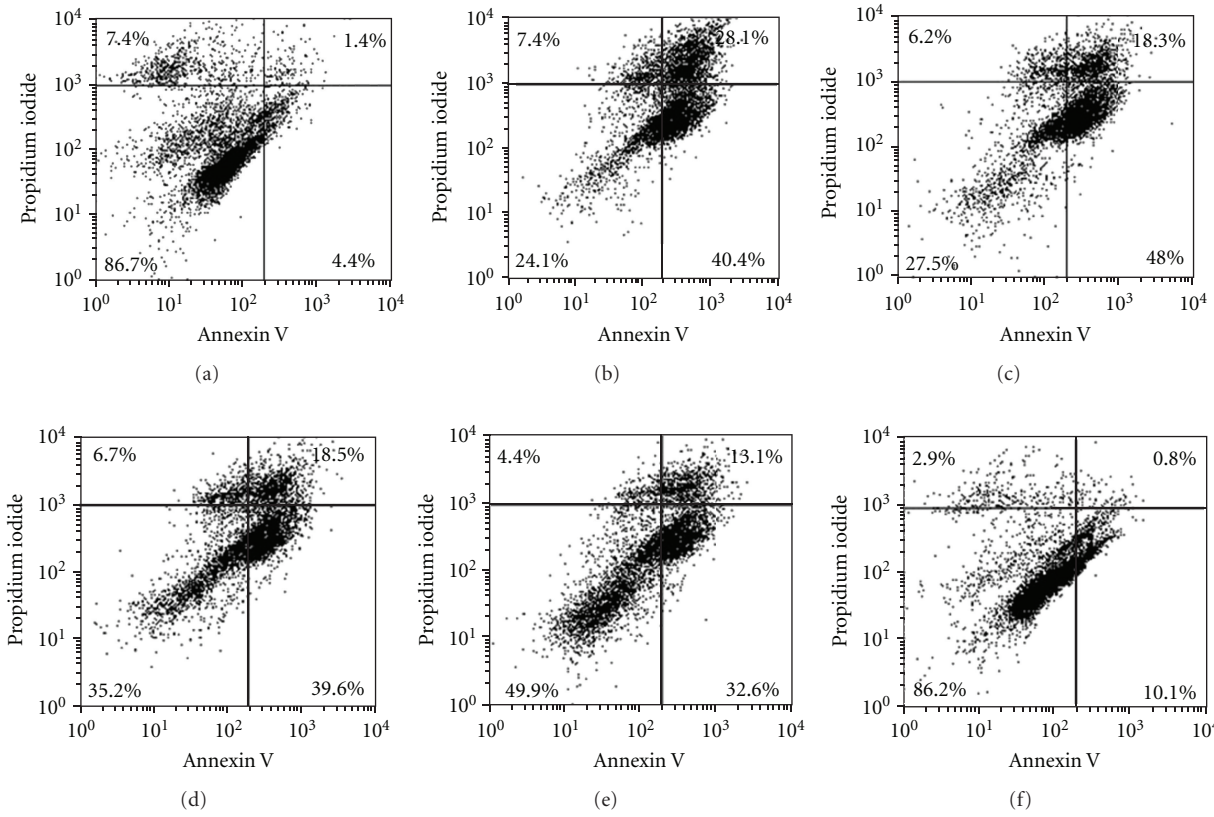

Figure 2: Effects of gliclazide and NAC on dRib-induced apoptosis of HIT-T15 cells. Cells were preincubated with gliclazide or NAC for 30 min and then cultured with 50 mM dRib for 24 h. Cells were stained with annexin V-FITC (horizontal axis) and PI (longitudinal axis) and analyzed using flow cytometry. The graph is representative of four independent experiments. (a) Vehicle (0.04% DMSO); (b) 50 mM dRib; (c) 10 μM gliclazide + 50 mM dRib; (d) 50 μM gliclazide + 50 mM dRib; (e) 100 μM gliclazide + 50 mM dRib; (f) 1 mM NAC + 50 mM dRib.

obtained fluorescence was corrected for intracellular protein in each well. The final results were expressed as the percentage of corrected fluorescence compared with vehicle-treated controls.

