

Original Paper

Effect of Salusin- β on Peroxisome Proliferator-Activated Receptor Gamma Gene Expression in Vascular Smooth Muscle Cells and its Possible Mechanism

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Background/Aims: salusin- β is considered to be a potential pro-atherosclerotic factor. Regulation and function of vascular smooth muscle cells (VSMCs) are important in the progression of atherosclerosis. Peroxisome proliferator-activated receptor gamma (PPAR γ) exerts a vascular protective role beyond its metabolic effects. Salusin- β has direct effects on VSMCs. The aim of the present study was to assess the effect of salusin- β on PPAR γ gene expression in primary cultured rat VSMCs. **Methods:** Western blotting analysis, real-time PCR and transient transfection approach were used to determine expression of target proteins. Specific protein knockdown was performed with siRNA transfection. Cell proliferation was determined by 5-bromo-2'-deoxyuridine incorporation. The levels of inflammation indicators interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) were determined using enzyme-linked immunosorbent assay. **Results:** Salusin- β negatively regulated PPAR γ gene expression at protein, mRNA and gene promoter level in VSMCs. The inhibitory effect of salusin- β on PPAR γ gene expression contributed to salusin- β -induced VSMCs proliferation and inflammation *in vitro*. I κ B α -NF- κ B activation, but not NF- κ B p50 or p65, mediated the salusin- β -induced inhibition of PPAR γ gene expression. Salusin- β induced nuclear translocation of histone deacetylase 3 (HDAC3). HDAC3 siRNA prevented salusin- β -induced PPAR γ reduction. Nuclear translocation of HDAC3 in response to salusin- β was significantly reversed by an I κ B α inhibitor BAY 11-7085. Furthermore, I κ B α -HDAC3 complex was present in the cytosol of VSMCs but interrupted after salusin- β treatment. **Conclusion:** I κ B α -HDAC3 pathway may contribute to salusin- β -induced inhibition of PPAR γ gene expression in VSMCs.

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Introduction

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors including three members: PPAR α , PPAR γ , and PPAR β/δ [1]. PPAR γ which is most widely studied has well characterized roles in adipocyte differentiation, lipid metabolism, and glucose homeostasis. Recently, increasing observations and studies indicate PPAR γ is interesting therapeutic targets for the treatment of diseases related to vascular complications such as atherosclerosis and hypertension which are most common morbidity and mortality associated with diabetes and obesity [2]. Besides the reason that improving glucose and lipid homeostasis by treating diabetes or insulin resistance has beneficial effects on cardiovascular diseases, the PPARs and particularly PPAR γ have been implicated in direct actions at the vascular wall. It is clear that in addition to adipose tissue, PPAR γ is also expressed in vascular cells including vascular smooth muscle cells (VSMCs), endothelial cells, adventitial fibroblasts, and macrophages, where it regulates gene expression of key proteins involved in vascular inflammation and proliferation beyond its metabolic effects [3, 4].

Salusin- β is newly identified regulatory peptide of 20 amino acid residues, which is expressed and synthesized ubiquitously within human, rat, and mouse tissues, including the vasculature, central nervous system, and kidneys [5]. Circulating salusin- β levels increase in patients with coronary artery disease [6] and salusin- β immunoreactivity has been detected in human coronary atherosclerotic plaques, with dominance of salusin- β in macrophage foam cells, VSMCs, and fibroblasts [7]. Chronic salusin- β infusion into apolipoprotein E-deficient mice enhances atherosclerotic lesions [8]. Further studies found that salusin- β accelerates inflammatory responses in human endothelial cells and monocyte-endothelial adhesion, stimulates human macrophage foam cell formation, and exerts potent mitogenic effects on both VSMCs and fibroblasts, thus leading to the progression of atherosclerotic diseases [7-11]. These findings suggest that salusin- β is a potential pro-atherosclerotic factor and could be candidate biomarkers and therapeutic targets for atherosclerotic cardiovascular diseases.

Atherosclerosis is a pathologic process occurring within the artery, in which many cell types participate in its initiation and progression in response to various inner- or outer-cellular stimuli. Regulation and function of vascular smooth muscle cells are important in the progression of atherosclerosis [12]. Several groups, including our own, have shown that salusin- β stimulates VSMCs proliferation and cell cycle progression *in vitro* and intimal hyperplasia *in vivo* [5, 7, 11, 13]. But the effects of salusin- β on VSMCs still remain largely unexplored. Growing evidence supports the role of PPAR γ in transcriptional control of vascular smooth muscle cell function. Interference with PPAR γ function in smooth muscle cells causes vascular dysfunction and exacerbates atherosclerosis [14-17]. On the other hand, many preclinical and clinical studies have suggested that PPAR γ agonists protect against the development of atherosclerosis and reduce the development of intimal hyperplasia [4, 15, 18, 19]. Given the role of PPAR γ in VSMCs and atherosclerosis, the aim of the present study was to examine PPAR γ regulation by salusin- β in primary cultured VSMCs.

Materials and Methods

Materials

Salusin- β was purchased from Phoenix Pharmaceuticals, INC (Burlingame, CA, USA) and dissolved by distilled water. The aqueous solution was frozen at -20°C and used within 4 weeks. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). PD98059, SB203580, SP600125 and LY294002 were obtained from Calbiochem (San Diego, CA). Bay 11-7085 was obtained from Selleck Chemicals (Houston, TX, USA). Trichostatin A (TSA) was obtained from sigma (Saint Louis, MO, USA). Rosiglitazone was ordered from Cayman (Ann Arbor, MI). 15-deoxy- $\Delta^{12,14}$ -Prostaglandin J_2 (15d-PG J_2) was obtained from Enzo Biochem (New York, USA). Antibodies against phospho-I κ B α (p-I κ B α), I κ B α , NF κ B p65, NF κ B p50 and histone H $_2$ A

were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody against PPAR γ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against HDAC1, HDAC2 and HDAC3 were obtained from *Enogene* Biotech Co. Ltd (Jiangsu, China). Antibody against GAPDH was obtained from Kangchen Bio-technology (Shanghai, China).

Cell culture

Male Sprague-Dawley rats were obtained from the Animal Center of Nantong University (Nantong, China). All procedures were performed with approval and in accordance with the guidelines of the Animal Care and Use Committee of Nantong University. VSMCs were prepared by the explant method from the thoracic aorta of 2-3 month old male Sprague-Dawley rats as described previously [11]. Confluent cells between passages 3 and 6 were used for the experiments. After 24 h of serum starvation in DMEM with 0.5% FBS, cells were treated with salusin- β in DMEM with 0.5% FBS.

