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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Key Words
Salusin-β • Smooth muscle cells • Signal transduction • Vascular biology

Abstract
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\textgamma) 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\textgamma 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\textgamma gene expression at protein, mRNA and gene promoter level in VSMCs. The inhibitory effect of salusin-β on PPAR\textgamma gene expression contributed to salusin-β-induced VSMCs proliferation and inflammation in vitro. \textit{IκBα-NF-κB} activation, but not NF-κB p50 or p65, mediated the salusin-β-induced inhibition of PPAR\textgamma gene expression. Salusin-β induced nuclear translocation of histone deacetylase 3 (HDAC3). HDAC3 siRNA prevented salusin-β-induced PPAR\textgamma reduction. Nuclear translocation of HDAC3 in response to salusin-β was significantly reversed by an IκBα inhibitor BAY 11-7085. Furthermore, IκBα-HDAC3 complex was present in the cytosol of VSMCs but interrupted after salusin-β treatment. Conclusion: IκBα-HDAC3 pathway may contribute to salusin-β-induced inhibition of PPAR\textgamma gene expression in VSMCs.
Introduction

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors including three members: PPARα, PPARγ, and PPARβ/δ [1]. PPARγ which is most widely studied has well characterized roles in adipocyte differentiation, lipid metabolism, and glucose homeostasis. Recently, increasing observations and studies indicate PPARγ 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 PPARγ have been implicated in direct actions at the vascular wall. It is clear that in addition to adipose tissue, PPARγ 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 PPARγ in transcriptional control of vascular smooth muscle cell function. Interference with PPARγ 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 PPARγ agonists protect against the development of atherosclerosis and reduce the development of intimal hyperplasia [4, 15, 18, 19]. Given the role of PPARγ in VSMCs and atherosclerosis, the aim of the present study was to examine PPARγ 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-Δ12,14-Prostaglandin J2 (15d-PGJ2) was obtained from Enzo Biochem (New York, USA). Antibodies against phospho-IκBα (p-IκBα), IκBα, NFκB p65, NFκB p50 and histone H2A
were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody against PPARγ 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 2 h 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: PPARγ 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 ACA-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**

HDAC1 siRNA, HDAC2 siRNA, HDAC3 siRNA, NFκB p50 siRNA and NFκB 65 siRNA were purchased from GenePharma RNAi Company (Shanghai, China). The HDAC1 siRNA were: sense 5’-GAC GAC UAC AUC AAC UUC UTT-3’ and antisense 5’-AGA UGU AGU UGU AGU CTT-3’. The HDAC2 siRNA were: sense 5’-CUU ACA UCG UUC UAA ATT-3’ and antisense 5’-UUA UAGAACAAGUAUAGGTT-3’. The HDAC3 siRNA were: sense 5’-GCU UCC AUU CUG AGG ACU ATT-3’ and antisense 5’-UAG UCC UCA GAA UGG AGG CTT-3’. The NFκB p50 siRNA were: sense 5’-GCC CAU ACCU UAAA UACU TT-3’ and antisense 5’-AGU AUU UAG AGA UGG CTT-3’. The NFκB p65 siRNA were: sense 5’-GCC CAU ACCU UAAA UACU TT-3’ and antisense 5’-AGU AUU UAG AGA UGG CTT-3’. Nonspecific control siRNA were: sense 5’-UCC UCC GGU GGU UCC CTT-3’ and antisense 5’-GUU UUAGG GGU GGU CTT-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 PPARγ promoter luciferase reporter plasmid pPPARγ-Luc contains the 5’-flanking region (-2333 bp) of the PPARγ gene promoter in a luciferase reporter plasmid, and was a gift from Yajun Zhou.
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(Department of Biochemistry and Molecular Biology, Medical College, Nantong University, Nantong, China) [21]. Murine pCMX-PPARγ 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 ± 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 PPARγ gene expression in VSMCs
To examine whether salusin-β regulates PPARγ gene expression in VSMCs, the cultured VSMCs were stimulated with or without salusin-β. Western blot analysis showed that 10⁻⁷mol/L salusin-β significantly reduced PPARγ protein expression in a time-dependent manner (Fig. 1A). VSMCs were then stimulated with salusin-β at different doses (10⁻⁹-10⁻⁷mol/L) for 24h. A dose-dependent inhibitory effect of salusin-β on PPARγ expression at protein and mRNA levels was demonstrated in Fig. 1B, C. To further verify the role of salusin-β in PPARγ gene expression, VSMCs were transfected with a PPARγ promoter luciferase reporter plasmid pPPARγ-Luc and incubated with salusin-β for 24h. Measurement of luciferase activities demonstrated that salusin-β caused a dose-dependent reduction in PPARγ 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 PPARγ gene expression in primary cultured rat VSMCs.

