The Antidiabetic Agent Rosiglitazone Upregulates SERCA2 and Enhances TNF- α - and LPS-Induced NF- κ B-Dependent Transcription and TNF- α -Induced IL-6 Secretion in Ventricular Myocytes

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Key Words

Rosiglitazone • TZD • Ventricular myocytes • Calcium transients • SERCA2 • NF- κ B • IL-6 • Cardioprotection

Abstract

Positive hemodynamic effects of the antidiabetic agent rosiglitazone on perfused whole hearts have recently been described, but the mechanisms regulating these effects are not well understood. This study reports the effects of rosiglitazone on calcium regulation in isolated neonatal rat ventricular myocytes by measurement of Ca²⁺ transient decay rates and SERCA2 gene expression, and shows that rosiglitazone enhances known cardioprotective signaling pathways. Myocyte treatment with 10 μ M/L rosiglitazone accelerated Ca²⁺ transient decay rates by ~30%, enhanced SERCA2 mRNA levels by ~1.5-fold and SERCA2 production by ~3-fold. Rosiglitazone treatment (1, 5, and 10 μ M/L) also led to a dose-dependent increase (~1.2-1.5-fold) in SERCA2 promoter activity. Comparable levels of cardiac SERCA overexpression have been associated with physiologically relevant and compensatory effects *in vivo*. These data link thiazolidinedione-induced

improvement in cardiac myocyte function to an upregulation of SERCA2 gene expression. Since NF- κ B-dependent pathways, including the upregulation of IL-6 secretion, were shown to protect neonatal rat ventricular myocytes from apoptosis upon TNF- α stimulation, additional experiments were designed to determine whether rosiglitazone enhances TNF- α -induced NF- κ B-dependent transcription and IL-6 secretion. Because the endotoxin stress response in ventricular myocytes involves the upregulation of TNF- α , and the activation of NF- κ B, the effects of rosiglitazone on lipopolysaccharide-induced NF- κ B-dependent transcription were also investigated. Treatment of neonatal rat ventricular myocytes with 10 μ M/L rosiglitazone enhanced TNF- α - and lipopolysaccharide-induced NF- κ B-dependent transcription by ~1.8- and ~1.4-fold respectively, and TNF- α -induced IL-6 secretion by ~1.5-fold. Rosiglitazone had no significant effects on basal levels of NF- κ B-dependent transcription and IL-6 secretion. Thus, cardioprotective effects of rosiglitazone may be partly mediated by NF- κ B.

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Introduction

Rosiglitazone belongs to the thiazolidinedione (TZD) family of insulin-sensitizing drugs used to treat Type 2 diabetes. Because cardiovascular disease is the leading cause of death among diabetic patients [1], studies have been aimed at exploring the effects of TZDs on the heart. Cardioprotective effects of TZDs have only recently been discovered [2-9], and little is known about the cellular and molecular mechanisms underlying these effects. Although rosiglitazone has been shown to have positive hemodynamic effects in isolated perfused rat hearts [6], its direct effects on contractile function and calcium handling in isolated ventricular myocytes have not been previously reported.

We hypothesized that rosiglitazone would improve the contractile function of ventricular myocytes by enhancing cytosolic calcium removal. Because the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA2) is responsible for most of the calcium resequestration into the sarcoplasmic reticulum during the contraction-relaxation cycle of cardiomyocytes [10], we proposed that rosiglitazone may improve calcium regulation at least partly through enhancing SERCA2 expression.

In addition to its positive hemodynamic effects in isolated perfused rat hearts [6], rosiglitazone has been shown to protect cardiac function, inhibit cell damage, and reduce myocardial infarct size in rat hearts following ischemia/reperfusion injury [7-9]. Although cumulative evidence has strongly linked the activation of nuclear factor- κB (NF- κB)-dependent signaling mechanisms to protective pathways in many cell types, including ventricular myocytes [11-17], no published studies have explored a possible link between TZD-induced cardioprotection and such signaling mechanisms.

