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# Celastrol Attenuates Adipokine Resistin-Associated Matrix Interaction and Migration of Vascular Smooth Muscle Cells

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## ABSTRACT

Obesity instigates various health problems such as atherosclerosis, diabetes, and cancer. Resistin, an adipose tissue-specific secretory adipokine, operates endocrine functions through increasing insulin resistance. Vascular smooth muscle cells (SMC) migrate into the subendothelial space and proliferate, thereby contributing to neointimal formation in atherosclerosis and restenosis. The aim of this study was to elucidate whether celastrol obtained from *Tripterygium wilfordii Hook*, inhibited human aortic SMC migration. Celastrol capable of antagonizing inflammatory responses attenuated the resistin secretion from THP-1-derived macrophages. The macrophage-conditioned media promoted SMC proliferation and MMP-2 production, which was dampened by 10–100 nM celastrol. Celastrol encumbered the SMC migration in response to 50 ng/ml resistin, concomitant with the inhibition of induction of connective tissue growth factor and collagen I/IV. In addition, celastrol disabled human aortic SMC exposed to resistin from migrating. The resistin-induced shedding of integrin β2/β3 expression was demoted by celastrol, thereby contributing to the inhibition of collagen matrix-SMC interaction. Next, resistin-induced Toll-like receptor-4 (TLR-4) expression was abrogated by celastrol, indicating that TLR-4 was the resistin signaling receptor that was blocked by celastrol. Collectively, these results demonstrate that anti-inflammatory celastrol blunted the macrophage secretion of the adipokine resistin, and suppressed the SMC migration by disturbing the interaction between SMC and intimal collagen matrix. Therefore, celastrol may inhibit atherogenic migration of vascular SMC upon resistin loading by intimal macrophages within atherosclerotic lesions. J. Cell. Biochem. 114: 398–408, 2013. 

**KEY WORDS:** CELASTROL; MACROPHAGE RESISTIN; TOLL-LIKE RECEPTOR-4; SMOOTH MUSCLE CELL MIGRATION

Obesity is one of metabolic syndrome risk factors that cause heart disease and other health problems such as atherosclerosis, diabetes, and cancer [Espinola-Klein et al., 2011]. Adipokines, active proteins secreted from adipose, play roles in the regulation of physiological and pathological processes including inflammation and insulin resistance [Mangge et al., 2010; Espinola-Klein et al., 2011]. Resistin is known as an adipose tissue-specific secretory adipokine to serve endocrine functions likely involved in the insulin resistance [Steppan et al., 2001]. Rodent resistin is produced in adipocytes, whereas macrophages are a major source of human resistin [Jung et al., 2006]. It has recently been found that resistin participates in the inflammatory responses [Nagaev et al., 2006]. Resistin increases the expression of several pro-inflammatory cytokines in an NF-κB-mediated fashion [Silswal et al., 2005]. In addition, resistin up-regulates chemotactic proteins responsible for leukocyte recruitment to sites of infection and inflammation [Verma et al., 2003]. Thus, resistin may be a link in the well-known association between inflammation and insulin resistance.

The adipokine resistin has been recently described in atherosclerosis but not yet extensively studied [Burnett et al., 2005]. Resistin has been defined as a novel inflammatory marker in atherosclerosis. The patho-physiology underlying this interplay, however, remains to be fully characterized. Plasma resistin levels are correlated with inflammatory markers and are predictive of coronary atherosclerosis in humans, independent of C-reactive protein levels [Reilly et al., 2005]. There is not much information on whether resistin could

Abbreviations used: CTGF, connective tissue growth factor; ECM, extracellular matrix; Hsp70, heat shock protein 70; MMP-2, matrix metalloproteinase-2; NF-κB, nuclear factor-κB; SMC, smooth muscle cells; TLR-4, Toll-like receptor-4.

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affect vascular SMC [Jung et al., 2006]. Vascular inflammatory responses promote SMC proliferation, migration, and matrix synthesis which are the major factors contributing to narrowing of the vessel lumen [Raines and Ferri, 2005; Schober, 2008]. Adipocyte-derived resistin is shown to promote vascular SMC migration and its migratory action on vascular SMC may provide evidence of the adipose-vascular interaction in metabolic disorders [Jiang et al., 2009]. Resistin-stimulated vascular SMC migration is associated with increased MMP expression, which is dependent on PKCε activation [Ding et al., 2011].

Celastrol, an active quinone methide triterpene extracted from the root bark of the chinese medicine "Thunder of God Vine" (tripterygium wilfordii Hook F.), was used for years as a natural remedy for inflammatory and auto-immune diseases [Liu et al., 2011]. Celastrol has recently attracted great interest especially for its potential anti-inflammatory and anti-cancer activities [Salminen et al., 2010; Kannaiyan et al., 2011]. Celastrol suppresses inflammatory and innate immunity responses in human retinal pigment epithelial cells via nuclear factor-κB (NF-κB) and heat shock protein 70 (Hsp70) regulation [Paimela et al., 2011]. Celastrol has potential in suppressing invasion and metastasis of cancer cells by down-modulation of expression of CXCR4 chemokine receptor [Yadav et al., 2010]. In addition, celastrol attenuates hypertension-induced inflammation and oxidative stress in vascular SMC via hemoxygenase-1 induction [Yu et al., 2010]. However, the atheroprotection of celastrol has not yet been characterized. Reinioside C, a triterpene of Polygala aureocauda Dunn, inhibits cholesteryl ester accumulation in macrophages and decreases vascular SMC proliferation [Li et al., 2008].

This study investigated that celastrol (Fig. 1A) possessed atheroprotective activity in terms of adipokine-associated vascular SMC proliferation and migration. Human aortic SMC were cultured with 50 ng/ml resistin in the absence and presence of 10–100 nM celastrol. In addition, human aortic SMC were cultured in conditioned media of THP-1-derived macrophages. This study attempted to investigate whether celastrol manipulated the SMC migration induced by resistin secreted from neighbor macrophages. Collagen formation, extracellular matrix (ECM)-degrading MMP-2 activity and integrin induction were examined in resistin-exposed and celastrol-treated human aortic SMC. In addition, the SMC transmigration and the adhesive interaction of SMC with collagen were elucidated. Furthermore, the involvement of Toll-like receptor-4 (TLR-4) signaling in the atheroprotection of celastrol was elucidated.

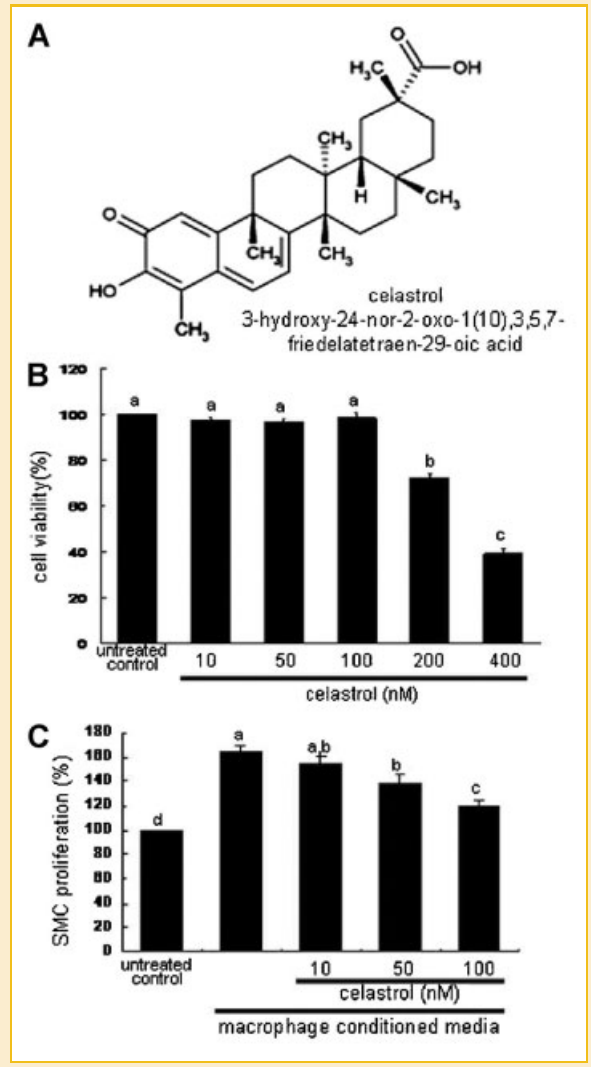


Fig. 1. Chemical structure (A) and human aortic SMC toxicity (B) of celastrol, and inhibition of THP-1 macrophage conditioned media-induced SMC proliferation by celastrol (C). THP-1 monocytes were incubated with 50 ng/ml PMA for 24 h, continuously incubated in PMA-free and serum-free media for 36 h, and THP-1 macrophage conditioned media were obtained. After human aortic SMC were cultured for 24 h in normal culture media or in THP-1 macrophage conditioned media in the presence of 10–100 nM celastrol, MTT assay was performed. The bar graph data represent mean ± SEM from five independent experiments with multiple estimations. Values are expressed as percent cell survival relative to untreated control cells (cell viability = 100%). Values not sharing a letter are different at $P < 0.05$.