IκBα-NF-κB activation, but not NF-κB p50 or p65, mediated salusin-β-induced inhibition of PPARγ 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-κB signals in aortic endothelial cells [8, 9]. To determine which pathway is required for the salusin-β-induced inhibition of PPARγ gene expression, kinase-specific chemical inhibitors were used to evaluate each pathway. The result showed that pretreatment of VSMCs with a NF-κB inhibitor, BAY 11-7085 which suppresses IκB phosphorylation and thus stabilizes NF-κB, attenuated salusin-β-induced reductions in PPARγ protein, mRNA expression and PPARγ promoter activity, whereas pretreatment with inhibitors of ERK1/2, JNK, p38 or AKT had no significant effect (Fig. 2A,
NF-κB pathway was involved in salusin-β-induced inhibition of PPARγ gene expression. We next confirmed that NF-κB pathway could be activated by salusin-β in VSMCs. Salusin-β treatment of VSMCs resulted in robust phosphorylation and loss of cytoplasmic IκBα 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-κB by classical pathway of phosphorylation and degradation of IκBα.

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

HDAC3 was required for salusin-β-induced inhibition of PPARγ 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 PPARγ and IκBα, we further analyzed the role of them in salusin-β–mediated reduction in PPARγ 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. 1. Salusin-β reduced PPARγ gene expression in VSMCs. A and B, Serum-starved VSMCs were treated with 10⁻⁷mol/L salusin-β for the indicated time periods (A) and various concentration of salusin-β for 24h (B). Nuclear extracts were obtained and PPARγ protein levels were measured by western blot analysis. Data are expressed as means ± SD (n=3). *P < 0.05 versus the control with no treatment (0h or 0mol/L salusin-β). C, Real-time PCR analysis of PPARγ mRNA level. VSMCs were stimulated with different concentrations of salusin-β for 24 h. Data are expressed as means ± SD (n=6). *P < 0.05 versus the control with no treatment (0mol/L salusin-β). D, Transfection assay for analysis of PPARγ promoter activity. VSMCs in 12-well plastic plates were transfected with pPPARγ-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 ± SD (n=6). *P < 0.05 versus the control with no treatment (0mol/L salusin-β).
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. 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, JNK and AKT [BAY 11-7085 (BAY, 10μmol/L), PD98059 (PD, 20μmol/L), SB203580 (SB, 20μmol/L), SP600125 (SP, 20μmol/L) and LY294002 (LY, 20μmol/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).
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. Representations 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 10⁻⁷mol/L salusin-β for 24h to detect PPARγ expression by western blot analysis. Data are expressed as means ± 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 10⁻⁷mol/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 ± SD (n=3). *P< 0.05 versus the control with no treatment (0min).
IκBα 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 IκBα 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 IκBα degradation. To test this possibility, the association of HDAC3 with IκBα was investigated using coimmunoprecipitation. Cytoplasmic extract of VSMCs were immunoprecipitated with either anti-IκBα or HDAC3 antibody. As expected, HDAC3 was found in the immunoprecipitation product of IκBα antibody, and IκBα was identified in the immunoprecipitation product of HDAC3 antibody strongly indicating that IκBα and HDAC3 coexist in the same protein complex in cells (Fig. 6C). However, treatment with salusin-β for 30min, IκBα was little detected in the immunoprecipitation product of HDAC3 (Fig. 6D), consisting with the observation that IκBα was degraded by salusin-β stimulation at early time (Fig. 2D). Taken together, these data suggest that IκBα may mediate nuclear translocation of HDAC3 in response to salusin-β in VSMCs.
Increased cellular levels of PPARγ protein or PPARγ ligands down-regulated salusin-β-induced VSMCs proliferation and inflammation

To directly assess the functional significance of salusin-β-induced PPARγ suppression, we tested whether increasing PPARγ expression antagonizes the proliferative and inflammatory effects of salusin-β in VSMCs. Transfection of VSMCs with 0.4μg of PPARγ-expression plasmid significantly increased PPARγ protein expression (Fig. 7A). The result showed that increasing amounts of transfected PPARγ 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 PPARγ expression plasmid (Fig. 7C and D).

