The PPARγ Agonist Protects Cardiomyocytes from Oxidative Stress and Apoptosis via Thioredoxin Overexpression

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Oxidative stress has been implicated in the pathogenesis of various cardiovascular diseases, including ischemic heart disease and heart failure. The peroxisome proliferator-activated receptor gamma (PPARγ) agonist improves insulin sensitivity and limits tissue inflammation and cellular apoptosis, but there are few data on the relationship between the PPARγ agonist, rosiglitazone (RSG), and the thioredoxin (TRx) system in oxidatively stressed cardiomyocytes (CMCs). Here we provide evidence that the PPARγ agonist RSG protects rat CMCs from hydrogen peroxide (H2O2)-induced apoptosis by TRx overexpression. The expression levels of pAkt/Akt, pErk/Erk, survivin, Bel-2/Bax-α, and manganese-superoxide dismutase were increased by RSG pretreatment in H2O2-injured rat CMCs. On the contrary, the expression levels of caspase-3 and p53 were decreased by RSG pretreatment. These effects of RSG were reversed by chemical inhibitors of TRx and the PPARγ antagonist. This suggests that TRx protects CMCs from H2O2-induced oxidative stress through TRx overexpression and a PPARγ-dependent mechanism.

Key words: rosiglitazone; thioredoxins; cardiomyocytes; oxidative stress; apoptosis

The thioredoxin (TRx) system regulates the levels of intracellular ROS and modulates intracellular oxidation-reduction statuses. TRX is a ubiquitous 12-kDa protein that has disulfide reducing moieties. The two cysteine residues (Cys32 and Cys35) of the active site (-Cys-Gly-Cys-) serve as reducing agents.8) Two isoforms of TRx have been discovered in mammalian cells. Each isoform has specific sites of action in the cells. TRx1 localizes within the cytosol and translocates to the nucleus during periods of oxidative stress. TRx2 resides exclusively in the mitochondria and affects mitochondrial redox homeostasis.9) TRx has diverse protective biological functions in cells. It has not only cytoprotective function against oxidative stress, but also regulates cell survival signaling pathways. Clinical and experimental results have demonstrated that inhibition of TRX1 promotes apoptosis, especially under conditions of oxidative stress.10) Recent in vitro studies have found that TRX1 action is inhibited by direct interaction between apoptosis signal-regulating kinase-1 and mitogen-activated protein (MAP), and that these reactions activate pro-apoptotic kinases such as p38 MAP kinase and c-Jun N-terminal kinase.11) This suggests that TRX1 plays a critical role in regulating the balance between cell proliferation and cell death.12)

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear receptor family which also includes hormone receptors, particularly steroid hormone receptors.13) There are three subtypes of PPARs that are encoded by three distinct genes, designated PPAR-alpha, PPAR-beta (as known as beta, PPARβ/δ), and PPAR-gamma (PPARγ).14) PPARs regulate the metabolism of glucose and lipids by modulating gene expression by direct binding to target genes or by interfering with other cellular signaling pathways. They also influence inflammatory, fibrotic, and hypertrophic responses in the body.15–17) They are expressed in several cell types in the cardiovascular system, including endothelial cells, vascular smooth muscle cells, and monocytes or macrophages in blood or tissues where they exert their anti-
inflammatory, anti-fibrotic, anti-atherogenic, and cardiovascular protective effects. RSG also protects endothelial cells from glucose-induced oxidative stress through an AMP-Activated Protein Kinase-dependent molecular pathway. In addition, PPARγ agonist reduces the fraction of neonatal rat CMCs (rCMCs) that undergo apoptosis under oxidative stress, through upregulation of Bcl-2 expression. Given this background, we hypothesized that PPARγ agonist RSG can reduce the apoptosis of rCMCs in the oxidative state created by H2O2, and that this protective action results from TRX overexpression. Here, we provide evidence that PPARγ agonist RSG protects rCMCs from H2O2-induced apoptosis by upregulating TRX expression, suggesting that RSG is cardioprotective during ischemia-reperfusion injury.