Western blot and Immunoprecipitation

The whole cell lysate, nuclear, and cytoplasmic extracts were prepared by using commercial kits (Beyotime Co., Jiangsu, China) and protein concentration was determined using a BCA Protein Assay Reagent (Beyotime Co., Jiangsu, China) according to the manufacturer's instructions. Equal amounts of protein were separated and then electro-transferred to nitrocellulose membranes (Bio-Rad, USA). Membranes were incubated with the appropriate primary antibodies, and incubated in the dark with the appropriate IRDye 680RD secondary antibodies (1:15000). The blot was imaged and quantitated using the Odyssey infrared imaging system (LI-COR Biosciences, Inc.). To detect multiple signals from one membrane, the membrane was treated with a stripping buffer (Beyotime Co., Jiangsu, China) for 20 min at 37 °C before the next blotting.

For immunoprecipitation, cellular extracts were precleared with protein A/G-agarose (Santa Cruz, CA, USA) for 2h at 4°C. After preclearing, the lysate was incubated with antibody overnight at 4°C. The immune complexes were washed with immunoprecipitation buffer and resolved by SDS-PAGE. Proteins were detected by western blot using specific antibodies.

RNA isolation and real-time PCR

Quantitative real time PCR analysis was used to measure mRNA expression. Total RNA was extracted using Trizol reagent (Takara, Otsu, Shiga, Japan). RNA was added as a template to reverse-transcriptase reactions carried out using the PrimeScript RT Master Mix Kit (Takara, Otsu, Shiga, Japan). PCRs were carried out with the resulting cDNAs using the SYBR Green Premix (Takara, Otsu, Shiga, Japan) with ABI steponeplus real time pcr system (Applied Biosystems, Foster City, CA). The primers are quoted as follows: PPAR γ 5'-ATT CTG GCC CAC CAA CTT CGG -3' (forward), 5'-TGG AAG CCT GAT GCT TTA TCC CCA -3' (reverse); 18S 5'-AGT CCC TGC CCT TTG TAC ACA-3' (forward), 5'-GAT CCG AGG GCC TCA CTA AAC-3' (reverse). Experimental cycle threshold (Ct) values were normalized to housekeeping gene 18S and relative mRNA expression was calculated versus a reference sample. Each sample was run and analyzed in triplicate.

Transient transfection with siRNAs

HDAC1siRNA, HDAC2 siRNA, HDAC3 siRNA, NF κ B p50 siRNA and NF κ B 65 siRNA were purchased from GenePharma RNAi Company (Shanghai, China). The HDAC1 siRNA were: sense 5'-GAC GAC UAC AUC AAG UUC UTT-3' and antisense 5'-AGA ACU UGA UGU AGU CGU CTT-3'. The HDAC2 siRNA were: sense 5'-CUC AUA ACU UGC UUC UAA ATT-3' and antisense 5'-UUUAGAAGCAAGUUAUGAGTT-3'. The HDAC3 siRNA were: sense 5'-GCU UCC AUU CUG AGG ACU ATT-3' and antisense 5'-UAG UCC UCA GAA UGG AAG CTT-3'. The NF κ B p50 siRNA were: sense 5'-GCC CAU ACC UUC AAA UAC UTT-3' and antisense 5'-AGU AUU UGA AGG UAU GGG CTT-3'. The NF κ B p65 siRNA were: sense 5'-GGA GUA CCU GAA GCU AUA TT-3' and antisense 5'-UAU AGC UUC AGG GUA CUC CTT-3'. Nonspecific control siRNA were: sense 5'-UUC UCC GAA CGU GUC ACG UTT-3' and antisense 5'-ACG UGA CAC GUU CGG AGA ATT-3'. siRNAs were reversely transfected into VSMCs using Lipofectamine 2000 according to manufacturer's guidelines as described previously [20]. Protein or mRNA levels were examined by western blot or real-time PCR assays.

Plasmid and transient transfection assays

The PPAR γ promoter luciferase reporter plasmid pPPAR γ -Luc contains the 5'-flanking region (-2333 bp) of the PPAR γ gene promoter in a luciferase reporter plasmid, and was a gift from Yajun Zhou

(Department of Biochemistry and Molecular Biology, Medical College, Nantong University, Nantong, China) [21]. Murine pCMX-PPAR γ was kindly provided by Jianxin Sun (Center for Translational Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania).

VSMCs were cultured in 12-well plastic plates and were transiently transfected with a reporter plasmid expressing Photinus luciferase plus 30 ng of control vector expressing Renilla luciferase (pRL-TK; Promega, Madison, WI, USA) by using LipofectAMINE reagent (Life Technologies, New York, NY, USA) according to manufacturer's instructions. Cell lysates were assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) and the data were expressed as the ratios of Photinus to Renilla luciferase activity for normalization of Photinus luciferase activity.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-6 and TNF- α in the VSMCs culture supernatants were measured with ELISA kits according to the manufacturer's instructions (Shanghai Westang Biotechnology Co. Ltd, Shanghai, China). The OD value of each well was measured at a 450 nm wavelength, and their concentrations were determined by interpolation of a standard calibration curve.

Cell proliferation was measured by 5-bromodeoxyuridine (BrdU)-incorporation assay using a cell proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany). Briefly, after the incubation period, the cells were incubated with a monoclonal anti-BrdU antibody during the last 4 h incubation period, followed by a peroxidase-conjugated secondary antibody for 1.5 h and substrates for detection. BrdU incorporation into DNA was determined by a standard elisa reader.

Statistical analysis

Data were reported as mean \pm SD. All values were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test using Graphpad Prism 5 software; $P < 0.05$ was taken to indicate a significant difference between groups.

Results

Salusin- β reduced PPAR γ gene expression in VSMCs

To examine whether salusin- β regulates PPAR γ gene expression in VSMCs, the cultured VSMCs were stimulated with or without salusin- β . Western blot analysis showed that 10^{-7} mol/L salusin- β significantly reduced PPAR γ protein expression in a time-dependent manner (Fig. 1A). VSMCs were then stimulated with salusin- β at different doses (10^{-9} - 10^{-7} mol/L) for 24h. A dose-dependent inhibitory effect of salusin- β on PPAR γ expression at protein and mRNA levels was demonstrated in Fig. 1B, C. To further verify the role of salusin- β in PPAR γ gene expression, VSMCs were transfected with a PPAR γ promoter luciferase reporter plasmid pPPAR γ -Luc and incubated with salusin- β for 24h. Measurement of luciferase activities demonstrated that salusin- β caused a dose-dependent reduction in PPAR γ promoter activity (Fig. 1D). This result was consistent with that shown in Fig. 1B, C. Taken together, these results strongly suggested that salusin- β might inhibit PPAR γ gene expression in primary cultured rat VSMCs.