Intracellular total glutathione was measured using a glutathione assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) based on an enzymatic recycling method using glutathione reductase. HIT-T15 cells were cultured in 6-well culture plates at a density of 5×10^5 per well. The cells were treated as described above. Cells were washed with ice-cold DPBS and sonicated followed by centrifugation at 10,000 g for 15 min. The resulting supernatants were used immediately for measurement of total glutathione. The concentration of total glutathione was expressed as micromoles per liter in reference to a standard curve.

2.8. Statistical Analysis. All data were expressed as the mean ± SD. Comparisons between groups were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's *post hoc* test. All analyses were performed using SPSS software (version 14.0; SPSS Inc., Chicago, IL, USA), and $P < 0.05$ was considered significant.

3. Results

3.1. dRib-Induced Oxidative Damage Is Slightly Reduced by Gliclazide and Markedly Decreased by NAC. The protective effects of gliclazide and NAC against dRib-induced cell death were assessed by trypan blue exclusion tests. A 24-hour incubation with 35 mM dRib induced an extensive death of HIT-T15 cells. Pretreatment with gliclazide slightly but significantly prevented this dRib-induced cell death in a dose-dependent manner. Addition of 1 mM NAC reduced the level of cell death markedly more than gliclazide treatment did (Figure 1). Flow cytometry using double staining with annexin V and PI showed that 50 mM dRib stimulation for 24 h produced large increases in the rates of early and late apoptosis. Pretreatment with 50 or 100 μM gliclazide partially attenuated the dRib-induced apoptosis, and 1 mM NAC almost decreased the apoptosis to that of the vehicle-treated control (Figure 2, Table 1). ROS production by HIT-T15 cells was measured following a 6-hour incubation with 50 mM dRib in a flow cytometric assay using DHR 123 staining. The dRib stimulation produced a nearly 100-fold increase in intracellular ROS amounts compared with the vehicle-treated control. The addition of gliclazide dose dependently inhibited the rise in intracellular ROS level to

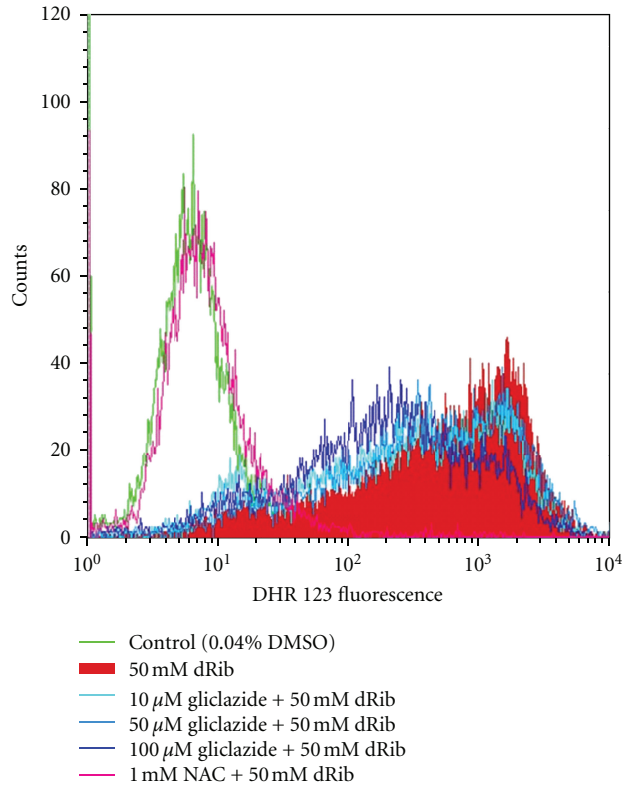

Figure 3: Effects of gliclazide and NAC on the dRib-induced rise in intracellular ROS levels. Cells were preincubated with gliclazide or NAC for 30 min and then cultured with 50 mM dRib for 6 h. Relative ROS levels were quantified by flow cytometry using the ROS-sensitive dye DHR 123. Cells were incubated with 5 μM DHR 123 during the final 30 min. The histogram is representative of four independent experiments.

