The Inhibition of Insulin Stimulated Proliferation of Vascular Smooth Muscle Cell by Rosiglitazone is Mediated by the Akt-mTOR-p70S6K Pathway

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The Inhibition of Insulin Stimulated Proliferation of Vascular Smooth Muscle Cell by Rosiglitazone is Mediated by the Akt-mTOR-p70S6K Pathway

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Thiazolidinediones are known to inhibit vascular smooth muscle cell (VSMC) proliferation by increasing the activity of $p27^{Kip1}$ and retinoblastoma protein (RB). However, the upstream signaling mechanisms associated with this pathway have not been elucidated. The Akt-mTOR-p70S6K pathway is the central regulator of cell growth and proliferation and increases cell proliferation by inhibiting the activities of $p27^{Kip1}$ and retinoblastoma protein (RB). Therefore, in this study, we hypothesize that rosiglitazone inhibits VSMC proliferation through the inhibition of Akt-mTOR-p70S6K signaling pathway. Rat aortic SMCs (RAoSMCs) were treated with 10 μM rosiglitazone 24 hours before the addition of insulin as a mitogenic stimulus. Western blot analysis and RT-PCR were performed to determine the inhibitory effect of rosiglitazone treatment on the Akt-mTOR-p70S6K signaling pathway. Carotid balloon injury was also performed in OLETF diabetic rats that were pretreated for 1 week with 3mg/kg of rosiglitazone. Western blot analysis demonstrated significant inhibition of expression and activation of Akt, *p*-Akt, m-TOR, *p*-m-TOR, p70S6K and *p*-p70S6K in cells treated with rosiglitazone. RT-PCR revealed significantly decreased transcripts of Akt, m-TOR and p70S6K in cells treated with rosiglitazone. The inhibition of the expression of Akt-mTOR-p70S6K signaling pathway was demonstrated *in vivo* in OLETF diabetic rats by immunohistochemistry. The inhibition of the activation of the *p*-mTOR-*p*-p70S6K pathway seemed to be mediated

upstream by both the PI3K pathway and the MEK-ERK complex. In conclusion, the inhibitory effect of rosiglitazone on RAoSMC proliferation *in vitro* and *in vivo* is mediated by the inhibition of Akt-mTOR-p70S6K pathway.

Key words: Vascular smooth muscle cell, mammalian target of Rapamycin, Insulin, Rosiglitazone

I. Introduction

It is well known that thiazolidinediones (TZDs, e.g. rosiglitazone, troglitazone and pioglitazone) are insulin-sensitizing agents that possess *in vivo* anti-diabetic activities. TZDs are ligands of peroxisome proliferator activated receptor-γ (PPAR-γ), a member of a larger family of ligand-activated nuclear receptor transcription factors. Recent evidence suggests that thiazolidinediones exert potent anti-proliferative and anti-inflammatory effects on the vascular wall with inhibitory effects on vascular smooth muscle cell proliferation, inhibition of monocyte inflammatory cytokines, inhibition of macrophage activation, and inhibition of the expression of cell adhesion molecules being suggested as mechanisms of these effects.[1-6] Thiazolidinediones are known to inhibit vascular smooth muscle cell proliferation by diminishing mitogen-induced degradation of $p27^{Kip1}$ thereby resulting in increased activity of retinoblastoma protein (RB).[7] However, the upstream signaling mechanisms associated with the above mentioned mechanism has not been elucidated. The mammalian target of rapamycin, mTOR, is an enzyme located in the non-particulate region of the cell cytoplasm that plays an essential role in connecting extracellular signals with intracellular pathways that regulate cell cycle and proliferation. Rapamycin eluting stents have proven to significantly reduce the rate of coronary stent restenosis in human clinical trials.[8] Through inactivation of p70S6 kinase (p70S6K) and eukaryocytic initiation factor 4E (eIF4E), rapamycin increases the activity of $p27^{Kip1}$ and Rb.[9,10] mTOR itself is known to be activated by the phosphoinositide 3–kinase (PI3K)-Akt pathway.[11,12]

We used insulin as a mitogenic stimulus due to the important role of hyperinsulinemia in the development of neointima hyperplasia and the known concomitant increase in translation initiation factor activities, phosphorylation of 4EBP-1 and p70S6K, and critical cell cycle

regulators downstream of mTOR. Insulin and angiotensin II are known to induce activation of the PI3K/Akt/mTOR/p70S6K pathway and to phosphorylate 4E-BP1.[13-15]

In this study, we hypothesized that rosiglitazone, a PPAR-γ agonist, inhibits insulin-stimulated vascular smooth muscle cell (VSMC) proliferation through the inhibition of the Akt-mTOR-p70S6K system and that the Akt-mTOR-p70S6K system is a specific, major pathway for inhibition of VSMC proliferation.