Some of the adverse effects of diabetes on the heart are similar to those of the endotoxin stress response (septic shock), and both disease states are characterized by elevated levels of circulating tumor necrosis factor- α (TNF- α) [17-19]. In neonatal rat ventricular myocytes (NRVMs), lipopolysaccharide (endotoxin) leads to the induction of TNF- α and the activation of the NF- κB pathway [17]. Interestingly, rosiglitazone has been shown to inhibit lipopolysaccharide-induced release of TNF- α in NRVMs [20]. Thus, in the interest of establishing a link between TZD-induced cardioprotection and NF- κB -dependent pathways, experiments were designed to test whether treatment of NRVMs with rosiglitazone would induce NF- κB -dependent transcription in response to

TNF- α or lipopolysaccharide stimulation. Because IL-6 was shown to be a NF- κB target gene that protects NRVMs from apoptosis [13], the effects of rosiglitazone on basal and TNF- α -induced IL-6 secretion by these cells were also tested.

Materials and Methods

Neonatal Rat Ventricular Myocyte Isolation and Culture

NRVMs were isolated from one- to four-day old Harlan Sprague Dawley rats (*Rattus norvegicus albinus*), and cultured as described by Sprenkle et al. [21]. All animal procedures were in accordance with the San Diego State University Animal Subjects Committee (UASC) and NIH Animal Welfare Assurance A3728-01. Myocytes were pooled, and incubated overnight in DMEM/F-12 containing 10% FBS to allow recovery [21]. To initiate treatments with rosiglitazone, cells were incubated in ITS medium (minimal medium (DMEM/F-12, 1 mg/ml BSA) supplemented with 1X ITS (Insulin-Transferrin-Selenium, Life Technologies), 0.4X MEM Nonessential Amino Acids mixture (Sigma), 0.1X MEM Vitamins medium (Life Technologies)) in the presence or absence of agonist(s), unless stated otherwise. For all assays, control cultures were incubated with 0.1% DMSO (vehicle for rosiglitazone). For luciferase reporter assays, following recovery, cells were incubated in minimal medium for 24 hours before initiating treatments, as indicated. For ELISA, cells were incubated for 48 hours in ITS medium $\pm$ agonist(s), as indicated. All culture media were supplemented with 50 $\mu\text{g}/\text{ml}$ ampicillin and 100 $\mu\text{g}/\text{ml}$ kanamycin, and NRVMs were incubated at 37°C (5% CO_2).

Plasmids and Rosiglitazone

pSERCA2-luc encodes a luciferase reporter driven by the rat SERCA2 promoter (-556 to +60) [22] (kindly provided by A.M. Samarel, Loyola University, Chicago Stritch School of Medicine). p2xNF- κB -luc encodes a luciferase reporter driven by a minimal prolactin promoter with two nearby, upstream NF- κB consensus sites (kindly provided by M. Karin, University of California San Diego) [23]. pCH110 (Pharmacia), an SV40- β -galactosidase expression plasmid, was used as a marker for transfection efficiency and normalization in all luciferase reporter assays. Rosiglitazone was a generous gift from GlaxoSmithKline, and stock solutions were prepared in DMSO.

Calcium Transient Measurements

Calcium transient measurements were made using a dual emission photometry system (PTI, Inc.), as described by McDonough et al. [24]. Cells were loaded with indo-1/AM and monitored for fluorescence during electrical pacing of contractions (0.3 Hz, 10 ms duration, 60 V). Intracellular Ca^{2+} transient decay was evaluated by curve fitting to a single exponential equation. Since SERCA2 accounts for 92% of calcium resequestration in rat ventricular myocytes [10], the curve was not fitted to a second exponential to take into account

the slower removal of the remaining calcium by sodium-calcium exchangers and mitochondrial uniporters; this contribution was assumed to be negligible to the calcium transient signal.

Quantitative Real-Time RT-PCR

Total RNA was extracted using RNeasy 96 (Qiagen). The final RT-PCR reaction mixes contained 300 nmol/L each of forward and reverse primers and 100 nmol/L of fluorescent probes (Integrated DNA Technologies, Inc.). The following sequences were used in the RT-PCR reactions for rat SERCA2:

Forward Primer:

5'-GAGATCACAGCTATGACTGGTGATG-3'

Reverse Primer: 5'-CCCATTTCGACTTCTTCA-3'

Probe: 5'-/56-FAM/TGTGAACGACGCGCCCGC/
36-TAMRA/-3'

SERCA2 mRNA was quantified through the dual-labeled fluorogenic probe method for real-time RT-PCR (ABI PRISM 7000, Applied Biosystems). Results were normalized to total RNA quantified by Ribogreen RNA Quantitation Reagent (Molecular Probes).