Similarly, pretreatment with the PPARγ natural ligand 15d-PGJ2 and synthetic ligand rosiglitazone significantly blocked salusin-β-induced VSMCs proliferation and inflammation (Fig. 8).
Fig. 7. Increased cellular levels of PPARγ protein down-regulated salusin-β-induced VSMCs proliferation and inflammation. VSMCs were transfected with increased amount of pCMX-PPARγ expression plasmid or pCMX empty vector for 24 h. 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 PPARγ protein levels were measured by western blot 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 ± SD (n=6). *P< 0.05 versus the 0.4μg pCMX empty vector without salusin-β (the first column on the left); #P< 0.05 versus 0.4μg pCMX empty vector with 10^{-7}mol/L salusin-β treatment (the second column on the left).

Fig. 8. PPARγ ligands inhibited salusin-β-induced VSMCs proliferation and inflammation. A-C, VSMCs were pretreated with PPARγ natural ligand 15d-PGJ_2 (5μmol/L) or synthetic ligand rosiglitazone (5μmol/L) for 1h and further treated with or without 10^{-7}mol/L salusin-β for 24 h. 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 ± SD (n=6). *P< 0.05 versus the control with no treatment; #P<0.05 versus 10^{-7}mol/L salusin-β treatment alone.
IκBα-NF-κB, but decreased PPARγ gene expression through HDAC3 without involving NF-κB p50 and p65. In addition, the inhibitory effect of salusin-β on PPARγ gene expression contributes to salusin-β-induced VSMCs proliferation and inflammation in vitro.

PPARγ has been recognized as an important regulator in the vasculature and maintaining an appropriate expression level in vascular wall cells is essential for ensuring 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, PPARγ is a good candidate because it is present in all cells composing blood vessels including VSMCs [4]. Many studies suggest that PPARγ 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 PPARγ suppresses production of inflammatory cytokines, and there is accumulating data that PPARγ ligands exert anti-inflammatory, antioxidative, and antiproliferative effects on VSMCs [2, 4]. In addition to its atheroprotective effects, PPARγ ligands identifying a potential role for PPARγ as a regulator of VSMCs biology, many genetic evidences further support a normal role for PPARγ in inhibiting VSMCs proliferation and inflammation in the context of restenosis or atherosclerosis. For example, dominant-negative loss-of-function PPARγ mutations have also been introduced into in vivo and in vitro experiments. It is shown that transfer of a PPARγ-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 PPARγ-wild-type gene was found to inhibit smooth muscle proliferation and reduce neointima formation after balloon injury [15, 17]. In addition, VSMCs-specific PPARγ 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 PPARγ 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 PPARγ gene expression at protein and mRNA levels and reduced PPARγ gene promoter activity in VSMCs. Since vascular PPARγ plays a pivotal role in protection from vascular disease, we speculate that the inhibitory action of salusin-β on PPARγ gene expression contributes to its proliferative and inflammatory effects on VSMCs. This postulate is confirmed by the result that increased cellular levels of PPARγ protein down-regulated salusin-β-induced VSMCs proliferation and inflammation in the present study.

