The effect of salusin-β on expression of pro- and anti-inflammatory cytokines in human umbilical vein endothelial cells (HUVECs)

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Abstract

BACKGROUND: Atherosclerosis is one of the predominant causes of cardiovascular disease (CVD). Several studies indicated the significant pathophysiological role of salusin-β in atherosclerosis. Cytokines are involved in all stages of atherosclerosis. Therefore, we aimed to assess the effect of salusin-β on interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 18 (IL-18) (as inflammatory cytokines) and interleukin 1Ra (IL-1Ra) (as anti-inflammatory cytokines) levels in human umbilical vein endothelial cells (HUVECs).

METHODS: The HUVECs were cultured in HUVEC completed medium and treated with different doses of salusin-β for 6 and 12 hours. For the investigation of nuclear factor κβ (NF-κβ) signaling pathway involvement, cells were treated in the presence or absence of Bay 11-7082 (as NF-κβ inhibitor). The mRNA expression and protein level of cytokines were measured by a real-time polymerase chain reaction (PCR) system and enzyme-linked immunosorbent assay (ELISA) method, respectively.

RESULTS: Salusin-β increased mRNA expression and protein level of IL-6, IL-8 and IL-18. This protein decreased mRNA and protein level of IL-1Ra in HUVECs. NF-κβ signaling pathway was involved in the up-regulatory effect of salusin-β on mRNA expression of pro-inflammatory cytokines. The down-regulatory effect of salusin-β on IL-1Ra expression could not be influenced by Bay 11-7082 pre-treatment.

CONCLUSION: It seems that salusin-β may participate in a cascade pathway in vascular inflammation. Our findings suggested that salusin-β has potential use as a therapeutic target for atherosclerosis.

Keywords: Atherosclerosis, Cardiovascular Diseases, Cytokines, Endothelial Cells, Inflammation, Salusin-Beta

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Introduction

Atherosclerosis is one of the predominant causes of cardiovascular disease (CVD). More than 17.5 million people die each year because of CVDs.1 Due to the global growing rate of diabetes and obesity, it is expected that morbidity and mortality of CVD will increase.2 Therefore, it is of great significance to understand the precise mechanisms which are involved in atherosclerosis. Atherosclerosis is now regarded as a chronic inflammatory disorder of large and medium arteries.3 Cytokines are particularly significant in inflammatory processes,4 and many of them are believed to be complicated in atherogenesis.5 Several cytokines are detected in atherosclerotic plaque, on the other hand, all the cells involved in the disease can produce cytokines.6 These proteins are involved in initial stages of atherosclerosis, recruitment and activation of leukocytes, foam cell and fatty streak formation, development of complex lesions, plaque stability and rupture.2,4 It is noted that some cytokines have an anti-atherosclerotic effect.2 Several signaling pathways are involved in cytokines expression, among which nuclear factor κβ (NF-κβ) signaling pathway involvement is of great importance.

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transcription factor is an important signaling pathway inducing the expression of some cytokines.\textsuperscript{7}

Salusin-β (with 20 amino acids) is a novel peptide which is discovered via bioinformatics analysis of a human full-length cDNA library.\textsuperscript{8} This protein is synthesized from pre-pro-salusin which is expressed at a great level in human vascular smooth muscle cells (VSMCs) and endothelial cells.\textsuperscript{8} It is indicated that salusin-β is released greatly from cell lines such as THP-1 (a human monocytic cell line) and U937 (a model cell line for the study of behavior and differentiation of monocytes), at the time of stimulation to be differentiated into macrophages.\textsuperscript{9} Several data indicated that this protein has a pathophysiological role in atherosclerosis via overexpression of acyl-CoA, cholesterol acyltransferase-1 (ACAT-1) and increase in macrophage foam cell formation (an important step in atherosclerosis),\textsuperscript{10} stimulation of the proliferation of VSMCs and fibroblasts and induction of the expression of c-myc, c-fos.\textsuperscript{8} Also in vivo and in vitro studies have shown that Salusin-β accelerates inflammatory responses in vascular endothelial cells.\textsuperscript{11}