Table 1: Percentage of annexin V-positive cells in vehicle- and dRib-treated HIT-T15 cells, with or without gliclazide or NAC.

	Annexin V-positive cells (%)
Control	8.2 ± 3.6
50 mM dRib alone	72.9 ± 5.4**
10 μM gliclazide + 50 mM dRib	70.9 ± 5.2
50 μM gliclazide + 50 mM dRib	64.4 ± 4.4†
100 μM gliclazide + 50 mM dRib	57.7 ± 8.7††
1 mM NAC + 50 mM dRib	13.3 ± 3.4††

Group data from the experiments illustrated in Figure 2 are expressed as the mean ± SD of four independent experiments. $^{**}P < 0.01$ versus control (0.04% DMSO) and $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ versus 50 mM dRib group evaluated by one-way ANOVA with Duncan's *post hoc* test.

a small extent. However, pretreatment with NAC completely blocked this dRib-induced ROS increase (Figure 3, Table 2).

3.2. Gliclazide and NAC Scavenge Fenton Reaction-Driven Hydroxyl Radicals to a Similar Degree. For direct assessment of the ROS-scavenging capacities of gliclazide and NAC, we performed ESR spectroscopy in cell- and dRib-free conditions. Hydroxyl radicals were generated in a Fenton reaction system with DMPO as the trapping agent in a 0.4% ethanol-treated control. The addition of gliclazide caused significant decreases in the levels of DMPO-hydroxyl radical adduct compared with the control. Pretreatment with NAC also significantly reduced the hydroxyl radical signal. In terms of the capacity for scavenging hydroxyl radicals generated via the Fenton reaction, there was no significant difference between gliclazides and NAC (Figure 4).

Table 2: Relative intracellular ROS levels in vehicle- and dRib-treated HIT-T15 cells, with or without gliclazide or NAC.

	Relative fluorescence (fold change from control)
Control	1.0
50 mM dRib alone	97.2 ± 15.7**
10 μM gliclazide + 50 mM dRib	84.0 ± 22.7
50 μM gliclazide + 50 mM dRib	63.7 ± 17.5††
100 μM gliclazide + 50 mM dRib	50.3 ± 9.0††
1 mM NAC + 50 mM dRib	1.7 ± 0.3††

Data are expressed as the mean ± SD of four independent experiments. $^{**}P < 0.01$ versus control (0.04% DMSO) and $^{\dagger\dagger}P < 0.01$ versus 50 mM dRib alone evaluated by one-way ANOVA with Duncan's *post hoc* test.

3.3. NAC but Not Gliclazide Treatment Restores the Level of Intracellular Glutathione Depleted by dRib. When HIT-T15 cells were stimulated with various concentrations of dRib for 6 h, intracellular reduced and total glutathione levels were significantly and dose dependently decreased (See Supplementary Figure 1 in Supplementary Material available online at doi:10.1155/2011/390678). Additions of 10, 50 and 100 μM gliclazide never reversed the dRib-induced depletion of reduced and total glutathione in HIT-T15 cells. However, pretreatment with 1 mM NAC completely restored the dRib-induced depletions and increased glutathione levels to more than those seen in the vehicle-treated control (Figure 5).

