Rosiglitazone Attenuates Endothelial Progenitor Cell Apoptosis Induced by TNF-α via ERK/MAPK and NF-κB Signal Pathways

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Abstract. The discovery of endothelial progenitor cells (EPCs) provides us with a novel treatment strategy for complications requiring therapeutic revascularization and vascular repair. However, the feasibility of this strategy may be limited due to reduced number and impaired function of EPCs under stimulation of TNF-α. The present study was designed to investigate the effect of rosiglitazone on EPC apoptosis induced by TNF-α and the molecular mechanisms involved. Rosiglitazone attenuated apoptosis of TNF-α-stimulated EPCs in a dose-dependent manner. Rosiglitazone decreased caspase-3 activity and cleavages of caspase-3, caspase-7, and parp. Rosiglitazone also moderated the dissipation of mitochondrial membrane potential caused by TNF-α treatment and reduced the expression of bax and the release of cytochrome c. Furthermore, rosiglitazone inhibited phosphorylations of ERK/MAPK and NF-κB signal molecules. Both ERK and NF-κB inhibitors decreased TNF-α–induced apoptosis of EPCs, as well as the expression of cleaved caspase-3 and parp. These results suggest that rosiglitazone may mediate the inhibitory effect on EPCs apoptosis under TNF-α stimulation through suppression of ERK/MAPK and NF-κB signal pathways.

Keywords: endothelial progenitor cell, TNF-α, rosiglitazone, apoptosis, molecular mechanism

Introduction

Endothelial progenitor cells (EPCs) play an essential role in cardiovascular protection. EPCs are proved to be involved in the maintenance of endothelium homeostasis and the process of new vessel formation. Evidence suggests that EPCs contribute to about 25% of new vessels (1), and transplantation of EPCs into patients has been indicated to induce blood flow recovery in ischemic limbs (2). However, the number and function of EPCs are impaired in patients with diabetes (3) and cardiovascular diseases (4). In addition, allotransplantation of EPCs from healthy donors bears the problem of immunologic incompatibilities. Therefore rehabilitation of autologous EPC amount and function may represent a promising therapeutic strategy in future clinical application of EPC-based treatment.

TNF-α is a contributing risk factor in atherosclerosis and common metabolic disturbances including insulin resistance and dyslipidemia (5). Increased plasma levels of TNF-α in diabetic patients not only impair the function of endothelial cells (6, 7) but also enhance aging and apoptosis of EPCs (8, 9). However, the molecular mechanisms by which TNF-α impaired EPCs are still under investigation. Rosiglitazone (RSG), a peroxisome proliferator-activated receptors (PPARs) agonist, has been used in the treatment of type 2 diabetes for a decade and its actions have been convincingly shown to reach far beyond its use as an insulin sensitizers. Pretreatment with rosiglitazone increases survival of endothelial cells and myocardial cells (10, 11). In recent years, the effects of rosiglitazone on EPCs have been also studied and it was reported to promote EPC number and migratory activity (12). Nevertheless, no research has surveyed whether rosiglitazone could attenuate dysfunction of EPCs induced by TNF-α. We hypothesize that the detrimental effects of TNF-α on EPCs could be reversed by rosiglitazone.
We performed this study to determine whether rosiglitazone could attenuate TNF-α–induced apoptosis of EPCs and the corresponding signal transduction pathways involved in this process. Our findings provide evidence that rosiglitazone may attenuate TNF-α–induced apoptosis of EPCs via apoptosis-related proteins such as caspase-3/7, bax, and cytochrome c by inhibiting ERK/MAPK and NF-κB phosphorylations.

Materials and Methods

Materials
Peripheral blood mononuclear cells were obtained from healthy volunteers. The informed consents were obtained from all volunteers. Endothelial Cell Growth Medium-2 (EGM-2) was from Lonza (Walkersville, MD, USA). Lympholyte-H cell separation media was from Cedarlane (Burlington, VT, USA). Fetal bovine serum (FBS) was purchased from Gibco (Los Angeles, CA, USA). TNF-α was purchased from Peprotech (Princeton, NJ, USA). Rosiglitazone was from Cayman (Ann Arbor, MI, USA). Dimethyl sulfoxide (DMSO) was obtained from Sigma Chemical (St. Louis, MO, USA). FITC Annexin V Apoptosis Detection kit was from BD Biosciences (San Diego, CA, USA). SP600125, SB203580, and Bay11-7082 were purchased from Calbiochem (Darmstadt, Germany). Anti-IκB, anti-phospho-IκB, anti-ERK1/2, anti-phospho-ERK1/2, anti- phospho-p38, anti-phospho-p38, anti-caspase-3, anti-casped caspase-7, anti-cleaved caspase-7, anti-parp, anti-p38, anti-phospho-p38, anti-JNK, anti-phospho-JNK, anti-ERK1/2, anti-phospho-ERK1/2, anti-NF-κB, anti-phospho-NF-κB, anti-NF-κB inhibitor α (IkB-α), anti-phospho-IκB-α, and anti-histone for western blotting were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-cytochrome c antibody was purchased from Merek Millipore (Billerica, MA, USA). Anti-bax antibody was acquired from Santa Cruz (Santa Cruz, CA, USA). HRP-conjugated monoclonal mouse anti-GAPDH antibody was purchased from Kangchen (Shanghai, China).