II. Materials and Methods

1. Materials and experimental animals

Experimental materials

Rosiglitazone was a generous gift from GlaxoSmithKlein Korea (Seoul, Korea). All other materials were purchased from commercial suppliers.

Experimental animals

Spontaneous diabetic OLETF rats were kindly supplied by the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. Four weeks old male Otsuka Long-Evans Tokushima Fatty (OLETF) rats (70-80 g initial weight) were housed in a temperature-controlled environment under a 12-hour light/dark cycle and were given standard chow and water *ad libitum*. Animals were handled in compliance with the *Guiding Principles in the Care and Use of Animals*.[16] All rats were fed standard rat chow until they were 28 weeks of age. Rosiglitazone, 3 mg/kg, was mixed with pulverized standard chow to a final concentration of 0.015% (wt/wt), and rats were treated for 1 week. After 1 week, carotid artery balloon injury was performed. After 3 weeks of treatment, the rats were sacrificed for analysis.

2. Methods

Primary culture of RAoSMCs

Rat aortic smooth muscle cells (RAoSMCs) were isolated and purified by previously described methods.[17] The thoracic aortas from 6- to 8-week-old Sprague-Dawley rats were removed and transferred to serum-free Dulbecco's modified Eagle medium (DMEM; Invitrogen Co, Carlsbad, CA, USA) containing 100 units/ml penicillin and 100 g/ml streptomycin. The aorta was freed from 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, St. Louis, MO, USA) and 0.5 μg/ml elastase (USB Bioscience, Cleveland, OH, USA), and incubated for 30 min at 37 °C. The aorta was then transferred into DMEM and the adventitia was stripped off with forceps under a binocular microscope. The aorta was transferred into a plastic tube containing 5 ml of the enzyme dissociation mixture and incubated for 2 h at 37°C. The suspension was centrifuged (1,500 rpm for 10 min) and the pellet was resuspended in DMEM with 10% fetal bovine serum (FBS). RAoSMCs were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 g/ml streptomycin in 75-cm^2 flasks at 37°C in a humidified atmosphere of 95% air and 5% CO_2 (Forma Scientific, Inc., Marjetta, OH, USA.).

Proliferation assay

RAoSMCs were plated in triplicate in wells of 96-well plates at a density of 1 x 10^4 per well, and treated with rosiglitazone prior to insulin stimulation. Cell viability was determined by the MTT assay. After the incubation period, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma, St. Louis, MO, USA) was added to each well to a final concentration of 0.5 mg/mL and was incubated at 37℃ for 3 hr to allow MTT reduction. The formazan crystals were dissolved by adding dimethylsulfoxide (DMSO) and absorbance was

measured at 570 nm with a spectrophotometer.

Immunoblot analysis

Protein-treated cells were washed once in PBS and lysed in a lysis buffer (Cell signaling, Beverly, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM PMSF. Protein concentrations were determined using the Bradford protein assay kit (BioRad, Hercules, CA, USA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co, Bedford, MA, USA). After blocking the membrane with Tris-buffered saline-tween 20 (TBS-T, 0.1% tween 20) containing 5% non-fat dried milk for 1 hr at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibody for 1 hr at room temperature or overnight at 4°C. The membrane was washed three times with TBS-T for 10 min and then incubated for 1 hr at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using the Photo-Image System (Molecular Dynamics, Uppsala, Sweden). For treatment with PPAR-γ ligand, sub-confluent RAoSMCs (passage 4 and 8) were made quiescent by serum starvation (0.1% FBS) for 3 days. The cells were treated with rosiglitazone at 10μM/L 24 hr before the addition of insulin (1μM). Independent preparations of VSMCs were used for each experiment.

Perfusion of vessels and H&E staining.

OLETF rats treated with carotid balloon injury were sacrificed 3 weeks after the balloon injury, and their carotid artery was excised. The heart was perfused with Krebs-Ringer Bicarbonate Buffer (KRBB : 120 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2

mM $MgSO_4$, 2.5 mM $CaCl_2$, and 20 mM MOPS) for 10 min to wash out the blood and then fixed with 10% formalin. The carotid artery was sliced transversely. Within 24 hr of fixation, each section was embedded in paraffin. Serial 2μm carotid artery sections were cut with a microtome and mounted on siliconized slides. Paraffin sections were stained with hematoxylin and eosin (HE) and evaluated with light microscopy to assess the histological effects. Normal and neointimal areas were measured with NIH Image.