PPARγ 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 transduct extracellular stimuli into cells [11]. However, the present study showed that they were not involved in salusin-β inhibition of PPARγ gene expression. Other studies have demonstrated that salusin-β is a powerful activator of the NF-κB pathway in both cultured endothelial cells and in intact arteries [8, 9, 10]. NF-κB is a transcriptional factor that participates in vascular damage through the regulation of several genes involved in proliferation and immune responses. The prototypical NF-κB heterodimer consists of RelA (p65) and p50 that contain the transcriptional activation domain. The classical NF-κB activation model involves cytoplasmic sequestration of NF-κB by inhibitory protein IκBα, preventing NF-κB from migrating to the nucleus. When IκBα is phosphorylated, it is degraded, allowing NF-κB 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 IκBα phosphorylation [30], markedly suppressed salusin-β-induced reduction in PPARγ 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-κB by classical pathway of phosphorylation and degradation of IκBα with parallel induction of
nuclear translocation of NF-κB p65 and p50 in VSMCs. However, silencing with NF-κB p50 or p65 did not impact salusin-β–induced down-regulation of PPARγ gene expression. These findings suggest that IκBα may have a new and NF-κB-independent role in salusin-β–induced PPARγ 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]. Therefore, 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 PPARγ. For example, in adipose cells, recruitment of HDAC1 and HDAC3 are involved in the regulation of PPARγ function [34, 35], leading to the inhibition of adipocyte differentiation. siRNA–mediated inhibition of HDAC3 attenuated angiotensin II mediated reductions in PPARγ abundance in VSMCs [26]. TGF-β1 down-regulates PPARγ gene expression through recruitment of HDAC1 at the PPARγ promoter in cardiac fibroblasts [36]. Importantly, it has been reported that cytoplasmic IκBα increases NF-κB-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 PPARγ. Our results showed that salusin-β induced nuclear translocation HDAC3, which was required for salusin-β–induced inhibition of PPARγ 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 PPARγ in the present study. It has been reported that nuclear translocation of HDAC3 is controlled by IκBα in the IκBα-HDAC3 model. IκBα binds HDAC3 in the cytosol through the ankyrin repeats [37], and IκBα degradation leads to nuclear translocation of HDAC3 [38]. In the present study, the same mechanism is shown to be responsible for the PPARγ inhibition by salusin-β in VSMCs by four lines of evidence: (a) Either IκBα phosphorylation inhibitor Bay11-7085 or siRNA knockdown targeting HDAC3 can significantly inhibit salusin-β–induced reduction in PPARγ gene expression; (b) salusin-β–induced nuclear translocation of HDAC3 was coupled with IκBα phosphorylation and IκBα 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 IκBα-HDAC3 complex was also present in the cytosol of VSMCs but interrupted after salusin-β treatment in parallel with IκBα degradation and HDAC3 translocation.

Conclusion: The present study indicated that salusin-β negatively regulated PPARγ gene expression at protein, mRNA and gene promoter level in VSMCs and IκBα-HDAC3 pathway may contribute to salusin-β–induced inhibition PPARγ 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.
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Disclosure Statement

The authors report no conflicts of interest.

References

1. Tyagi S, Gupta P, Saini AS, Kaushal C, Sharma S: The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. J Adv Pharm Technol Res 2011;2:236-240.

2. Hasegawa H, Takano H, Komuro I: Therapeutic Implications of PPARgamma in Cardiovascular Diseases. PPAR Res DOI:10.1155/2010/876049.

3. Ketsawatsomkron P, Pelham CJ, Groh S, Keen HL, Faraci FM, Sigmund CD: Does peroxisome proliferator-activated receptor-gamma (PPAR gamma) protect from hypertension directly through effects in the vasculature? J Biol Chem 2010;285:9311-9316.

4. Duan SZ, Usher MG, Mortensen RM: Peroxisome proliferator-activated receptor-gamma-mediated effects in the vasculature. Circ Res 2008;102:283-294.

5. Shichiri M, Ishimaru S, Ota T, Nishikawa T, Isogai T, Hirata Y: Salusins: newly identified bioactive peptides with hemodynamic and mitogenic activities. Nat Med 2003;9:1166-1172.

6. Liu J, Ren YG, Zhang LH, Tong YW, Kang L: Serum Salusin-β Levels Are Associated With the Presence and Severity of Coronary Artery Disease. J Investing Med 2015;63:632-635.

7. Watanabe T, Sato K, Itoh F, Iso Y, Nagashima M, Hirano T, and Shichiri M: The roles of salusins in atherosclerosis and related cardiovascular diseases. J Am Soc Hypertens 2011;5:359-365.