Materials and Methods

Isolation and culture of neonatal rat cardiomyocytes. Isolation and primary culturing of rCMCs was performed by modification of previously reported protocols. All animal experiments were performed with the approval of the Institutional Review Board of the Chungbuk National University Laboratory Animal Research Center. Hearts of 2 to 3-old rats (Sprague-Dawley, Orient Bio, Seongnam, Republic of Korea) were removed, and the left ventricles were cut into small pieces and minced with a surgical scissors. After filtering, the cells were collected and washed twice with cold ADS buffer (in 116 mM NaCl, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM MgSO4, pH 7.4). The rCMCs were then plated at 5 x 10^5 cells/mL and grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco BRL, Rockville, MD) supplemented with Medium 199 (M199, Sigma-Aldrich, St. Louis, MO), 10% horse serum, 5% fetal bovine serum, 2% penicillin/streptomycin, and 0.5% of glucose. After 2–3 d before exposure to experimental conditions, the cultures were transferred to serum-free media containing DMEM:M199 (4:1). The rCMCs were cultured in 5% CO2 at 37 °C for 2–3 d before exposure to experimental conditions.

Generation of oxidative stress by H2O2. The raw chemical of RSG was supplied by GlaxoSmithKline (Brentford, London, UK). The rCMCs were incubated in 1 μM of RSG for 1 h, and then exposed to 0.5 mM H2O2 for 4 h in the presence of RSG. Another two groups of rCMCs were pretreated with 10 μM of PX (12 (1-methylpropyl)-2-imidazoyl di sulfide, a specific chemical inhibitor of TRx, Toeris Bioscience, Bristol, UK), or 10 μM of GW 9662 (2-chloro-5-nitrobenzamide, a specific PPARγ antagonist, Cayman, Ann Arbor, MI) for 16 h, and then RSG was added.

Quantification of apoptosis by fluorescence-activated cell sorting. Cultured rCMCs were processed using Annexin V: Phycoerythrin (PE) Apoptosis Detection Kit I (BD Sciences) to assess cell membrane permeability and on the intercalation of double-stranded DNA. In brief, the rCMCs collected were washed twice with cold phosphate buffered saline and then resuspended in 1X binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) at a concentration of 1 x 10^5 cells/mL. The mixture was then transferred to a culture tube, and 5 μL of Annexin-V and 5 μL of 7-AAD were added. After gentle vortexing, the cell line was incubated for 10 min at room temperature in a dark environment, and was analyzed using a FACS instrument (FACS Calibur-S, BD Sciences) equipped with an argon ion laser system at excitation and emission wavelengths of 488 and 578 nm respectively.

Western blot analysis. We examined the expression pattern of the TRX system in rCMCs stressed by H2O2. Western blotting was performed on samples at baseline and at 4 h after the addition of 0.5 mM of H2O2, as follows: Harvested rCMCs were immersed in PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Seongnam, Korea) and incubated at −20 °C for 12 h. The extracts were centrifuged at 4 °C for 15 min at 12,000 rpm to remove insoluble material. The Bradford assay was used to quantify protein expression.

Protein concentrations were measured using a dye-binding assay (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA). Equal amounts of total protein (25 μg) were diluted with sample buffer containing 100 mM di thiothreitol and heated to 95 °C for 5 min, then subjected to 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a gel apparatus ( Hoefer Scientific, San Francisco, CA), and then transferred to polyvinylidene fluoride membranes. Blots were blocked with 5% skim milk in Tris-buffered Saline Tween-20 (TBST) for 2 h and then incubated at 1:2,000 in TBST, with shaking at room temperature for 1 h, with antibodies against TRX (ABFRONTIER, Seoul, Korea), manganese-superoxide dismutase (Mo-SOD), TxNip, Akt, pAkt, Erk, pErk, Bcl-2, Bax, caspase-3, p53, and survivin (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then washed with TBST, incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000–1:10,000) for 1 h, and treated with West-Zol kits (iNtRON Biotechnology) for 1 min. Chemiluminescence was detected using a LAS-3000 imaging system (Fujiﬁlm, Tokyo) for 10–300 s. Optical densities (OD) were calculated using the Multi-Gauge v.3.1 program (Fujiﬁlm, Tokyo). The experiments were repeated 5 times, and the results were expressed as means±SD of the OD, as percentages of the baseline values. Percentage changes in OD versus baseline were also calculated with respect to time.

