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Rosiglitazone protects human neuroblastoma SH-SY5Y cells against acetaldehyde-induced cytotoxicity

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Abstract

Acetaldehyde, an inhibitor of mitochondrial function, has been widely used as a neurotoxin because it elicits a severe Parkinson's disease-like syndrome with elevation of the intracellular reactive oxygen species level and apoptosis. Rosiglitazone, a peroxisome proliferator-activated receptor-γ agonist, has been known to show various non-hypoglycemic effects, including anti-inflammatory, anti-atherogenic, and anti-apoptotic. In this study, we investigated the protective effects of rosiglitazone on acetaldehyde-induced apoptosis in human neuroblastoma SH-SY5Y cells and attempted to examine its mechanism. Acetaldehyde-induced apoptosis was moderately reversed by rosiglitazone treatment. Our results suggest that the protective effects of rosiglitazone on acetaldehyde-induced apoptosis may be ascribed to ability to induce the expression of anti-oxidant enzymes and to regulate Bcl-2 and Bax expression. These data indicate that rosiglitazone may provide a useful therapeutic strategy for the prevention of progressive neurodegenerative disease such as Parkinson's disease.

Keywords: Acetaldehyde; Parkinson's disease; PPAR-γ; Rosiglitazone; ROS; Apoptosis

Parkinson's disease (PD) is a neurodegenerative disease characterized by the progressive loss of dopaminergic neurons in the substantia nigra. Although the specific cause of Parkinson's disease remains unclear, an evidence points to the involvement of mitochondrial dysfunction and oxidative stress [1].

Acetaldehyde, the metabolite of alcohol, has been shown to selectively and potently damage mitochondria when combined with 1-methyl-4-phenyl-2,3,6-tetrahydropyridine (MPTP), [2] and it induces a syndrome resembling Parkinson's disease in animal and cellular models [3,4]. Therefore, it is classically used as a Parkinson's neurotoxin to study the mechanisms of Parkinson's disease. It is thought that neuronal cell death induced by acetaldehyde is mediated by the opening of mitochondrial permeability transition (MPT) pores [5].

While there is more than one pathway to apoptosis, Bcl-2 plays a significant role in acetaldehyde-induced apoptosis [6]. The interplay between pro- and anti-apoptotic Bcl-2 family members may determine the fate of cells by regulating MPT pores and controlling the release of cytochrome *c* from mitochondria [7,8]. Cytochrome *c* forms an apoptotic complex with apoptosis activating factor, Apaf-1 and procaspase-9, and then activates caspase-3 [9]. Caspase-3 has been demonstrated to participate in aldehyde-induced apoptosis [10].

Rosiglitazone, a thiazolidinedione (TZD), is a peroxisome proliferator-activated receptor (PPAR)-γ agonist. It has been widely used for treating hyperglycemia and improving insulin resistance [11]. The metabolic effects of TZDs are mediated by receptor-dependent activation of the PPAR-γ–retinoid X receptor (RXR) complex and

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subsequent transcriptional activation of target genes [12]. Interestingly, TZDs may be also potent inhibitors of inflammation in part by receptor-independent mechanisms [13–16]. It is also thought that the anti-inflammatory effects of TZDs may reduce the cardiovascular disease risk [17]. Recently, it was suggested that rosiglitazone could inhibit apoptosis by regulating the expression of Bcl-2 [18].

It is now believed that damage to dopaminergic neurons, involving oxidative stress and/or mitochondrial impairment, culminates in the activation of an apoptotic cascade [1]. Regulation of intracellular ROS and modification of the apoptotic cascade may control apoptosis and provide new strategies for prevention and treatment of Parkinson's disease. The purpose of this study was to investigate the effects of rosiglitazone on acetaldehyde-induced cytotoxicity in human neuroblastoma SH-SY5Y cells in order to find a possible therapeutic application of the effective compound for degenerative disease by evaluating the protective effect of rosiglitazone on SH-SY5Y cells against acetaldehyde-induced cytotoxicity. This study demonstrated that rosiglitazone inhibits loss of cell viability by overcoming oxidative stress and regulating apoptotic genes.

Materials and methods

Chemicals. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin–EDTA, and antibiotics for cell culture were obtained from Gibco-BRL-Life Technologies (Grand Island, NY). Rosiglitazone maleate tablets were purchased from SmithKline Beecham Pharmaceuticals (West Sussex, UK). All other chemicals and reagents, unless otherwise noted, were obtained from Sigma Chemical.

