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Salsalate ameliorates the atherosclerotic response through HO-1- and SIRT1-mediated suppression of ER stress and inflammation

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Abstract

Objective and design Inflammation plays a causative role in atherosclerosis development. Salsalate is an anti-inflammatory drug used to treat atherosclerosis, but the mechanisms by which it affects atherosclerotic progression remain unclear.

Methods Human umbilical vascular endothelial cells (HUVECs) and THP-1 human monocytes were treated with salsalate. Heme oxygenase 1 (HO-1) and sirtuin 1 (SIRT1) small interfering RNAs (siRNAs) were used to suppress each gene expression. Protein analyses were performed for measuring the expression of HO-1, SIRT1, nuclear factor kappa B (NFκB), cell adhesion molecules, and endoplasmic reticulum (ER) stress markers. Furthermore, cell adhesion assay, caspase 3 activity assay, and ELISA were also performed.

Results In this study, we show that salsalate increases the expression of HO-1 and SIRT1 in HUVEC and suppresses lipopolysaccharide (LPS)-induced atherosclerotic responses via HO-1- and SIRT1-mediated pathways. Salsalate treatment of HUVEC and THP-1 cells reduced LPS-induced phosphorylation of NFκB and secretion of the proinflammatory cytokines TNFα and MCP-1. Salsalate treatment of HUVEC reduced the expression of the adhesion molecules ICAM, VCAM, and E-selectin and the LPS-induced adhesion of THP-1 cells to HUVEC. Salsalate treatment also attenuated LPS-induced ER stress and cell apoptosis. These anti-atherosclerotic effects were reversed by treating cells with siRNA for HO-1 and SIRT1.

Conclusions Salsalate ameliorates LPS-induced atherosclerotic reactions via HO-1 and SIRT1-dependent reduction of inflammation and ER stress. Activation of these pathways by salsalate may provide therapeutic strategies for treating atherosclerosis.

Keywords Salsalate · SIRT1 · Heme oxygenase 1 · Inflammation · ER stress · Apoptosis · HUVEC · THP-1

Abbreviations

SIRT1	Sirtuin 1
HO-1	Heme oxygenase 1
ER	Endoplasmic reticulum
siRNA	Small interfering RNA
HUVEC	Human umbilical vein endothelial cells

Introduction

Atherosclerosis is the primary cause of coronary artery disease and the leading cause of mortality in developed countries [1]. The mechanisms underlying the pathogenesis of atherosclerosis are complicated, with endothelial dysfunction and inflammation playing crucial roles [2]. Inflammation causes vascular lesions in blood vessels, and this endothelial damage contributes to the progression of atherosclerosis [3, 4]. Thus, attenuation of inflammation and endothelial damage represents a potential therapeutic approach to treat atherosclerosis.

Inflammation is pivotal in atherosclerosis development [5]. Macrophages play an important role in the rupture of fibrous caps of atherosclerotic plaques in fatal acute myocardial infarction (AMI). Macrophage infiltration and T

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lymphocyte activation contribute to plaque destabilization [6], causing up-regulation of proinflammatory cytokines and lytic enzymes, leading to thinning and rupture of the fibrous cap [7]. Therefore, suppression of inflammation is a therapeutic strategy for treating atherosclerosis. Anti-inflammatory reagents such as sodium salicylate and aspirin have anti-atherogenic effects [8, 9]. However, side effects such as gastric irritation and bleeding limit their usage. Salsalate is a salicylate prodrug that is well tolerated and considered safe for long-term treatment of atherosclerosis [10]. We previously reported that salsalate reduces hepatic insulin resistance through AMP-activated protein kinase (AMPK)-mediated suppression of selenoprotein P expression [11] and ameliorates hepatic steatosis via attenuation of nuclear factor kappa B (NFκB)-associated fetuin-A expression [12]. Here, we investigated the effects of salsalate on atherosclerotic response development and explored the molecular mechanisms by which salsalate influences LPS-induced atherogenic activity in human umbilical vascular endothelial cells (HUVEC).