8. Koya T, Miyazaki T, Watanabe T, Shichiri M, Atsumi T, Kim-Kaneyama JR, Miyazaki A: Salusin-β accelerates inflammatory responses in vascular endothelial cells via NF-κB signaling in LDL receptor-deficient mice in vivo and HUVECs in vitro. Am J Physiol Heart Circ Physiol 2012;303:96-105.

9. Zhou CH, Liu L, Liu L, Zhang MX, Guo H, Pan J, Yin XX, Ma TF, Wu YQ: Salusin-β not salusin-α promotes vascular inflammation in ApoE-deficient mice via the I-κBα/NF-κB pathway. PLoS One 2014;9:e91468.

10. Zhou CH, Pan J, Huang H, Zhu Y, Zhang M, Liu L, Wu Y: Salusin-β, but not salusin-α, promotes umbilical vein endothelial cell inflammation via the p38 MAPK/JNK-NF-κB pathway. PLoS One 2014;9:e107555.

11. Xu XL, Zeng Y, Zhao C, He MZ, Wang E, Zhang W: Salusin-β Induces Smooth Muscle Cell Proliferation by Regulating Cyclins D1 and E Expression Through MAPKs Signaling Pathways. J Cardiovasc Pharmacol 2015;65:377-385.

12. Chistiakov DA, Orekhov AN, Bobrovshiev YV: Vascular smooth muscle cell in atherosclerosis. Acta Physiol (Oxf) DOI: 10.1111/apha.12466.

13. Liu LL, Wu YQ, Song Z, Zhou CH, Xing SH: Effect of salusin-β on the proliferation of vascular smooth muscle cells and the underlying mechanisms. Chin Pharm Bull 2010;2:666-669.

14. Halabi CM, Beyer AM, de Lange WJ, Keen HL, Baumbach GL, Faraci FM, Sigmund CD: Interference with PPARgamma function in smooth muscle causes vascular dysfunction and hypertension. Cell Metab 2008;7:215-226.

15. Meredith D, Panchatcharam M, Mirivala S, Tsai YS, Morris AJ, Maeda N, Stouffer GA, Smyth SS: Dominant-negative loss of PPARgamma function enhances smooth muscle cell proliferation, migration, and vascular remodeling. Arterioscler Thromb Vasc Biol 2009;29:465-471.

16. Liu JZ, Lyon CJ, Hsueh WA, Law RE: A Dominant-Negative PPARgamma Mutant Promotes Cell Cycle Progression and Cell Growth in Vascular Smooth Muscle Cells. PPAR Res 2009;2009:438673.

17. Lim S, Jin CJ, Kim M, Chung SS, Park HS, Lee IK, Lee CT, Cho YM, Lee HK, Park KS: PPARgamma gene transfer sustains apoptosis, inhibits vascular smooth muscle cell proliferation, and reduces neointima formation after balloon injury in rats. Arterioscler Thromb Vasc Biol 2006;26:808-813.