Cell culture. Human blastoma SH-SY5Y cells were obtained from ATCC and cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin. Cells were kept at 37 °C in humidified 5% CO_2 and 95% air. All experiments were carried out 24–48 h after cells were seeded. During acetaldehyde studies, the growth medium was supplemented with 100 μM acetaldehyde, 10 μM rosiglitazone, 15 mM sodium diethyldithiocarbamate trihydrate, and 13 mM 3-amino-1,2,4-triazole as anti-oxidant enzyme inhibitors.

Cell viability assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved in a PBS solution at a concentration of 5 mg/ml, filtered through a 0.22 μm filter to sterilize, and remove insoluble residues, and then stored in amber vials at 4 °C for a month. After 48 and 72 h incubations, 25 μl of the MTT solution was added to each well of 96-well plates and incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. At the end of the incubation period, the media were discarded using a suction pump. The extraction buffer of 20% w/v sodium dodecyl sulfate (SDS) in a solution of 50% *N*,*N*-dimethylformamide (DMF) in demineralized water (50:50, v/v) was prepared at pH 4.7 and filtered through a 0.22 μm filter to remove insoluble residues. The absorbance was determined at 570 nm. The A_{570} was taken as an index of the cell viability. The net absorbance from the plates of cells cultured with the control medium (not treated) was considered as 100% cell viability.

Measurement of anti-oxidant enzyme activity. SOD activity was measured using assay kits purchased from Dojindo (Kumamoto, Japan). The caspase activity assay was performed as described previously [19].

Measurement of intracellular reactive oxygen species. Intracellular ROS was monitored using the fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA) [20]. Intracellular H_2O_2 and low-molecular-weight peroxides oxidize DCFH-DA to the highly fluorescent compound dichlorofluorescein (DCF). SH-SY5Y cells were seeded in 96-well plates and were incubated with increasing concentrations of acetaldehyde and/or rosiglitazone for 36 h. Cells were incubated with 10 μM DCFH-DA at 37 °C for 30 min, then washed twice with PBS, and finally the fluorescence intensity of DCF was measured in a microplate-reader at excitation wavelength 485 nm and emission wavelength 538 nm.

Nuclear staining with Hoechst 33258. After being treated with acetaldehyde and/or rosiglitazone for 48 h, the cells were incubated with 3 μg/ml Hoechst 33258, a DNA fluorochrome, for 20 min. Cells were washed with PBS and analyzed by fluorescent microscopy. Cells that exhibited reduced nuclear size, chromatin condensation, intense fluorescence, and nuclear fragmentation were considered as apoptotic.

Messenger RNA analysis for Bcl-2 and Bax. The messenger RNA (mRNA) levels of Bcl-2 and Bax were assessed by semi-quantitative RT-PCR analysis using β-actin as a control. Total RNA was extracted from SH-SY45Y cells using Trizol (Invitrogen, Carlsbad, CA, USA). Contaminating DNA was removed by treatment of each sample with DNAse I, according to the manufacturer's instructions (Promega, Madison, WI, USA). cDNA was prepared using SuperScript II first strand synthesis system, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). PCR primers were designed as follows: Bcl-2 sense, 5′-actttgcagagatgtccagt-3′; Bcl-2 anti-sense, 5′-cggttcaggtactcagtcat-3′; Bax sense, 5′-actggacagtaacatggagc-3′; Bax anti-sense, 5′-tcttcttccagatg gtgagt-3′; β-actin sense, 5′-gacctgacagactacctca-3′; β-actin anti-sense, 5′-tc tcttgctcgaagtctagg-3′. RT-PCR products were electrophoresed on a 1.5% (w/v) agarose gel, stained with ethidium bromide, and bands were visualized by UV light.

Immunoblot analysis for cytochrome c and caspase-3. Immunoblot analysis was performed using the cytosol or whole cell lysates. Samples were resolved by 12% SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and blotted with appropriate primary antibodies. The membrane was incubated with peroxidase-conjugated secondary antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the bound antibody was visualized using chemiluminescence (ECL, Intron, Korea) and X-ray film (FUJI FILM, Japan). Primary antibodies to cytochrome *c* and caspase-3 were purchased from BD Sciences (San Jose, CA, USA) and BioLegend (San Diego, CA, USA).