Real-time RT-PCR analysis. Total ribonucleic acid (RNA) was extracted from the cultured cells using TRIzol Reagent (Invitrogen, San Diego, CA) following the manufacturer’s instructions. Real-time polymerase chain reaction (PCR) was performed by using a Qagen QuantiTect SYBR Green PCR Kit (Qagen, Hilden, Germany) and a 7500 Realtime PCR System (Applied Biosystems, Foster, CA). Gene-speciﬁc primers for the rat TRx gene (forward, 5’-TCC AAT GTG GTG TTC CTT GA-3’; reverse, 5’-ATA GAA CTG GAA GGT CGG CA-3’) and the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (forward, 5’-GTG AAG GTC GTG GTG AAC G-3’; reverse, 5’-GTT TCA CAC CCA TCA CAA AC-3’) were synthesized (Bioneer, Daejeon, Korea). A master mix was prepared containing 10 μL of QuantiTect SYBR Green PCR master mix, 7 μL of cDNA, 0.5 μL of forward and reverse primers (20 pm) per reaction. 18 μL of this master mix was added to 2 μL of cDNA inside LightCycler capillaries (Roche, Indianapolis, IN). The real-time PCR conditions were as follows: 20 min at 50 °C (stage 1, carryover prevention), 15 min at 95 °C (stage 2, PCR initial activation), and then 42 cycles of amplification for 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C (stage 3, PCR). We performed a melting curve analysis of the real-time PCR products. TRX mRNA expression was evaluated.

Statistical analysis. Each experiment was performed independently a minimum of 3 times in cell culture, or as otherwise stated in the ﬁgure legends. Results were expressed as mean±SD. Statistical analysis was performed by Student’s two-tailed t-test using SPSS v.16.0 (SPSS, Chicago, IL). Differences were considered statistically significant at p < 0.05.

Results

RSG increased the expression of TRX mRNA

Prior to the main experiment, we conﬁrmed that the degrees of H2O2-induced signiﬁcant oxidative stress and
apoptosis in the rCMCs occurred in a concentration-dependent manner. The apoptosis fractions were $1.08 \pm 0.03$ in $0.1 \text{mM} \text{H}_2\text{O}_2$, $1.37 \pm 0.21$ in $0.3 \text{mM} \text{H}_2\text{O}_2$, and $2.09 \pm 0.02$ in $0.5 \text{mM} \text{H}_2\text{O}_2$ over 4 h under each condition (Fig. 1, $p < 0.05$ compared to control). With 1 h of pretreatment of the rCMCs with RSG, the expression of TRx mRNA in the rCMCs increased. An increase was observed with $0.5 \mu\text{M}$ of RSG, which reached a plateau with $1 \mu\text{M}$ and higher concentrations of RSG. TRx mRNA expression in rCMCs with $0.5, 1$, or $5 \mu\text{M}$ of RSG was higher than that under control conditions of RSG (Fig. 2, $1.49 \pm 0.45$, $1.95 \pm 0.23$, $1.73 \pm 0.24$ vs. control, $p < 0.05$), TRx inhibitor PX 12 and PPARγ antagonist GW 9662 did not affect CMCs apoptosis as compared to control condition (Supplemental Fig. 1; see Biosci. Biotechnol. Biochem. Web site).