I κ B α -NF- κ B activation, but not NF- κ B p50 or p65, mediated salusin- β -induced inhibition of PPAR γ gene expression

Our previous study has shown that mitogen-activated protein kinases (ERK1/2, JNK, and p38) and AKT pathways can be activated by salusin- β in VSMCs [11] and other researchers reported that salusin- β facilitated NF- κ B signals in aortic endothelial cells [8, 9]. To determine which pathway is required for the salusin- β -induced inhibition of PPAR γ gene expression, kinase-specific chemical inhibitors were used to evaluate each pathway. The result showed that pretreatment of VSMCs with a NF- κ B inhibitor, BAY 11-7085 which suppresses I κ B α phosphorylation and thus stabilizes NF- κ B, attenuated salusin- β -induced reductions in PPAR γ protein, mRNA expression and PPAR γ promoter activity, whereas pretreatment with inhibitors of ERK1/2, JNK, p38 or AKT had no significant effect (Fig. 2A,

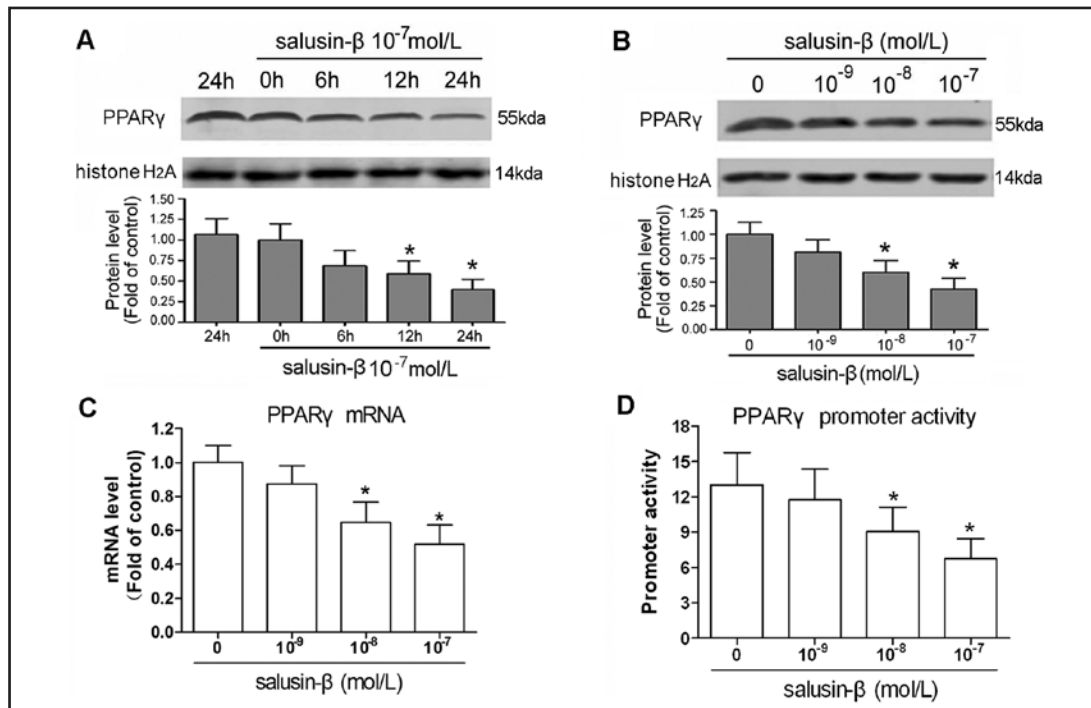


Fig. 1. Salusin- β reduced PPAR γ gene expression in VSMCs. A and B, Serum-starved VSMCs were treated with 10^{-7} mol/L salusin- β for the indicated time periods (A) and various concentration of salusin- β for 24h (B). Nuclear extracts were obtained and PPAR γ protein levels were measured by western blot analysis. Data are expressed as means \pm SD (n=3). * P < 0.05 versus the control with no treatment (0h or 0mol/Lsalusin- β). C, Real-time PCR analysis of PPAR γ mRNA level. VSMCs were stimulated with different concentrations of salusin- β for 24 h. Data are expressed as means \pm SD (n=6). * P < 0.05 versus the control with no treatment (0mol/Lsalusin- β). D, Transfection assay for analysis of PPAR γ promoter activity. VSMCs in 12-well plastic plates were transfected with pPPAR γ -Luc plus control vector pRL-TK and treated with different concentrations of salusin- β for 24 h. Luciferase assay was performed. Data are expressed as means \pm SD (n=6). * P < 0.05 versus the control with no treatment (0mol/Lsalusin- β).

B, C). These findings suggested that it is possible that NF- κ B pathway is involved in salusin- β -induced inhibition of PPAR γ gene expression.

We next confirmed that NF- κ B pathway could be activated by salusin- β in VSMCs. Salusin- β -treatment of VSMCs resulted in robust phosphorylation and loss of cytoplasmic I κ B α at early time period, between 15min and 60min, with robust accumulation of p50 and p65 in the nucleus (Fig. 2D). These results indicate that the salusin- β could activate NF- κ B by classical pathway of phosphorylation and degradation of I κ B α .

On the basis of the above observations, the role of the NF- κ B pathway was further examined. We used RNA interference to knock down NF- κ B p50 and NF- κ B p65 gene expression in VSMCs. However, not as expected, silencing neither NF- κ B p50 nor p65 impacted salusin- β -induced reduction in PPAR γ protein (Fig. 3), mRNA expression and PPAR γ promoter activity (data not shown).

HDAC3 was required for salusin- β -induced inhibition of PPAR γ gene expression

HDACs are repressive transcriptional regulators and involved in various signaling pathways. Because class I members HDAC1, HDAC2 and HDAC3 are associated with both PPAR γ and I κ B α , we further analyzed the role of them in salusin- β -mediated reduction in PPAR γ in VSMCs. Western blot analysis revealed salusin- β treatment had no effect on HDAC1,2 and 3 protein level in whole cell lysates (Fig. 4A). However, with salusin- β treatment, the HDAC3 level was significantly decreased in the cytoplasm, but increased in nuclear