Results and discussion

Rosiglitazone ameliorated acetaldehyde-induced loss of neuronal cell viability and oxidative stress

The anti-oxidant effect is a possible mechanism for rosiglitazone-mediated neuroprotection. Previous data demonstrated that oxidative damage occurs in the Parkinsonian brain [1]. Overproduction of ROS can cause severe impairment of cellular functions. ROS are involved in apoptotic mechanisms [21] and may contribute to the apoptotic process found in Parkinson's disease [22]. In this study, the effect of rosiglitazone on acetaldehyde-induced SH-SY5Y cell viability loss was assessed with a MTT assay. As shown in Fig. 1A, cell viabilities decreased to 51% after treatment with acetaldehyde for 48 h. The acetaldehyde-induced loss of viability was moderately attenuated by rosiglitazone treatment. The involvement of ROS in the cytotoxic effect of acetaldehyde was also explored. As shown in Fig. 1B, we measured the relative activities of SOD and catalase. The activities of SOD and catalase decreased to 61% and 39%, respectively, after acetaldehyde treatment. The inhibitory effect of acetaldehyde on SOD and catalase expressions suggests that the cytotoxic effect of acetaldehyde may be mediated by oxidative stress in SH-SY5Y cells. To determine suppression of ROS production by rosiglitazone, we measured ROS production in

