Anti-proliferative effect of rosiglitazone on angiotensin II-induced vascular smooth muscle cell proliferation is mediated by the mTOR pathway

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Abstract

VSMC (vascular smooth muscle cell) proliferation contributes significantly to intimal thickening in atherosclerosis, restenosis and venous bypass graft diseases. Ang II (angiotensin II) has been implicated in VSMC proliferation though the activation of multiple growth-promoting signals. Although TZDs (thiazolidinediones) can inhibit VSMC proliferation and reduce Ang II-induced fibrosis, the mechanism underling the inhibition of VSMC proliferation and fibrosis needs elucidation. We have used primary cultured rat aortic VSMCs and specific antibodies to investigate the inhibitory mechanism of rosiglitazone on Ang II-induced VSMC proliferation. Rosiglitazone treatment significantly inhibited Ang II-induced rat aortic VSMC proliferation in a dose-dependent manner. Western blot analysis showed that rosiglitazone significantly lowered phosphorylated ERK1/2 (extracellular-signal-regulated kinase 1/2), Akt (also known as protein kinase B), mTOR (mammalian target of rapamycin), p70S6K (70 kDa S6 kinase) and 4EBP1 (eukaryotic initiation factor 4E-binding protein) levels in Ang II-treated VSMCs. In addition, PPAR-γ (peroxisome-proliferator-activated receptor γ) mRNA increased significantly and CTGF (connective tissue growth factor), Fn (fibronectin) and Col III (collagen III) levels decreased significantly. The results demonstrate that the rosiglitazone directly inhibits the pro-atherosclerotic effect of Ang II on rat aortic VSMCs. It also attenuates Ang II-induced ECM (extracellular matrix) molecules and CTGF production in rat aortic VSMCs, reducing fibrosis. Importantly, PPAR-γ activation mediates these effects, in part, through the mTOR-p70S6K and -4EBP1 system.

Keywords: angiotensin II; mammalian target of rapamycin (mTOR); proliferation; rosiglitazone; smooth muscle cell

1. Introduction

VSMC (vascular smooth muscle cell) proliferation and migration are essential features of vasculogenesis and blood vessel maturation. Furthermore, these processes clearly play a role in the pathophysiology of several prominent cardiovascular disease states, such as atherosclerosis and restenosis (Lee et al., 2004). Following migration, VSMCs proliferate in the intima and secrete matrices and proteases to form atheromatous plaques under the influence of stimulatory cytokines. Various stimuli induce VSMC proliferation and migration; Ang II (angiotensin II), the effector molecule of the renin–Ang system, has profound effects on SMC (smooth muscle cell) proliferation and migration (Perlegas et al., 2005; Zuo et al., 2005). These effects are not only haemodynamic in nature but also comprise inflammation, thrombosis and cell proliferation by stimulating cytokine and growth factor production (Horuchi et al., 2003). ARBs [AT1 (Ang II type 1 receptor) blockers] (Zuo et al., 2005) and knockdown experiments have shown that intracellular signals involving Rac1 activation are important for the Ang II-mediated hypertrophy of SMCs and neointimal formation in injured arteries (Horuchi et al., 2003). Rosiglitazone is an agonist of PPAR-γ (peroxisome-proliferator-activated receptor γ) and is the most potent of the TZD (thiazolidinedione) anti-diabetic agents. The FDA (Food and Drug Administration) recently approved rosiglitazone for the treatment of type II diabetes mellitus. Rosiglitazone is thought to attenuate Ang II-induced vascular inflammation (Ji et al., 2009). In addition, PPAR-γ activators decrease BP (blood pressure) and cell growth, and improve endothelial dysfunction in mesenteric resistance arteries from Ang II-infused rats. Thus, PPAR-γ could contribute to the regulation of different vascular genes in hypertension (Ryan et al., 2004). Furthermore, rosiglitazone inhibits VSMC proliferation by blocking growth factor-induced phosphorylation of retinoblastoma tumour suppressor proteins (Bremmer and Law, 2003).

However, the role of mTOR (mammalian target of rapamycin) in rosiglitazone-inhibited VSMC proliferation and fibrosis induced by Ang II is unknown. We demonstrate that rosiglitazone directly

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Abbreviations: AMV, avian myeloblastosis virus; Ang II, angiotensin II; Col III, collagen III; CTGF, connective tissue growth factor; DMEM, Dulbecco’s modified Eagle’s medium; 4EBP, eukaryotic initiation factor 4E-binding protein; ECM, extracellular matrix; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; Fn, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PISK, phosphoinositide 3-kinase; PPAR, peroxisome-proliferator-activated receptor; p70S6K, 70 kDa S6 kinase; RT–PCR, reverse transcription PCR; siRNA, small interfering RNA; SMC, smooth muscle cell; TBS-T, TBS-Tween 20; TZD, thiazolidinedione; VSMC, vascular smooth muscle cell.
inhibits the pro-atherosclerotic effect of Ang II in rat aortic VSMCs through an mTOR-mediated pathway and down-regulates the expressions of Ang II-induced ECM (extracellular matrix) molecules and CTGF (connective tissue growth factor), reducing fibrosis.