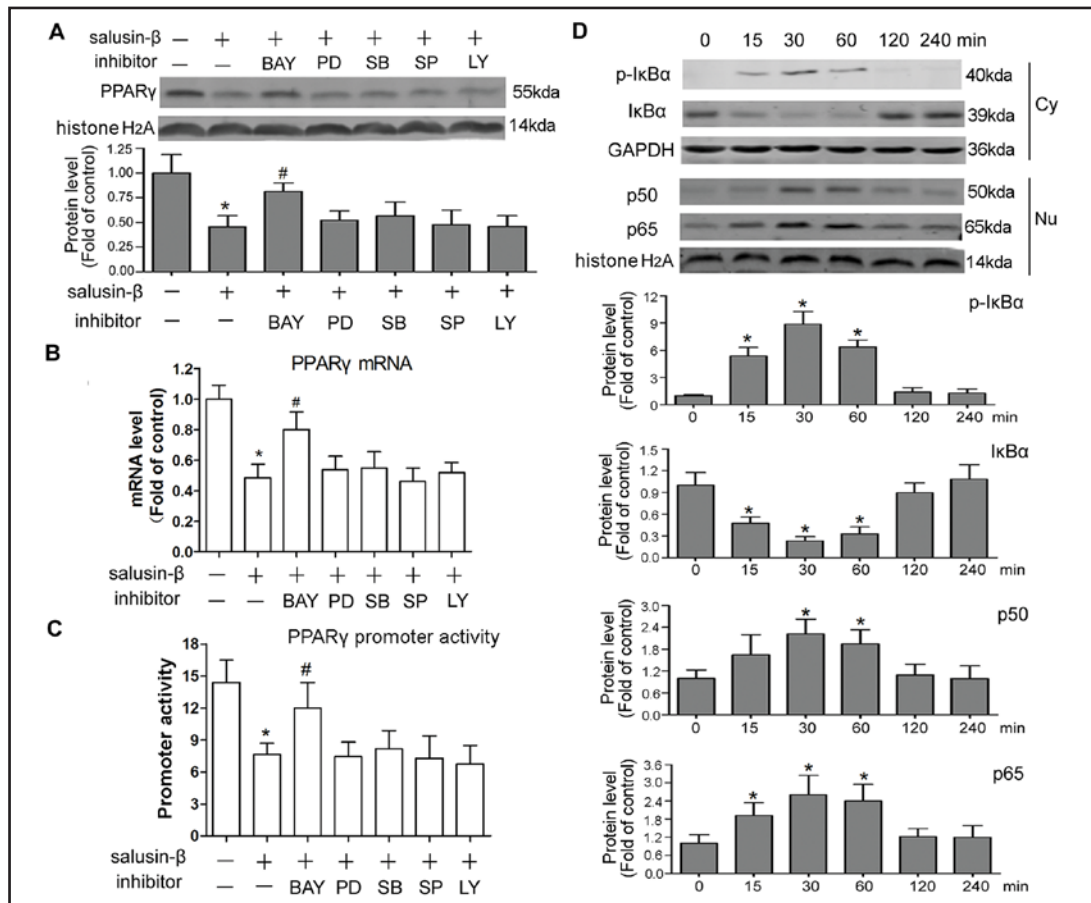


Fig. 2. IκBα-NF-κB activation mediated salusin-β-induced inhibition of PPARγ gene expression. A-C, VSMCs were pretreated with signaling inhibitors for NF-κB, ERK1/2, p38, JNK and AKT [BAY 11-7085 (BAY, 10 μmol/L), PD98059 (PD, 20 μmol/L), SB203580 (SB, 20 μmol/L), SP600125 (SP, 20 μmol/L) and LY294002 (LY, 20 μmol/L), respectively] for 1h and further treated with or without 10⁻⁷mol/L salusin-β for 24h to detect PPARγ expression. A, Nuclear extracts were obtained and protein levels were measured by western blot analysis (n=3). B, mRNA levels were examined by Real-time PCR analysis (n=6). C, Transfection assay for analysis of PPARγ promoter activity (n=6). Data are expressed as means ± SD. *P < 0.05 versus the control with no treatment; #P < 0.05 versus 10⁻⁷mol/L salusin-β treatment alone. D, salusin-β induced IκBα phosphorylation/degradation or p50 and p65 nuclear translocation. Quiescent VSMCs were treated with 10⁻⁷mol/L salusin-β for indicated time periods and then immunoblotted with antibodies against phosphorylated IκBα and IκBα in cytoplasmic extracts (Cy) or p50 and p65 in nuclear extracts (Nu). Data are expressed as means ± SD (n=3). *P < 0.05 versus the control with no treatment (0min).

extracts at 30 min (Fig. 4B and C). Such a quick and correlated HDAC3 change in the two different subcellular compartments suggested nuclear translocation of HDAC3 in response to salusin-β. In the experiment, HDAC1 was only detected in the nucleus, and subcellular distribution of HDAC1 and HDAC2 was not influenced by salusin-β (Fig. 4B and C).

Next, VSMCs were preincubated with a HDAC inhibitor, trichostatin A, for 30 minutes followed by incubation with salusin-β for 24h. Trichostatin A significantly attenuated the inhibitory effect of salusin-β on PPARγ gene expression at protein, mRNA and gene promoter level (Fig. 5A, B and C). To study HDAC proteins specifically, interference RNA (RNAi) knockdown was used in VSMCs (Fig. 5D). The result showed that HDAC3 silencing significantly prevented salusin-β-induced reduction of PPARγ protein, mRNA and promoter activity (Fig. 5E, F and G). Under the same conditions, inhibition of HDAC1 and HDAC2 failed to generate the effect. These data suggest that salusin-β-induced inhibition of PPARγ gene expression is dependent on HDAC3 function.

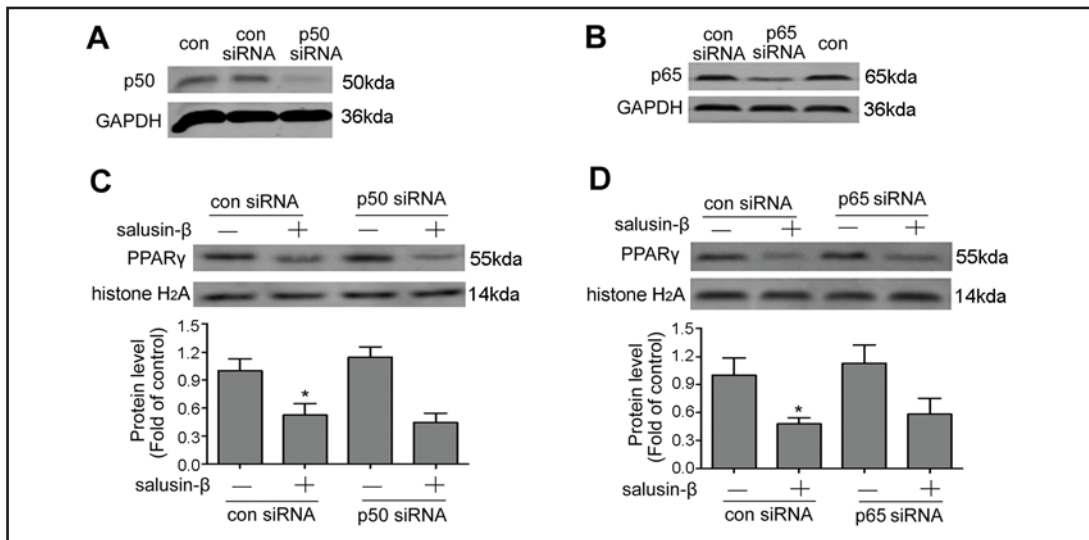


Fig. 3. Effects of depletion of NF-κB p50 and p65 on salusin-β-induced reduction in PPARγ. A and B, VSMCs were transfected with no siRNA (con), control siRNA (con siRNA), p50 siRNA or p65 siRNA. p50 (A) or p65 (B) protein level were assessed 24h after transfection by western blot. Representatives were shown here from three independent experiments. C and D, VSMCs were transfected with control siRNA (con siRNA), p50 siRNA or p65 siRNA for 24h, serum-starved for 24h, and treated with or without 10^{-7} mol/L salusin-β for 24h to detect PPARγ expression by western blot analysis. Data are expressed as means \pm SD (n=3). * $P < 0.05$ versus the con siRNA without salusin-β (the first column on the left).