Transfection by Electroporation

Immediately following the final dissociation step, cells were pooled by centrifugation, and 6×10^6 (for pSERCA2-luc) or 9×10^6 (for p2xNF- κ B-luc) cells were resuspended in 300 μ L of minimal medium per electroporation cuvette, and combined with the appropriate plasmids: 30 μ g of promoter/luciferase reporter plasmid and 12 μ g of pCH110 normalization plasmid. Cells were electroporated at 500 V, 25 μ F and 100 Ω in 0.2 cm gap cuvettes (Bio-Rad Laboratories) using a Gene Pulser II (Bio-Rad Laboratories) [21]. The electroporated cells were then divided evenly between three fibronectin-coated 24 mm wells in 12-well tissue culture plates, and incubated in DMEM/F-12 containing 10% FBS overnight to allow recovery. A subsequent 24-hour incubation in minimal medium was followed by the appropriate treatments for 48 hours.

Luciferase and β -Galactosidase Assays

Luciferase activity in cell lysates was quantified by measuring light emission for 10 seconds using a BioOrbit 1251 Luminometer, and was normalized to β -galactosidase activity to calculate relative luciferase activities [21].

ELISA

Following treatments, IL-6 secretion was measured using a kit according to the manufacturer's protocol (IL-6 DuoSet® ELISA Development Kit, R&D Systems).

Western Blot Analysis of SERCA2 and Sarcomeric Actin

Myocytes were cultured in DMEM/F-12 containing 2% FBS plus either 0.1% DMSO or 10 μ mol/L rosiglitazone for 72 hours. Cellular SERCA2 and alpha-actin levels were determined via the method of Suarez et al. [25] modified for cultured cells. In brief, the cells were rinsed with chilled PBS, and harvested in lysis buffer (50 mM Tris pH 7.4, 20 mM NaCl, 100 μ M EDTA, 1% Triton X-100, 13 mM deoxycholic acid, 0.04% beta

mercaptoethanol (v/v)). The samples were subjected to 3 freeze/thaw cycles, and particulates were pelleted by microcentrifugation. Protein concentrations were determined using the BioRad Bradford kit, and 20 μ g of soluble protein per sample were analyzed by SDS-PAGE using a 4-12% bis-Tris-HCl-buffered gel (Invitrogen, Carlsbad, CA). Samples were electrophoresed, transferred to nitrocellulose membranes, and probed with a rabbit polyclonal antibody raised against the cytoplasmic segment of the SERCA2 protein [26], followed by a donkey anti-rabbit antibody conjugated to horseradish peroxidase (Amersham). Blots were then re-probed utilizing a monoclonal antibody against sarcomeric alpha-actin (Sigma), and an HRP-conjugated goat anti-mouse IgG (Amersham) as the secondary antibody. Detection was accomplished with ECL reagents (Western Lighting) from Perkin Elmer.

Immunocytofluorescence Analysis of p65 (NF- κ B)

Myocytes were cultured for 72 hours in the presence of either 0.1% DMSO, 10 μ mol/L rosiglitazone, 2 ng/ml TNF- α , or the combination of rosiglitazone and TNF- α . The cells were then fixed in 4% paraformaldehyde, permeabilized with 1% Triton X-100 and stained for the p65 subunit of NF- κ B using a rabbit polyclonal antibody against p65 (Santa Cruz), followed by a goat anti-rabbit antibody conjugated to FITC (Jackson). During incubations with the secondary antibody, DAPI (Sigma) was included to visualize the nuclei, and a Texas-Red-phalloidin conjugate (Molecular Probes) was also included to stain the myofibrillar apparatus, allowing identification of the cardiac myocytes (vs. fibroblasts). Images were acquired by a digital video camera interfaced to an Olympus epifluorescence microscope with a 60X oil-immersion objective. Images were converted to gray scale and optimized for brightness and fluorescence in Adobe Photoshop.

Statistical Analysis

All experiments were performed at least three times ($n=20$ for calcium transient experiments; $n=3$ for all other experiments). Data are expressed as means, $\pm$ SEM. Statistical significance was analyzed through ANOVA, followed by Tukey's *post hoc* test, using SPSS for Windows (version 10.1) statistical package (SPSS, Inc.). Results were considered significant if $p < 0.05$.