Histology and Immunohistochemistry

OLETF rats treated with balloon injury were killed and their carotid artery excised. The vessels were perfusion-fixed with 10% (v/v) neutral buffered formaldehyde for 24 hr, transversely sectioned into serial thick sections, and embedded in paraffin by routine methods. Sections 2 μm in thickness were mounted on gelatin-coated glass slides to ensure different stains could be used on successive sections of tissue cut through the areas of ballon injury. After de-paraffinization and rehydration, the sections were analyzed with rabbit anti-mTOR obtained from Cell Signaling Technology (MA, USA). Texas Red-conjugated goat anti-rabbit IgG from Jackson ImmunoResearch Lab (PA, USA) was used as secondary antibody. All images were made by using an excitation filter under reflected light fluorescence microscopy and transferred to a computer equipped with MetaMorph software v. 4.6 (Universal Imaging Corp, Downingtown, PA, USA).

Induction of neointima formation after Balloon denudation

Balloon denudation of the common carotid artery endothelium was evoked in OLETF rats. Under IM ketamine (10mg/kg, Yuhan, Seoul, Korea) and Xylazine (5mg/kg, Bayer Korea, Seoul, Korea) anesthesia, a neck midline incision was made and, after exposure of the left

carotid artery, a 2F Fogarty balloon catheter (Edwards Lifesciences, Mississauga, ON) was inserted into the external carotid branch to the aortic arch, insufflated to produce slight resistance, and withdrawn three times. They were compared with sham-operated controls in which the same procedure was performed with the exception of the balloon insertion. Animal housing and experimentation in accordance with Animal Care and NIH guidelines were approved by the local animal care committee.

RT-PCR analysis

The expression levels of various proteins were analyzed by the reverse transcription polymerase chain reaction (RT-PCR) technique. For RNA preparation confluent rat aortic smooth muscle cells were cultured for 48 h in serum-free α-MEM. Total RNA was prepared by the UltraspectTM-II RNA system (Biotecx Laboratories, Inc., USA) and single-stranded cDNA was then synthesized from isolated total RNA by Avian Myeloblastosis virus (AMV) reverse transcriptase. A 20 µl reverse transcription reaction mixture containing 1µg of total RNA, 1X reverse transcription buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs) 0.5 unit of RNase inhibitor, 0.5µg of oligo(dT)15, and 15 units of AMV reverse transcriptase was incubated at 42℃ for 15 min, heated to 99℃ for 5 min, and then incubated at 0-5℃ for 5 min. PCR reactions were performed for 35 cycles with 3' and 5' primers based on the sequences of various genes. The actin primers (5'-catcacatactcacaacgctcaac-3' and 5'-catagcacgatggtcgattgtcgt-3') were used as the internal standard. The signal intensity of the amplification product was normalized to its respective actin signal intensity.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed by Student's t-test. Relationships were considered statistically significant when the *p* value was less than 0.05.

III. Results

1. Antiproliferative effect of rosiglitazone in insulin-stimulated RAoSMC

In order to investigate whether rosiglitazone affects RAoSMC proliferation upon insulin treatment, RAoSMCs were treated with 1μM insulin in the presence or absence of rosiglitazone and a cell proliferation assay was performed. Figure 1 shows that cell proliferation gradually increased upon treatment with insulin within 3 days, but 10μM of rosiglitazone inhibited the augmentation of cell proliferation upon insulin treatment, suggesting that rosiglitazone affects cell proliferation due to insulin treatment.

2. Inhibitory effects of rosiglitazone on expression of mTOR in insulin-stimulated RAoSMC

To confirm the inhibitory effect of various concentrations of rosiglitazone on mTOR, the changes in mTOR levels were estimated by immunoblot analysis. RAoSMCs at 70% confluence were made quiescent by serum starvation with 0.1% FBS for three days. Cells were treated with various concentrations of rosiglitazone before a 1 μM insulin stimulus. The expression of mTOR upon insulin stimulus was significantly inhibited by rosiglitazone in a dose-dependent manner (Figure 2). Compared to cells treated with insulin alone, cells treated with rosiglitazone before insulin stimulus demonstrated significant inhibition of mTOR.

3. Inhibitory effects of rosiglitazone on mTOR expression in balloon-injured OLETF rats

We then performed immunohistochemistry to determine the expression of mTOR during

neointima expansion in OLETF rats. The inhibitory effect of rosiglitazone was detected not only *in vitro* but also *in vivo*. As shown in Figure 3, there was no immunoreactivity for mTOR in normal uninjured OLETF rats. In response to vascular injury, expression of mTOR was increased after balloon injury due to proliferation of RAoSMCs. However, the intensity of mTOR immunostaining was markedly decreased in OLETF rats treated with rosiglitazone after balloon injury compared with balloon-injured OLETF rat treated without rosiglitazone.