Materials and methods

Cell cultures, reagents, and antibodies

Human umbilical vein endothelial cells (HUVEC; ATCC, Manassas, VA, USA) were cultured on 0.2% gelatin-coated culture plates in M200PRF medium (Invitrogen, Carlsbad, CA, USA) with a low serum growth supplement (Invitrogen). The human monocyte THP-1 (Korean Cell Line Bank, Seoul, Korea) cell line was maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were confirmed to be free from mycoplasma. Cells at passages 3–4 were used for all experiments. Salsalate (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma). Lipopolysaccharide (LPS) (Sigma) was dissolved in phosphate-buffered saline (PBS; Biosesang, Seoul, Korea). Zinc protoporphyrin-9 (Santa Cruz, CA, USA) and ex527 (Santa Cruz) were dissolved in DMSO. Anti-phospho NFκBp65 (1:1000) and anti-NFκBp65 (1:2500) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-ICAM-1 (1:1000), anti-VCAM-1 (1:1000), anti-E-selectin (1:1000), anti-phospho eIF2α (1:1000), anti-eIF2α (1:1000), anti-phospho IRE-1 (1:1000), anti-IRE-1 (1:1000), anti-CHOP (1:1000), anti-HO-1 (1:1000), anti-SIRT1 (1:1000), and anti-β actin (1:5000) were purchased from Santa Cruz Biotechnology (Santa Cruz).

Western blot analysis

Cells were harvested, and proteins were extracted with lysis buffer (PRO-PREP; Intron Biotechnology, Seoul Korea) for 60 min at 4 °C. Protein samples (30 μg) were subjected to 10% SDS-PAGE, transferred to a nitrocellulose membrane (Amersham Bioscience, Westborough, MA, USA), and probed with the indicated primary antibody followed by secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). The signals were detected using enhanced chemiluminescence (ECL) kits (Amersham Bioscience). Image J (National Institute of Mental Health, Bethesda, MD, USA) was used for densitometry.

Enzyme linked immunosorbent assay (ELISA)

Cell-secreted TNFα and MCP-1 were measured with the respective ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Transfection with siRNA for gene silencing

Small interfering (si) RNA oligonucleotides (20 nM) specific for heme oxygenase 1 (HO-1) and sirtuin 1 (SIRT1) were purchased from Santa Cruz Biotechnology. To suppress gene expression, cell transfection was performed with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions.

Cell adhesion assay

According to a previous report [13], HUVECs were treated with LPS (100 ng/ml) and CHI3L1 (100 ng/ml) for 24 h. Next, the treated cells were co-cultured with THP-1 cells labeled with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester (BCECF/AM; Invitrogen) green fluorescent dye. After 1 h, the co-cultured cells were washed three times with PBS and counted.

Cell viability assay

Cell viability was measured by MTT assay. In brief, MTT solution was added to experimental cell cultures in 96-well plates and incubated at 37 °C for 1 h. After washing three times with PBS, accumulated red formazan in the

experimental cells was dissolved in DMSO. The optical density was used as an indicator of cell viability and was determined at 550 nm.

Caspase 3 activity assay

The caspase 3 activity assay was performed using a Caspase 3 Colorimetric Assay kit (Abcam, Cambridge, MA, USA) following the manufacturer's directions.

Statistical analysis

All analyses were performed using the SPSS/PC statistical program (version 23 for Windows; SPSS, Chicago, IL, USA). Results are presented as the fold of the highest value. All experiments were performed at least three times. Tukey's test after one-way ANOVA was used for statistical analysis.

Results

Salsalate reduces LPS-induced NFκB phosphorylation via HO-1/SIRT1-dependent signaling

Heme oxygenase 1 (HO-1), SIRT1, and NFκB play key roles in atherosclerosis development. HO-1 suppresses inflammation and the atherosclerotic response [14]. SIRT1 has a protective role in atherosclerosis development [15] and interacts with HO-1 [16, 17]. Therefore, we hypothesized that salsalate attenuates LPS-induced NFκB phosphorylation through HO-1/SIRT1-mediated pathway. Since monocyte–endothelial cell interactions contribute to atherosclerosis development [18], we used human endothelial cells (HUVEC) and human monocytes (THP-1 cells). Treatment of HUVEC and THP-1 cells with salsalate suppressed LPS-induced NFκB phosphorylation in a dose-dependent manner (Fig. 1a, d) and increased the expression of HO-1 and SIRT1 (Fig. 1b, e). Small interfering RNAs (siRNA) suppression of HO-1 and SIRT1 mitigated the effects of salsalate on LPS-induced NFκB phosphorylation (Fig. 1c, f).