Results

To investigate the effects of rosiglitazone on calcium handling in NRVMs, intracellular Ca^{2+} transients were measured in cells that were incubated in 1 or 10 μ mol/L rosiglitazone, or 0.1% DMSO (vehicle for rosiglitazone) for 48 hours. Myocytes treated with up to 10 μ mol/L rosiglitazone displayed basal (diastolic) and systolic indo-1 ratios that were similar to controls but displayed significantly faster Ca^{2+} transient decay kinetics (Figure 1A). To display the decay kinetics more clearly, the indo-

Table 1. Effects of rosiglitazone on intracellular Ca^{2+} transients. Results from Figure 1 are summarized. Each value represents the mean for 20 samples, $\pm$ SEM. * $p < 0.05$ vs. Untreated and DMSO. † $p < 0.01$ vs. Untreated and DMSO.

	T_{peak} (sec)	Decay Rate (sec^{-1})	T_{decay} (sec)
Untreated	0.351 ± 0.02	-1.142 ± 0.07	0.662 ± 0.05
DMSO	0.308 ± 0.03	-1.072 ± 0.08	0.682 ± 0.05
1 μM Rosi	0.309 ± 0.01	-1.392 ± 0.09	0.542 ± 0.04
10 μM Rosi	0.334 ± 0.02	$-1.534 \pm 0.10^*$	$0.481 \pm 0.02^\dagger$

1 transients from cells treated at each condition were aligned, averaged, and normalized to their respective diastolic (0%) and systolic (100%) values (Figure 1B). Cells treated with 10 $\mu\text{mol/L}$ rosiglitazone displayed significantly faster Ca^{2+} transient decay rates and faster times to 50% decay (T_{decay}) than those of untreated and DMSO-treated myocytes (Table 1); there was also a trend for 1 $\mu\text{mol/L}$ rosiglitazone to accelerate decay of the Ca^{2+} transients, but this did not reach statistical significance vs. control cells. No significant difference was observed in times to peak (T_{peak}) between the treatment groups.

The acceleration of intracellular Ca^{2+} transient decay rates by rosiglitazone suggests that enhanced SERCA2 gene expression may be involved. Thus, NRVMs were treated with 1 or 10 $\mu\text{mol/L}$ rosiglitazone, or 0.1% DMSO for 48 hours, and SERCA2 mRNA levels were assessed through quantitative real-time RT-PCR. Treatment with 10 $\mu\text{mol/L}$ rosiglitazone led to a ~ 1.5 -fold increase over control values in cellular SERCA2 mRNA levels; 1 $\mu\text{mol/L}$ rosiglitazone and DMSO had no significant effects (Figure 2A). To determine if rosiglitazone could enhance SERCA2 gene expression at the transcriptional level, NRVMs were transfected with a rat SERCA2 promoter/luciferase reporter construct (pSERCA2-luc) [22] and subsequently treated with 1, 5, or 10 $\mu\text{mol/L}$ rosiglitazone, or 0.1% DMSO for 48 hours before measuring luciferase activity in cell lysates. Treatment with rosiglitazone led to a ~ 1.2 - 1.5 -fold dose-dependent increase in SERCA2 promoter activity as compared to control values (Figure 2B); DMSO had no significant effect (data not shown).

The up-regulation of SERCA2 gene expression by rosiglitazone was further confirmed via western blotting analysis. NRVMs exposed to 10 $\mu\text{mol/L}$ rosiglitazone for 72 hours expressed approximately 3-fold more SERCA2 protein than control cultures exposed to vehicle (Figure 3). Rosiglitazone had no effect on the expression of sarcomeric actin.

To examine the effects of rosiglitazone on basal and TNF- α - or LPS-induced NF- κB -dependent transcription, NRVMs were transfected with p2xNF- κB -luc, then incubated in the absence or presence of TNF- α (2 or

Fig. 1. Rosiglitazone accelerates Ca^{2+} transient decay in NRVMs. A, Indo-1 ratio traces obtained from untreated myocytes, or myocytes exposed to 10 μM rosiglitazone. Myocytes were paced for 30 sec (at 0.3 Hz), to obtain 10 contractile cycles, which were aligned and averaged to obtain an “average” transient for each cell; the traces from all cells were then further aligned and averaged ($n = 20$ cells per condition) for display purposes. B, Indo-1 traces normalized to their respective diastolic and systolic values to compare transient kinetics. Data are for untreated myocytes, for myocytes exposed to 0.1% DMSO (vehicle), and for myocytes exposed to either 1 μM or 10 μM rosiglitazone. Data are for $n = 11$ to 20 myocytes per condition and are representative of 3 independent experiments.