## MATERIALS AND METHODS

### CHEMICALS

M199, RPMI 1640, human epidermal growth factor (hEGF), hydrocortisone, phorbol 12-myristate 13-acetate (PMA), and 3-(4, 5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT), celastrol and rat tail collagen IV were obtained from the Sigma–Aldrich Chemical (St. Louis, MO), as were all other reagents, unless specifically mentioned elsewhere. Human aortic SMC, fetal bovine serum (FBS), penicillin–streptomycin and trypsin–EDTA were provided by Lonza Walkersville (Walkersville, MD). THP-1 monocytic cells were purchased from the American Type Culture Collection (Manassas, VA). Human MMP-2 antibody was purchased from R&D systems (Minneapolis, MN). Human collagens I and IV antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA). CTGF antibody was obtained from AbCam (Cambridge, UK). Human resistin protein and resistin antibody were provided from Phoenix Pharmaceuticals (Belmont, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG, donkey anti-goat IgG, and goat anti-mouse IgG were obtained from Jackson Immuno-Research Laboratories (West Grove, PA).

### CULTURE OF HUMAN AORTIC SMC AND THP-1-DERIVED MACROPHAGES

Human aortic SMC were cultured in growth media (Clonetics SmGM-2 Bullet Kit, Lonza Walkersville) containing growth supplements of hEGF, insulin, human fibroblast growth factor-B, FBS and GA-1000. THP-1 monocytes were cultured in RPMI 1640 containing 10% FBS. Both SMC and THP-1 monocytes were plated at 90–95% confluence and sustained at 37°C in an atmosphere of 5% $CO_2$. To differentiate into macrophages, THP-1 monocytes were cultured in RPMI 1640 containing 50 ng/ml PMA for 24 h. Cells were continuously incubated for 36 h in PMA-free and serum-free RPMI 1640 in the absence and presence of 10–100 nM celastrol. Culture media of THP-1-derived macrophages were collected for 36 h to measure resistin production. Macrophage conditioned media for culturing SMC were prepared from serum-free RPMI 1640 collected for 36 h. SMC were incubated for 48 h in conditioned media of THP-1 macrophages in the absence and presence of 10–100 nM celastrol.

For the measurement of SMC proliferation, at the end of incubation with 10–100 nM celastrol the MTT assay was performed to determine cellular viability. SMC were incubated in a fresh medium containing 1 mg/ml MTT at 37°C for 3 h. After elimination of MTT-unconverted, the purple formazan product was dissolved in 0.5 ml of isopropanol via gentle shaking. Absorbance of formazan dye was measured at $\lambda = 570$ nm using a microplate reader (Bio-Rad Model 550, Hercules, CA). Non-toxic concentrations of celastrol in serum-free culture for 24 h were $\leq$100 nM.

### HUMAN AORTIC SMC PROLIFERATION

The SMC proliferation was assessed by using an in vitro scratch assay. SMC were seeded at 50,000 cells onto a 24-well plate and incubated for 24 h in a growth media containing 10% FBS. Human aortic SMC on well were scratched away horizontally with a P100 pipette tip. After scratch, injured cells were incubated for another 24 h in serum free medium containing conditioned media of THP-1

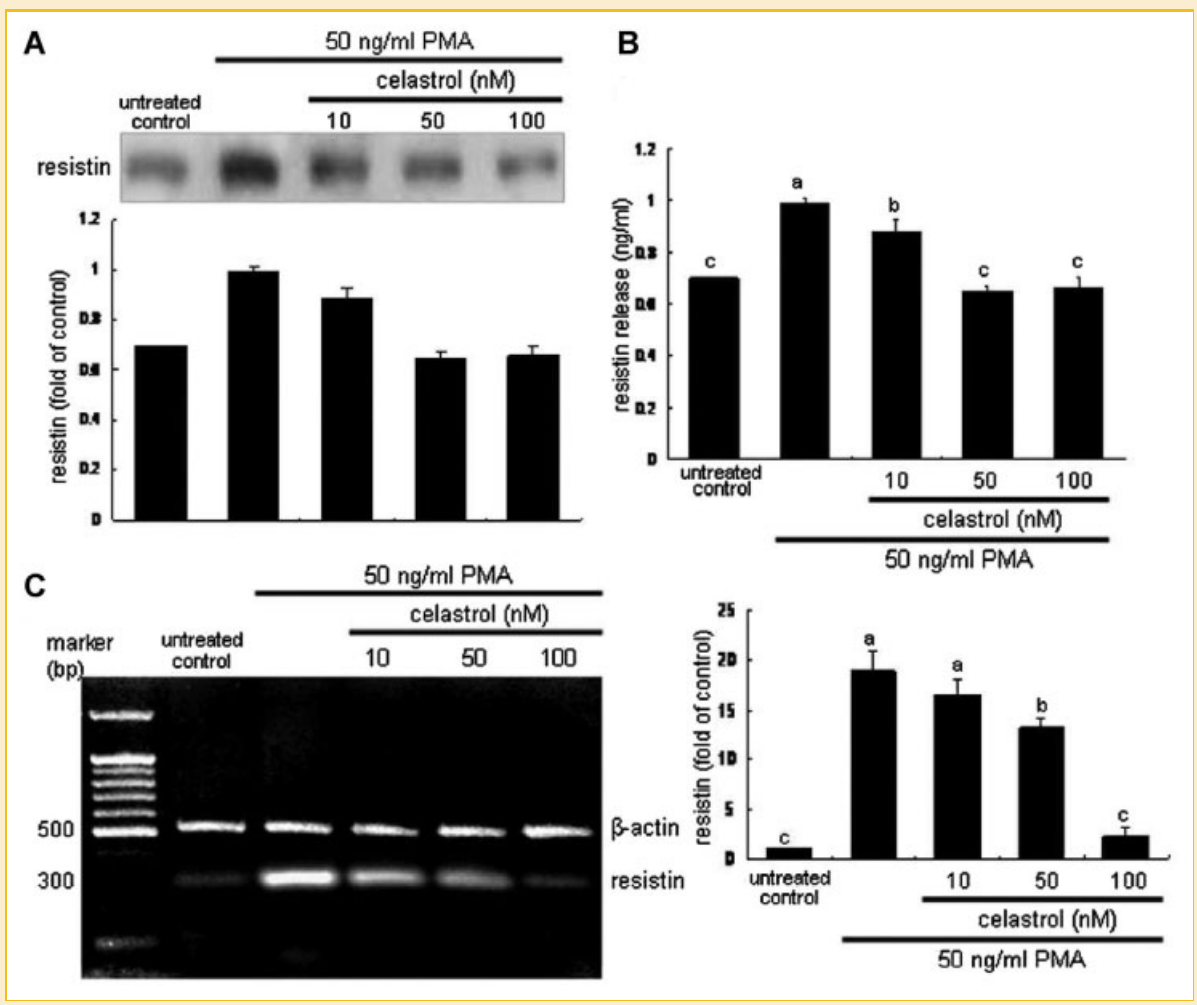


Fig. 2. Inhibitory effects of celastrol on expression (A) and secretion (B) of resistin and on its mRNA levels in THP monocytes (C). THP-1 cells were incubated with 50 ng/ml PMA for 24 h and continuously incubated in PMA-free and serum-free media for 24 h in the absence and presence of 10–100 nM celastrol. Culture media were subject to 12% SDS–PAGE with a primary antibody against resistin (A). Secretion of resistin into culture media was measured by using ELISA (B). The mRNA levels of resistin were analyzed by using RT-PCR (C). β-Actin was used as a housekeeping gene for the co-amplification with resistin. The bar graphs (mean ± SEM, n = 3) represent quantitative densitometric results of upper and left bands (A,C). Values in bar graphs not sharing a letter differ at $P < 0.05$.

macrophages in the absence and presence of 10–100 nM celastrol. A reduction in the scratched area indicates a sign of cell proliferation. Cell proliferation was measured by using MTT assay.