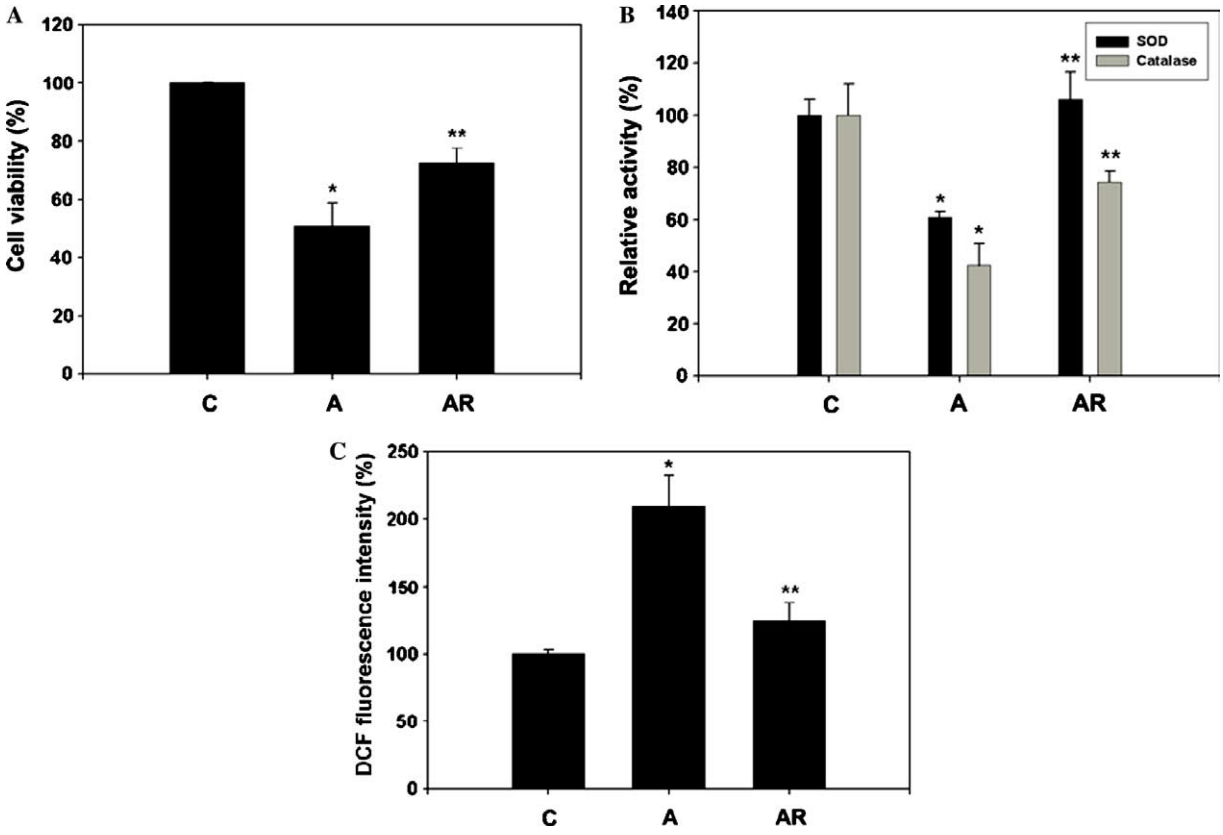

Fig. 1. Effect of rosiglitazone on the acetaldehyde-induced decrease in SH-SY5Y cell viability. Cell viability was assessed by the MTT assay as described in Materials and methods. (A) Cells were treated with 100 μM acetaldehyde in the absence or presence of 10 μM rosiglitazone for 48 h. (B) SOD and catalase activities were measured under the conditions of (A). (C) ROS production was measured under the conditions of (A). Data are expressed as the percentage of values in untreated control cultures and are means ± SD ($n = 3$). *$P < 0.05$, compared with the control group. **$P < 0.05$, compared with acetaldehyde treatment group. *Abbreviations:* C, control; A, acetaldehyde treatment; AR, acetaldehyde plus rosiglitazone treatment.

SH-SY5Y under several conditions. As expected, rosiglitazone suppressed acetaldehyde-induced ROS production (Fig. 1C). The depressant effects of anti-oxidant enzymes strongly suggest the involvement of ROS in the cytotoxic effect of acetaldehyde on SH-SY5Y cells. SOD and catalase promoters contain PPAR response element [23]. Thus, expression of SOD and catalase could potentially be regulated by rosiglitazone. Our present results have shown that rosiglitazone decreases acetaldehyde-induced cell death and ROS production in SH-SY5Y cells by inducing anti-oxidant enzymes. Based on these findings, we postulate that the anti-oxidative properties of rosiglitazone may contribute to the protection of SH-SY5Y cells from ROS caused by acetaldehyde.

Rosiglitazone rescued acetaldehyde-induced changes in nuclear morphology

Change in nuclear morphology after acetaldehyde treatment was assessed by Hoechst 33258 staining. As shown in Fig. 2, the control SH-SY5Y cell's nuclei had a regular and ovum shape. However, apoptotic nuclei, characterized by nuclear condensation and fragmentation, appeared after exposure to acetaldehyde for 48 h. Rosiglitazone treatment blocked the acetaldehyde-induced nuclear damage.

Rosiglitazone affected the expression of Bcl-2 and Bax in acetaldehyde-treated cells by regulation of anti-oxidant enzyme expressions

Other mechanisms could also be pertinent in the protective mechanism of rosiglitazone. It is increasingly apparent that mitochondria lie at the centre of the process of cell death regulation. Induction of apoptosis often converges on the mitochondria to induce MPT and release of apoptotic proteins into the cytoplasm, resulting in a biochemical and morphological alteration of apoptosis. Although the precise mechanism by which the Bcl-2 family acts remains unclear, it has been established that the Bcl-2 family plays a key role in the mitochondrial apoptotic pathway [24]. Bax and Bcl-2, the two main members of Bcl-2 family, affect the permeability of the mitochondrial membrane. Bax is a pore-forming cytoplasmic protein, translocates to the outer mitochondrial membrane, influences its permeability, and induces cytochrome *c* release from the intermembrane space of the mitochondria into the cytosol, and subsequently leads to apoptosis [8]. Bcl-2, which has anti-apoptotic properties, is associated with the outer mitochondrial membrane where it stabilizes membrane permeability, thus preserving mitochondrial integrity, suppressing the release of cytochrome *c*, and inhibiting apoptosis [7]. Cell survival