4. Discussion

This study showed that gliclazide treatment had only minor protective effects on dRib-induced oxidative damage, but that NAC treatment almost completely prevented oxidative damage in HIT-T15 cells. We hypothesize that gliclazide and NAC have different degrees of protection against dRib-induced oxidative injury because they have different antioxidative mechanisms. In this experiment, gliclazide could scavenge hydroxyl radicals generated by a Fenton reaction but did not regenerate the cellular glutathiones depleted by dRib. Then, gliclazide only slightly suppressed the dRib-induced rises in intracellular ROS and apoptosis. However, NAC possessed both radical-scavenging and glutathione-regenerating capacities and completely reversed the dRib-induced oxidative stress and cell death. The elevation of intracellular glutathione was more important than free radical scavenging in preventing dRib-induced β-cell damage because NAC was more effective than gliclazide in protecting them against dRib-induced damage. Further, we surmise

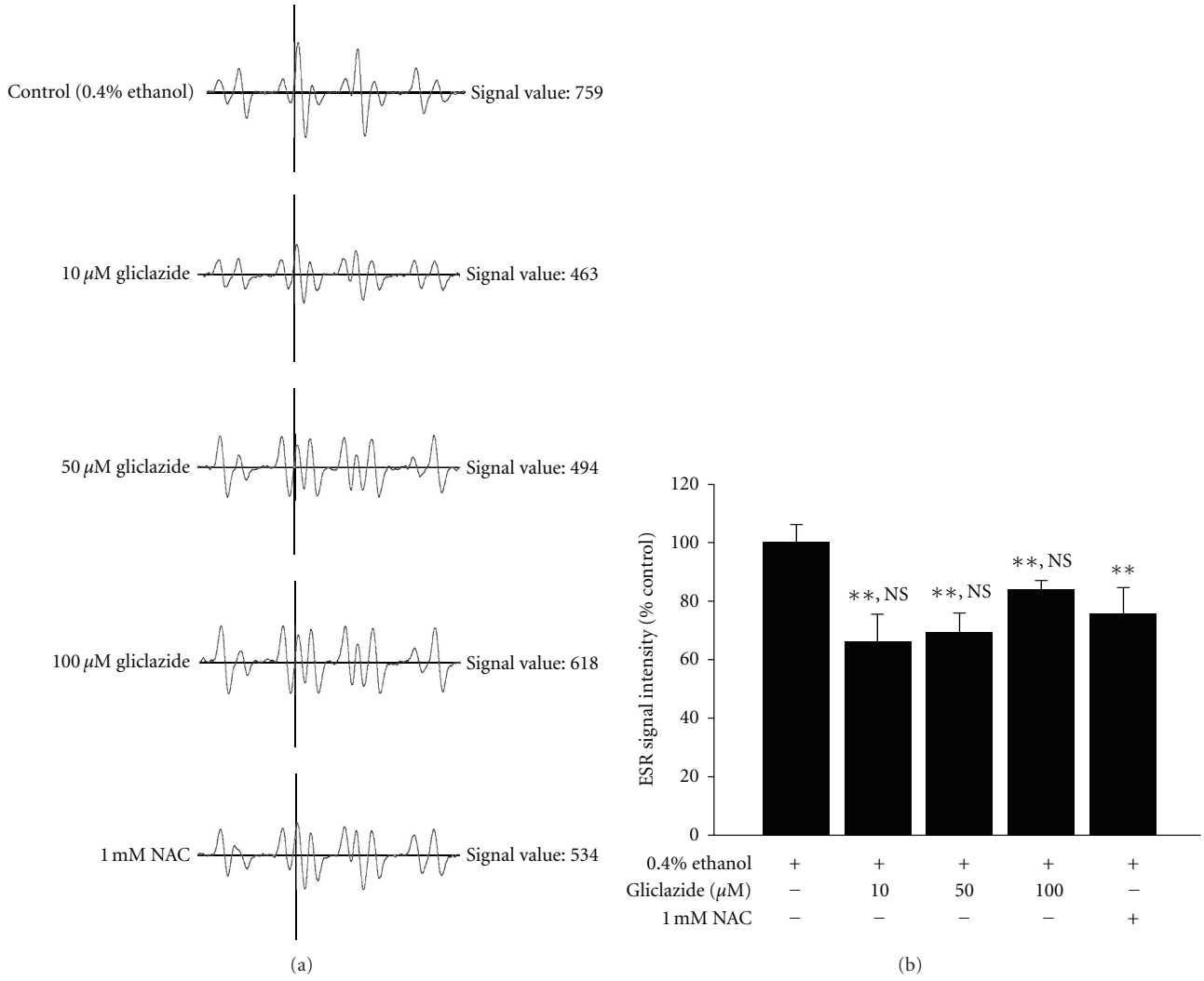

Figure 4: Scavenging effects of gliclazide and NAC on hydroxyl radical generation. (a) Representative ESR spectra of DMPO-hydroxyl radical conjugates measured in the Fenton reaction. (b) Comparison of hydroxyl radical-scavenging activity between gliclazide and NAC in the reaction as in (a). Reaction mixtures contained 60 mM DMPO, 2 mM $FeSO_4$, and 2 mM H_2O_2 in DPBS with and without various concentrations of gliclazide or NAC dissolved in 0.4% ethanol. The results are the mean ± SD from four independent experiments. $^{**}P < 0.01$ versus vehicle- (0.4% ethanol)-treated control evaluated by one-way ANOVA with Duncan's *post hoc* test. NS: no significant difference from the 1 mM NAC group.