Isolation and cultivation of EPCs
EPCs were isolated, cultured, and characterized according to previously described techniques (13–15). Briefly, peripheral blood mononuclear cells (PBMNCs) were isolated from healthy volunteers by Ficoll density gradient centrifugation and then cultured on human FN-coated dishes in EGM-2 containing 10% FBS, vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), insulin-like growth factor (IGF), and ascorbic acid. After 3 days in culture, non-adherent cells were removed by washing with phosphate-buffered saline (PBS), and adherent cells were maintained in fresh medium for another 4 days. In addition, cells prepared for flow cytometry were seeded in a 25-cm² flask (Corning, Corning, NY, USA), and those for western blot analysis were seeded in a 6-well plate.

Apoptosis detection with FITC-conjugated Annexin V
After washing with FBS-free EGM-2 and 12-h synchronization, cells were further cultured in the absence or presence of TNF-α, rosiglitazone, and various signal inhibitors for another 24 h. Cells were then washed, resuspended in the staining buffer, and stained with FITC-conjugated Annexin V and propodium iodide. The apoptotic rates of EPCs after treatment were analyzed by fluorescence-activated cell sorting (FACS).

Mitochondrial membrane potentials assay
JC-1 probe (Beyotime, Jiangsu, China) was employed to measure variation in mitochondrial transmembrane potential of apoptotic EPCs. Briefly, Cells cultured in six-well plates after the indicated treatments were resuspended and incubated with an equal volume of JC-1 staining solution (5 μg/ml) at 37°C for 20 min and washed twice with 1× incubation buffer. Mitochondrial membrane potentials were monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using a Flow Cytometer under 488-nm laser excitation. Mitochondrial depolarization was indicated by an increase in the green/red fluorescence intensity ratio.

Caspase-3 activity assay
Activity of caspase-3 was measured using a colorimetric kit (Keygen, Nanjing, China). In brief, cells were washed with cold PBS, resuspended in lysis buffer, and left on ice for 15 min. The lysate was centrifuged at 16,000 × g at 4°C for 15 min. The protein concentration was determined by the Bradford method. Each sample was adjusted to 200 μg of protein and then incubated for 1 h at 37°C with 10 μl caspase-3 substrate (Ac-DEVDpNA) (2 mM). The absorbance was determined at 405 nm and the activity of caspase-3 was assessed by calculating the ratio of the OD 405 nm of the drug-treated cells to the untreated cells.

Preparation of whole cell lysate for western blot analysis
Following treatment, EPCs were washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA) supplemented with protease inhibitor cocktail (Mercck, Germany), 1 mM PMSF, 1 mM Na3VO4, and 10 mM NaF.
The protein concentration was determined by the Bradford method. After being denatured at 95°C for 5 min, 15 – 30 μg of the protein was loaded in each lane and subjected to 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% skim milk and then incubated overnight with primary antibody in the appropriate dilution, before incubation for 1 h with a secondary antibody conjugated to horseradish peroxidase (1:10,000 dilution). After reaction with enhanced chemiluminescence reagent (Amersham, Haemek, Isreal), the images were captured on an image reader LAS-4000 system (Fujifilm, Tokyo).

Preparation of plasma extracts for cytochrome c western blotting

The cells were collected and lysed with lysis buffer (20 mM HEPES/NaOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail, 1 mM PMSF) for 15 min on ice. The samples were homogenized by repeated passages through a 27-gauge needle, before it was centrifuged at 12,000 rpm for 15 min at 4°C.