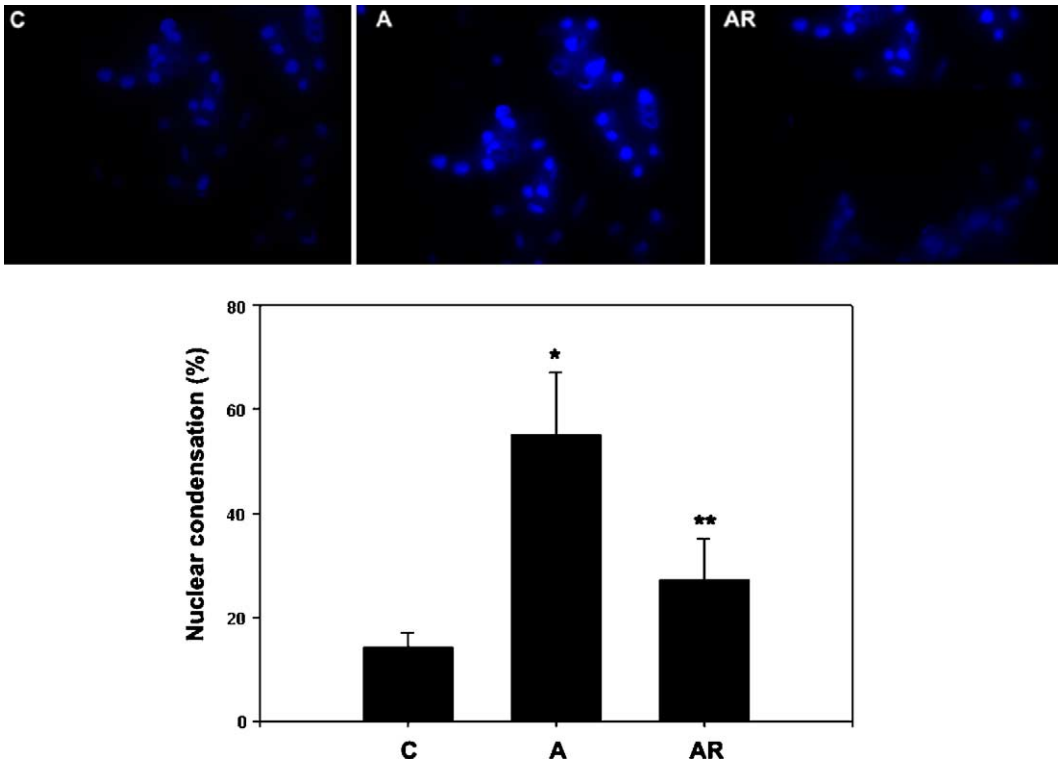

Fig. 2. Nuclear morphological assessment of SH-SY5Y cells by fluorescence microscopy. The figures show the fluorescence micrographs of Hoechst 33258 stained nuclear morphology. Percentage of condensed nuclei as assessed by Hoechst 33258 staining of adherent and floating cells. SH-SY5Y cells were untreated (C), treated with 100 μM acetaldehyde for 48 h (A), and treated with 10 μM rosiglitazone, and acetaldehyde (AR) for 48 h. *Abbreviations:* C, control; A, acetaldehyde treatment; AR, acetaldehyde plus rosiglitazone treatment.

in the early phases of the apoptotic cascade depends mostly on the balance between the pro- and anti-apoptotic proteins of the Bcl-2 family. In this regard, the Bax/Bcl-2 ratio may be a better predictor of apoptotic fate than the absolute concentrations of either Bax or Bcl-2 alone [24]. Any shift in the balance of pro- and anti-apoptotic proteins will affect cell death. Bcl-2 family members are intimately involved in cell death processes caused by acetaldehyde [25]. In this study, we investigated whether rosiglitazone has any effect on the expressions of Bcl-2 and Bax in acetaldehyde-treated cells using semi-quantitative RT-PCR analysis. As shown in Fig. 3A, Bax mRNA expression increased significantly in the acetaldehyde-treated group, compared with that of the control group. However, rosiglitazone treatment could decrease the Bax mRNA expression level almost to the normal values. Differently a case of Bax, the level of Bcl-2 in the acetaldehyde-treated group was significantly decreased compared with that of the control group. However, expression of Bcl-2 was recovered with rosiglitazone treatment. The Bax/Bcl-2 ratio increased to levels 20-fold higher than those of the control group following treatment with acetaldehyde, and rosiglitazone reversed the acetaldehyde-induced increase of the Bax/Bcl-2 ratio (Fig. 3B). To explore the relationship of rosiglitazone, expressions of anti-oxidant enzymes, and regulation of Bax/Bcl-2, we used anti-oxidant enzyme inhibitors. As expected, these inhibitors eliminated the effects of rosiglitazone (Fig. 3). Treatment with 0.1, 2, 10, and 50 μM rosiglitazone altered the Bax/Bcl-2 ratio by 53%, 52%, 11%, and 10% compared to that of the acetaldehyde treatment group, respectively (Fig. 4). These data show that rosiglitazone treatment is dose dependent. It ameliorates the acetaldehyde-induced Bax/Bcl-2 ratio elevation in SH-SY5Y cells. Therefore, the effect of rosiglitazone on acetaldehyde-induced apoptosis may be, at least in part, mediated by regulation of Bax and Bcl-2 expression, and regulation of anti-oxidant enzymes may exist between effects of rosiglitazone and regulation of Bax and Bcl-2 in an anti-apoptotic mechanism.