Fig. 2. Rosiglitazone increases SERCA2 mRNA levels and enhances SERCA2 promoter activity. A, SERCA2 mRNA levels as assessed through quantitative real-time RT-PCR. Each value represents the mean of 6 samples, $\pm$ SEM. * p <0.001 vs. Untreated, DMSO, and 1 μ M Rosi. B, SERCA2 promoter activity

as measured by luciferase reporter assays. Each value represents the mean of 6 samples, $\pm$ SEM. * p <0.01 vs. Untreated. † p <0.001 vs. Untreated. ‡ p <0.01 when compared to each other, as indicated. § p <0.05 when compared to each other, as indicated.

10 ng/ml) or LPS (10 μ g/ml) $\pm$ rosiglitazone (1 or 10 μ mol/L). Rosiglitazone had no significant effect on basal luciferase activity in cells that were not treated with TNF- α or LPS (Figure 4). However, in cells that were treated with TNF- α or LPS, co-treatment with 10 μ mol/L rosiglitazone enhanced luciferase activity by 1.7-1.8-fold (Figure 4A) and 1.4-fold (Figure 4B), respectively, over levels in cells that were not treated with rosiglitazone. One μ mol/L rosiglitazone and 0.1% DMSO had no significant effects (Figure 4).

As an additional test to determine the relative effects of rosiglitazone and TNF- α on the NF- κ B signaling pathway, myocytes were exposed to these agents, either each alone or in combination, and then processed for immunocytofluorescence staining for the p65 NF- κ B subunit (Figure 5A). In control myocytes (exposed to DMSO), p65 NF- κ B staining was cytoplasmic, punctate, and perinuclear, leading to a "speckled" pattern surrounding the nucleus. Importantly, the nuclei were generally devoid of p65 staining in the control cells. Myocytes treated with 10 μ mol/L rosiglitazone exhibited p65 staining that was similar to that of controls. For myocytes exposed to 2 ng/ml TNF- α , p65 staining was primarily perinuclear, but some nuclear fluorescence was sometimes observed. On the other hand, for myocytes exposed to the combination of TNF- α and rosiglitazone,

Fig. 3. Effect of rosiglitazone on SERCA2 protein expression in cultured cardiac myocytes. Neonatal rat ventricular myocytes were cultured for 72 hours in the presence of either 10 μ M rosiglitazone (Rosi) or vehicle (DMSO), harvested, and assayed for SERCA2 and sarcoplasmic actin. Independent samples were tested in duplicate.

bright nuclear staining was observed, indicating clear nuclear localization of NF- κ B p65. These results are generally in agreement with the results obtained with the NF- κ B-sensitive luciferase reporter construct and are consistent with the hypothesis that rosiglitazone potentiates the activation of NF- κ B by TNF- α .

Fig. 4. Enhancement of TNF- α - and LPS-induced NF- κ B-dependent transcription by rosiglitazone. Neonatal myocytes were transfected with p2xNF- κ B-luc and pCH110. A, cells were incubated in ITS medium $\pm$ TNF- α (2 or 10 ng/ml) and $\pm$ rosiglitazone (1 or 10 μ M) for 48 hours. Each value represents the mean of 3 samples, $\pm$ SEM. * p <0.001 vs. respective TNF- α only treatment. B, cells were incubated in ITS medium $\pm$ 10 μ g/

ml LPS $\pm$ 10 μ M rosiglitazone (Rosi) or 0.1% DMSO, for 48 hours. Each value represents the mean of 3 samples, $\pm$ SEM. * p <0.001 vs. Untreated, DMSO, and Rosi. ** p <0.001 vs. LPS and LPS+DMSO. For A and B, luciferase activity was normalized to β -galactosidase activity, and results are representative of three different experiments.

Fig. 5. Rosiglitazone enhances TNF- α -induced p65 translocation. Neonatal cardiac myocytes were exposed to 0.1% DMSO (Con), 10 μ M rosiglitazone, 2 ng/ml TNF- α , or the combination of rosiglitazone and TNF- α for 72 hours, then

fixed and stained for the NF- κ B p65 subunit. Myocytes (as opposed to nonmyocytes) were positively identified by myofibrillar staining (images not shown). A, p65 staining. B, DAPI staining to identify the nuclei (same fields as in A).