As noted, salusin-β contributes to the pathogenesis of atherosclerosis, but little is known about the effect of this protein on pro- and anti-inflammatory cytokines expression. We aimed to assess the effect of salusin-β on interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 18 (IL-18) (as inflammatory cytokines) and interleukin 1Ra (IL-1Ra) (as anti-inflammatory cytokines) levels in human umbilical vein endothelial cells (HUVECs). Further, we assessed the probable involvement of NF-κβ signaling pathway in salusin-β effect on cytokines.

**Materials and Methods**

This study was performed at Isfahan University of Medical Sciences, Iran, (grant number 394287) and Hamadan University of Medical Sciences, Iran, (2015-2016). Salusin-β was provided by PeptaNova (Cat No: 4417-s, Japan), and E)-3-(4-methylphenyl sulfonyl)-2-propenonitrile (Bay 11-7082) was supplied from Cayman chemical (CAY10010266, USA). All other substances were acquired with best attainable purity grade.

HUVEC (ATCC® CRL-1730) was purchased from Pasteur Institute, Tehran, Iran. The cells were cultured in cell culture treated flasks and grown in HUVEC completed medium, containing Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F12), endothelial cell growth factor (ECGF), non-essential amino acid (NEAA), Heparin, Insulin, Nap and 10% fetal bovine serum, in a humidified 5% CO₂ incubator at 37 °C. The cells were pretreated with or without various concentrations of salusin-β (3, 10, 30, 90 nM) for 6 and 12 hours. Also, HUVECs were treated in the presence or absence of Bay 11-7082 (3 and 10 μM) as NF-κβ signaling pathway inhibitor.

The cell viability was assessed by the reduction of MITT to its insoluble formazan.\textsuperscript{12} HUVECs (5000 cells/well) were seeded in 96-well microplates. After overnight incubation, the cells were treated with different concentrations of salusin-β (1, 3, 10, 30, 90 and 180 nM) for 24 hours. Afterward, the cells were incubated at 37 °C with 15 μl of MITT (5 mg/ml) in phosphate-buffered saline for 4 hours. Then, the medium was removed and 150 μl of dimethyl sulfoxide (DMSO) was added and the absorbance at 570 nm and 630 nm was measured by an enzyme-linked immunosorbent assay (ELISA) reader.

After RNA extraction by TRIzol® Reagent (Thermo Fisher Scientific, Cat No: 15596-026, USA) according to manufacturer's protocol, RNA concentration and purity were determined by microspectrophotometer (A & E Lab, UK). RNA was converted to first-strand cDNA by Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Cat No: K-1622, USA). The mRNA expression of IL-6, IL-8, IL-18 and IL-1Ra were measured by real-time polymerase chain reaction (PCR) system (BioRAD CFX 96) and Syber® Premix Ex TaqTMII (Takara, Cat.RR820L, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control gene. Real-time PCR data were quantified by 2\textsuperscript{ΔΔCT} method,\textsuperscript{13} it should note that the PCR efficiency of the target gene was similar to the internal control gene. All samples and no template controls were examined in duplicates. All amplicons (Table 1) were confirmed by sequencing (sequencing service-Bioneer, South Korea).