Rosiglitazone inhibited acetaldehyde-induced cytochrome c release

Given the key role of the ratio between Bax and Bcl-2 proteins in the apoptotic cascade, it is not surprising that in our experiments treatment with rosiglitazone is also associated with the prevention of the downstream apoptotic signaling pathways, finally preventing the release of cytochrome *c* from mitochondria. In this study, the involvement of mitochondria in acetaldehyde-induced apoptosis was investigated by taking into account the loss of mitochondrial function assessed by the release of cytochrome *c*. The opening of the MPT pores causes a release of apoptogenic substances such as cytochrome *c* from

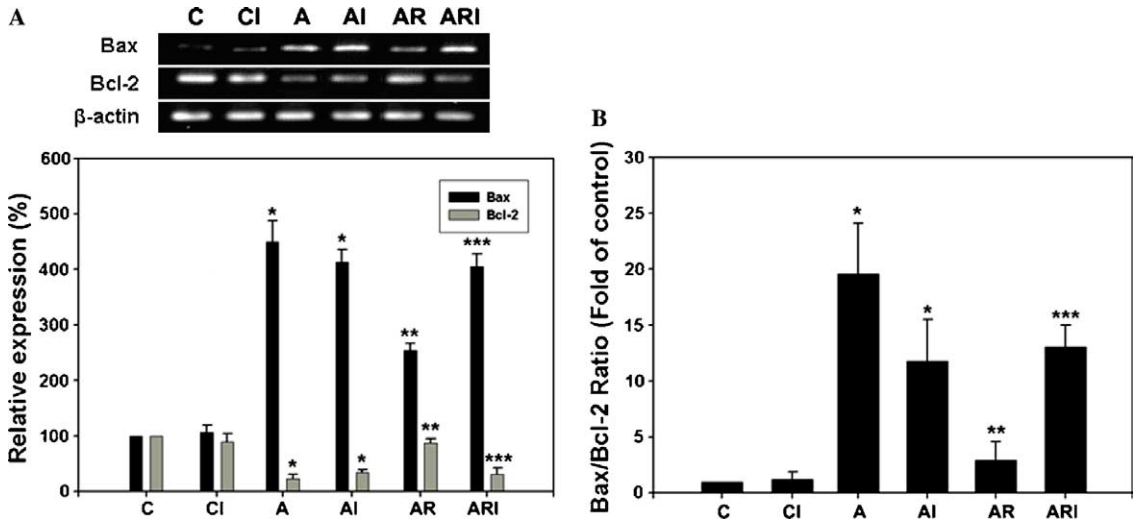

Fig. 3. Effect of rosiglitazone on the expression of Bcl-2 and Bax in SH-SY5Y cells. Cells were treated with 100 μM acetaldehyde in the absence and presence of 10 μM rosiglitazone for 48 h, and total RNA was collected for semi-quantitative RT-PCR. The levels of Bax and Bcl-2 were quantitated by densitometric analysis (A) and the Bax/Bcl-2 ratio was determined (B). Data are means ± SD ($n = 3$). *$P < 0.05$, compared with the control group. **$P < 0.05$, compared with acetaldehyde treatment group. ***$P < 0.05$, compared with rosiglitazone plus acetaldehyde treatment group. *Abbreviations:* C, control; A, acetaldehyde treatment; AR, acetaldehyde plus rosiglitazone treatment; ARI, acetaldehyde, rosiglitazone, and antioxidant enzyme inhibitor treatment; CI, anti-oxidant enzyme inhibitor treatment; AI, acetaldehyde and anti-oxidant enzyme inhibitor treatment.