Fig. 6. Rosiglitazone enhances TNF- α -induced IL-6 secretion. NRVMs were incubated $\pm$ TNF- α (2, 5, or 10 ng/ml) $\pm$ 1 or 10 μ M rosiglitazone for 48 hours in ITS medium. The culture media were analyzed for IL-6 secretion by ELISA. Results from 3 similar experiments were combined, and each value represents the mean of 9 samples, $\pm$ SEM. * p <0.05 vs. 0 μ M Rosi of respective group. p <0.001 for 2, 5 and 10 ng/ml TNF- α groups vs. 0 ng/ml TNF- α group.

To test the effects of rosiglitazone on IL-6 secretion by NRVMs, cells were incubated $\pm$ TNF- α (2, 5, or 10 ng/ml) $\pm$ rosiglitazone (1 or 10 μ M/L) for 48 hours before IL-6 secretion was assessed by ELISA. Rosiglitazone had no significant effect on IL-6 secretion in the absence of TNF- α . In agreement with previous reports [13], all three doses of TNF- α tested increased IL-6 secretion by approximately 2- to 3-fold over that of untreated cells (Figure 6). Co-treatment with 10 μ M/L rosiglitazone enhanced IL-6 secretion by approximately 1.5-fold over that of cells treated with TNF- α alone (Figure 6). DMSO had no significant effect (data not shown). These results further suggest that rosiglitazone may play a role in enhancing NF- κ B-mediated cardioprotective pathways.

Discussion

The data presented in this study show that rosiglitazone improves calcium handling in ventricular myocytes and indicate that this improvement may be mediated, at least in part, by an up-regulation of SERCA2 gene expression, as assessed at both the SERCA mRNA and protein levels. This study is the first to link TZD-induced improvement in the physiological function of ventricular myocytes to a concomitant upregulation of SERCA2. Previous studies in which cardiac SERCA2 protein was overexpressed through adenoviral-mediated or transgenic approaches reported approximately 1.5- to

2-fold overexpression over control levels [27, 28]. Furthermore, a 20% increase in SERCA levels in transgenic animals expressing a SERCA2 transgene in the heart resulted in accelerated Ca^{2+} resequestration and improved contractile function, and had a compensatory effect by preventing contractile dysfunction caused by hypothyroidism, aortic stenosis-induced hypertrophy, and diabetes [28]. Thus, the degree of rosiglitazone-induced upregulation of SERCA2 is comparable to overexpression levels reported in other studies, and is of a physiologically relevant magnitude shown to be sufficient in providing a compensatory enhancement of function in vivo [28].

Further support for a positive effect of rosiglitazone on the contractile function of cardiomyocytes comes from studies in our laboratory, in which pre-treatment of isolated ventricular myocytes with rosiglitazone prevented lipopolysaccharide-induced contractile depression (data not shown). In light of such studies, our finding that rosiglitazone can enhance calcium resequestration and SERCA2 gene expression in cardiomyocytes provides evidence that rosiglitazone could play a potential role in alleviating the adverse effects of diabetes on the heart; these effects include ventricular dysfunction and diabetic cardiomyopathy, often leading to congestive heart failure [1]. Complicating matters, however, are recent reports that rosiglitazone and pioglitazone have been shown to increase fluid retention, which, in certain cases, has led to congestive heart failure [29, 30]. Thus, much additional research is required in order to take advantage of the potential cardioprotective effects of rosiglitazone.

The mechanisms by which rosiglitazone and other TZDs might confer cardioprotection are not yet understood. Rosiglitazone is the highest affinity known ligand for the peroxisome proliferator-activated receptor gamma (PPAR γ), a member of the subfamily of nuclear receptors [31]. Because PPAR γ is expressed in low levels in the heart [9, 32, 33], and because it has been observed that not all TZDs have positive effects on cardiac hemodynamics [6], it is reasonable to consider that the observed effects of rosiglitazone may be, at least in part, PPAR γ -independent. This notion is supported by the apparent lack of a PPAR γ response element (PPRE) consensus site (AGGTCA γ AGGTCA) [31] in the SERCA2 promoter sequence used [22]. However, the lack of a PPRE in the SERCA2 promoter cannot yet be confirmed with absolute certainty because certain PPREs described in the literature vary somewhat from the consensus PPRE sequence [34]. SERCA2 promoter deletion mutants will be used in future studies to identify possible rosiglitazone/PPAR γ -response elements.