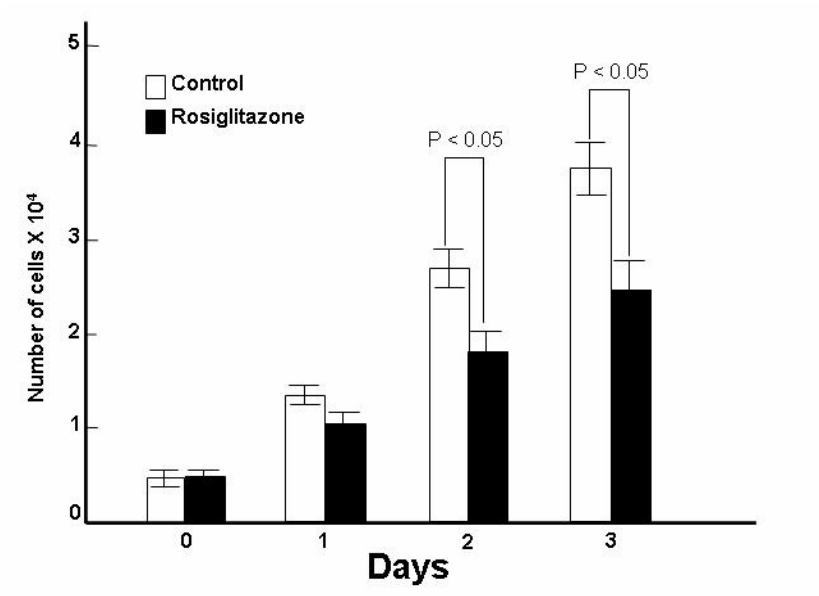

Figure 1. Effect of rosiglitazone on the proliferation of insulin-induced RAoSMCs. Quiescent RAoSMCs (0.5 x 10^4 cells per well) were stimulated with 1μM of insulin. After three days in culture in the absence or presence of rosiglitazone cell viability was determined by the MTT assay.

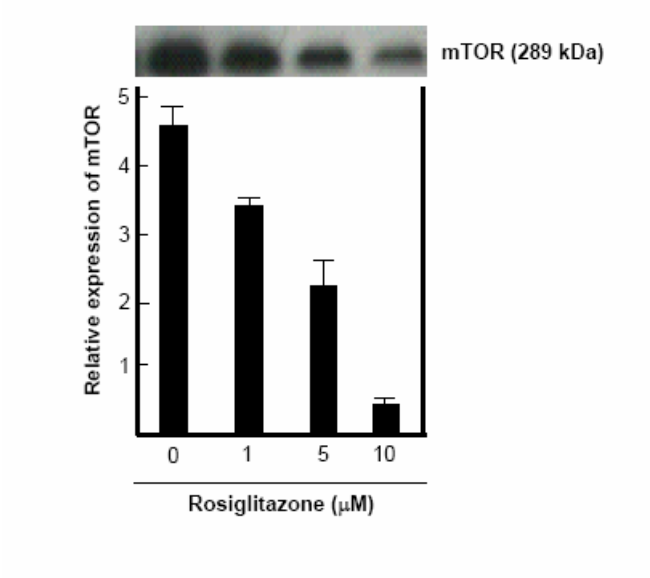

Figure 2. Effect of rosiglitazone on the expression of mTOR in RAoSMCs. Western blot analysis of mTOR in RAoSMCs exposed to 10μM rosiglitazone for thee days in DMEM with 0.5% FBS. Each signal was quantified by scanning densitometry and the figure shows the levels of each activity as the relative value of the maximal level of mTOR. Each western blot was repeated three times.

4. Akt-mTOR-p70S6K Signaling pathway in RAoSMC treated with rosiglitazone

Binding of insulin to its receptor activates the receptor tyrosine kinase which phophorylates the insulin receptor substrate. Insulin promotes neointima hyperplasia and stimulates proliferation-related signaling pathways such as the Akt-mTOR-p70S6K pathway. The expression and activation of the Akt-mTOR-p70S6K pathway was significantly increased after treatment with 1μM of insulin for 15 min. The expression of mTOR in RAoSMCs treated with insulin was significantly inhibited by rosiglitazone. As Figure 4 shows, compared to cells treated with insulin alone, cells treated with rosiglitazone before insulin stimulus demonstrated a significant inhibitory effect on proteins upstream, Akt, and downstream, p70S6K, of mTOR.

This suggests that rosiglitazone significantly inhibits both the expression and activation of the Akt-mTOR-p70S6K system.