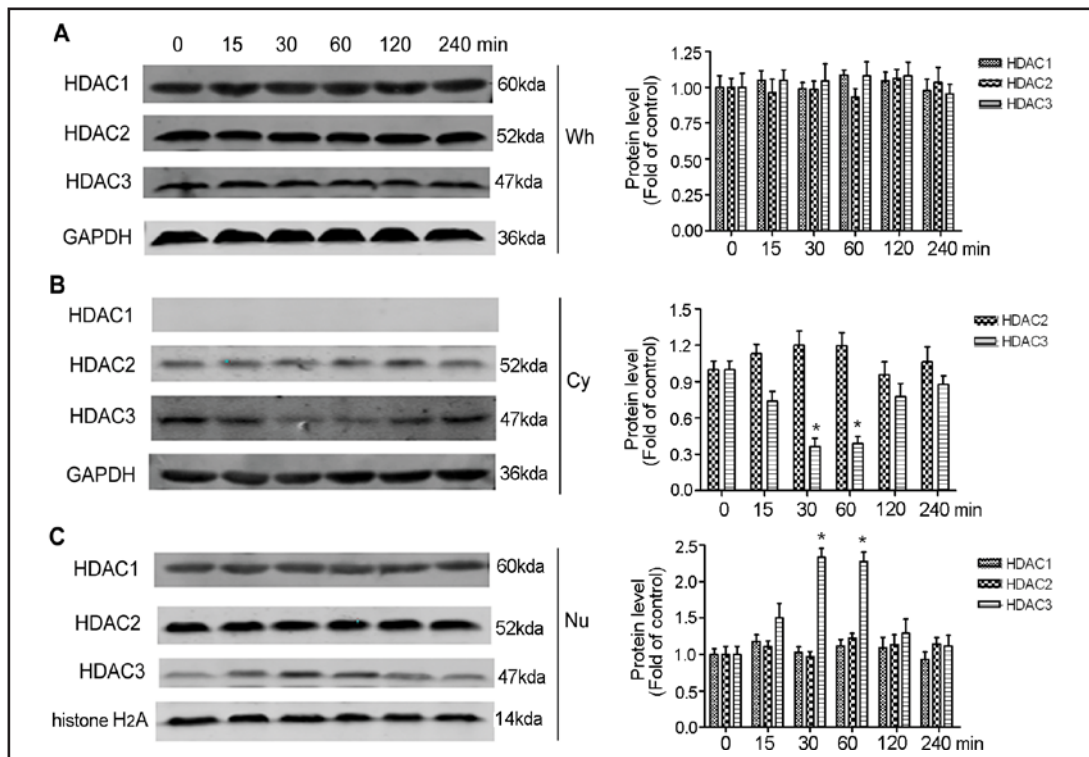


Fig. 4. HDAC3 translocation to the nucleus in response to salusin-β. Quiescent VSMCs were treated with 10^{-7} mol/L salusin-β for indicated time periods, and then the whole cell lysates (Wh) (A), cytoplasmic lysates (Cy) (B) and nuclear lysates (Nu) (C) were analyzed by western blot using the HDAC1, HDAC2 or HDAC3 antibody. Data are expressed as means \pm SD (n=3). * $P < 0.05$ versus the control with no treatment (0min).

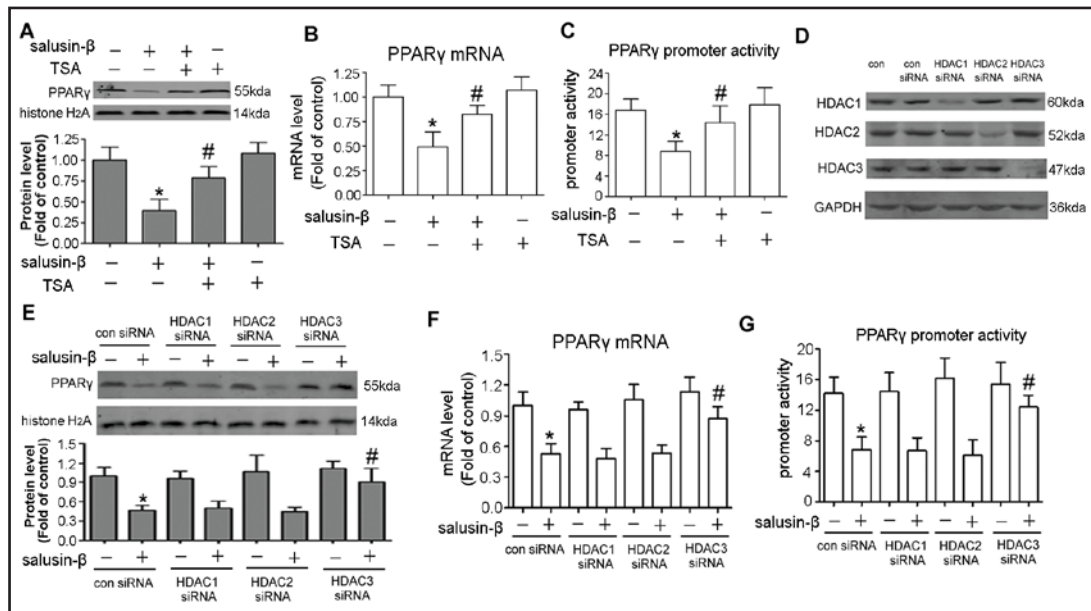


Fig. 5. Inhibition of salusin- β activity by HDAC inhibitor and HDAC siRNA. A-C, VSMCs were pretreated with HDAC Inhibitor TSA (1 μ mol/L) for 30min and further treated with or without 10⁻⁷mol/L salusin- β for 24h to detect PPAR γ expression. A, Nuclear extracts were obtained and protein levels were measured by western blot analysis (n=3). B, mRNA levels were examined by Real-time PCR analysis (n=6). C, Transfection assay for analysis of PPAR γ promoter activity (n=6). Data are expressed as means \pm SD. **P* < 0.05 versus the control with no treatment; #*P* < 0.05 versus 10⁻⁷mol/L salusin- β treatment alone. D, VSMCs were transfected with no siRNA (con), control siRNA (con siRNA), HDAC1 siRNA, HDAC2 siRNA and HDAC3 siRNA. HDAC1, HDAC2 and HDAC3 protein level were assessed 24h after transfection by western blot. Representatives were shown here from three independent experiments. E-G, VSMCs were transfected with control siRNA (con siRNA), HDAC1 siRNA, HDAC2 siRNA or HDAC3 siRNA for 24h, serum-starved for 24h, and treated with or without 10⁻⁷mol/L salusin- β for 24h to detect PPAR γ expression. E, Nuclear extracts were obtained and protein levels were measured by western blot analysis (n=3). F, mRNA levels were examined by Real-time PCR analysis (n=6). G, Transfection assay for analysis of PPAR γ promoter activity (n=6). Data are expressed as means \pm SD. **P* < 0.05 versus the con siRNA without salusin- β (the first column on the left); #*P* < 0.05 versus con siRNA with 10⁻⁷mol/L salusin- β treatment (the second column on the left).

