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PPAR gamma protects cardiomyocytes against oxidative stress and apoptosis via Bcl-2 upregulation

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ABSTRACT

Cardiovascular disease (CVD) is a leading cause of death and disabilities worldwide. Peroxisome proliferator-activated receptor gamma (PPARγ) agonists possess potent anti-inflammatory actions and have recently emerged as potential therapeutic agents for CVD. Here we show that H_2O_2 induced apoptosis in cardiomyocytes with a marked down-regulation of Bcl-2 protein. The PPARγ agonist rosiglitazone protected cardiomyocytes from oxidative stress and apoptosis. Cardiomyocytes constitutively overexpressing PPARγ were resistant to oxidative stress-induced apoptosis and protected against impairment of mitochondrial function. On the contrary, cells expressing a dominant negative mutant of PPARγ were highly sensitive to oxidative stress. Cells overexpressing PPARγ exhibited an almost 3 fold increase in Bcl-2 protein content; whereas, in PPARγ dominant negative expressing cells, Bcl-2 was barely detected. Bcl-2 knockdown by siRNA in cells overexpressing PPARγ results in increased sensitivity to oxidative stress, suggesting that Bcl-2 up-regulation mediated the protective effects of PPARγ. These data suggest that, in oxidative stress-induced cardiomyocyte apoptosis, PPARγ protects cells from oxidative stress through upregulating Bcl-2 expression. These findings provide further support for the use of PPARγ agonists in ischemic cardiac disease.

1. Introduction

Cardiovascular disease (CVD) accounts for more than one third of global morbidity and remains a major cause of death in developed countries (Khor, 2001; Schnall et al., 2000; Smith, 2000). It also increasingly contributes to mortality in developing countries. CVD includes such familiar but debilitating disease entities as heart disease, atherosclerosis, stroke and hypertension. Many generalized risk factors predispose the development of CVD in individuals and these include those that are modifiable by life style changes like smoking and sedentary lifestyle, and also those that are modifiable by pharmacotherapeutic interventions like lipid disorders, diabetes and chronic inflammation. Plasma lipoprotein abnormalities and lipid metabolism derangements rank among the most firmly established and best understood risk factors for CVD. Fat- and carbohydrate-rich diets coupled with a sedentary lifestyle contribute to increasing incidences of dyslipidemia, type 2 diabetes mellitus and obesity around the world.

Peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors and belong to the nuclear receptor family, which also includes the thyroid hormone receptors and the steroid hormone receptors (Cresci, 2007; Kuusisto et al., 2007; Nikolaidis and Levine, 2004). There are three subtypes of PPARs, which are encoded by three distinct genes designated as PPAR-alpha (PPARα), PPAR-delta (also known as beta) (PPARβ/δ) and PPAR-gamma (PPARγ). PPARs regulate both glucose and lipid metabolism by modulating gene expression through direct binding to target genes or by interfering with other cellular signaling pathways. They also influence inflammatory, fibrotic and hypertrophic responses in the body. PPARs are expressed in the cardiovascular system such as endothelial cells, vascular smooth muscle cells and monocytes/macrophages where they exert their anti-inflammatory, anti-fibrotic, anti-atherogenic and cardiovasculoprotective actions.

The PPAR family (PPARα, β/δ, γ) of nuclear receptor transcription factors is an important regulator of cardiac metabolism and has been targeted for pharmacologic therapies aimed at modulating metabolism in the body (Kersten and Wahli, 2000). Drugs targeting PPARα and PPARγ are used to treat dyslipidemia and atherosclerosis (Scott et al., 2007). Rosiglitazone (RGZ) is one of the synthetic ligands of PPARγ and acts as an agonist. Treatment with RGZ enhanced the expression of down-regulated PPARγ. In a rat model, RGZ prevented development of hypertension and improved endothelial function

Abbreviations: CVD, cardiovascular disease; PPAR, peroxisome proliferator activated receptors; RGZ, rosiglitazone; ROS, reactive oxygen species; siRNA, small interfering RNA.

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(Diep et al., 2004). However, the long-term outcome of RGZ on cardiovascular mortality is currently not available and studies are needed to fully characterize the effect of RGZ on CVD events.

Oxidative stress has been implicated in the pathogenesis of a variety of CVDs including ischemic heart disease and heart failure (Kersten et al., 2000). Increased generation of reactive oxygen species (ROS) during both the ischemic and reperfusion phase is associated with dysfunction of myocardial contractility and cardiomyocyte death and is likely a major contributing factor to apoptotic cell death during cardiac ischemia/reperfusion injury and in congestive heart failure (Lopez-Neblina et al., 2005). H_2O_2 has been used to induce oxidative stress and apoptosis of cardiomyocytes *in vitro* (Khan et al., 2006; Lou et al., 2006). Mitochondrial damage, cytochrome c release and activation of caspase 3 and 9 have been implicated in apoptotic pathways (Suzuki et al., 2001). The Bcl-2 family proteins, which contain both pro-apoptotic and anti-apoptotic members, are increasingly recognized as important modulators of cardiac myocyte apoptosis. These proteins act at the mitochondrial level to regulate cytochrome c release. Bcl-2 family proteins act either as heterodimers or as homodimers and the dynamic equilibrium between such complexes appears to determine the fate of a cell (Gross et al., 1999).