The IL-6 level was determined by a human IL-6 ELISA kit, Affymetrix eBioscience (catalog No: 88-7066, USA) sensitivity < 2 pg/ml. The protein level of IL-8 was measured by Human IL-8 Elisa kit Affymetrix eBioscience (catalog No: 88-8086, USA), sensitivity 2 pg/ml. These ELISA kits were specifically engineered for accurate and precise measurement protein levels of IL-6 and IL-8, respectively. The protein level of IL-18 was quantified with Human IL-18 Elisa kit, Boster Bio (catalog No: EK0864, USA), with a sensitivity < 1 pg/ml. The protein level of IL-1Ra was determined by human IL-1Ra Elisa kit, Boster Bio (catalog No: EK0782, USA) with a sensitivity less than 2 pg/ml.
Table 1. Polymerase chain reaction (PCR) primers and the PCR product size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number (Variant)</th>
<th>Sequence</th>
<th>PCR product (bp)</th>
</tr>
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<tr>
<td>GAPDH</td>
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<td>F: AAGGCTGTGGGCAAGGTTCATC</td>
<td>248</td>
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<td></td>
<td>NM_001256799.2 (Variant 2)</td>
<td>R: GCGTCAAAAGGGGAGAGTGG</td>
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<tr>
<td></td>
<td>NM_001289745.1 (Variant 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NM_001289746.1 (Variant 4)</td>
<td></td>
<td></td>
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<tr>
<td>IL-1Ra</td>
<td>NM_173842.2</td>
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<td>185</td>
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<td></td>
<td>NM_173843.2</td>
<td>R: ACTTGACACAGGACAGGCACA T</td>
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<td>IL-6</td>
<td>NM_000584</td>
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<td></td>
<td></td>
<td>R: ATTTGTGGTGGGCTGAGG</td>
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<td>IL-8</td>
<td>NM_000584</td>
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<td></td>
<td></td>
<td>R: CACTGATCTTCCTGATAACC</td>
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<td>IL-18</td>
<td>NM_001562.3</td>
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<tr>
<td></td>
<td>NM_001243211.1</td>
<td>R: GGCATTATCTCTAAGTCCAG</td>
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</table>

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IL-1Ra: Interleukin 1Ra; IL-6: Interleukin 6; IL-8: Interleukin 8; IL-18: Interleukin 18; F: Forward; R: Reverse; bp: Base pair; NM: mRNA accession number; PCR: Polymerase chain reaction.

These ELISA kits were specific for natural and recombinant IL-18 and IL-1Ra, respectively. The procedures were done according to manufacturer’s instructions. All cytokine level was measured in cell culture supernatant treated with salusin-β after 6 and 12-hour treatments. All samples were analyzed in duplicates.

Data were presented as the mean ± standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) test. Bonferroni post-hoc test was done to access statistical differences between groups. The normality test was controlled by Kolmogorov-Smirnov test. The results supported normality of our variables. P < 0.050 was considered as statistically significant. The statistical analyses were accomplished by SPSS software (version 24, IBM Corporation, Armonk, NY, USA).

Results

The effect of salusin-β on HUVECs: HUVECs viability was assessed by the MTT assay. The results indicated that salusin-β had no cytotoxic effect in HUVECs in the concentration range of 1-180 nM.

The effect of salusin-β on mRNA expression of IL-6, IL-8, IL-18 and IL-1Ra: To assess whether salusin-β could exert an inflammatory effect on endothelial cells via modulating cytokines expression, mRNA expression of pro- and anti-inflammatory cytokines were determined. The results were reported base on Bonferroni post-hoc test. The mRNA expression of IL-6 was increased by salusin-β at 30 nM (2.86 ± 0.20, P < 0.001) and 90 nM (1.82 ± 0.02, P = 0.007) for 6-hour treatment, and 90 nM (2.17 ± 0.20, P = 0.013) for 12-hour treatment. Also, we observed that mRNA expression of IL-8 was increased at 10 nM (1.92 ± 0.08, P = 0.021), 30 nM (2.68 ± 0.19, P = 0.001) and 90 nM (5.44 ± 0.24, P < 0.001) for 6-hour treatment, and at 90 nM (2.00 ± 0.09, P = 0.002) for 12-hour treatment. The mRNA expression of IL-18 was increased at 30 nM (2.55 ± 0.01, P = 0.014) and 90 nM (3.17 ± 0.40, P = 0.003) for 6-hour treatment, and at 90 nM (1.70 ± 0.11, P = 0.012) for 12-hour treatment.