I κ B α may mediate nuclear translocation of HDAC3 in response to salusin- β in VSMCs

We next investigated whether BAY 11-7085 influences nuclear translocation of HDAC3 in response to salusin- β . VSMCs were pretreated with BAY 11-7085 for 1h before incubation with salusin- β for an additional 30min. As shown in Fig. 6A, salusin- β -induced I κ B α phosphorylation, degradation and nuclear translocation of p50 and p65 were significantly reversed by BAY 11-7085. Furthermore, Bay11-7085 pretreatment caused evidently increased protein expression of HDAC3 in the cytoplasm and accordingly decreased protein expression of HDAC3 in the nuclear compartment (Fig. 6B). Thus, the nuclear translocation of HDAC3 may be a consequence of I κ B α degradation. To test this possibility, the association of HDAC3 with I κ B α was investigated using coimmunoprecipitation. Cytoplasmic extract of VSMCs were immunoprecipitated with either anti-I κ B α or HDAC3 antibody. As expected, HDAC3 was found in the immunoprecipitation product of I κ B α antibody, and I κ B α was identified in the immunoprecipitation product of HDAC3 antibody strongly indicating that I κ B α and HDAC3 coexist in the same protein complex in cells (Fig. 6C). However, treatment with salusin- β for 30min, I κ B α was little detected in the immunoprecipitation product of HDAC3 (Fig. 6D), consisting with the observation that I κ B α was degraded by salusin- β stimulation at early time (Fig. 2D). Taken together, these data suggest that I κ B α may mediate nuclear translocation of HDAC3 in response to salusin- β in VSMCs.

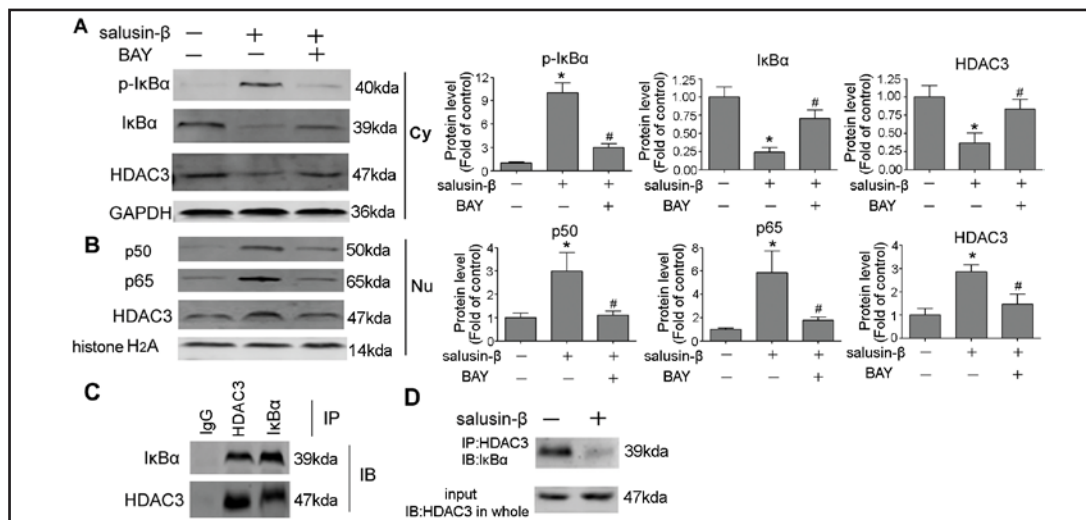


Fig. 6. I κ B α may mediate nuclear translocation of HDAC3 in response to salusin- β in VSMCs. A and B, VSMCs were pretreated with BAY 11-7085 (BAY, 10 μ mol/L) for 1h and further treated with or without 10⁻⁷mol/L salusin- β for 30min. A, Cytoplasmic extracts (Cy) were obtained and the phosphorylated I κ B α , I κ B α and HDCA3 protein levels were measured by western blot analysis. B, nuclear extracts (Nu) were obtained and the nuclear p50, p65 and HDAC3 protein levels were measured by western blot analysis. Data are expressed as means \pm SD (n=3). *P < 0.05 versus the control with no treatment; #P < 0.05 versus 10⁻⁷mol/L salusin- β treatment alone. C, association of I κ B α and HDAC3 in coimmunoprecipitation. The cytoplasmic extract was made from VSMCs and used in the coimmunoprecipitation. IgG was a control for nonspecific signal. Representatives were shown here from three independent experiments. D, Serum-starved VSMCs were treated with 10⁻⁷mol/L salusin- β for 30min, The amount of I κ B α associated with the HDAC3 protein in cytoplasmic extract was assessed by immunoprecipitation. Input samples were loaded as 10% of whole cell lysates used for the immunoprecipitation. Representatives were shown here from three independent experiments.

Increased cellular levels of PPAR γ protein or PPAR γ ligands down-regulated salusin- β -induced VSMCs proliferation and inflammation

To directly assess the functional significance of salusin- β -induced PPAR γ suppression, we tested whether increasing PPAR γ expression antagonizes the proliferative and inflammatory effects of salusin- β in VSMCs. Transfection of VSMCs with 0.4 μ g of PPAR γ -expression plasmid significantly increased PPAR γ protein expression (Fig. 7A). The result showed that increasing amounts of transfected PPAR γ expression plasmid reduced salusin- β -induced VSMCs proliferation as indicated by BrdU incorporation into DNA in a dose-dependent manner (Fig. 7B). Moreover, salusin- β could promote the release of inflammation indicators interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) from VSMCs, while this release was also inhibited in VSMCs transfected with the PPAR γ expression plasmid (Fig. 7C and D).

Similarly, pretreatment with the PPAR γ natural ligand 15d-PGJ₂ and synthetic ligand rosiglitazone significantly blocked salusin- β -induced VSMCs proliferation and inflammation (Fig. 8).