**RSG protected rCMCs from apoptosis associated with H$_2$O$_2$-induced oxidative stress**

RSG effectively reduced the fraction of rCMCs that underwent apoptosis induced by H$_2$O$_2$ without RSG treatment (Fig. 3, $1.39 \pm 0.20$ vs. $3.69 \pm 0.44$, $p < 0.05$). In addition, to exploit the mechanisms underlying the reduction in apoptosis by RSG in H$_2$O$_2$-induced oxidative stress, we examined two molecular pathways activated by TRx and PPARγ. We used PX 12 to limit the anti-oxidative activity of TRx and GW 9662 to block the effects of PPARγ agonist RSG. PX 12 and GW 9662 increased the apoptotic fraction of rCMCs in H$_2$O$_2$ (Fig. 3, PX 12: $4.07 \pm 0.33$, GW 9662: $4.45 \pm 0.21$ vs. $1.39 \pm 0.20$, $p < 0.05$). Therefore, both PX 12 and GW 9662 reversed the rescue effect of RSG on the apoptosis of rCMCs induced by H$_2$O$_2$.

**RSG promoted TRx expression in the rCMCs**

RSG significantly increased TRx expression in H$_2$O$_2$-stressed rCMCs as compared to the rCMCs in H$_2$O$_2$ without RSG. The expression of TRx in the RSG group significantly increased as compared to the H$_2$O$_2$ group ($3.61 \pm 0.68$ vs. $1.78 \pm 0.32$, $p < 0.05$). RSG-induced TRx overexpression was significantly reversed by PX 12 ($1.44 \pm 0.35$ vs. $3.61 \pm 0.68$, $p < 0.05$), and GW 9662 also inhibited the expression of TRx ($1.41 \pm 0.50$ vs. $3.61 \pm 0.68$, $p < 0.05$). The expression of TRx-interacting protein (TxNip), an intrinsic inhibitor of TRx in the TRx system, did not significantly change in either RSG group as compared to the H$_2$O$_2$ group ($1.49 \pm 0.27$ vs. $1.93 \pm 0.18$). The TRx/TxNip ratio, a crude indicator of anti-oxidative capability in the TRx system, was significantly higher in the RSG group than in the H$_2$O$_2$ group ($2.23 \pm 0.38$ vs. $1.18 \pm 0.24$, $p < 0.05$), and this ratio was also attenuated by PX 12 and by GW 9662 (Fig. 4, PX 12: $1.56 \pm 0.27$, GW 9662: $1.23 \pm 0.32$ vs. $2.23 \pm 0.38$, $p < 0.05$).

**RSG limited mitochondrial apoptosis and stimulated survival-pathway protein expression**

RSG increased the expression of survival protein Bcl-2 and decreased the expression of apoptotic protein Bax-α in the mitochondrial apoptotic pathway. On the other hand, the expression levels of pAkt/Akt, pErk/Erk, and survivin also increased in the RSG group (Figs. 5 and 6, densitogram ratio of RSG group compared to H$_2$O$_2$ group, Bcl-2/Bax-α: $2.89 \pm 0.59$ vs. $1.20 \pm 0.15$, pAkt/Akt: $1.07 \pm 0.06$ vs. $0.76 \pm 0.06$, pErk/Erk: $1.05 \pm 0.33$ vs. $0.55 \pm 0.09$, survivin: $1.82 \pm 0.29$ vs. $1.18 \pm 0.11$, $p < 0.05$). Along these lines, hallmark apoptotic proteins such as caspase-3 and p53 decreased in the RSG group as compared to the H$_2$O$_2$ control group (Figs. 5 and 6, densitogram ratio of RSG group compared to H$_2$O$_2$ group, caspase-3: $2.88 \pm 1.00$ vs. $4.94 \pm 0.83$, p53: $0.47 \pm 0.34$ vs. $3.72 \pm 0.89$, $p < 0.05$). In addition, expression of Mn-superoxide dismutase (Mn-SOD) significantly increased in the RSG group as compared to the H$_2$O$_2$ control group ($1.62 \pm 0.36$ vs. $0.56 \pm
Overexpression of Mn-SOD and survivin due to RSG treatment on H$_2$O$_2$ injured rCMCs was significantly reversed by PX 12 and by GW 9662 (Supplemental Fig. 2).