5. mRNA levels of Akt, mTOR and p70S6K in RAoSMC treated with rosiglitazone

The inhibition of expression of the Akt-mTOR-p70S6K system at the mRNA level was demonstrated by RT-PCR. The treatment of RAoSMCs with 1 μM insulin resulted in dramatically increased expression levels of Akt-mTOR-p70S6K that was reduced by treatment of 10 μM rosiglitazone (Figure 5). The results indicated that rosiglitazone has an effect on the protein expression levels of Akt-mTOR-p70S6K.

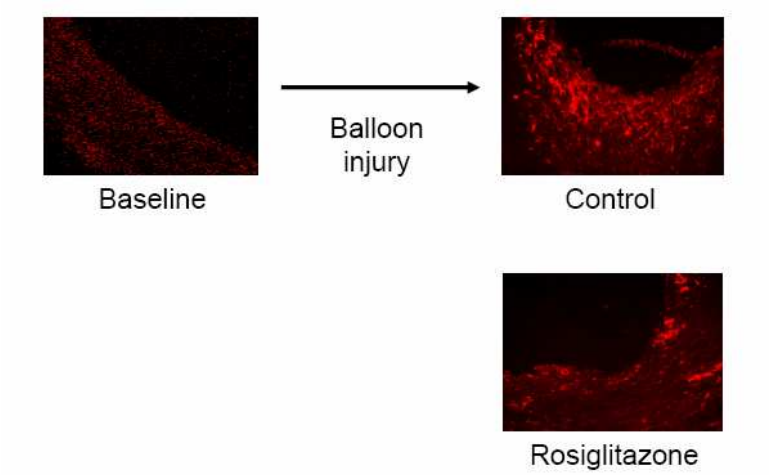

Figure 3. Effect of rosiglitazone on expression of mTOR in balloon-injured OLETF rats. After exposure of the left carotid artery to a 2F Fogarty balloon catheter, the expresson of mTOR was detected through immunostaining of sections of tissue cut through the areas of balloon injury after treatment of 3 mg/kg of rosiglitazone. The immunostain was visualized with Texas Red-conjugated goat anti-rabbit IgG secondary antibody.

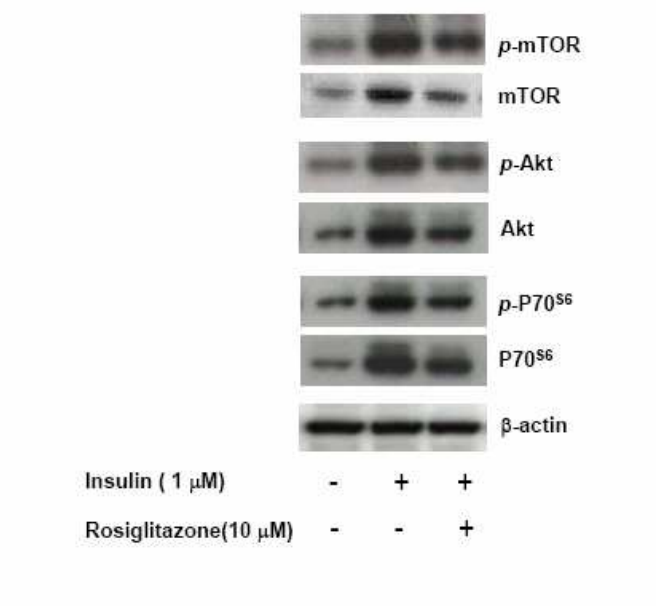

Figure 4. Effect of rosiglitazone on the phosphorylation and expression of Akt, mTOR, and p70S6K in RAoSMCs with 10μM rosiglitazone. (A) Equivalent amounts of protein lysate from RAoSMCs were subjected to SDS-PAGE, transferred to a PVDF membrane, and incubated with antibody. The immunoreactive protein was visualized by use of an alkaline phosphatase detection system.

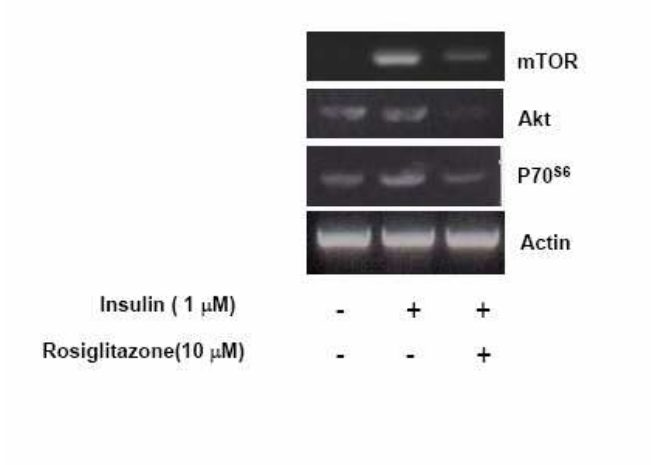

Figure 5. Effect of rosiglitazone on the mRNA expression of Akt, mTOR and p70S6K in RAoSMCs treated with 10μM rosiglitazone. The expressions of genes were analyzed by RT-PCR. Amplication products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