Salusin-β reduced mRNA expression of IL-1Ra at 10 nM (0.41 ± 0.08, P = 0.005), 30 nM (0.57 ± 0.11, P = 0.021) and 90 nM (0.60 ± 0.03, P = 0.026) for 6-hour treatment. The mRNA expression of IL-1Ra was decreased at 10 nM (0.22 ± 0.03, P = 0.006), 30 nM (0.26 ± 0.01, P = 0.008) and 90 nM (0.37 ± 0.04, P = 0.017) for 12-hour treatment (Table 2).

Table 2. The expression ratio of each target gene normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in treated cells with different doses of salusin-β compared to untreated cells

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Salusin-β</th>
<th>6 hour (3 nM)</th>
<th>12 hour (3 nM)</th>
<th>6 hour (10 nM)</th>
<th>12 hour (10 nM)</th>
<th>6 hour (30 nM)</th>
<th>12 hour (30 nM)</th>
<th>6 hour (90 nM)</th>
<th>12 hour (90 nM)</th>
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<tr>
<td>IL-6</td>
<td>0.89 ± 0.04</td>
<td>1.31 ± 0.20</td>
<td>1.31 ± 0.20</td>
<td>2.86 ± 0.20*</td>
<td>2.86 ± 0.20*</td>
<td>1.26 ± 0.04</td>
<td>1.26 ± 0.04</td>
<td>2.17 ± 0.20</td>
<td>2.17 ± 0.20</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.60 ± 0.09</td>
<td>1.26 ± 0.13</td>
<td>1.92 ± 0.08</td>
<td>0.71 ± 0.11</td>
<td>2.68 ± 0.19*</td>
<td>1.03 ± 0.02</td>
<td>5.44 ± 0.24***</td>
<td>2.00 ± 0.09***</td>
<td>2.00 ± 0.09***</td>
</tr>
<tr>
<td>IL-18</td>
<td>1.26 ± 0.20</td>
<td>1.50 ± 0.12</td>
<td>1.97 ± 0.11</td>
<td>0.96 ± 0.13</td>
<td>2.55 ± 0.01*</td>
<td>0.94 ± 0.06</td>
<td>3.17 ± 0.40**</td>
<td>1.70 ± 0.11*</td>
<td>1.70 ± 0.11*</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>0.58 ± 0.05</td>
<td>0.61 ± 0.41</td>
<td>0.41 ± 0.08</td>
<td>0.22 ± 0.03</td>
<td>0.57 ± 0.11</td>
<td>0.26 ± 0.01</td>
<td>0.60 ± 0.03*</td>
<td>0.37 ± 0.04</td>
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</tbody>
</table>

Data are shown as mean ± standard deviation (SD); One-way analysis of variance and Bonferroni post-hoc tests are used; “P < 0.050, **P < 0.010, ***P < 0.001; IL-6: Interleukin 6; IL-8: Interleukin 8; IL-18: Interleukin 18; IL-1Ra: Interleukin 1Ra.
Salusin-β on protein level of IL-6, IL-8, IL-18 and IL-1Ra: In line with the changes in cytokines mRNA, we observed that protein level of pre- and anti-inflammatory cytokines were changed. The production of IL-6 was increased at 90 nM for 6-hour and 12-hour treatments (82.62 ± 4.81 pg/ml vs. 42.89 ± 4.11 pg/ml in untreated cells, P = 0.004 and 118.06 ± 15.02 pg/ml vs. 65.92 ± 5.21 pg/ml in untreated cells, P = 0.040, respectively) (Figures 1A and 1B). The results indicated that salusin-β at 90 nM increased protein level of IL-8 (238.82 ± 3.98 pg/ml vs. 178.10 ± 1.80 in untreated cells) for 6-hour treatment (P = 0.036); however, 12-hour treatment had no effect on protein level of IL-8 (dose 3 nM: P = 0.074, dose 10 nM: P = 0.841, dose 30 nM: P = 0.188, and dose 90 nM: P = 0.072) (Figure 2A and 2B). Treatment of HUVECs with different doses of salusin-β increased protein level of IL-18 at 90 nM (84.82 ± 0.76 pg/ml vs. 35.49 ± 1.41 pg/ml untreated cells) (P < 0.001) for 12-hour treatment. It was noted that 6-hour treatment had no effect on protein level of IL-18 (dose 3 nM: P > 0.999, dose 10 nM: P > 0.999, dose 30 nM: P = 0.330, dose 90 nM: P = 0.055) (Figures 3A and 3B). The protein level of IL-1Ra was decreased at 30 and 90 nM (7.33 ± 1.20 pg/ml and 5.82 ± 0.76 pg/ml, respectively vs. 16.52 ± 0.70 pg/ml in untreated cells) for 12-hour treatment (P = 0.020 and P = 0.010, respectively). Salusin-β at 6-hour treatment had no effect on IL-1Ra protein level, (dose 3 nM: P > 0.999, dose 10 nM: P > 0.999, dose 30 nM: P > 0.999, dose 90 nM: P = 0.594) (Figure 4A and 4B).