Discussion

There were two major findings in this study. First, RSG attenuated H$_2$O$_2$-induced apoptosis in rCMCs. Second, the antiapoptotic effects of RSG on oxidatively stressed rCMCs were transmitted through TRx and PPAR$\gamma$-dependent pathways.

PPARs are ligand-binding transcription factors belonging to the nuclear receptor superfamily, which also includes receptors for steroids, thyroid hormone, and retinoids. The functions of PPARs are diverse, including regulation of lipid metabolism, immune function, cell growth, cell differentiation, and apoptosis. PPARs are involved in the pathomechanisms of several diseases, including obesity, diabetes, cardiovascular disease, and malignancy. In recent years, the significance of PPARs as therapeutic targets in cardiovascular disease has been recognized. RSG is a synthetic PPAR$\gamma$ ligand that was developed as an insulin-sensitizing agent to control diabetes mellitus. Besides glucose control, RSG shows diverse therapeutic effects in the regulation of endothelial function, cellular apoptosis, tissue fibrosis, and the control of blood pressure.
Fig. 4. Expression of TRx in rCMCs after Treatment with RSG.

RGS significantly increased TRx expression in H$_2$O$_2$-induced oxidatively stressed rCMCs compared to the H$_2$O$_2$ control group (3.61 ± 0.68 vs. 1.78 ± 0.32, p < 0.05). This effect was clearly reversed by 10 µM of PX 12 (TRx inhibitor) and by 10 µM of GW 9662 (PPARγ antagonist) (1.44 ± 0.35, 1.40 ± 0.50 vs. 3.61 ± 0.68, p < 0.05). The TRx/TxNip ratio was significantly elevated by RSG as compared to the H$_2$O$_2$ control group (2.23 ± 0.38 vs. 1.18 ± 0.24, p < 0.05). This effect was also hindered by PX 12 or GW 9662 (1.56 ± 0.27, 1.23 ± 0.32 vs. 2.23 ± 0.38, p < 0.05). Data expressed as mean ± SE for four independent experiments. (*Compared to H$_2$O$_2$ control, p < 0.05, **Compared to RGS + H$_2$O$_2$, p < 0.05)

Fig. 5. Expression of Cell Survival and Apoptotic Signals in the rCMCs Treated with RSG.

RSG significantly increased the expression of Mn-SOD (1.62 ± 0.36 vs. 0.56 ± 0.21, p < 0.05), pErk/Erk (1.05 ± 0.33 vs. 0.55 ± 0.09, p < 0.05), and pAkt/Akt (1.07 ± 0.06 vs. 0.76 ± 0.06, p < 0.05) as compared to the H$_2$O$_2$ group. Data expressed as mean ± SE for three independent experiments. (*Compared to H$_2$O$_2$ control, p < 0.05)
addition, it limits cardiac hypertrophy in diet-induced hypercholesterolemic rats by the renin-angiotensin system.28) Furthermore, current evidence suggests that it has beneficial effects on CMCs under oxidative stress. For example, it decreases rCMC apoptosis in a hypoxia/re-oxygenation model by enhanced Akt re-phosphorylation and in oxidative stress through a Bcl-2-dependent pathway.19,21) Our study suggests that RSG has a cardioprotective effect in limiting the apoptosis of CMCs during the excessive oxidative stimulation that can be generated by ischemia-reperfusion.28)