18. Cariou B, Charbonnel B, Staels B: Thiazolidinediones and PPARγ agonists: time for a reassessment. Trends Endocrinol Metab 2012;23:205-215.
19 Soskic SS1, Dobutovic BD, Sudar EM, Obradovic MM, Nikolic DM, Zatic BL, Stojanovic SD, Stolic EJ, Mikhalilidis DP, Isenovic ER: Peroxisome proliferator-activated receptors and atherosclerosis. Angiology 2011;62:523-534.
20 Chen Y, Gao H, Yin Q, Chen L, Dong P, Zhang X, Kang J: ER stress activating ATF4/CHOP-TNF-α signaling pathway contributes to alcohol-induced disruption of osteogenic lineage of multipotential mesenchymal stem cell. Cell Physiol Biochem 2013;33:2:743-754.
21 Zhou Q, Guan W, Qiao H, Cheng Y, Li Z, Zhai X, Zhou Y: GATA binding protein 2 mediates leptin inhibition of PPARγ1 expression in hepatic stellate cells and contributes to hepatic stellate cell activation. Biochim Biophys Acta 2014;1842:2367-2377.
22 Lacolley P, Regnault V, Nicoletti A, Li Z, Michel JB: The vascular smooth muscle cell in arterial pathology: a cell that can take on multiple roles. Cardiovasc Res 2012;95:194-204.
23 Subramanian V, Gollege J, Ijaz T, Bruemmer D, Daugherty A: Pioglitazone-induced reductions in atherosclerosis occur via smooth muscle cell-specific interaction with PPARγ. Circ Res 2010;107:953-958.
24 Marchesi C, Rehman A, Rautureau Y, Kasal DA, Briet M, Leibowitz A, Simeone SM, Ebrahimian T, Neves MF, Offermanns S, Gonzalez FJ, Paradis P, Schiffelin EL: Protective role of vascular smooth muscle cell PPARγ in angiotensin II-induced vascular disease. Cardiovasc Res 2013;97:562-570.
25 Fu M, Zhang J, Lin Y, Zhu X, Zhao L, Ahmad M, Ehrengruber MU, Chen YE: Early stimulation and late inhibition of peroxisome proliferator-activated receptor γ (PPAR gamma) gene expression by transforming growth factor beta in human aortic smooth muscle cells: role of early growth-response factor -1 (Egr-1), activator protein 1 (AP1) and Smads. Biochem J 2003;370:1019-1025.
26 Subramanian V, Gollege J, Heywood EB, Bruemmer D, Daugherty A: Regulation of peroxisome proliferator-activated receptor-γ by angiotensin II via transforming growth factor-β1-activated p38 mitogen-activated protein kinase in aortic smooth muscle cells. Arterioscler Thromb Vasc Biol 2012;32:397-405.
27 Cheang WS, Fang X, Tian XY: Pleiotropic effects of peroxisome proliferator-activated receptor γ and δ in vascular diseases. Circ J 2013;77:2664-2671.
28 Siebenlist U, Franzoso G, Brown K: Structure, regulation and function of NF-κB. Annu Rev Cell Biol 1994;10:405-455.
29 Gilmore TD: Introduction to NF-κB: players, pathways, perspectives. Oncogene 2006;25:6680-6684.
30 Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME: Novel inhibitors of cytokine-induced IκBα phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. J Biol Chem 1997;272:21096-21103.
31 Zhou B, Margariti A, Zeng L, Xu Q: Role of histone deacetylases in vascular cell homeostasis and arteriosclerosis. Cardiovasc Res 2011;90:413-420.
32 Montgomery RL, Potthoff MJ, Haberland M, Qi X, Matsuzaki S, Humphries KM, Richardson JA, Babes-Duby R, Olson EN: Maintenance of cardiac energy metabolism by histone deacetylase 3 in mice. J Clin Invest 2008;118:3588-3597.
33 Findeisen HM, Gizard F, Zhao Y, Qing H, Heywood EB, Jones KL, Cohn D, Bruemmer D: Epigenetic regulation of vascular smooth muscle cell proliferation and neointima formation by histone deacetylation inhibition. Arterioscler Thromb Vasc Biol 2011;31:851-860.
34 Fu M, Rao M, Bouras T, Wang C, Wu K, Zhan gX, Li Z, Yao TP, Pestell RG: Cyclin D1 inhibits peroxisome proliferator-activated receptor gamma-mediated adipogenesis through histone deacetylase recruitment. J Biol Chem 2005;280:16934-16941.
35 Guan HP, Ishizuka T, Chui PC, Lehrke M, Lazar MA: Corepressors selectively control the transcriptional activity of PPARγ in adipocytes. Genes Dev 2005;19:453-461.
36 Gong K, Chen YF, Lucas JA, Hage FG, Yang Q, Nozelle SE, Oparil S, Xing D: Transforming growth factor-β inhibits myocardial PPARγ expression in pressure overload-induced cardiac fibrosis and remodeling in mice. J Hypertens 2011;29:1810-1819.
37 Viau tor P, Legrand-Poels S, van Lint C, Warnier M, Merville MP, Gielen J, Piette J, Bours V, Chariot A: Cytoplasmic IkappaBalpha increases NF-kappaB-independent transcription through binding to histone deacetylase (HDAC) 1 and HDAC3. J Biol Chem 2003;278:46541-46548.
38 Gao Z, He Q, Peng B, Chiao PJ, Ye J: Regulation of nuclear translocation of HDAC3 by IkappaBalpha is required for tumor necrosis factor inhibition of peroxisome proliferator-activated receptor gamma function. J Biol Chem 2006;281:4540-4547.