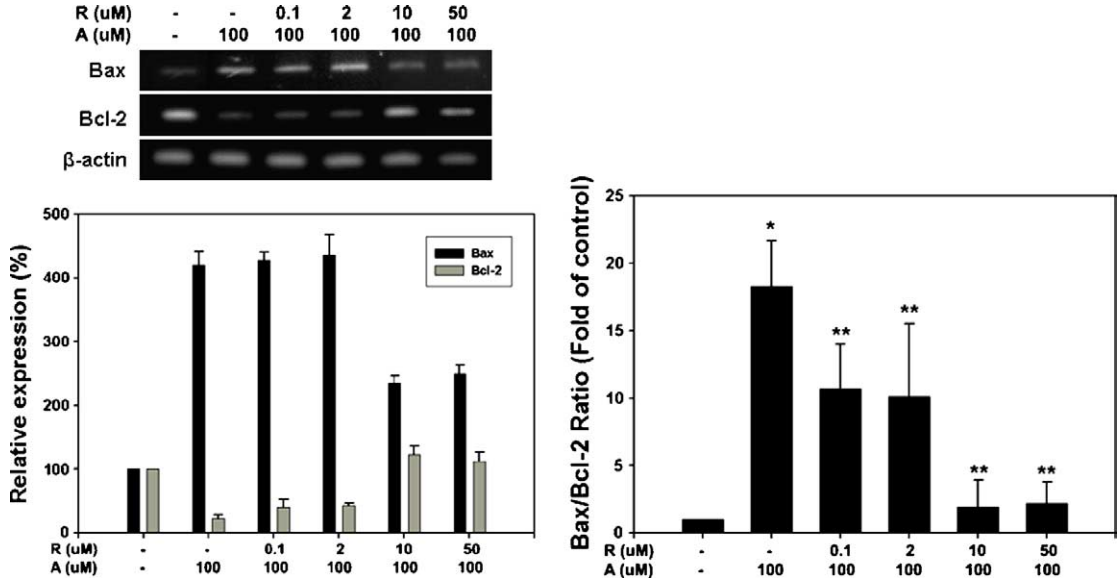

Fig. 4. Dose-dependent effect of rosiglitazone on acetaldehyde-induced apoptosis. The Bax/Bcl-2 ratio was assessed by semi-quantitative RT-PCR. Data are means ± SD ($n = 3$). *$P < 0.05$, compared with the control group. **$P < 0.05$, compared with acetaldehyde-treated group, and 0.1, 2, 10, and 50 μM rosiglitazone plus acetaldehyde-treated group. *Abbreviations:* A, acetaldehyde treatment; R, rosiglitazone treatment.

mitochondria into the cytosol [26]. Cytochrome *c* release from mitochondria was proven to play a critical role in apoptosis [27]. In the mitochondrial pathway, a variety of stimuli trigger the MPT and the release of cytochrome *c*. The opening of the MPT pores is associated with collapse of the membrane voltage [28]. Using Western blot analysis, we investigated the possible effect of rosiglitazone on acetaldehyde-induced cytochrome *c* release from mitochondria. As shown in Fig. 5, acetaldehyde treatment could significantly induce cytochrome *c* release to approximately 3-fold of that of the control group. However, the induction was inhibited in the presence of rosiglitazone. The release of cytochrome *c* was inhibited by 37% compared to that of the acetaldehyde treatment group when cells were treated with a combination of acetaldehyde and rosiglitazone. From our observation, an increase in cytochrome *c* release correlates well with an increase in the Bax/Bcl-2 ratio, as pro-apoptotic Bax is thought to be upstream of cytochrome *c* release in the mitochondria-mediated apoptosis pathway.

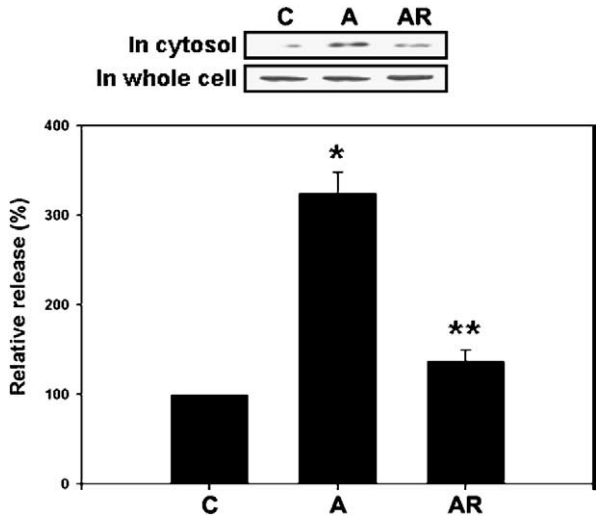

Fig. 5. Rosiglitazone blocked acetaldehyde-induced cytochrome *c* release. SH-SY5Y cells were treated with 100 μM acetaldehyde in the absence or presence of rosiglitazone for 48 h. The release of cytochrome *c* was determined by Western blot analysis. The amount of cytochrome *c* was estimated by densitometric analysis of each protein band. Data are means ± SD ($n = 4$). $^{*}P < 0.05$, compared with the control group. $^{**}P < 0.05$, compared with the acetaldehyde-treated group. *Abbreviations:* C, control; A, acetaldehyde treatment; AR, acetaldehyde plus rosiglitazone treatment.