2. Materials and methods

2.1. Isolation and culture of rat aortic VSMCs

Rat aortic VSMCs were isolated as previously described (Lee et al., 2004). All experimental procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine, and performed in accordance with the Committee’s guidelines and regulations for animal care. The thoracic aortas from 6- to 8-week-old Sprague-Dawley rats were removed and transferred into serum-free DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen) containing 100 units/ml penicillin and 100 μg/ml streptomycin. The aorta was freed from the connective tissue, transferred into a Petri dish containing 5 ml of an enzyme dissociation mixture containing DMEM with 1 mg/ml collagenase type I (Sigma) and 0.5 μg/ml elastase (USB Bioscience), and incubated for 30 min at 37°C. The aorta was transferred into DMEM, and the adventitia was stripped with forceps under a microscope. The aorta was transferred into a plastic tube containing 5 ml enzyme dissociation mixture and incubated for 2 h at 37°C. The suspension was centrifuged (1500 rev./min for 10 min), and the pellet was resuspended in DMEM with 10% FBS (fetal bovine serum). Rat aortic VSMCs were cultured in DMEM supplement with 10% FBS, 100 IU (international units)/ml penicillin and 100 μg/ml streptomycin in 75 cm² flasks at 37°C in a humidified atmosphere of 95% air and 5% CO₂ (Forma Scientific).

2.2. Cell proliferation assay

Rat aortic VSMCs were plated in triplicate wells of 96-well plates at 1 x 10⁴ per well. Cells were pretreated with rosiglitazone for 30 min prior to Ang II exposure (100 nM) for 30 min. Cells were washed once in PBS and lysed in a lysis buffer (Cell Signaling) containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₂VO₄, 1 mg/ml leupeptin and 1 mM PMSF. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore). After blocking the membrane with TBS-T (TBS-Tween 20; 0.1% Tween 20) containing 5% (w/v) non-fat dried skimmed milk powder for 1 h at room temperature, membranes were washed twice with TBS-T and incubated with primary antibody for 1 h at room temperature or overnight at 4°C. The following primary antibodies were used: rabbit anti-ERK (extracellular-signal-regulated kinase), mouse anti-phospho-ERK (Santa Cruz Biotechnology), rabbit anti-mTOR, rabbit anti-phospho-mTOR, rabbit anti-p70S6K (70 kDa S6 kinase), mouse anti-phospho-p70S6K, rabbit anti-4E-BP1 (eukaryotic initiation factor 4E-binding protein) and rabbit anti-phospho-4E-BP1 (Cell Signaling). The membrane was washed three times with TBS-T for 10 min and incubated with HRP (horseradish peroxidase)-conjugated secondary antibody for 1 h at room temperature. After extensive washing, the bands were detected using ECL® (enhanced chemiluminescence) reagent (Santa Cruz Biotechnology). The band intensities were quantified using a Photo-Image System (Molecular Dynamics).

2.5. Statistical analysis

All quantified data are the averages of at least triplicate samples. The error bars are S.D. of the mean. Statistical significance was determined by Student’s t test, with P<0.05 considered significant.

3. Results

3.1. Anti-proliferative effect of rosiglitazone on Ang II-stimulated rat aortic VSMCs

Ang II induces a wide variety of vascular events, including endothelial activation and dysfunction, cell proliferation and
monocyte chemoattraction, which play important roles in atherosclerosis development (Jia et al., 2008). We first confirmed that Ang II increased rat aortic VSMC proliferation in a dose-dependent manner, the highest proliferation being at 100 nM Ang II (Figure 1A). In addition, the effect of rosiglitazone on rat aortic VSMC proliferation was examined since decreased proliferation after rosiglitazone treatment may contribute to Ang II-induced decrease in rat aortic VSMC proliferation. Rosiglitazone significantly decreased rat aortic VSMC proliferation compared with that of the control cells at concentrations over 20 μM (Figure 1B). Therefore, 1 and 10 μM rosiglitazone were used in further experiments. To investigate whether rosiglitazone regulates the proliferation of rat aortic VSMCs stimulated by Ang II, cells were pretreated with 1 and 10 μM rosiglitazone for 30 min prior to Ang II exposure (100 nM). The drug inhibited Ang II-stimulated proliferation of rat aortic VSMCs dose-dependently (Figure 1C), indicating a specific effect.