Discussion

In the present study, we examined PPAR γ regulation by salusin- β in primary cultured rat VSMCs and investigated its possible mechanism. The findings of current study provided the first demonstration that salusin- β directly inhibited PPAR γ gene expression at protein level, mRNA level and PPAR γ gene promoter activity level. Furthermore, salusin- β activated

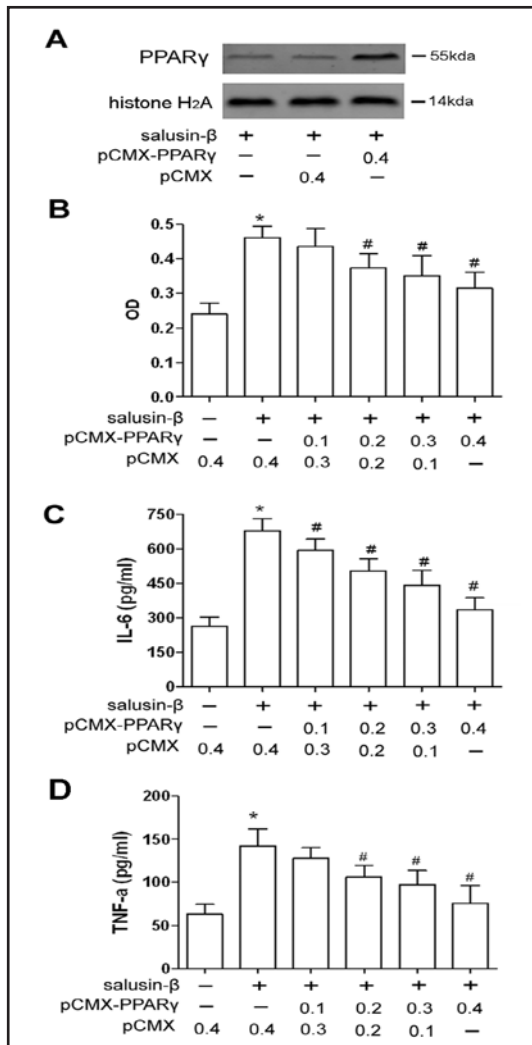


Fig. 7. Increased cellular levels of PPAR γ protein down-regulated salusin- β -induced VSMCs proliferation and inflammation. VSMCs were transfected with increased amount of pCMX-PPAR γ expression plasmid or pCMX empty vector for 24h. The empty vector was used to ensure the equal amount of total DNA in transfection assay. After transfection and recovery, cells were serum-starved for 24 h before the stimulation with or without 10^{-7} mol/L salusin- β for an additional 24 h. A, Nuclear extracts were obtained and PPAR γ protein levels were measured by westernblot analysis. Representatives were shown here from three independent experiments. B, Cell proliferation was determined by the Brdu assay. C and D, the levels of IL-6 and TNF- α were determined using an ELISA assay. Data are expressed as means \pm SD (n=6). * P < 0.05 versus the 0.4μ g pCMX empty vector without salusin- β (the first column on the left); # P < 0.05 versus 0.4μ g pCMX empty vector with 10^{-7} mol/L salusin- β treatment (the second column on the left).

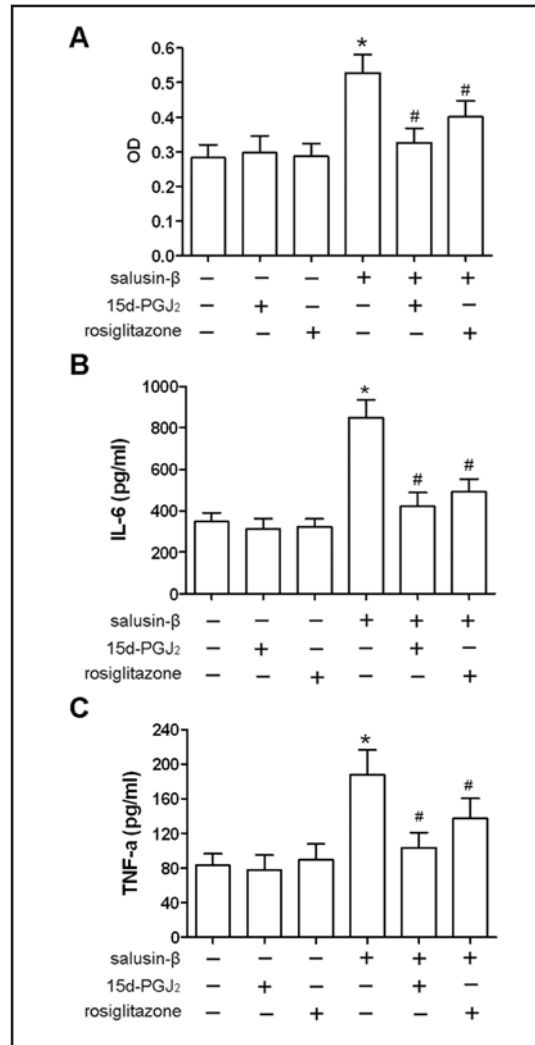


Fig. 8. PPAR γ ligands inhibited salusin- β -induced VSMCs proliferation and inflammation. A-C, VSMCs were pretreated with PPAR γ natural ligand 15d-PGJ $_2$ (5μ mol/L) or synthetic ligand rosiglitazone (5μ mol/L) for 1h and further treated with or without 10^{-7} mol/L salusin- β for 24h. A, Cell proliferation was determined by the Brdu assay. B and C, the levels of IL-6 and TNF- α were determined using an ELISA assay. Data are expressed as means \pm SD (n=6). * P < 0.05 versus the control with no treatment; # P < 0.05 versus 10^{-7} mol/L salusin- β treatment alone.

I κ B α -NF- κ B, but decreased PPAR γ gene expression through HDAC3 without involving NF- κ B p50 and p65. In addition, the inhibitory effect of salusin- β on PPAR γ gene expression contributes to salusin- β -induced VSMCs proliferation and inflammation *in vitro*.

PPAR γ has been recognized as an important regulator in the vasculature and maintaining an appropriate expression level in vascular wall cells is essential for assuring that vascular structure and function adapt to atherogenic factors acting on the vascular wall [4]. VSMCs are the main constitutive stromal cells of the vascular wall, continually exposed to mechanical signals and biochemical components generated in the blood compartment [22]. Various genes have been examined with respect to their abilities to prevent atherosclerosis, and of these, PPAR γ is a good candidate because it is present in all cells composing blood vessels including VSMCs [4]. Many studies suggest that PPAR γ ligands such as the thiazolidinediones not only ameliorate insulin sensitivity, but also have pleiotropic effects on the pathophysiology of vascular disease including atherosclerosis [18, 19]. Activation of PPAR γ suppresses production of inflammatory cytokines, and there is accumulating data that PPAR γ ligands exert anti-inflammatory, antioxidative, and antiproliferative effects on VSMCs [2, 4]. In addition to the atheroprotective effects of PPAR γ ligands identifying a potential role for PPAR γ as a regulator of VSMCs biology, many genetic evidences further support a normal role for PPAR γ in inhibiting VSMCs proliferation and inflammation in the context of restenosis or atherosclerosis. For example, dominant-negative loss-of-function PPAR γ mutations have also been introduced into *in vivo* and *in vitro* experiments. It is shown that transfer of a PPAR γ -dominant-negative gene promoted neointima formation in balloon injured animal arteries and enhances VSMCs proliferation, inflammation, and vascular remodeling [15-17]; in contrast transfer of the PPAR γ -wild-type gene was found to inhibit smooth muscle proliferation and reduce neointima formation after balloon injury [15, 17]. In addition, VSMCs-specific PPAR γ deficiency augmented angiotensin II-induced atherosclerosis [23, 24]. It should be noted that many growth factors and cytokines, such as platelet-derived growth factor (PDGF), TGF- β , or angiotensin II-induced atherosclerosis through interactions with PPAR γ expressed in VSMCs [25, 26]. As a new potential proinflammatory and proatherosclerotic factor, salusin- β has direct effects on VSMCs [5, 11, 13]. The present results showed that salusin- β caused a marked reduction in PPAR γ gene expression at protein and mRNA levels and reduced PPAR γ gene promoter activity in VSMCs. Since vascular PPAR γ plays a pivotal role in protection from vascular disease, we speculate that the inhibitory action of salusin- β on PPAR γ gene expression contributes to its proliferative and inflammatory effects on VSMCs. This postulate is confirmed by the result that increased cellular levels of PPAR γ protein down-regulated salusin- β -induced VSMCs proliferation and inflammation in the present study.