Preparation of nuclear extracts for p65 western blotting

The cells were collected and lysed with lysis buffer (20 mM HEPES/NaOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail, 1 mM PMSF) for 15 min on ice. The samples were centrifuged at 14,000 × g at 4°C. The samples were centrifuged at 14,000 × g at 4°C. The pellet was resuspended in nuclear protein extraction agent supplemented with 1 mM PMSF. Then the samples were centrifuged for 5 min at 14,000 × g at 4°C. The protein concentration was determined by the Bradford method. After being denatured at 95°C for 5 min, 15 – 30 μg of the protein was loaded in each lane and subjected to 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% skim milk and then incubated overnight with primary antibody in the appropriate dilution, before incubation for 1 h with a secondary antibody conjugated to horseradish peroxidase (1:10,000 dilution). After reaction with enhanced chemiluminescence reagent (Amersham, Haemek, Isreal), the images were captured on an image reader LAS-4000 system (Fujifilm, Tokyo).

Statistical analysis

The obtained data are expressed as means ± S.E.M. from at least three independent experiments and analyzed by the unpaired Student’s t-test for comparisons between two groups or one-way ANOVA for multiple comparisons. P values < 0.05 are considered statistically significant.

Results

Rosiglitazone dose-dependently decreased TNF-α–induced apoptosis of EPCs

Cells were pre-incubated with various concentrations of rosiglitazone (0, 1, 5, and 30 μM) for 30 min before TNF-α (10 ng/ml, 24 h) treatment. The apoptosis of EPCs was detected by flow cytometry after staining with FITC-conjugated Annexin V and propodium iodide. Rosiglitazone decreased apoptosis of EPCs in a dose-dependent manner (Fig. 1: A, B).

Rosiglitazone-mediated protection inhibited activation of caspase-3 activity and cleavage of caspase-3, caspase-7, and parp

TNF-α significantly increased the expressions of cleaved-caspase-3, cleaved-caspase-7 and cleaved parp, which were down-regulated by pre-treatment of EPCs with rosiglitazone for 30 min (Fig. 2: A – C). Moreover, rosiglitazone inhibited caspase-3 activity under the stimulation of TNF-α (Fig. 2D).

Rosiglitazone down-regulated bax and cytochrome c expression, which was consistent with changes of mitochondrial membrane potentials

We further examined the effects of rosiglitazone on the apoptosis of EPCs by a mitochondrial membrane potential assay kit. Similar apoptotic rates of EPCs were detected by JC-1 staining with TNF-α treatment, while pre-treated rosiglitazone allayed apoptosis (Fig. 3: A – D). Cytochrome c would be released from the mitochondria in response to pro-apoptotic stimuli and subsequently activated caspase-3 and caspase-7. Bax, a member of the Bcl-2 protein family, played an essential role in regulating cell apoptosis by controlling mitochondrial permeability and release of cytochrome c. As shown in Fig. 3, E and F, rosiglitazone reduced the expression of bax as well as the release of cytochrome c. These results indicated rosiglitazone may ameliorate the expression and activities of caspase-3 and caspase-7 through modulating mitochondrial function.

Rosiglitazone attenuated EPC apoptosis via p44/42 MAPK (ERK1/2) pathway

TNF-α transitorily activated MAPK (ERK1/2, p38, and JNK) pathways. The activation of MAPKs by TNF-α was decreased to the baseline level after 30 min (Fig. 4A). Pretreatment with PD98059, a selective inhibitor of MEK1/2, the upstream activator of ERK1/2 (20 μM), but not the JNK MARK inhibitor SP600125 (20 μM) or SB203580, a specific inhibitor of p38 MAPK (10 μM), protected EPCs from apoptosis caused by TNF-α, which was similar to the effect of rosiglitazone (Fig. 4B). PD98059 was also shown to inhibit activation of caspase-3 (Fig. 4C). In addition, PD98059 also exerted a similar protective effect, attenuating cleavage of caspase-7 and parp, as rosiglitazone did (Fig. 4E). We further examined whether rosiglitazone had inhibitory effects on TNF-α–induced MAPK activation and confirmed that pretreatment of rosiglitazone inhibited ERK1/2 activation under TNF-α stimulation (Fig. 4D). Together, the
data suggested that the inhibition of ERK1/2 MAPK may, at least partly, participate in the rosiglitazone-mediated anti-apoptotic effect on EPCs.

**NF-κB pathway also plays a role in rosiglitazone-mediated inhibitory effects on EPCs**

In addition, TNF-α was found to activate the NF-κB pathway (Fig. 5A). We examined the effect of rosiglitazone on NF-κB subunit p65 and IκB-α phosphorylation and the results inferred rosiglitazone attenuated phosphorylation of NF-κB and IκB-α (Fig. 5B). Moreover, rosiglitazone exerted an inhibitory effect on TNF-α-induced translocation of NF-κB to the nucleus, which was performed with nuclear extracts of EPCs (Fig. 5C). Pretreatment of EPCs with the NF-κB inhibitor Bay11-7082 also inhibited phosphorylation of NF-κB, IκB-α, and nuclear translocation of NF-κB (data not shown). Furthermore, Bay11-7082 added before TNF-α blocked the subsequent apoptosis (Fig. 5: D, E). Thus, the protective effect of rosiglitazone may also result from inactivating the NF-κB pathway.