that glutathione regeneration is a key mechanism in the prevention of β-cell damage produced by dRib because NAC, a glutathione precursor, completely restored the dRib-induced oxidative damage in HIT-T15 cells.

The progressive decrease in the glucose-lowering effect of sulfonylureas in patients with type 2 diabetes whose glycemia responds initially to treatment has been called secondary failure [10]. In clinical studies, it was demonstrated that gliclazide had a lower secondary failure rate compared with other sulfonylureas [11, 12]. This secondary failure is now recognized as a common progressive form of β-cell deterioration that develops in the late stage of type 2 diabetes on any antidiabetic regimen [13, 14]. Oxidative stress is known to be one of the mechanisms of β-cell failure in patients with type 2 diabetes. In this research, we could not elucidate the direct mechanism of the protective effects of gliclazide against dRib-induced oxidative β-cell damage. However, we suppose that free radical scavenging would be one of the mechanisms because gliclazide quenched Fenton reaction-driven hydroxyl radicals in a cell-free system. In previous studies, gliclazide proved to be a general free radical scavenger [4, 5] and an effective antioxidant that decreased oxidative stress markers *in vivo* [6–8]. Accordingly, we attribute the lower secondary failure rate of gliclazide to its antioxidative effects, which might result from ROS-scavenging activity.

We demonstrated here that intracellular glutathione depletion might be the most important mechanism of dRib-induced oxidative stress in HIT-T15 cells. However, we did not establish a mechanism for the exhaustion of