Salsalate suppresses LPS-induced expression of adhesion molecules and adhesion of monocytes to endothelial cells

The endothelial NFκB pathway contributes to atherosclerosis via up-regulation of the adhesion molecules intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin [19]. We investigated the effects of salsalate on the expression of these adhesion molecules in LPS-treated HUVEC. Salsalate reduced LPS-induced adhesion of THP-1 cells to HUVEC (Fig. 2a) and

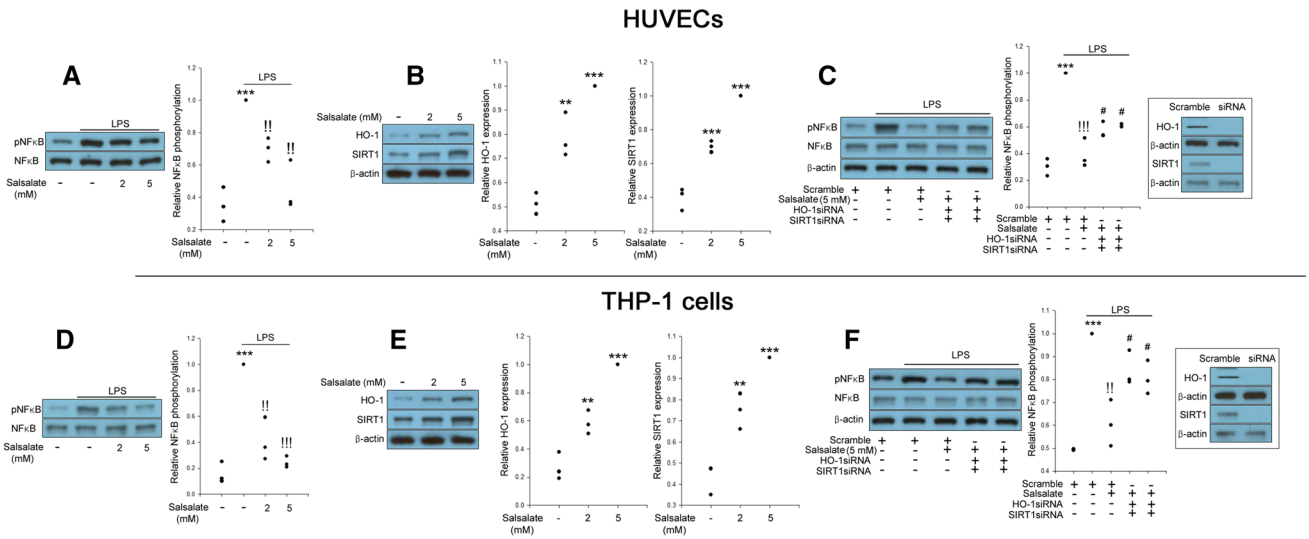

Fig. 1 Salsalate reduces LPS-induced NFκB phosphorylation through HO-1- and SIRT1-mediated pathways in HUVEC and THP-1 cells. Cells were treated with salsalate (0, 2, or 5 mM) and LPS (200 ng/ml). After 24 h, NFκB phosphorylation level was determined by Western blotting in HUVEC (**a**) and THP-1 cells (**d**). Western blot analysis of HO-1 and SIRT1 expression in salsalate-treated HUVEC (**b**) and THP-1 cells (**e**). Each of siRNAs (scrambled siRNA, HO-1, and SIRT1 siRNA)-transfected cells were treated with LPS or LPS plus salsalate. After 24 h, NFκB phosphorylation level was determined by Western blotting in HUVEC (**c**) and THP-1 cells (**f**). Experiments were performed three times. Statistics: one-way ANOVA ($P<0.001$) and Tukey's t test: $^{***}P<0.001$ and $^{**}P<0.01$ when compared to control. $^{!!!}P<0.001$ and $^{!!}P<0.01$ when compared to 5 mM salsalate or LPS treatment. $^{\#}P<0.05$ when compared to LPS plus salsalate treatment