The role of NF-κβ signaling pathway in Salusin-β treatment: To better understand the mechanisms of salusin-β-triggered cytokine expression, we measured mRNA expression of cytokines in the pretreatment of Bay 11-7082 (3, 10 μM) and salusin-β treatment. The results indicated that Bay 11-7082 (10 μM) can suppress the up-regulatory effect of salusin-β on mRNA expression of IL-6, 0.67 ± 0.11 (P = 0.002), Figure 5A. Also, Bay 11-7082 (10 μM) inhibited the up-regulatory effect of salusin-β on mRNA expression of IL-18 (0.95 ± 0.09) (P = 0.023) (Figure 5B).

Figure 1. The effect of salusin-β on protein level of interleukin 6 (IL-6) at different time points and concentrations. Salusin-β (90nM) increased IL-6 protein level at 6-hour (A) and 12-hour (B) treatment; * P < 0.050, ** P < 0.010 IL-6: Interleukin 6

Figure 2. The effect of salusin-β on protein level of interleukin 8 (IL-8). Salusin-β (90 nM) increased protein level of IL-8 at 6-hour treatment (A), and had no effect at 12-hour (B) treatment; * P < 0.050 IL-8: Interleukin 8
Also, we observed that Bay 11-7082 (3 and 10 μM) inhibits IL-8 mRNA expression induced by salusin-β (0.37 ± 0.09 and 0.45 ± 0.06, respectively) (P < 0.001) (Figure 5C). This inhibitor had no effect on the down-regulatory effect of salusin-β on IL-1Ra mRNA expression, data not indicated.

Discussion

Endothelial cells have a fundamental function in the inflammatory response. These cells can synthesize various pro-inflammatory cytokines in response to different stimuli. The present study, for the first time, demonstrated that salusin-β can increase mRNA and protein level of pro-inflammatory cytokines including IL-6, IL-8 and IL-18 and decrease mRNA and protein level of IL-1Ra in HUVECs. Several studies confirmed that salusin-β has a pro-atherogenic effect, considering the prominent role of cytokines in key pathogenic events in atherosclerosis, it is worth to recognize the relationship between salusin-β and cytokines.

IL-8 is an atherogenic chemokine. The high level of this protein has been reported in the arterial atherosclerotic wall, atherosclerotic plaques and macrophages. IL-8 is involved in firm adhesion of monocytes to vascular endothelium under flow condition, a crucial step in atherosclerosis initiation. Also, IL-8 can promote monocytes and neutrophils activation. Because of specific biochemical properties of IL-8, this cytokine is a perfect choice for sites of inflammation. IL-8 is a mitogenic and chemotactic factor for VSMCs, which up-regulates mRNA expression and production of matrix metallopeptidase 2 (MMP2) and matrix metallopeptidase 9 (MMP9) in endothelial cells. It also inhibited tissue inhibitor of metalloproteinase 1 (TIMP-1) expression which leads to an imbalance between matrix metalloproteinase and TIMP-1; consequently, resulting in atherosclerotic plaque rupture and thrombosis. Therefore, IL-8 can accelerate the initiation, progression and plaque destabilization.