3.2. PPAR-γ inhibits mTOR activation in Ang II-stimulated rat aortic VSMCs

To demonstrate that PPAR-γ activation is associated with rosiglitazone inhibition of Ang II-induced rat aortic VSMC proliferation, PPAR-γ expression in Ang II-stimulated rat aortic VSMCs was investigated. Ang II down-regulated the expression of PPAR-γ mRNA, and rosiglitazone treatment re-established this expression (Figure 2A). In addition, Ang II activated mTOR in rat aortic VSMCs, and rosiglitazone significantly inhibited altered mTOR phosphorylation (Figure 2B). Thus, the mTOR-associated signal pathways mediate the Ang II-induced control of rat aortic VSMC proliferation. Regarding cell survival and proliferation mechanisms, Akt and ERK activation plays an important role in gene regulation (Arellano-Plancarte et al., 2010). Akt phosphorylation was markedly increased following Ang II stimulation, and rosiglitazone inhibited the increase in a dose-dependent manner (Figure 2B). In addition, since Ang II is known to stimulate ERK activation in rat aortic VSMCs (Touyz et al., 1999a, 1999b), we investigated the effect of rosiglitazone on this effect. Ang II treatment markedly activated ERK after 30 min. Then 1 and 10 μM rosiglitazone pretreatment significantly inhibited this activation (Figure 2B). To confirm whether the inhibitory effects of rosiglitazone on Ang II-stimulated VSMCs proliferation is specifically mediated by PPAR-γ signal pathways, PPAR-γ expression was down-regulated using a specific siRNA (small interfering RNA), following which mTOR-associated signal molecules were assessed. PPAR-γ expression was inhibited by siRNA specifically and the expression levels of mTOR-associated signal molecules were slightly but significantly rescued by rosiglitazone (Figure 2C), supporting the result that PPAR-γ mediates the inhibitory effects of rosiglitazone in Ang II-induced VSMCs proliferation.

3.3. Effects of rosiglitazone on p70S6K and 4EBP1 signalling pathways in Ang II-stimulated rat aortic VSMCs

Rosiglitazone blocked Ang II-induced stimulation of cell proliferation in rat aortic VSMCs. Rosiglitazone inhibits the mTOR signalling pathway, which includes p70S6K, and this plays a key role in cell growth (Park et al., 2008). Furthermore, Ang II triggered dissociation of the translation initiation factor, eIF-4E (eukaryotic initiation factor 4E), from its regulatory binding protein 4EBP1 (Lu et al., 2005). As expected, p70S6K and 4EBP1 phosphorylation increased significantly in Ang II-stimulated rat aortic VSMCs. Rosiglitazone pretreatment completely suppressed excess phosphorylation (Figure 3). Therefore, rosiglitazone significantly inhibits the activation of both mTOR-p70S6K and -4EBP1 systems.

3.4. Inhibitory effects of rosiglitazone on the expression of CTGF and ECM components in Ang II-stimulated rat aortic VSMCs

To investigate the effects of rosiglitazone on the Ang II-induced fibrotic activity of VSMCs, the expression of CTGF and ECM components, such as Col III and Fn, were examined. CTGF regulates the expression of ECM components (Gao et al., 2007). CTGF, Col III and Fn mRNA levels increased markedly in Ang II-stimulated rat aortic VSMCs and were significantly dose-dependently inhibited by rosiglitazone treatment (Figure 4). The results show that rosiglitazone reduces Ang II-induced fibrosis.

4. Discussion

Hypertension and diabetic mellitus are risk factors for coronary diseases and atherosclerosis (Satani et al., 2006). We have demonstrated that a PPAR-γ activator, rosiglitazone, can directly inhibit the pro-atherosclerotic effect of Ang II on rat aortic VSMCs.
In addition, fibrosis is a vascular change caused by hypertension and diabetic mellitus. We also show that rosiglitazone can attenuate Ang II-induced ECM and CTGF production in rat aortic VSMCs. More importantly, PPAR-γ activation mediates these effects, in part, through mTOR-p70S6K and -4EBP1 systems.