Given the prominent role of PPARγ in regulating cardiac metabolism and its regulatory role in modulating inflammatory response, we hypothesized that PPARγ may be involved in cardiomyocyte apoptosis induced by H_2O_2, and that PPARγ may function through modulating the expression of Bcl-2 family members. Here we provide evidence that PPARγ protected cardiac myocytes from H_2O_2-induced apoptosis through upregulating Bcl-2 expression, suggesting that PPARγ agonist such as RGZ may be cardioprotective during ischemia/reperfusion injury.

2. Materials and methods

2.1. Cell culture

Cardiomyocytes were isolated from 1- or 2-day post-natal Sprague–Dawley rats by Percoll gradient centrifugation. These cells were cultured in minimal essential medium containing 5% calf serum in humidified air with 5% carbon dioxide at 37 °C for 24 h, and were then maintained in serum-free medium for another 24 h. The purity of cardiomyocytes was determined by immunostaining these cultured cells for the presence of cardiac cell-specific sarcomeric actin. Only cultures that contained over 95% cells positive for sarcomeric actin were employed in this study.

2.2. Cell viability assay and apoptosis studies

The viability of cardiomyocytes was studied by using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit according to the manufacturer's instruction (Promega, Madison, WI). Briefly, cells were plated at 5×10^4 cells per well of a 96-well plate and cultured for 1.5 h at 37 °C. CellTiter 96® AQueous Reagent was added and cells were incubated for 1 h before fluorescence was read at 490 nm using a Labsystems Fluoroskan Ascent plate reader (MTX Lab Systems, Vienna, VA).

Apoptosis was determined by staining cells with Annexin V-FITC and propidium iodide for flow cytometric analysis (BD, Carlsbad, CA). Briefly, cells were harvested at indicated time points after transfection, washed twice with cold phosphate-buffered saline and resuspended. The cells were incubated with Annexin V-FITC and propidium iodide for 15 min in the dark. Fluorescence was measured on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) with the quadrant settings set to allow the negative control less than 1% positivity.

2.3. Caspase-3 activity assay

Caspase-3 activity was determined by measuring the cleavage of fluorogenic caspase substrates, DEVD-AFC, as instructed by the manufacturer (Biovision, Mountain View, CA). Approximately 200 μg of total cellular protein was added to the reaction buffer containing DEVD-AFC (50 μm) and incubated for 2 h at 37 °C. The levels of AFC released were determined using FACS analysis and the specific caspase activity was normalized for total proteins of cell lysates and was expressed as fold over the baseline caspase activity of vehicle-treated control cells. Caspase-3 inhibitor, Z-DEVD-FMK (Imgenex, San Diego, CA), was used for inhibiting caspase-3 activity where indicated.

2.4. Plasmid DNA transfection and small interfering RNA (siRNA) transfection assays

Cardiomyocytes were separately transfected with plasmid DNA vectors PPARγ–pcDNA3 (PPAR+ cells), which encoded the wildtype PPARγ, or plasmid vectors dominant negative-PPARγ–pcDNA3 (PPAR- cells), which encoded the dominant negative form of mutant PPARγ. Stably transfected cardiomyocytes were selected under 0.5 mg/ml geneticin for 2 weeks until resistant colonies appeared.

The siRNAs against Bcl-2 were supplied by Ambion (Austin, TX). Non-specific control siRNA duplexes were synthesized using scrambled sequences as a negative control. For transfection experiments, logarithmically growing cardiomyocytes were plated in 60 mm dishes (1×10^6 cells per dish) in Opti-MEM (Gibco, Grand Island, NY). After 24 h, cells were transfected with the indicated siRNA duplexes at a final concentration of 67 nM by using Lipofectamine (Invitrogen, Carlsbad, CA). Cells were typically used for experiments after 48–72 h of siRNA treatment.

2.5. Western blot analysis

After treatment with plasmid or siRNA at the indicated time points, cells were harvested and whole-cell extracts for immunobloting analyses were prepared as described (Stommel and Wahl, 2004). Anti-Bcl-2 and anti-GADPH antibodies were used (Santa Cruz Biotechnology, Santa Cruz, Ca). Immunoblotting experiments were performed as previously described (Cui et al., 2009) and were repeated at least three times independently.

2.6. Quantitative real time RT-PCR assay

Total RNA was isolated with Trizol reagent according to the manufacturer's protocol (Invitrogen) and reverse transcription was carried out by using the M-MLV Reverse Transcriptase kit (Invitrogen). The PCR amplification was carried out using the QuantiTect SYBR green (Qiagen, Chatworth, CA) with the following primers for Bcl-2 (forward: 5′-TGCACCTGACGCCCTTCAC-3′ and reverse: 5′-AGACAGCCAGGAG-AAATCA-3′) and GAPDH (forward: 5′-ATGGGGAAGGTGAAGGTCG-3′ and reverse: 5′-GGGGTCATTGATGGCAACAATA-3′). The Ct (threshold cycle) value of Cx43 amplification was normalized to GAPDH control.

2.7. ROS measurements

Cells were incubated with the oxidant sensitive fluorescent probe 2, 7-dichlorofluorescein diacetate (10 μM) (Molecular Probe, Eugene, OR) for 30 min and were washed three times with phosphate-buffered saline solution. Fluorescence signal of treated cells was obtained using a 488 nm argon laser to excite 2,7-dichlorofluorescein and signals were collected at 505–530 nm.