Very recent studies revealed that salusin-β can elevate migration and intimal hyperplasia of VSMCs via reactive oxygen species (ROS)/NF-kβ/MMP-9 pathway.
Our results indicated that salusin-β increases mRNA and protein level of IL-8 in HUVECs. As noted above, salusin-β accelerates oxidative stress leading to activation of NF-κB signaling pathway in endothelial cells. Furthermore, salusin-β induces mRNA expression of IL-1β. It is well known that these factors are involved in IL-8 expression.

IL-18 is an important player in atherosclerotic processes. This cytokine is highly expressed in macrophages, endothelial cells and smooth muscle cells (SMCs) of atherosclerotic lesions. Recombinant IL-18 accelerates atherogenesis and increases cytokines level such as IL-1β, IL-8, IL-6. Also, IL-18 also intensifies adhesion molecules expression such as vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) in endothelial cells and fibroblasts. Thus, IL-18 results in endothelial dysfunction. IL-18 has a close association with interferon gamma (IFN-γ) induction and it is proposed that pro-atherogenic effect of IL-18 is mediated by this cytokine. IFN-γ has a key role in atherosclerosis especially in foam cell formation via up-regulation of scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX). IFN-γ inhibits collagen synthesis by SMCs, thereby can modulate collagen content of atherosclerotic plaques. IL-18 deficiency is associated with decreased recruitment of macrophages. Apoptosis of endothelial cells and acceleration of migration and proliferation of SMCs is mediated by IL-18. In vivo studies indicated that endogenous inhibitor of IL-18 impeded fatty streak development in the thoracic aorta and has slowing effect on progression of advanced atherosclerotic plaque.

IL-18 is a part of IL-1 superfamily; therefore, this cytokine has structural and functional similarity with IL-1β. It also has the identical signaling cascade in common with IL-1β. Salusin-β increased mRNA expression of IL-1β and this study showed that salusin-β increased mRNA and protein level of IL-18 in HUVECs, suggesting a crosstalk between salusin-β, IL-1β and IL-18.

IL-6 is expressed in human atherosclerotic plaque and increases inflammatory cascade. IL-6 is involved in the expression of acute phase proteins in SMCs, also in migration and differentiation of activated macrophages. IL-6 accelerates the proliferation of VSMCs and enhances the permeability of endothelial cells. These events are included in onset and development of atherosclerosis. Animal studies have shown that recombinant IL-6 caused atherosclerotic lesion development. IL-6 is a pro-coagulant cytokine, which can activate the tissue factor production. Therefore, IL-6 has a role in plaque stability and rupture. It is demonstrated that endothelial cells can express ICAM-1 when are exposed to several inflammatory cytokines such as IL-6. The studies indicated that salusin-β can increase mRNA level of ICAM-1. This study revealed that salusin-β increased mRNA and protein level of IL-6 in HUVECs. In line with our results, in vivo and in vitro studies indicated that salusin-β increased protein level of IL-6; however, these studies did not measure mRNA expression of IL-6.

IL-1 is an influential pro-inflammatory cytokine in vascular hemostasis. This cytokine induces the production of some cytokines and chemokines, and stimulates adhesion molecule expression which accelerates monocyte recruitment and permeation into the arterial wall. Also, IL-1 participates in the development of tissue damage via inducing cell proliferation and matrix metalloproteinases release.