6. Akt-mTOR-p70S6K expression and neointima formation by rosiglitazone in balloon-injury of OLETF rats

To test the role of rosiglitazone in neointima response after balloon injury, a daily dose of 3mg/kg of rosiglitazone was administered to the rats for one week before the balloon injury and for three weeks after the balloon injury. Withdrawal of the inflated balloon resulted in vascular injury leading to neointima formation. In the present study, neointima formation in balloon-injured arteries was confirmed by histology. Figure 6 shows that the density of neointima formation after balloon injury was much thicker than in the sham-treated rats. However, no further neointimal growth was observed in OLEFT rats treated with rosiglitazone after balloon injury. At the time of analysis, the neointima of OLEFT rats treated with rosiglitazone was thinner than that of controls.

7. Effect of PI3K or MEK inhibitor on Akt-mTOR-p70S6K in RAoSMC treated with rosiglitazone

It was shown that rosiglitazone inhibits not only expression but also phosphorylation of Akt-mTOR-p70S6K system in RAoSMCs stimulated with insulin. To determine whether factors upstream of the Akt-mTOR-p70S6K system regulate phosphorylation of this system, inhibitors of upstream molecules were added to cells prior to treatment with insulin. PI3K and MAPK are upstream molecules and related to the insulin mediated signaling pathway. Insulin can activate PI3K through the phosphorylation of IRS1/2 or MAPK by Ras-GTP. It is known that wortmannin is a PI3K inhibitor and U0126 inhibits MEK1/2 activation. Figure 7 shows the effect of inhibition of the MEK/ERK and PI3K pathways on the effect of rosiglitazone treatment. The expression of *p*-p70S6K after rosiglitazone administration was assessed before and after treatment with the MEK inhibitor U0126 (10μM) and the PI3K inhibitor wortmannin (10μM). Both MEK inhibition and PI3K inhibition resulted in partial reduction of pp70S6K expression that was associated with additional inhibition. The results show that both the MAPK and PI3K pathways have an effect on the activation of the Akt-mTOR-p70S6K system.

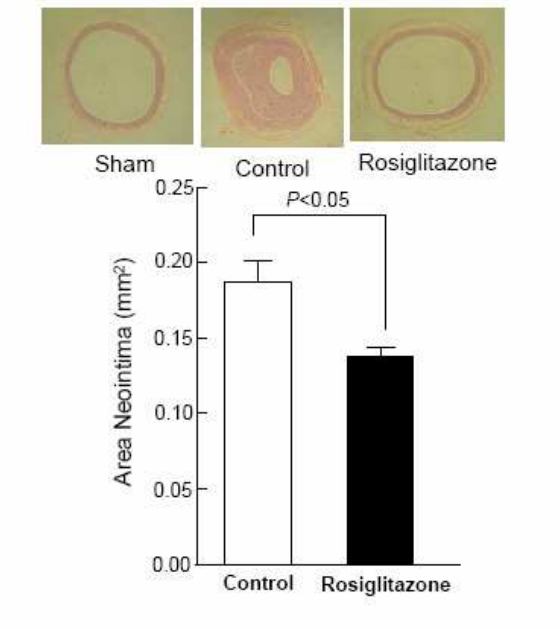

Figure 6. Effect of rosiglitazone on neointima formation in carotid balloon-injured OLETF rats. Injured and uninjured (intact) carotid arteries were excised at 7 days after injury. 3 mg/kg of rosiglitazone was administered for 7 days before the balloon injury and 21 days after the balloon injury. The sections were stained with H&E, and neointima formation was evaluated. Representative photographs of H&E staining was upper and bar graphs show neointima quantified by Imagescope 2.5. Data are expressed as means ± SEM (control n= 5, rosiglitazone group: n = 8).