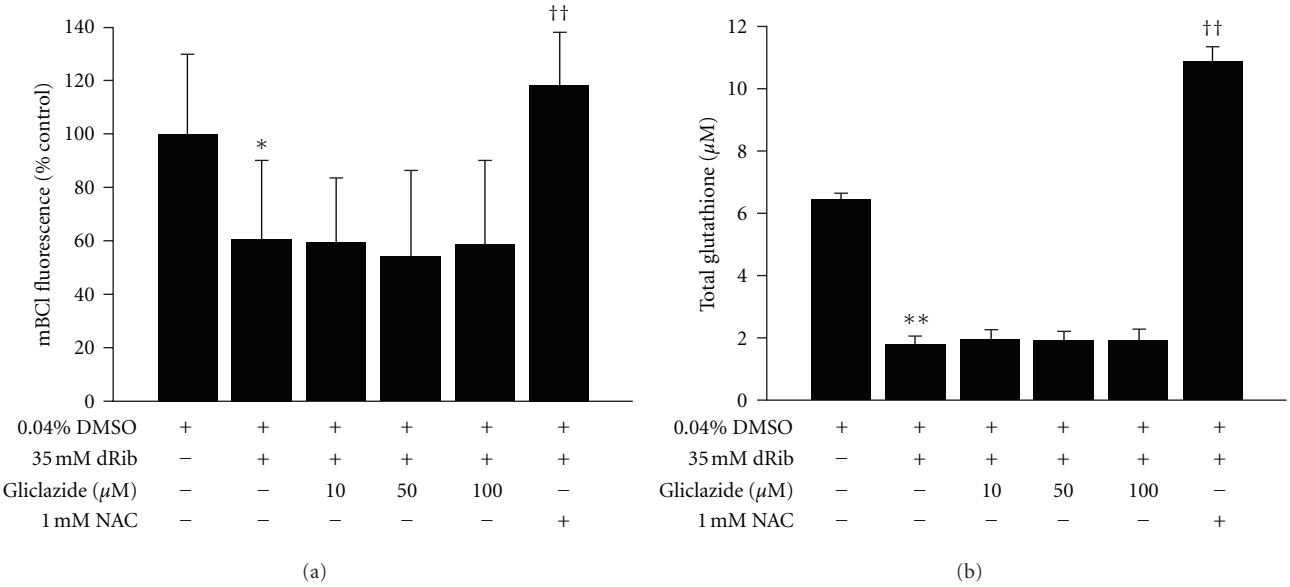

Figure 5: Effects of gliclazide and NAC on the dRib-induced depletion of intracellular glutathione. The intracellular glutathione levels were determined by measuring the fluorescent intensity of the GSH-mBCl adduct (a) or using a glutathione assay kit (b). Experimental conditions were as described in the Section 2. These experiments were performed twice in quadruplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control treated with vehicle alone (0.04% DMSO). $^{\dagger\dagger}P < 0.01$ versus 35 mM dRib group evaluated by one-way ANOVA with Duncan's *post hoc* test.

intracellular glutathione. Fico et al. [15] reported that dRib produced oxidative stress-induced apoptosis by inhibiting the synthesis of GSH and by increasing GSH efflux in a mouse embryonic stem cell line. We could not study the effect of dRib on glutathione synthesis, but we measured the extracellular levels of GSH and oxidized glutathione (GSSG) for indirect assessment of the glutathione efflux mediated by dRib. In our glutathione assay, dRib stimulation did not elevate the extracellular GSH and GSSG levels compared with control cells in the culture medium alone (data not shown). Recently, Schmidt et al. [16] also reported that the decline of intracellular glutathione observed after exposure of cultured astrocytes to dRib was not accompanied by increases in extracellular glutathione. Hence, the cellular mechanisms of dRib-induced glutathione depletion might vary with the cell types, so more studies for elucidating the exact mechanism of dRib are clearly needed.

Prolonged exposure to high glucose concentrations can lead to irreversible β-cell dysfunction and apoptosis, a phenomenon termed glucose toxicity [17]. Tanaka et al. [18] showed that NAC, a substrate for glutathione biosynthesis, prevented glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats. It was reported that the overexpression of glutathione peroxidase that catalyzes the reduction of hydrogen peroxide by GSH could protect pancreatic islets against oxidative stress and ameliorate hyperglycemia in db/db diabetic mice [19, 20]. These results suggest that glutathione might be a therapeutic target to prevent β-cell failure in the hyperglycemic conditions seen in subjects with type 2 diabetes.

In summary, gliclazide partially attenuated dRib-induced oxidative stress and apoptosis in HIT-T15 cells. However, its effects were much smaller than those of NAC because gliclazide did not restore the intracellular glutathione depleted by dRib, unlike NAC. The major protective mechanism against dRib-induced oxidative damage appears to be the elevation of intracellular glutathione rather than via ROS scavenging. Therefore, intracellular glutathione-elevating agents can completely prevent the dRib-induced oxidative damage; moreover, they might protect β-cells against glucose toxicity.

Acknowledgments

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