### PROTEIN ISOLATION AND WESTERN BLOT ANALYSIS

Western blot analysis was executed using whole cell lysates and culture media collected from human aortic SMC and THP-1-derived macrophages. Whole cell lysates and culture media were prepared in 1 M Tris–HCl (pH 6.8) lysis buffer containing 10% SDS, 1% β-glycerophosphate, 0.1 M $Na_3VO_4$, 0.5 M NaF and protease inhibitor cocktail. Cell lysates containing equal amounts of total proteins or equal volume of culture media were fractionated by electrophoresis on 8–15% SDS–PAGE and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in Tris buffered saline-Tween 20 (TBS-T) containing 5% skim milk for 3 h. The membrane was incubated overnight at 4°C with a primary anti-human antibody against human resistin, human CTGF, human collagen I, human collagen IV, human MMP-2, human integrin β2, and integrin β3. After triple washes with TBS-T, the membrane was then incubated with goat anti-rabbit IgG, goat anti-mouse IgG, or donkey anti-goat IgG conjugated to horseradish peroxidase for 1 h. Following another triple washing, each target protein was measured using the Supersignal West Pico chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL) and Agfa medical X-ray film blue (Agfa HealthCare NV, Mortsel, Belgium).

### ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The resistin secretion in THP-1 macrophages was determined by using ELISA. Collected culture media were assayed for the secretion of resistin using ELISA kits (R&D System) according to the manufacturer's instructions.

### CELL TRANSMIGRATION ASSAY

The transmigration assay was carried out using gelatin-coated transwell apparatus with 8 μm pore size filters (Costar, Corning, NY). The lower sides of transwell filters were coated with 10 μg/ml gelatin B and allowed to be dry at 25°C for 1 h. The transwells were assembled in a 24-well plate, and the lower chambers were filled with the culture media. Cells (30,000 cells/well) were added to each chamber and the plate was incubated at 37°C in 5% $CO_2$ for 24 h. Cells that had migrated to the lower surface of the insert were stained with toluidine blue, and cells migrated were counted and photographed in the 2–3 microscopic fields per well using a microscopy with CCD camera (Motic®, Wetzlar, Germany).

### REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Total RNA was isolated from differentiated THP-1 macrophages using a commercially available Trizol reagent kit (Invitrogen, Carlsbad, CA), as previously described [Li and Wang, 2011]. The RNA (5 μg) was reversibly transcribed with 200 units of reverse transcriptase and 0.5 μg/μl oligo-(dT)15 primer (Bioneer Company,

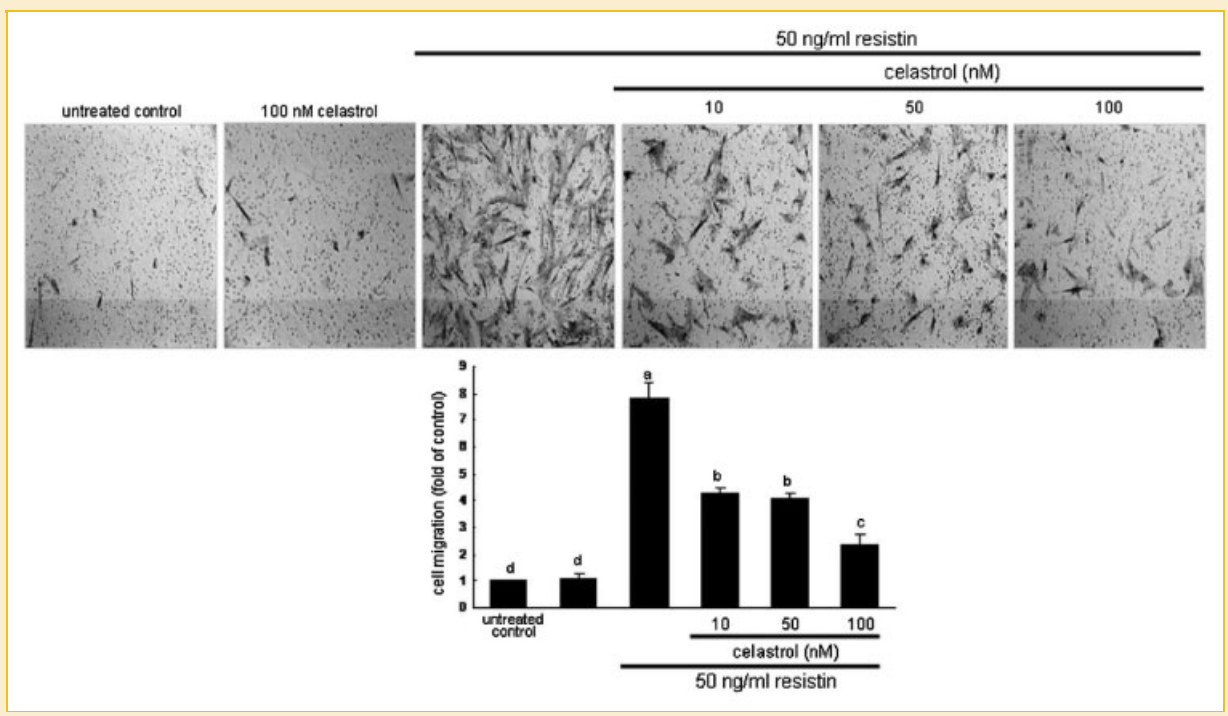


Fig. 3. Blockade of cell transmigration by celastrol in resistin-treated SMC. Human aortic SMC were cultured on gelatin-coated transwells with 8 μm pore inserts. Cells were incubated for 24 h in a medium containing 50 ng/ml resistin in the absence and presence of 10–100 nM celastrol. Cells transmigrated for 24 h onto the lower surface of filters were stained with toluidine blue and counted. The bar graphs (mean ± SEM, n = 4) represent the number of cells transmigrated. Means without common letters refer to significant different at $P < 0.05$.

Daejeon, Korea). The expression of mRNA transcripts of resistin (forward primer: 5′-ATGAAAGCTCTCTGTCTCCTCCTC, reverse primer: 5′-CCTCAG GGCTGCACACGACA) and β-actin (forward primer: 5′-GACTACCTCATGAAGATC-3′, reverse primer: 5′-GATC-CACATCTGCTGGAA-3′) was evaluated by RT-PCR with a slight modification. The PCR was performed in 25 μl of 10 mM Tris–HCl (pH 9.0), 25 mM $MgCl_2$, 10 mM dNTP, five units of Taq DNA polymerase, and 10 μM of each primer and started with 5 min denaturation at 95°C followed by 30 PCR cycles. Each cycle consisted of 30 s at 95°C, 30 s at 60°C and 45 s at 72°C, and the final extension was for 10 min at 72°C. After thermocycling and electrophoresis of the PCR products (15 μl) on 1.2% agarose gel containing 0.5 μg/ml ethidium bromide, the bands were visualized using a TFX-20 M model-UV transilluminator (Vilber-Lourmat, Marne-la-Vallée, France) and gel photographs were obtained. The absence of contaminants was routinely checked by the RT-PCR assay of negative control samples without a primer addition.