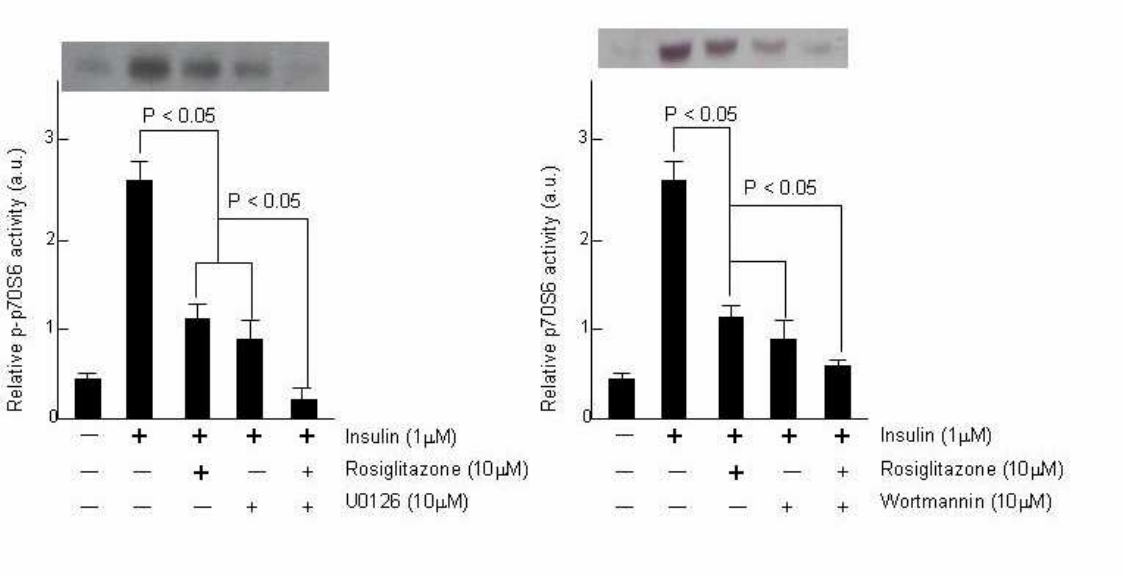

Figure 7. Effect of rosiglitazone on phosphorylation of p70S6K after treatment with U0126 or wortmannin in insulin-stimulated RaoSMCs. Equivalent amounts of protein lysate from RaoSMCs were subjected to SDS-PAGE, transferred to a PVDF membrane, and incubated with antibody. The immunoreactive protein was visualized by use of an alkaline phosphatase detection system.

IV. DISCUSSION

Thiazolidinediones are insulin sensitizing agents that are used to treat hyperglycemia by reduction of insulin resistance in type 2 diabetic patients.[1] In addition to improvements in insulin sensitivity, studies have shown significant anti-inflammatory and anti-proliferative effects on the vascular tissues both *in vitro* and *in vivo*.[2,12] However, the molecular mechanisms underlying the anti-proliferative effect have not been fully elucidated. In general, diabetes and hyperglycemia alone have not been associated with the process of cell cycle regulation, but it has been known that insulin is a key player in protein synthesis, a factor important in cell proliferation. Insulin induces increased expression of translation initiation factors and phosphorylation of 4EBP-1 and S6K, critical cell cycle regulators downstream of mTOR. The mammalian target of rapamycin (mTOR) is a key regulatory kinase that plays a major role in the mammalian cell cycle and is a member of a major pathway in the pathogenesis of neointima hyperplasia and stent restenosis. Previous studies have revealed that mTOR signaling is important in cell and organism growth, in the cell cycle and proliferation, in aspects of metabolism, as well as for modulating gene transcription and transcriptional regulators. By inactivating p70S6K and eukaryocytic initiation factor 4E (eIF4E), rapamycin increases the activity of $p27^{Kip1}$ and Rb resulting in subsequent inhibition of vascular smooth muscle cell proliferation. Because mTOR itself is known to be activated by the phosphoinositide-3-kinase (PI3Ks)-Akt pathway, we wanted to determine whether rosiglitazone inhibits vascular smooth muscle cell proliferation through inhibition of the Akt-mTOR-p70S6K pathway and whether the Akt-mTOR-p70S6K pathway system is a specific, major pathway for VSMC proliferation inhibition. This hypothesis was derived from the fact that the Akt-mTOR-p70S6K pathway may be the upstream mechanism that increases the activity of $p27^{Kip1}$ and Rb with administration of PPAR-γ agonists. The results from this study demonstrate that cells treated with rosiglitazone

before insulin stimulus demonstrate significant inhibition of mTOR, p-mTOR, Akt, p-Akt, p70S6K, p-p70S6K, suggestive of significant inhibition of both the expression and activation of the Akt-mTOR-p70S6K system.

The inhibition of the expression of these various proteins is most likely mediated by regulation of transcriptional levels of the Akt-mTOR-p70S6K pathway proteins, which was supported by the mRNA transcript levels of Akt, mTOR and p70S6K that were detected by RT-PCR. However, there may also be a PPAR-ɣ independent effect that inhibits the enzyme activity of this pathway, which is shown by the reduced levels of the phosphorylated forms of the pathway. Because the mTOR pathway members are activated not only by the PI3K-Akt pathway but also the mitogen activated protein kinase pathway,[18-20] the inhibitory effect of rosiglitazone on the Akt-mTOR-p70S6 kinase pathway is regulated by both the PI3K-Akt pathway and the mitogen activated protein kinase pathway.