In general, diabetes and hyperglycaemia alone have not been associated with cell cycle regulation, but Ang II triggers responses in VSMCs that lead to proliferation, migration and phenotypic modulation, resulting in growth factor and ECM production (Rosendorff, 1996; Brassard et al., 2005; Mehta and Griendling, 2007). Ang II induces increased translation initiation factors, 4EBP1 and S6K phosphorylation, and critical cell cycle regulators downstream of mTOR (Touyz et al., 1999a, 1999b; Dugourd et al., 2003; Yamakawa et al., 2003; Hafizi et al., 2004). A key regulatory kinase, mTOR, plays a major role in the mammalian cell cycle, and is a major pathway in neointimal hyperplasia pathogenesis and stent restenosis (Choi et al., 2004). Previous studies have revealed that mTOR signalling is important in cell and organism growth, the cell cycle, proliferation, aspects of metabolism, and gene transcription and transcriptional regulators (Hafizi et al., 2004). TZDs, such as rosiglitazone, are insulin-sensitizing agents used to treat hyperglycaemia by improving insulin resistance in type 2 diabetic patients (Berger and Moller, 2002). In addition to increasing insulin sensitivity, Berger and Moller (2002) have shown a significant anti-proliferative effect of TZDs on vascular tissues. The molecular mechanism underlying

Figure 2 Effects of rosiglitazone on mTOR-associated signals in Ang II-stimulated VSMCs
(A) PPAR-γ expression levels in the presence or absence of rosiglitazone in Ang II-stimulated VSMCs for 4 h were evaluated by RT-PCR. (B) Altered phosphorylated levels of mTOR-associated signals were assessed in the presence or absence of rosiglitazone in Ang II-stimulated VSMCs using Western blotting. (C) Altered phosphorylated levels of mTOR-associated signals were measured in the presence or absence of rosiglitazone in PPAR-γ specific siRNA treated VSMCs using Western blotting. Values are the average of three measurements with the S.D. indicated by error bars (*P<0.05, **P<0.01).

Figure 3 Effects of rosiglitazone on the downstream molecules of mTOR in Ang II-stimulated VSMCs
The altered phosphorylated levels of downstream molecules of mTOR, p70S6K and 4EBP1 were assessed in the presence or absence of rosiglitazone in Ang II-stimulated VSMCs for 30 min using Western blotting. Values are the average of three measurements with the S.D. indicated by error bars (*P<0.05, **P<0.01).
the anti-proliferative effect of the mTOR pathway has not been fully elucidated.

Because the PI3K (phosphoinositide 3-kinase)-Akt pathway activates mTOR, we determined whether rosiglitazone inhibits VSMC proliferation by inhibiting the Akt-mTOR-p70S6K or -4EBP1 pathway to check whether this pathway is a specific, major inhibitory pathway for Ang II-stimulated VSMCs (Dugourd et al., 2003; Hafizi et al., 2004). Previous studies have shown that PI3K/Akt-4EBP1 and ERK1/2 signalling pathway inhibitions mediated the effect of rosiglitazone on chronic Ang II-induced PI3K and MAPK (mitogen-activated protein kinase) signalling, but have not demonstrated a relationship between mTOR and rosiglitazone (Goetze et al., 1999; Benkirane et al., 2006a, 2006b; Cho et al., 2006). The results from our study demonstrate that cells treated with rosiglitazone before Ang II stimulation show significant p-mTOR, p-Akt, p-P70S6K and p-4EBP1 decreases, suggestive of significant Akt-mTOR-p70S6K and -4EBP1 system inhibition.

Ang II has been implicated in potential VSMCs growth and fibrosis through multiple growth promoting pathways and MMPs (matrix metalloproteinases) (Goetze et al., 1999; Brassard et al., 2005). Ang II plays a role in vascular fibrosis, a vascular change associated with hypertension and diabetes mellitus (Rosendorff, 1996). Gao et al. (2007) demonstrated that rosiglitazone inhibits Ang II-induced VSMC proliferation and ECM molecule production by reducing CTGF, which plays a role in cell proliferation and apoptosis. Our data demonstrate that rosiglitazone could significantly reduce Ang II-stimulated CTGF and ECM expression in rat aortic VSMCs.

In conclusion, rosiglitazone inhibits Ang II-induced VSMCs proliferation by blocking the mTOR-p70S6K and -4EBP1 systems. Reduced ECM production may also mediate the inhibitory effect of rosiglitazone on Ang II-induced rat aortic VSMCs proliferation.

These findings extend the understanding of the role of rosiglitazone in vascular fibrosis and provide novel evidence for its beneficial vascular effect.

Author contribution

Jung-Sun Kim, Il-Kwon Kim and Se-Yeon Lee designed the experiments and wrote the paper. Byeong-Wook Song, Min-Ji Cha, Heesang Song, Eunmi Choi, Soyeon Lim and Onju Ham performed the experiments and analysed data. Yangsoo Jang discussed and revised the paper. Ki-Chul Hwang designed, planned and supervised the study and contributed to the preparation of the paper.

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