**Anti-apoptotic effect through NF-κB pathway possibly depends on ERK/MAPK pathway**

To study the relationship between NF-κB and the ERK/MAPK pathway, we further compared PD98059 with Bay11-7082 on phosphorylation of NF-κB and ERK. Pretreatment of EPCs with the MEK/ERK inhibitor PD98059 had no influence on TNF-α-mediated NF-κB activation. On the contrary, the NF-κB inhibitor Bay11-7082 reduced phosphorylation of ERK as much as PD98059 (Fig. 6). These results revealed that NF-κB inhibition alleviated EPC apoptosis induced by TNF-α by preventing activation of caspase-3 via the ERK/MAPK pathway.

**Discussion**

Given the importance of EPCs in the repair process for vascular disorders, we performed the present study to test
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First, EPCs incubated with TNF-α for 24 h, at the concentration 10 ng/ml known to induce apoptosis, were protected from apoptosis in a dose-dependent manner by rosiglitazone. At concentration of 30 μM, rosiglitazone also achieved the maximum effect to inhibit activation of caspase-3 and caspase-7 with high-level cleaved segments. Second, TNF-α caused the decrease of mitochondrial membrane potential and enhanced bax expression and cytochrome c release from mitochondria. Rosiglitazone restored these pathological changes. Third, although TNF-α was shown to activate all MAPKs (ERK, p38, and JNK), the data presented here suggested the ERK/MAPKs pathway was the main signal transduction pathway for TNF-α to induce EPC apoptosis. NF-κB might also contribute to TNF-α-stimulated apoptosis via the ERK pathway. Finally, pretreatment of EPCs with rosiglitazone decreased phosphorylation of ERK and NF-κB and translocation of NF-κB. These data suggested a direct effect of rosiglitazone to reduce EPC apoptosis under TNF-α stimulation by restoring the membrane potential of mitochondria and inhibiting activation of effector caspase-3 and caspase-7. ERK and NF-κB pathways may play an important role for the rosiglitazone-mediated beneficial effect on EPC apoptosis induced by TNF-α.

EPCs were shown to promote endothelial repair and angiogenesis after injury in vast experimental studies (16, 17). The capacity of EPCs to maintain endothelium

Fig. 2. Rosiglitazone attenuated the activation of effector caspase-3/7. A–C) EPCs subjected to TNF-α exhibited increased expression of cleaved caspase-3 and caspase-7. Moreover, cleaved parp was produced by caspase cleavage. Rosiglitazone reduced cleavage of caspase-3, caspase-7, and parp in a dose-dependent manner. D) TNF-α upregulation of caspase-3 activity, which was abrogated by 30 μM rosiglitazone. *P < 0.05 vs. untreated cells; **P < 0.05, ***P < 0.01 vs. 10 ng/ml TNF-α without pretreatment of rosiglitazone.
homoeostasis and improve the development of new vessels would contribute to treatment of vascular disease and enhance compensatory angiogenesis, thus blocking the development and progression of ischemic syndromes. Microvascular complications and subsequent critical limb ischemia were major health care concerns related to diabetes. However, vascular complications in diabetes led to reduced EPC numbers, angiogenicity, and other functions including migration (3, 18). A progressive reduction of EPCs was further in parallel with increasing severity of peripheral vascular complications in those patients with type 2 diabetes (19). Additionally, the function of EPCs isolated from patients with type 2 diabetes and vascular disease is more damaged as compared to patients with diabetes but without vascular disease (20).