## GELATIN ZYMOGRAPHY

For the measurement of MMP-2 activity of culture media, gelatin zymography was conducted. Briefly, culture media were subject to electrophoresis on 8% SDS–PAGE (0.3 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol and 0.03% bromophenol blue) co-polymerized with 0.1% gelatin as a substrate. After completing electrophoresis, gels were incubated for 1 h at 37°C in a 2.5% Triton X-100 solution, washed in 50 mM Tris–HCl buffer (pH 7.5) for 30 min, and incubated for another 20 h in 50 mM Tris–HCl buffer (pH 7.5) containing 200 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij-35. The gels were stained with a solution containing 0.1% Coomassie Brilliant Blue G-250, 2% acetic acid and 45% methanol, and then destained in a solution with 30% methanol and 10% acetic acid.

## CELL ADHESION ASSAY

Human aortic SMC adhesion was determined to by using an adhesion method. Briefly, the bottom surface of 96-well plates was coated with collagen I overnight at 4°C. The wells were washed with PBS and nonspecific binding was blocked with 1% BSA at 37°C for 1 h. Human aortic SMC ($2.0 \times 10^4$) were treated with 50 ng/ml resistin in the absence and presence of 10–100 nM celastrol. The cells ($1 \times 10^4$) were seeded onto the surface of 96-well plates coated with collagen I and allowed to adhere for 30 min. After non-adhered cells were removed, the images were obtained by using a microscopy with CCD camera (Motic®).

For the adhesion quantification, SMC were stained with fluorescent calcein-AM, and the staining intensity was measured at 485 nm excitation and 538 nm emission by a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland).

## DATA ANALYSIS

Data were represented as mean ± SEM of separate experiments. Statistical analyses were carried out using Statistical Analysis Systems statistical software package (SAS Institute, Cary, NC). Significance was determined by one-way ANOVA followed by

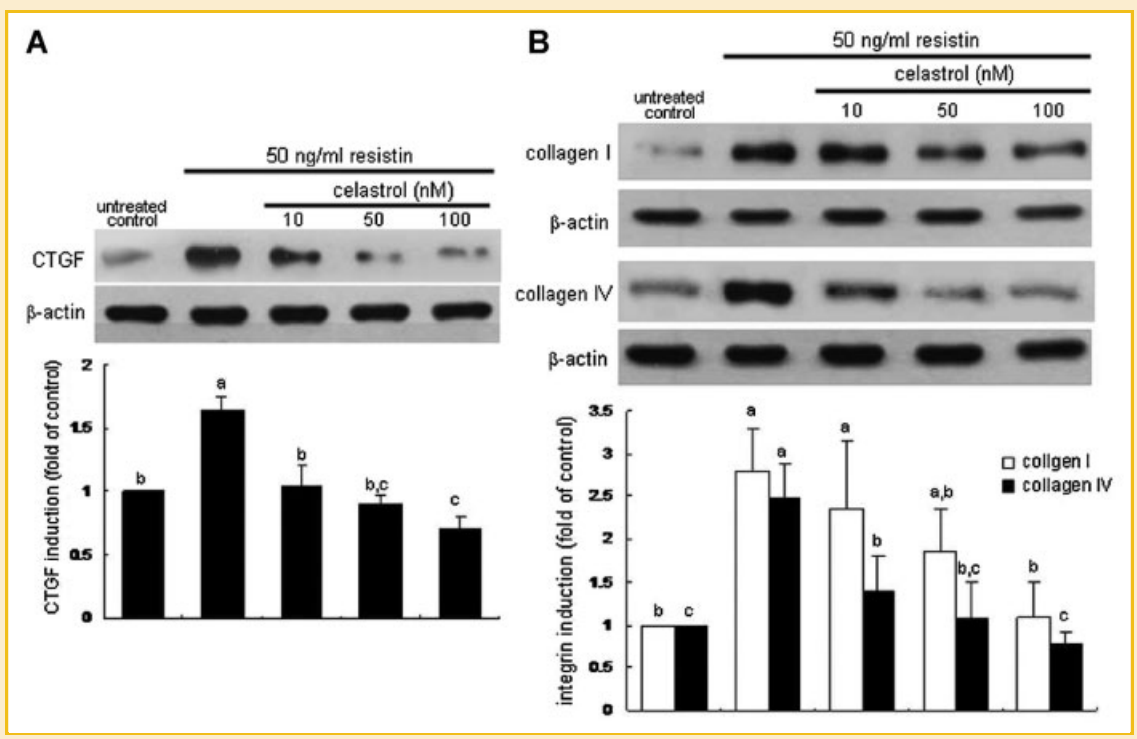


Fig. 4. Western blot data showing suppressive effects of celastrol on expression of CTGF, collagen I and collagen IV. Human aortic SMC were treated with 50 ng/ml resistin in the absence and presence of 10–100 nM celastrol. Cell lysate proteins were subject to 10% SDS–PAGE and Western blot analysis with a primary antibody against CTGF (A), and collagens I and IV (B). β-Actin protein was used as an internal control for cellular expression of CTGF, collagens I and IV. The bar graph (mean ± SEM, n = 3) represent quantitative densitometric results of upper bands. Values not sharing a letter refer to significant different at $P < 0.05$.

Duncan multiple range test for multiple comparisons and *P*-values $<0.05$ were considered as statistically significant.

## RESULTS

### SUPPRESSION OF HUMAN AORTIC SMC PROLIFERATION BY CELASTROL

Celastrol at the doses of 10–100 nM showed no SMC toxicity, as evidenced by MTT assay (Fig. 1B). This study attempted to investigate whether the presence of monocytes/macrophages promoted intimal SMC proliferation and whether such proliferation was inhibited by celastrol treatment. The addition of macrophage conditioned media to human aortic SMC substantially promoted SMC proliferation by ≈60%, evidenced by the in vitro scratch assay (Fig. 1C). However, 50–100 nM celastrol significantly attenuated the enhanced proliferation.

### INHIBITION OF RESISTIN INDUCTION OF MACROPHAGES BY CELASTROL

This study examined whether THP-1-derived macrophages produced the adipokine resistin, which was attenuated by celastrol. The resistin secretion increased substantially within 2 days after differentiating THP-1 monocytes with 50 ng/ml PMA (data not shown). When 10–100 nM celastrol was treated to THP-1 cells differentiating to macrophages, the resistin secretion of THP-1-derived macrophages was dampened (Fig. 2A,B). In addition, $\geq$50 nM celastrol inhibited the expression of resistin mRNA (Fig. 2C). Accordingly, celastrol attenuated the resistin secretion at its transcriptional level.

### INHIBITORY EFFECTS OF CELASTROL ON SMC MIGRATION

Vascular SMC migration is well-documented events in atherosclerosis [Raines, 2004; Ishigaki et al., 2011]. This study investigated