Previous studies have demonstrated that PPAR-γ activation reduces mitogen-induced VSMC growth by inhibition of Akt activity and increased phosphorylated forms of 4E-BP1. It had been demonstrated that the sequence of events between the activation of Akt and increased phosphorylation of 4E-BP1 involves the activation of mTOR signaling. However, studies regarding the role of PPAR-γ agonists in modulating the activity of mTOR have not been done. This study demonstrates that the inhibition of the activity of mTOR-p70S6K pathway may be an important mechanism in modulating the effects of various downstream signaling factorssuch as the 4E-BP1 and CDK/CK complexes.

The interruption of blood flow caused by ligation of the common carotid artery induces the migration and proliferation of SMCs from the media to the intima, with the concomitant infiltration and activation of circulating leukocytes, leading to neointima formation. OLETF rats characteristically begin to gain weight rapidly from 5 weeks of age, show insulin resistance at 8

to 9 weeks, and finally develop obese type 2 diabetes. The cumulative incidence of diabetes in male OLETF rats over age 23 weeks is 86%. In OLETF rats treated with rosiglitazone after balloon injury, the intensity of neointima formation and immunoreactivity of mTOR was markedly decreased.

In conclusion, rosiglitazone inhibits neointima hyperplasia after carotid balloon injury because it blocks the Akt-mTOR-p70S6K pathway resulting in inhibition of vascular smooth muscle cell proliferation. The inhibitory effect of rosiglitazone on the activity of the Akt-mTOR-p70S6K pathway may be mediated by the MAPK and PI3K pathway.

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국문초록

Akt-mTOR-p70S6K 신호전달체계의 억제를 통한 Rosiglitazone 의 인슐린 촉진 혈관 평활근 증식 억제작용

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Thiazolidinediones은 $p27^{Kip1}$과 retinoblastoma protein(RB)의 활성도를 억제해서 혈관 평활근세포의 증식을 억제하는 것으로 현재까지 보고되고 있다. 그렇지만 이러한 기전과 연관된 상위 신호절달체계는 현재까지 밝혀진 바가 없다. Akt-mTOR-p70S6K는 $p27^{Kip1}$ 그리고 retinoblastoma protein(RB)의 활성도의 조절을 매개로 세포성장 및 증식을 조절하는데 중추적인 역할을 하는 신호전달체계이다. 따라서 본 연구의 가설은 thiazolidinedione인 rosiglitazone이 Akt-TOR-p70S6K 전달체계의 억제를 통해 혈관 평활근세포의 증식을 억제한다는 것이다. In vitro 실험에서는 인슐린을 mitogenic stimulus로 사용하였으며 백서 대동맥 혈관 평활근세포에 인슐린을 처치하기 24시간전 rosiglitazone 10μM를 처치하였다. Rosiglitazone이 Akt-mTOR-p70S6K 신호전달체계를 억제하는지를 분석하기 위해 western blot 분석과 RT PCR을 시행하였다. Western blot 분석결과 rosiglitzone이 전처치된 혈관 평활근세포에서는 Akt, p-Akt, m-TOR, p-m-TOR, p70S6K and p-p70S6K의 표현과 활성도가 현저하게 감소됨을 관찰하였고 RT-PCR 결과 Akt, m-TOR 및 p70S6K의 mRNA transcript가 rosiglitazone이 전처치된 세포에서 현저히 감소됨을 관찰하였다. 또한 in vivo에서는 OLETF 당뇨쥐에서 1주일간 rosiglitazone 3mg/kg 전처치후 경동맥 풍선손상술을 시행하였다. Rosiglitazone이 투여된 OLETF 당뇨쥐에서는 신생내막의 형성이 현저히 억제되었고 신생내막조직에서 Akt-mTOR-p70S6K의 표현이 억제되었음을 확인하였다. 또한 본 연구결과에서 rosiglitazone에 의한 p-mTOR,

pp70S6K 신호전달체계의 억제는 그 upstream에 있는 PI3K 신호전달체계 및 MEK-ERK 신호전달체계와 연관이 있는 것으로 밝혀졌다. 따라서 결론적으로 본 연구에서는 rosiglitazone에 의한 혈관 평활근세포 증식 억제효과는 in vitro 및 in vivo에서 Akt-mTOR-p70S6K 신호전달체계의 억제를 통해서 이루어짐을 밝혀냈다.

핵심되는 말: 혈관 평활근세포, mammalian target of Rapamycin, 인슐린, Rosiglitazone