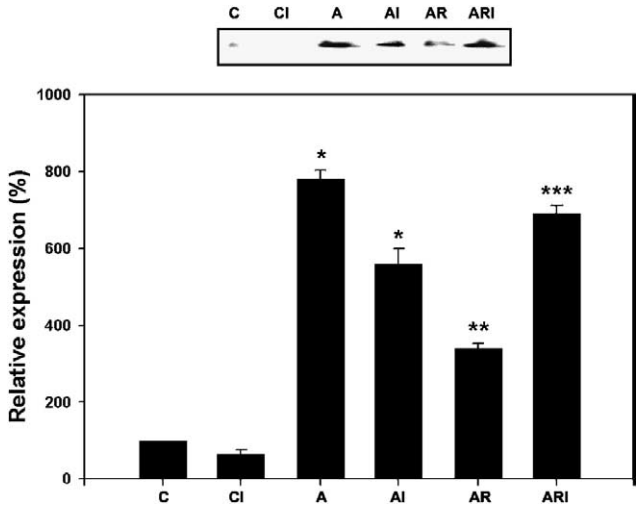

Fig. 6. Rosiglitazone inhibited acetaldehyde-induced caspase-3 expression. SH-SY5Y cells were treated with 100 μM acetaldehyde in the absence or presence of rosiglitazone fo 48 h. Caspase-3 expression was determined by Western blot analysis. The expression of caspase-3 was estimated by densitometric analysis of each protein band. Data are means ± SD ($n = 4$). $^{*}P < 0.05$, compared with the control group. $^{**}P < 0.05$, compared with acetaldehyde treatment group. $^{***}P < 0.05$, compared with acetaldehyde plus rosiglitazone treatment group. *Abbreviations:* C, control; A, acetaldehyde treatment; AR, acetaldehyde plus rosiglitazone treatment; ARI, acetaldehyde, rosiglitazone, and anti-oxidant enzyme inhibitor treatment; CI, anti-oxidant enzyme inhibitor treatment; AI, acetaldehyde and anti-oxidant enzyme inhibitor treatment.

Rosiglitazone inhibited acetaldehyde-induced caspase-3 expression

Because caspase-3 is an important apoptotic biomarker of the apoptotic [29], its expression was examined in this study. The effect of rosiglitazone on acetaldehyde-induced caspase-3 up-regulation is shown in Fig. 6. Following 48 h treatment of SH-SY5Y cells with acetaldehyde, we detected a dramatic increase in caspase-3 expression. Addition of rosiglitazone attenuated acetaldehyde-induced caspase-3 expression. To confirm the effects of rosiglitazone by inducing anti-oxidant enzyme expressions in an expression of caspase-3, we also treated cells with anti-oxidant enzyme inhibitors and they eliminated the effect of rosiglitazone as expected.

The prospects for developing an anti-apoptotic compound which modifies progression of Parkinson's disease appear favorable. An evidence from both postmortem Parkinson's disease brain tissue and cellular and animal models suggests that pathways involving p53/Bcl-2 family members/mitochondrial membrane permeabilization may represent suitable targets, although death receptor-mediated pathways may also play a role. The effects of rosiglitazone presented here resemble those of neuroprotective drugs such as green tea polyphenol, epigallocatechin-3-gallate, and rasagilin, which similarly alter Bcl-2 and Bax expression [30]. Based on these reports and our observations, we hypothesized that rosiglitazone modulates SOD, catalase, and the Bcl-2 family protein levels in response to acetaldehyde treatment and then regulates mitochondria-mediated downstream molecular events including cytochrome *c* release and caspase-3 expression. Thus, we verified that rosiglitazone decreased morphological changes of nuclei, production of ROS, impairment of anti-oxidant enzymes expression, the Bax/Bcl-2 ratio, cytochrome *c* release, caspase-3 expression, and cell death in acetaldehyde-treated SH-SY5Y cells. These findings, taken together, support the theory that rosiglitazone-mediated cytoprotection is due, in part, to inhibition of the oxidative stress resulting from the mitochondrial apoptotic pathway. Our study also shows that rosiglitazone inhibits nuclear condensation and increases cell viability despite controversial reports [31].

In summary, rosiglitazone protects SH-SY5Y cells against acetaldehyde-induced cytotoxicity. Its anti-oxidative and anti-apoptotic properties render this effective molecule potentially protective against acetaldehyde-induced cytotoxicity. Further studies of the neuroprotective mechanisms of rosiglitazone will be necessary and it will also be needing more researches about the delivery of rosiglitazone to the brain through the blood–brain barrier. This report may offer a new clinical strategy for treatment of progressive neurodegenerative diseases such as Parkinson's.

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