TRx is a small, ubiquitous, thiol protein that is one of the important regulators of the reduction-oxidation balance and redox-controlled cell functions. The regulation of TRx is closely related to control of the cellular redox balance, promotion of cell growth, inhibition of apoptosis, and modulation of inflammation. The redox activity of TRx depends entirely on two cysteine residues, Cys32 and Cys35.33) These residues exist as a dithiol in reduced form and a disulfide in oxidized form. TRx is oxidized when it transfers reducing equivalents to disulfide groups in target proteins, and reduced back to the dithiol form by an NAD(P)H-dependent protein, TRx reductase.34) Moreover, it is markedly expressed in another anti-oxidant, Mn-SOD. Mn-SOD is an enzyme that catalyzes superoxide into H₂O₂, and it is present in almost all cells exposed to oxygen.35) In our results, Mn-SOD was significantly raised in the rCMCs of the RSG-treated groups as compared to the H₂O₂ control group. Data expressed as mean ± SE for three independent experiments. (* Compared to H₂O₂ control, p < 0.05)

In this study, we found that RSG significantly decreased H₂O₂-induced oxidative stress, and this effect was dependent on its ability to activate PPARγ. Our data also suggest that most of the antioxidative activity of RSG is determined by its ability to activate TRx. This was confirmed by the reversal of the anti-apoptotic effect of RSG in H₂O₂-induced oxidative stress treatment by PX 12, a specific chemical inhibitor of TRx (Figs. 3 and 4). The gross antioxidative effects of TRx depend mainly on the expression and balance of TRx and TxNip in the TRx system.37) TxNip is an endogenous inhibitor of TRx that binds to the redox-specific active site of TRx, thereby negatively regulating TRx activity. TxNip is believed to be strongly induced by oxidative stress.34) In this study TxNip expression in RSG groups decreased more than in the H₂O₂ group. These results suggest that RSG regulated the expression of TRx and TxNip in the TRx system, but PX 12 decreased the expression of TxNip more than the other chemical groups. This finding may have been caused by a feedback mechanism involving transient increases of oxidative stress related to intracellular TRx reduction by PX 12 (Fig. 4). TRx also induces gene expression of another anti-oxidant, Mn-SOD. Mn-SOD is an enzyme that catalyzes superoxide into H₂O₂, and it is present in almost all cells exposed to oxygen.35) In our results, Mn-SOD was significantly raised in the rCMCs of the RSG-treated groups as compared control and the H₂O₂ group (Fig. 5). These results suggest that treatment by RSG increased anti-oxidant enzymes and improved the survival of rCMCs under oxidative stress.

The PI3K/Akt pathway is crucial for the cell survival mechanism that attenuates apoptosis and regulates glycojen synthesis and glucose transport. Phosphorylation and activation of Akt limits ischemia/reperfusion...
injury and ischemic preconditioning. According to previous results, RSG affects the Akt signal[19,30–42] and increased expression of pAkt/Akt, pErk/Erk, survivin, and Bcl-2/Bax-α was observed in the RSG group but not the H2O2 group in our study. On the contrary, expression of caspase-3 and p53 was decreased in the RSG group as compared to the H2O2 group (Figs. 5 and 6). These results suggest that RSG activates Akt signaling, as well as TRX expression, under H2O2-induced oxidative stress. Activated Akt signaling improves the survival and functioning of rCMCs under oxidative stress.

Collectively, our results indicate that PPARγ agonist RSG reduced the harmful effects of oxidative stress-related apoptosis in cultured rCMCs.12) Furthermore, our experiments also indicate that H2O2-induced significant levels of apoptosis in cultured rCMCs, and that RSG effectively attenuated this process through overexpression of the TRX protein (Fig. 4). Additionally, we found that the ability of RSG to attenuate oxidative stress was mediated through activation of Akt and its effector proteins. Our results thus suggest that RSG treatment can limit H2O2-induced oxidative stress in rCMCs.

In conclusion, TRX and PPARγ are closely associated in protecting rCMCs from the apoptosis induced by oxidative stress. Targeting of TRX through the PPARγ agonist RSG might constitute a new strategy to limit secondary myocardial damage related to reperfused oxidative stress in acute myocardial infarction treatment.

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