Figure 5. The involvement of nuclear factor κB (NF-κB) signaling pathway in up-regulating effect of salusin-β on interleukin 6 (IL-6) (A), interleukin 18 (IL-18) (B), and interleukin 8 (IL-8) (C) mRNA expression; * P < 0.050, ** P < 0.010
IL-1Ra is a negative regulator of IL-1 signaling and has a role in maintaining vascular hemostasis.\textsuperscript{44,45} It is proved that treatment of apolipoprotein E (ApoE)\textsuperscript{-/-} mice with recombinant IL-1Ra is an impressive therapy for atherosclerosis.\textsuperscript{46} Several lines of evidence indicated that IL-1Ra has an anti-atherosclerotic effect.\textsuperscript{47,48} Animal studies indicated that lack of IL-1β causes less atherosclerotic lesions development;\textsuperscript{49} On the other hand, partial deficiency of IL-1Ra changes the composition of atherosclerotic plaques with a higher level of membrane cofactor protein 1 (MCP-1), ICAM-1 and VCAM-1 mRNA and accelerates vascular inflammation.\textsuperscript{42} Salusin-β increased mRNA level of MCP-1, ICAM-1, VCAM-1 and IL-1β in HUVECs.\textsuperscript{11} Our results indicated that salusin-β decreased mRNA/protein level of IL-1Ra in HUVECs. Because the balance between IL-1 and IL-1Ra may have a role in atherogenesis development,\textsuperscript{42} we suggested the other role for salusin-β via disturbance in IL-1 and IL-1Ra equilibrium.

NF-κβ is an important transcription factor in inflammatory processes.\textsuperscript{47} This transcriptional factor regulates transcriptions of several genes with a well-known function in atherosclerosis including cytokines, chemokines and adhesion molecules.\textsuperscript{58} The promoters of IL-6 and IL-8 genes have functional NF-κβ binding sites which have been proven to be essential for the transcriptional activation of these genes.\textsuperscript{14} Also, several studies confirmed the involvement of NF-κβ signaling pathway in IL-18 expression.\textsuperscript{48-50} The studies demonstrated that salusin-β accelerated vascular inflammatory responses via NF-κβ signaling pathway.\textsuperscript{11} We found that this complex protein involved in the up-regulatory effect of salusin-β on pro-inflammatory cytokines.

This study was performed with some limitations. We only studied mRNA expression of cytokines in the involvement of NF-κβ signaling pathway and further studies are necessary to confirm these results. Also, we could not study another cytokine-associated signaling pathway. Regarding no involvement of NF-κβ signaling pathway on the down-regulatory effect of salusin-β on IL-1Ra, it seems that the other cytokine-associated signaling pathways must be investigated.

**Conclusion**

Primary prevention of atherosclerosis, which is important for the management of atherosclerotic CVD, requires comprehensive assessment and modification of molecular cardiovascular risk factors. Cytokines are a central player in atherosclerotic processes. To the best of our knowledge, this is the first study to indicate the association of salusin-β with pro- and anti-inflammatory cytokines. This work indicated that salusin-β increased mRNA/protein level of IL-6, IL-8 and IL-18 as pro-inflammatory cytokines and decreased mRNA/protein level of IL-1Ra as an anti-inflammatory cytokine in HUVECs. The previous studies demonstrated the accelerator effect of salusin-β on vascular inflammation. It seems that salusin-β may participate in a cascade pathway in vascular inflammation (Figure 6). Our novel results can help to open up a new vista into the potential use of salusin-β as a therapeutic target for the prevention of atherosclerosis. Prospective studies to determine the mechanisms latent down-regulatory effect of salusin-β on IL-1Ra and in vivo researches are recommended.

**Figure 6.** Salusin-β intensifies vascular inflammation via several mechanisms. It increases oxidative stress, adhesion molecules and inflammatory cytokine expression. On the other hand, salusin-β decreases anti-inflammatory cytokine level

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**Conflict of Interests**

Authors have no conflict of interests.

**References**

1. World Health Organization. Cardiovascular disease: New initiative launched to tackle cardiovascular disease, the world’s number one
Salusin-β: pro- & anti-inflammatory cytokines


25. Kim YG, Lee JW, Ryu IC, Wei JD, Seong CM, Kim JH. Proinflammatory cytokine IL-1beta
stimulates IL-8 synthesis in mast cells via a leukotriene B4 receptor 2-linked pathway, contributing to angiogenesis. J Immunol 2010; 184(7): 3946-54.


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