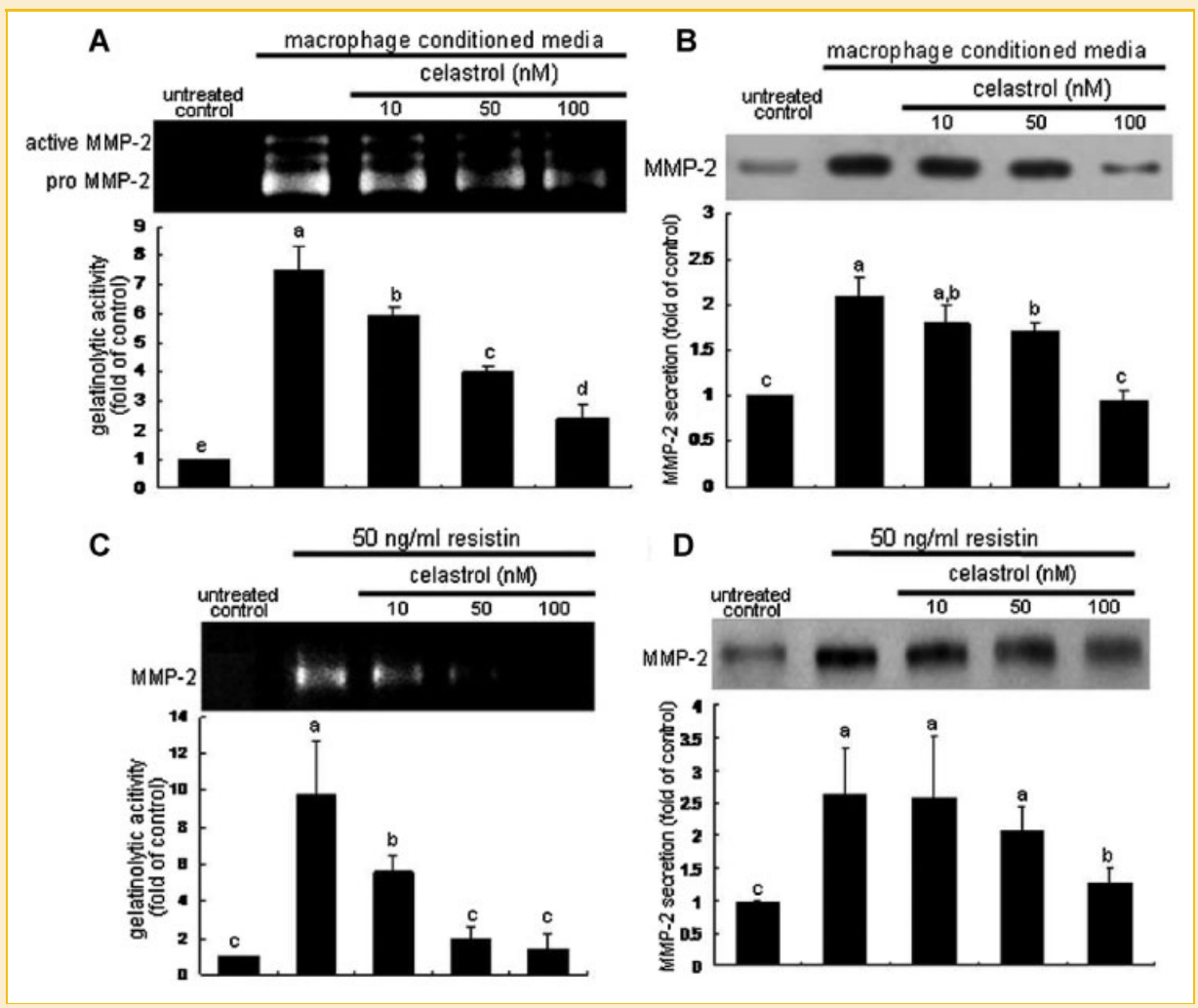


Fig. 5. Suppression of MMP-2 activity and its secretion by celastrol. THP-1 monocytes were incubated with 50 ng/ml PMA for 24 h and continuously incubated in PMA-free and serum-free media for 36 h. Human aortic SMC were incubated with and without 10–100 nM celastrol in THP-1-derived macrophage conditioned media collected for 36 h. Collected culture media were subject to gelatin zymography for MMP-2 enzyme activity (A,C) and Western blot analysis with a primary antibody against MMP-2 for its secretion (B,D). Gelatinolytic activity was detected as unstained bands against the background of Coomassie blue-stained gelatin. The bar graphs (mean ± SEM, n = 3) represent quantitative densitometric results of upper bands. Values not sharing a letter refer to significant different at $P < 0.05$.

whether the presence of the adipokine resistin promoted vascular SMC migration and whether adding celastrol to human aortic SMC retarded such migration. As expected, resistin markedly enhanced the SMC migration by ≈8-fold (Fig. 3). However, nontoxic celastrol at ≥10 nM significantly demoted the SMC migration. It should be noted that celastrol per se did not influence vascular SMC migration.

### RETARDATION OF COLLAGEN IV AND CTGF INDUCTION BY CELASTROL

CTGF is involved in ECM production and is expressed in stable atherosclerotic plaques [Leeuwis et al., 2010]. This study elucidated the suppressive effects of celastrol on induction of matrix proteins. This study examined that resistin secreted by macrophages in the vessel wall modulated CTGF expression and subsequent secretion of ECM components. CTGF was markedly induced in human aortic SMC exposed to 50 ng/ml resistin (Fig. 4A). In addition, the secretion of collagen I and collagen IV was elevated by culturing SMC with 50 ng/ml resistin (Fig. 4B). It is assumed that resistin secreted from macrophages in the collagen complex may play a role in the ECM production of vascular SMC. When 10–100 nM celastrol was applied to human aortic SMC, the CTGF expression was fully abolished, and the collagens I and IV secretion was dose-dependently diminished (Fig. 4B). Collectively, the diminution of the matrix collagen proteins by celastrol was most likely due to the decrement in the CTGF induction of vascular SMC.

### REDUCTION OF MMP-2 ACTIVITY AND SECRETION BY CELASTROL

Conditioned media of THP-1-derived macrophages strikingly elevated gelatinolytic activity and secretion of MMP-2 in vascular SMC (Fig. 5A,B). When macrophage conditioned media-exposed