Because several studies have identified NF- κ B as a mediator of protective pathways in the myocardium [11-17], it was reasonable to speculate that rosiglitazone and other TZDs might confer cardioprotective effects through up-regulating NF- κ B-dependent pathways. This study has demonstrated that rosiglitazone enhances TNF- α - and LPS-induced NF- κ B-dependent transcription and TNF- α -induced IL-6 secretion in NRVMs, and suggests a link between rosiglitazone-induced effects and NF- κ B-dependent pathways. Consistent with previously reported cardioprotective effects of TZDs [6-9] is our finding that rosiglitazone enhances TNF- α -induced IL-6 secretion. Cardiac-derived cytokines are believed to serve protective functions in the heart in response to certain stresses [13, 15], and treatment of cultured NRVMs with TNF- α or IL-6 was shown to confer protection from apoptosis [13]. Moreover, NF- κ B has been implicated in mediating these protective effects [13, 15].

In our experiments, TNF- α alone increased NF- κ B-dependent gene expression by approximately 40% (Figure 4) but this was not statistically different from controls in this multiple comparison trial. Other investigators [35] have observed a greater effect of TNF- α on NF- κ B-dependent gene expression in NRVMs (approximately 5-fold). Procedural differences might account for this discrepancy. For example, Condorelli et al. [35] employed a higher concentration of TNF- α than used here, and a

reporter gene containing a 5X-concatamer of the NF- κ B binding site (as opposed to the 2X concatamer employed in the present study). In our hands, the nuclear staining of the p65 NF- κ B subunit was most prevalent in the presence of TNF- α and rosiglitazone together, providing further evidence that rosiglitazone enhances the ability of TNF- α to activate NF- κ B.

The signaling pathways through which rosiglitazone may enhance the observed NF- κ B-dependent effects remain to be elucidated. Because p38 MAPK- and NF- κ B-dependent pathways collaborate to induce IL-6 release upon TNF- α stimulation of NRVMs [13], it is conceivable that p38 may play a role in the rosiglitazone-induced effects observed in this study. The findings that rosiglitazone confers cardioprotection from ischemia/reperfusion injury [7-9], and that during cardiac ischemia p38 activates NF- κ B [36] and IL-6 is induced [37], further support a possible role for p38 in rosiglitazone-induced cardioprotection. The observation that the rosiglitazone-conferred protection from ischemia-reperfusion injury was accompanied by the inhibition of the Jun NH $_2$ -terminal kinase (JNK)/activating protein 1 (AP-1) signaling pathways [8] suggests a role for these pathways, as well, in regulating the effects of rosiglitazone on the heart. The MAP kinases ERK2 and JNK can phosphorylate a MAP kinase consensus site in the amino terminal A/B domain of PPAR γ . This phosphorylation was shown to inhibit PPAR γ 's ligand-dependent and ligand-independent transactivation functions [38]. Thus, studies utilizing known inhibitors of the MAP kinases, as well as antisense oligonucleotides targeted at PPAR γ , may be useful in determining the role of PPAR γ in the observed rosiglitazone-induced effects, within the context of MAP kinase signaling pathways. Our future efforts will be aimed at such studies.

The ability of rosiglitazone to enhance TNF- α - and LPS-induced NF- κ B-dependent mechanisms, including the enhancement of IL-6 secretion, may prove to be beneficial in protecting ventricular myocytes from apoptosis in both diabetes and septic shock by helping to moderate the adverse effects of high circulating levels of TNF- α . Since the leading cause of death among patients with Type 2 diabetes is cardiovascular disease [1], identifying cardioprotective effects of rosiglitazone and other TZDs, and the mechanisms through which they are exerted may allow for modification of therapies in order to take advantage of these beneficial effects.

Abbreviations

BSA (bovine serum albumin); DMEM (Dulbecco's Modified Eagle's Medium); DMSO (dimethyl sulfoxide); ELISA (enzyme-linked immunosorbent assay); FBS (fetal bovine serum); IL-6 (interleukin-6); ITS (insulin-transferrin-selenium); JNK (Jun NH₂-terminal kinase); MAPK (mitogen-activated protein kinase); NF- κ B (nuclear factor-kappa B); NRVM (neonatal rat ventricular myocyte); PBS (phosphate-buffered saline); PPAR γ (peroxisome proliferator-activated receptor gamma); TNF- α (tumor necrosis factor-alpha); TZD (thiazolidinedione); μ M (μ mole/L).

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