Elevated plasma levels of TNF-α were associated with the pathogenesis of diabetes and atherosclerosis (21). TNF-α was related to endothelial dysfunction in patients with type 2 diabetes mellitus after myocardial infarction (6) and actively involved in the progression of atherosclerosis (22). Moreover, exposure to TNF-α resulted in premature senescence of EPCs via the p38 MAPK pathway (8). However, TNF-α increased the apoptotic rate of EPCs without a clear signal transduction pathway. According to the results, we suggest TNF-α activates ERK/MAPK and NF-κB pathways to cause activation of effector caspases, caspase-3/7, in EPCs. In addition, TNF-α was revealed here to affect mitochondrial regulation by reducing mitochondrial membrane potential apart from directly initiating the pathways via the intermediate membrane proteins TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD). In accord with previous studies, the rapid drop of mitochondrial inner trans-membrane potential caused succeeding release of cytochrome c, a pro-apoptotic molecule. Cytosolic cytochrome c then could bind with Apoptotic protease activating factor-1 (Apaf-1) and ATP, which further bind to pro-caspase-9 to create a protein

![Graphs and images](image-url)
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The apoptosome in turn orchestrates activation of execution caspases, including caspase-3. In this complicated process, Bax plays an important role to trigger the release of cytochrome c (24). The increased expression of Bax and cytochrome c in our study further implied their effects in apoptosis of EPCs.

In contrast, rosiglitazone improved EPC number and migratory activity of diabetic patients and reduced NADPH oxidase activity to restore EPC re-endothelialization capacity (12, 25). Furthermore, rosiglitazone increased EPC survival, differentiation, and function, which were impaired by C-reactive protein (CRP) (26). Another study reported that rosiglitazone ameliorated AGE-induced dysfunction of EPCs via activation of the PI3K–Akt–eNOS signal pathway (27). However, the current findings of mechanisms of rosiglitazone on EPCs mainly included activation of the PI3-kinase/Akt pathway and endothelial nitric oxide synthase, as well as inhibition of the NAD(P)H oxidase activity of progenitor cells. Besides, rosiglitazone was shown to inhibit ERK and NF-κB pathways in HUVECs and thereby decrease the levels of sICAM-1 and TNF-α induced by homocysteine. However, it was not revealed whether rosiglita-
Rosiglitazone exerted therapeutic effects to promote cell survival through the above pathways under stimulation of TNF-α (28). Therefore, in the present study, we demonstrated for the first time that rosiglitazone might alleviate TNF-α–induced apoptosis of EPCs by inhibiting activation of the ERK/MAPK pathway. Our data also revealed that rosiglitazone inhibited cleavages of caspase-3/7 and parp. Furthermore, the data indicated rosiglitazone restored the balance of mitochondrial membrane potential and reduced release of cytochrome c.

Rosiglitazone is also documented to have an anti-inflammatory effect by inhibiting phosphorylation of IkB-α and nuclear translocation of NF-κB. A subfamily of NF-κB proteins include P50/p105, p52/p100, RelA (p65), RelB, and c-Rel. Before activation, NF-κB complexes are retained in the cytoplasm because of their interaction with IkB-α. Once stimulated, IkB-α undergoes phosphorylation and degradation in a proteasome. Meanwhile, the free NF-κB dimer is translocated into the nucleus to regulate the transcription of target genes (29). Nevertheless, for some type of cells, activation of NF-κB protected cells from apoptosis induced by TNF-α (30, 31), while for other cells, NF-κB pathway activation led to apoptosis (32, 33). A recent study demonstrated Bay11-7082 and knockdown of NF-κB decreased TNF-α–induced apoptosis of EPCs. Caspase-3 activity was also restored by pretreatment of Bay11-7082 and NF-κB SiRNA (34). The results here indicated a similar effect by Bay11-7082, which confirmed the hypothesis that rosiglitazone may also attenuate apoptosis of EPCs induced by TNF-α via inhibiting phosphorylation of IkB-α and nuclear translocation of NF-κB. Moreover, we revealed ERK...
might be a downstream target of NF-κB and thus rosiglitazone might inhibit TNF-α–induced EPC apoptosis mainly through the ERK/MAPK pathway, yet more detailed mechanisms remained to be determined.

Rosiglitazone was reported to reduce glucose-induced oxidative stress (35) and stimulate nitric oxide synthesis (36) in endothelial cells via AMP-activated protein kinase (AMPK). AMPK inhibited homocysteine-induced apoptosis in EPCs and reduced reactive oxygen species (ROS) accumulation and endothelial nitric oxide synthase (eNOS) down-regulation (37). Therefore, we hypothesize that rosiglitazone may also attenuate TNF-α–induced EPC apoptosis via AMPK by reducing oxidative stress.

In conclusion, rosiglitazone ameliorated TNF-α–induced apoptosis of EPCs via reducing expression and activity of caspase-3 and caspase-7. Rosiglitazone also participated in mitochondrial regulation and inhibited release of cytochrome c. ERK/MAPK and NF-κB signal pathways played important roles in these beneficial effects. These findings suggest a potential therapeutic role of rosiglitazone in EPC survival under TNF-α stimulation and warrant further EPCs-based cytotherapy in ischemic vascular diseases.

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References