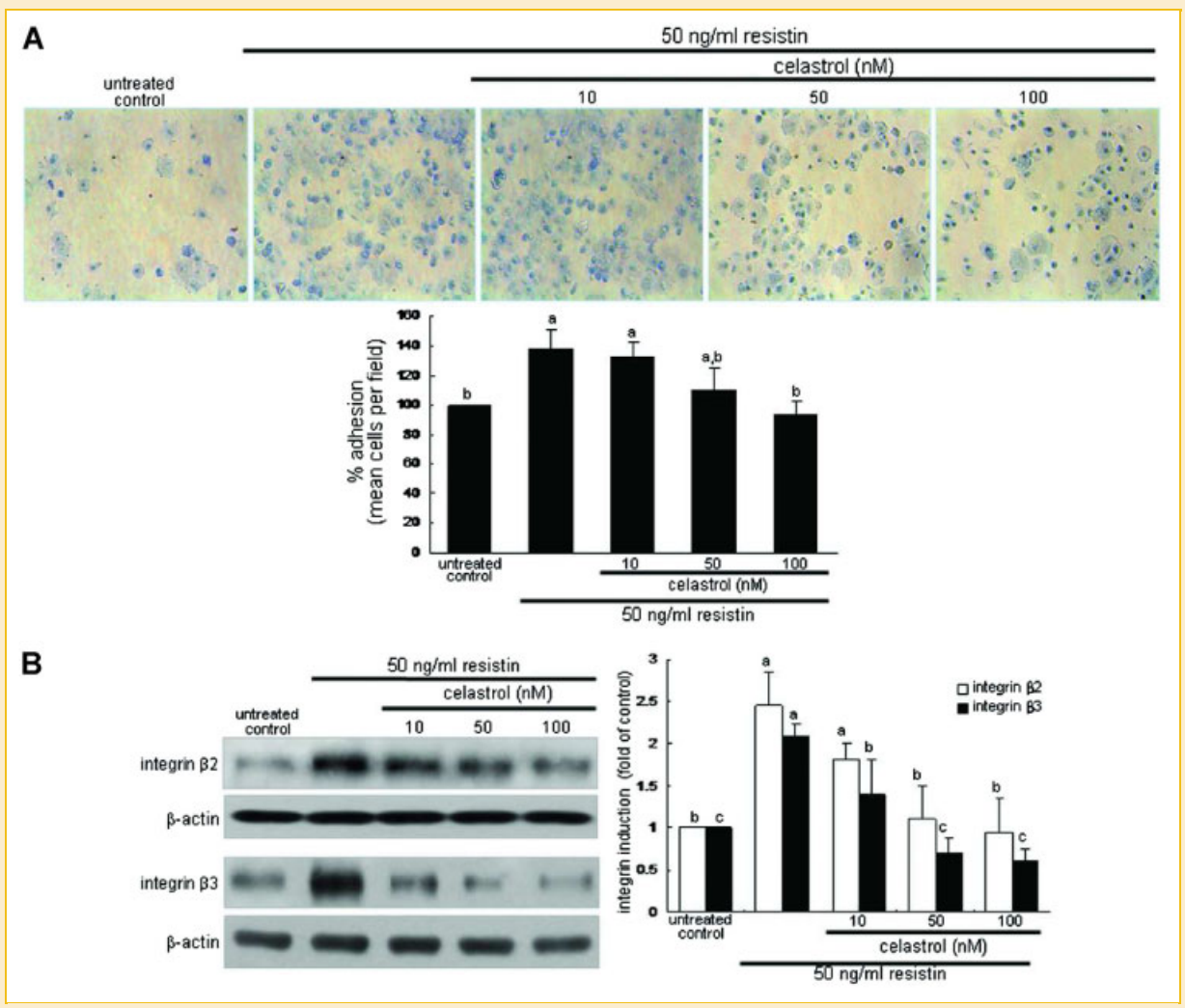


Fig. 6. Celastrol inhibition of resistin-induced SMC adhesion onto the collagen I-coated well plate (A) and resistin-stimulated integrin induction (B). Human aortic SMC were treated with 10–100 nM celastrol for 20 min and exposed to 50 ng/ml resistin. Cells adhered onto collagen I-coated plate for 30 min were stained with toluidine blue and the images were obtained by using a light microscopy (A). For the quantification, SMC were stained with fluorescent calcein-AM. The bar graphs in the bottom panel represent quantitative results obtained by using a Fluoroscan ELISA plate reader at $\lambda = 485$ nm excitation and $\lambda = 538$ nm emission. Equal amounts of cell lysate proteins were subject to 10% SDS–PAGE and Western blot analysis with a primary antibody against integrin β2 or integrin β3 (A), and integrin β2 and integrin β3 (B). β-Actin protein was used as an internal control for cellular expression of integrin β2 and integrin β3. The bar graphs (mean ± SEM, n = 3) represent quantitative densitometric results of upper bands. Means without common letters refer to significant different at $P < 0.05$.

human aortic SMC were treated with ≥50 nM celastrol for 24 h, the gelatinolytic activity of MMP-2 was near-completely abolished (Fig. 5C). It was also found that the macrophage conditioned media-elicited MMP-2 secretion was diminished by treating subnanomolar celastrol (Fig. 5D). Thus, celastrol was effective in encumbering ECM stabilization enhanced by resistin secreted from neighbor monocytes/macrophages.

### SUPPRESSIVE EFFECTS OF CELASTROL ON INTERACTION OF INTIMAL ECM–SMC

Adhesive interactions between vascular SMC and ECM proteins play a vital role in atherosclerotic lesions [Ström et al., 2006]. Resistin enhanced the adhesive interaction of vascular SMC with collagen I, which was markedly inhibited by ≥50 nM celastrol (Fig. 6A). This indicates that celastrol may hamper the assembly of collagen fibrils during SMC phenotypic modulation.

Integrins are known to be a family of cell surface receptors mediating these interactions [Li et al., 2010; Ishigaki et al., 2011]. Resistin promoted the expression of integrin β2 and integrin β3 on human aortic SMC (Fig. 6B). In contrast, ≥10 nM celastrol markedly inhibited the expression of both integrin β2 and integrin β3. Accordingly, the treatment with celastrol that blocked the interaction between ECM proteins and integrins may inhibit SMC migration (Fig. 3).

This study further examined whether resistin induced TLR-4 in vascular SMC and such induction was blocked by celastrol. TLR-4 was induced in human aortic SMC exposed to 50 ng/ml resistin (Fig. 7A). This indicates that TLR-4 was a resistin receptor triggering subsequent activation of downstream signaling. However, ≥10 nM celastrol blunted the TLR-4 induction. In addition, the integrin protein levels enhanced by resistin were downregulated in the presence of 10 μM OxPAPC, a TLR-2/4 signaling inhibitor (Fig. 7B). These results reveal a mechanism whereby celastrol failed to mediate vascular SMC migration through disturbing TLR-4 activation and subsequent interaction with integrin β2/3.

## DISCUSSION

Four major findings were observed in this study. (1) subnanomolar celastrol, a natural terpenoid present in *Tripterygium wilfordii Hook*, inhibited resistin secretion from THP-1-derived macrophages at its transcriptional level. (2) Nontoxic celastrol diminished human aortic SMC transmigration promoted by resistin through demoting the

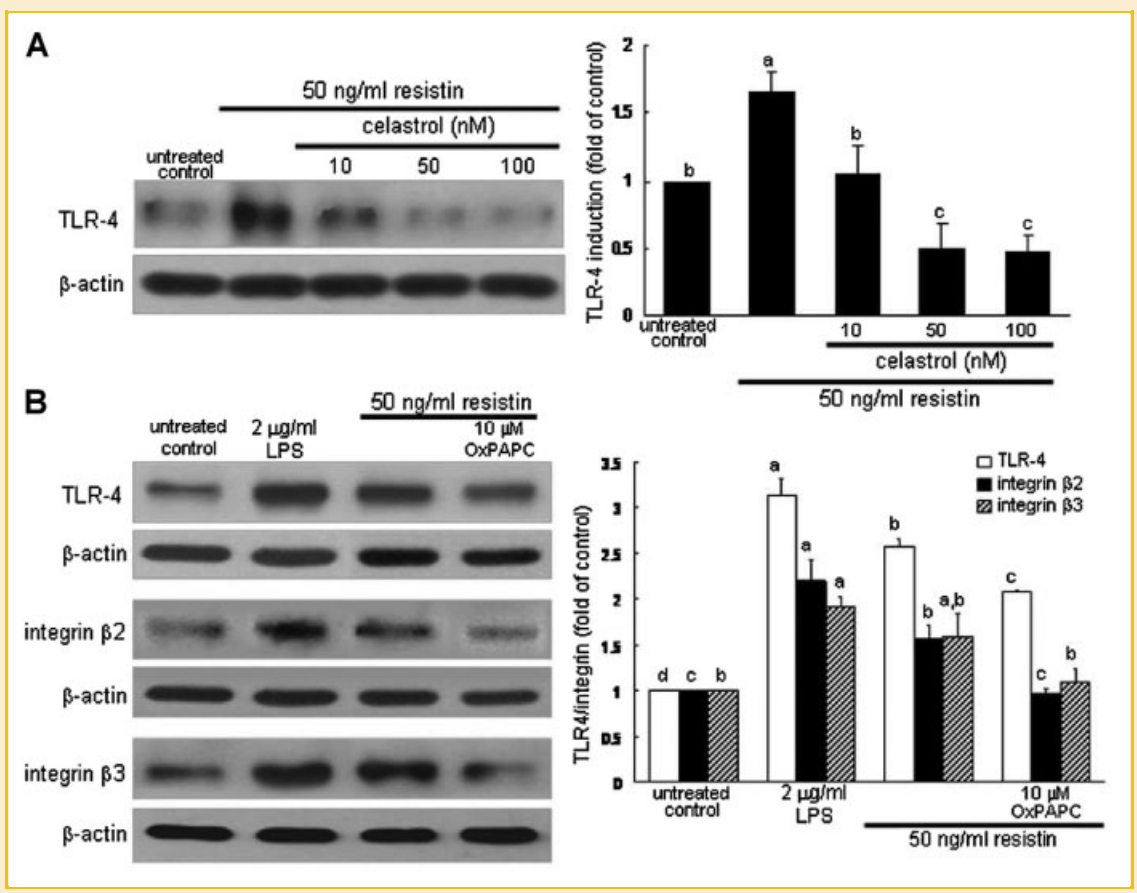


Fig. 7. Suppressive effects of celastrol (A) and OxPAPC (B) on induction of TLR-4, integrin β2 and integrin β3. Human aortic SMC were incubated with and without 10–100 nM celastrol or 10 μM OxPAPC, and exposed to 50 ng/ml resistin. Equal amounts of cell lysate proteins were subject to 10% SDS–PAGE and Western blot analysis with a primary antibody against TLR-4, integrin β2 or integrin β3. β-Actin protein was used as an internal control for cellular expression of TLR-4, integrin β2 and integrin β3. The bar graphs (mean ± SEM, n = 3) represent quantitative densitometric results of upper bands. Values not sharing a letter refer to significant different at $P < 0.05$.

expression of collagen I/IV and attenuating MMP-2 activity. (3) Celastrol at the concentrations of 10–100 nM dose-dependently reduced resistin-enhanced adhesive interaction of SMC to collagen I, concomitant with a marked attenuation of integrin β2/β3 expression. (4) The celastrol treatment attenuated the resistin induction of TLR-4. These observations demonstrate that non-toxic celastrol blocked macrophage-derived resistin-associated SMC proliferation and migration through disturbing the interaction between integrin proteins and ECM proteins via TLR-4 signaling pathway. Accordingly, celastrol was a potential therapeutic agent exerting atheroprotection.