PPAR γ has also been shown *in vitro* to function at the nodal point of various cell-signaling pathways [17, 27]. Our previous study revealed that salusin- β stimulation in VSMCs invokes mitogen-activated protein kinases (MAPKs) and AKT signaling pathways which are key components of the signals that transduce extracellular stimuli into cells [11]. However, the present study showed that they were not involved in salusin- β inhibition of PPAR γ gene expression. Other studies have demonstrated that salusin- β is a powerful activator of the NF- κ B pathway in both cultured endothelial cells and in intact arteries [8, 9, 10]. NF- κ B is a transcription factor that participates in vascular damage through the regulation of several genes involved in proliferation and immune responses. The prototypical NF- κ B heterodimer consists of RelA (p65) and p50 that contain the transcriptional activation domain. The classical NF- κ B activation model involves cytoplasmic sequestration of NF- κ B by inhibitory protein I κ B α , preventing NF- κ B from migrating to the nucleus. When I κ B α is phosphorylated, it is degraded, allowing NF- κ B to translocate to the nucleus, followed by the activation of specific target genes [28, 29]. The present study showed that BAY 11-7085, a known pharmacologic inhibitor of I κ B α phosphorylation [30], markedly suppressed salusin- β -induced reduction in PPAR γ gene expression. Furthermore, to our knowledge, the present study is the first to report that like in endothelial cells, salusin- β could also activate NF- κ B by classical pathway of phosphorylation and degradation of I κ B α with parallel induction of

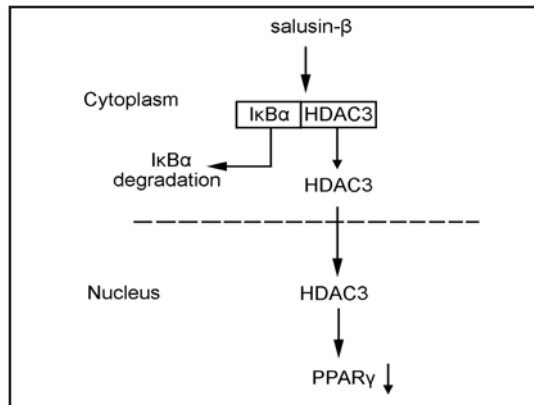
nuclear translocation of NF- κ B p65 and p50 in VSMCs. However, silencing with NF- κ B p50 or p65 did not impact salusin- β -induced down-regulation of PPAR γ gene expression. These findings suggest that I κ B α may have a new and NF- κ B-independent role in salusin- β -induced PPAR γ reduction.

HDACs are enzymes that remove acetylation from lysine residues within histones, resulting in a closed chromatin structure and an overall reduction in transcriptional potential. The interplay between histone acetyltransferases and HDACs permits rapid changes in gene expression in response to extrinsic or intrinsic signals [31, 32]. There-

fore, although originally shown to be involved in cancer and neurological disease, HDACs have been found to be able to modulate most steps of atherosclerosis in the traditional view, e.g. inflammation, proliferation and apoptosis of both ECs and VSMCs [31]. There are 18 characterized members of HDACs, which have been classified into four groups. Class I members HDAC1, HDAC2 and HDAC3 are considered as mitogen-responsive genes in VSMCs [33]. Moreover, HDAC1 and HDAC3 are well known to be transcriptional corepressors of PPAR γ . For example, in adipose cells, recruitment of HDAC1 and HDAC3 are involved in the regulation of PPAR γ function [34, 35], leading to the inhibition of adipocyte differentiation. siRNA-mediated inhibition of HDAC3 attenuated angiotensin II mediated reductions in PPAR γ abundance in VSMCs [26]. TGF- β 1 down-regulates PPAR γ gene expression through recruitment of HDAC1 at the PPAR γ promoter in cardiac fibroblasts [36]. Importantly, it has been reported that cytoplasmic I κ B α increases NF- κ B-independent transcription through binding to HDAC1 and HDAC3 [37]. Thus, we next examined the effects of salusin- β on HDAC1, HDAC2 and HDAC3 and the relationship between them and PPAR γ . Our results showed that salusin- β induced nuclear translocation HDAC3, which was required for salusin- β -induced inhibition of PPAR γ gene expression. HDAC3 is found to shuttle between nucleus and cytoplasm [31]. Since it is generally believed that HDACs could suppress gene expression only when they are in the nucleus [31], modulation of cellular localization is considered to be critical in the effect of HDAC3 on PPAR γ in the present study. It has been reported that nuclear translocation of HDAC3 is controlled by I κ B α in the I κ B α -HDAC3 model. I κ B α binds HDAC3 in the cytosol through the ankyrin repeats [37], and I κ B α degradation leads to nuclear translocation of HDAC3 [38]. In the present study, the same mechanism is shown to be responsible for the PPAR γ inhibition by salusin- β in VSMCs by four lines of evidence: (a) Either I κ B α phosphorylation inhibitor Bay11-7085 or siRNA knockdown targeting HDAC3 can significantly inhibit salusin- β -induced reduction in PPAR γ gene expression; (b) salusin- β -induced nuclear translocation of HDAC3 was coupled with I κ B α phosphorylation and I κ B α degradation; (c) nuclear translocation of HDAC3 in response to salusin- β was significantly reversed by BAY 11-7085; (d) The present study firstly demonstrated that I κ B α -HDAC3 complex was also present in the cytosol of VSMCs but interrupted after salusin- β treatment in parallel with I κ B α degradation and HDAC3 translocation.

Conclusion: The present study indicated that salusin- β negatively regulated PPAR γ gene expression at protein, mRNA and gene promoter level in VSMCs and I κ B α -HDAC3 pathway may contribute to salusin- β -induced inhibition PPAR γ gene expression (Fig. 9). Given that serum salusin- β levels are significantly higher in patients undergoing coronary artery disease than those in healthy controls [6], the results in the present study might have potential implications for clarifying the mechanisms of presence and severity of coronary artery disease associated with elevated salusin- β level.

Fig. 9. Mechanism of inhibitory action of salusin- β on PPAR γ gene expression in VSMCs.



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Disclosure Statement

The authors report no conflicts of interest.

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