Adipokines, active proteins secreted from adipose tissues, are associated with inflammatory responses and insulin resistance characterized by abnormal cytokine production [Fantuzzi, 2005; Tilg and Moschen, 2006]. Resistin is known as an adipose tissue-specific secretary adipokine to serve various endocrine functions including insulin resistance [Steppan et al., 2001]. It was shown that macrophages are a major source of human resistin [Jung et al., 2006]. In this study, monocyte-derived macrophages secreted resistin that can manipulate vascular SMC through a paracrine fashion. This study found that the macrophage conditioned media containing resistin increased SMC proliferation. This provided the evidence of the crosstalk of vascular SMC and macrophages through resistin in metabolic disorders such as atherosclerosis. Resistin induces human aortic SMC proliferation through both ERK 1/2 and Akt signaling pathways, accounting in part for causing restenosis in diabetes patients [Calabro et al., 2004].

Celastrol, an active quinone methide triterpene present in the root bark of *tripterygium wilfordii Hook F.*, has been employed as an anti-inflammatory therapeutic agent [Liu et al., 2011]. Celastrol suppresses inflammatory and innate immunity responses in human retinal pigment epithelial cells [Paimela et al., 2011]. Especially, celastrol has attracted great interest as a potential anti-cancer agent [Salminen et al., 2010; Kannaiyan et al., 2011]. Celastrol antagonizes invasion and metastasis of cancer cells by down-modulation of expression of CXCR4 chemokine receptor [Yadav et al., 2010]. In addition, celastrol possesses anti-oxidative effect on attenuating hypertension-induced oxidative stress via hemoxygenase-1 induction in vascular SMC [Yu et al., 2010]. A recent study conducting quantitative proteomics reveals numerous cellular targets of celastrol as a valuable biomarker tool of disease treatment strategies [Hansen et al., 2011]. Quantitative proteomics reveals that celastrol activates cellular antioxidant defense systems and mediates induction of stress response pathways. However, the vascular protection of celastrol beneficially modulating neointimal formation in atherosclerosis and restenosis has not yet been defined. The current study revealed that celastrol inhibited the mitogenic and migratory effects of resistin on vascular SMC adjacent to intimal macrophages secreting the adipokine resistin.

Proliferation and migration of vascular SMC into the sub-endothelial space contribute to neointimal formation in atherosclerosis and restenosis [Panchatcharam et al., 2010]. Thus, celastrol hampering resistin-stimulated SMC proliferation and migration may be a therapeutic agent obesity-associated atherosclerosis and restenosis. Also, celastrol attenuated the promoting effect of macrophage conditioned media on matrix-degrading MMP production, suggesting a paracrine action of resistin secreted from intimal macrophages. Mechanisms manipulating the paracrine function of resistin, however, remain to be fully characterized. Recent studies have shown that resistin promotes vascular SMC migration associated with increased MMP expression, which is dependent on PKCε activation [Jiang et al., 2009; Ding et al., 2011].

Celastrol attenuated the collagen matrix synthesis of SMC promoted by resistin, contributing to the impairment of the

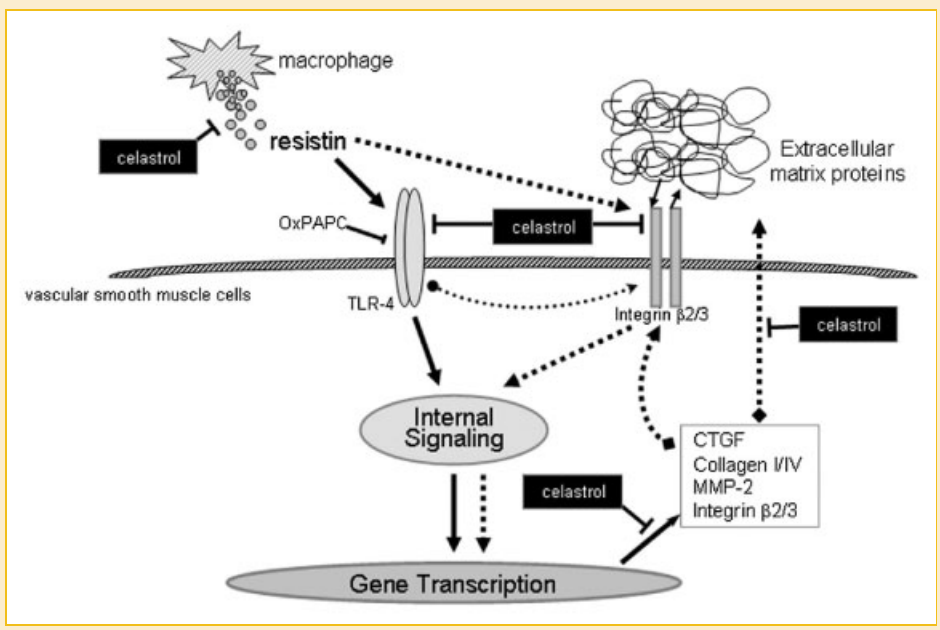


Fig. 8. Schematic diagram showing actions of celastrol blocking impact of macrophage and consequent resistin intimal matrix interaction responsible for SMC migration. The symbol indicates stimulation (→) due to resistin or blockade (⊥) due to treatment with celastrol.

migration of vascular SMC. Collagen may participate in the ECM organization that regulates SMC migration during atherogenic process. In addition, expression of CTGF, known to stimulate vascular SMC proliferation and ECM production in atherosclerosis [Game et al., 2007], was blunted by celastrol. In addition, celastrol blocked the SMC induction of integrin β2/β3 by resistin. The SMC integrin-matrix interaction is functionally essential in many cell migration-dependent processes including wound healing, metastasis and immune defense [Jean et al., 2011; Schultz et al., 2011]. The binding of integrins to molecules present in ECM such as fibrinogen, collagen and laminin contributes to cell migration, adhesion and growth. It was shown that celastrol inhibited the adhesion of SMC to collagen most likely through retarding the induction of integrins. Accordingly, this study revealed that celastrol encumbered vascular SMC migration through disturbing adhesive interaction of vascular SMC with ECM present in the vascular wall.

This study attempted to address the mechanism(s) by which resistin induced SMC migration and the migratory effect was attenuated by celastrol. In this study TLR-4 was induced by resistin, suggesting that TLR-4 may be a SMC receptor for resistin. In addition, the resistin-promoted TLR-4 induction diminished by celastrol appeared to stimulate the expression of integrin β2/β3 proteins. Accordingly, the interplay between SMC integrins and ECM that regulated SMC migration was blocked by celastrol through disturbing the TLR-4 pathway triggered by resistin. On the other hand, a recent study has shown that enhanced proliferation and migration of vascular SMC in response to vascular injury under hyperglycemic conditions is controlled by integrin β3 signaling [Panchatcharam et al., 2010]. It can be assumed that resistin enhanced SMC migration through acting as an integrin β2/β3 ligand and activating its downstream signaling. This study suggests a mechanism whereby celastrol dampened the elevated SMC migration in response to resistin activating integrin β2/β3 pathway.

In summary, the current report demonstrated that celastrol abrogated resistin-induced vascular SMC migration by retarding ECM stabilization and fibril formation and by disturbing the interaction with SMC integrins. The adipokine resistin secreted by intimal macrophages adjacent to vascular SMC may in turn influence the migration of the neighboring SMC as a paracrine mediator (Fig. 8). Furthermore, celastrol mitigated the stimulated TLR-4 induction in response to resistin and subsequent activation of integrin pathway. Therefore, celastrol may be a potential therapeutic agent modulating SMC migration and targeting atheroprotection.

## REFERENCES


Burnett MS, Lee CW, Kinnaird TD, Stabile E, Durrani S, Dullum MK, Devaney JM, Fishman C, Stamou S, Canos D, Zbinden S, Clavijo LC, Jang GJ, Andrews JA, Zhu J, Epstein SE. 2005. The potential role of resistin in atherogenesis. Atherosclerosis 182:241–248.

Calabro P, Samudio I, Willerson JT, Yeh ET. 2004. Resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways. Circulation 110:3335–3340.

Ding Q, Chai H, Mahmood N, Tsao J, Mochly-Rosen D, Zhou W. 2011. Matrix metalloproteinases modulated by protein kinase Cε mediate resistin-induced migration of human coronary artery smooth muscle cells. J Vasc Surg 53:1044–1051.

Espinola-Klein C, Gori T, Blankenberg S, Munzel T. 2011. Inflammatory markers and cardiovascular risk in the metabolic syndrome. Front Biosci 16:1663–1674.

Fantuzzi G. 2005. Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol 115:911–919.

Game BA, He L, Jarido V, Nareika A, Jaffa AA, Lopes-Virella MF, Huang Y. 2007. Pioglitazone inhibits connective tissue growth factor expression in advanced atherosclerotic plaques in low-density lipoprotein receptor-deficient mice. Atherosclerosis 192:85–91.

Hansen J, Palmfeldt J, Vang S, Corydon TJ, Gregersen N, Bross P. 2011. Quantitative proteomics reveals cellular targets of celastrol. PLoS ONE 6:e26634.

Ishigaki T, Imanaka-Yoshida K, Shimojo N, Matsushima S, Taki W, Yoshida T. 2011. Tenascin-C enhances crosstalk signaling of integrin αvβ3/PDGFR-β complex by SRC recruitment promoting PDGF-induced proliferation and migration in smooth muscle cells. J Cell Physiol 226:2617–2624.

Jean C, Gravelle P, Fournie JJ, Laurent G. 2011. Influence of stress on extracellular matrix and integrin biology. Oncogene 30:2697–2706.

Jiang C, Zhang H, Zhang W, Kong W, Zhu Y, Zhang H, Xu Q, Li Y, Wang X. 2009. Homocysteine promotes vascular smooth muscle cell migration by induction of the adipokine resistin. Am J Physiol Cell Physiol 297:C1466–C1476.

Jung HS, Park KH, Cho YM, Chung SS, Cho HJ, Cho SY, Kim SJ, Kim SY, Lee HK, Park KS. 2006. Resistin is secreted from macrophages in atheromas and promotes atherosclerosis. Cardiovasc Res 69:76–85.

Kannaiyan R, Shanmugam MK, Sethi G. 2011. Molecular targets of celastrol derived from Thunder of God Vine: Potential role in the treatment of inflammatory disorders and cancer. Cancer Lett 303:9–20.

Leeuwis JW, Nguyen TQ, Theunissen MG, Peeters W, Goldschmeding R, Pasterkamp G, Vink A. 2010. Connective tissue growth factor is associated with a stable atherosclerotic plaque phenotype and is involved in plaque stabilization after stroke. Stroke 41:2979–2981.

Li C, Wang MH. 2011. Anti-inflammatory effect of the water fraction from hawthorn fruit on LPS-stimulated RAW 264.7 cells. Nutr Res Pract 5:101–106.

Li H, Wang QJ, Zhu DN, Yang Y, Reinioside C. 2008. A triterpene saponin of Polygala aureocauda Dunn, exerts hypolipidemic effect on hyperlipidemic mice. Phytother Res 22:159–164.

Li G, Jin R, Norris RA, Zhang L, Yu S, Wu F, Markwald RR, Nanda A, Conway SJ, Smyth SS, Granger DN. 2010. Periostin mediates vascular smooth muscle cell migration through the integrins alphavbeta3 and alphavbeta5 and focal adhesion kinase (FAK) pathway. Atherosclerosis 208:358–365.

Liu Z, Ma L, Zhou GB. 2011. The main anticancer bullets of the Chinese medicinal herb, thunder god vine. Molecules 16:5283–5297.

Mangge H, Almer G, Truschnig-Wilders M, Schmidt A, Gasser R, Fuchs D. 2010. Inflammation, adiponectin, obesity and cardiovascular risk. Curr Med Chem 17:4511–4520.

Nagaev I, Bokarewa M, Tarkowski A, Smith U. 2006. Human resistin is a systemic immune-derived proinflammatory cytokine targeting both leukocytes and adipocytes. PLoS ONE 1:e31.

Paimela T, Hyttinen JM, Viiri J, Ryhänen T, Karjalainen RO, Salminen A, Kaarniranta K. 2011. Celastrol regulates innate immunity response via NF-κB and Hsp70 in human retinal pigment epithelial cells. Pharmacol Res 64:501–508.

Panchatcharam M, Miriyala S, Yang F, Leitges M, Chrzanowska-Wodnicka M, Quilliam LA, Anaya P, Morris AJ, Smyth SS. 2010. Enhanced proliferation and migration of vascular smooth muscle cells in response to vascular injury under hyperglycemic conditions is controlled by beta3 integrin signaling. Int J Biochem Cell Biol 42:965–974.

Raines EW. 2004. PDGF and cardiovascular disease. Cytokine Growth Factor Rev 15:237–254.

Raines EW, Ferri N. 2005. Thematic review series: The immune system and atherogenesis. Cytokines affecting endothelial and smooth muscle cells in vascular disease. J Lipid Res 46:1081–1092.

Reilly MP, Lehrke M, Wolfe ML, Rohatgi A, Lazar MA, Rader DJ. 2005. Resistin is an inflammatory marker of atherosclerosis in humans. Circulation 111:932–939.

Salminen A, Lehtonen M, Paimela T, Kaarniranta K. 2010. Celastrol: Molecular targets of Thunder God Vine. Biochem Biophys Res Commun 394:439–442.

Schober A. 2008. Chemokines in vascular dysfunction and remodeling. Arterioscler Thromb Vasc Biol 28:1950–1959.

Schultz GS, Davidson JM, Kirsner RS, Bornstein P, Herman IM. 2011. Dynamic reciprocity in the wound microenvironment. Wound Repair Regen 19:134–148.

Silswal N, Singh AK, Aruna B, Mukhopadhyay S, Ghosh S, Ehtesham NZ. 2005. Human resistin stimulates the pro-inflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kappaB-dependent pathway. Biochem Biophys Res Commun 334:1092–1101.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. 2001. The hormone resistin links obesity to diabetes. Nature 409:307–312.

Ström A, Olin AI, Aspberg A, Hultgårdh-Nilsson A. 2006. Fibulin-2 is present in murine vascular lesions and is important for smooth muscle cell migration. Cardiovasc Res 69:755–763.

Tilg H, Moschen AR. 2006. Adipocytokines: Mediators linking adipose tissue, inflammation and immunity. Nat Rev Immunol 6:772–783.

Verma S, Li SH, Wang CH, Fedak PW, Li RK, Weisel RD, Mickle DA. 2003. Resistin promotes endothelial cell activation: Further evidence of adipokine–endothelial interaction. Circulation 108:736–740.

Yadav VR, Sung B, Prasad S, Kannappan R, Cho SG, Liu M, Chaturvedi MM, Aggarwal BB. 2010. Celastrol suppresses invasion of colon and pancreatic cancer cells through the downregulation of expression of CXCR4 chemokine receptor. J Mol Med (Berl) 88:1243–1253.

Yu X, Tao W, Jiang F, Li C, Lin J, Liu C. 2010. Celastrol attenuates hypertension-induced inflammation and oxidative stress in vascular smooth muscle cells via induction of heme oxygenase-1. Am J Hypertens 23:895–903.