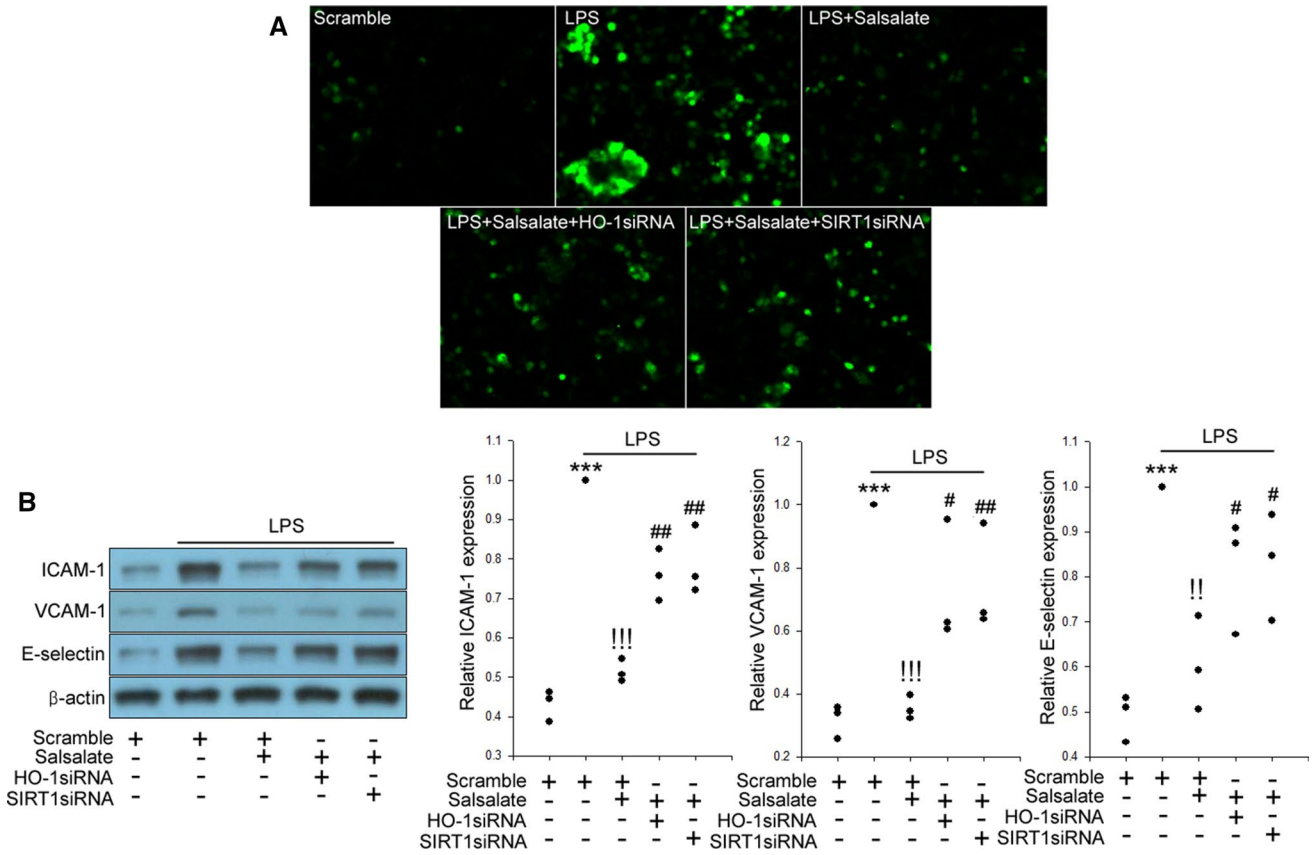

Fig. 2 Salsalate prevents LPS-induced adhesion molecule expression and THP-1 cell adhesion via HO-1- and SIRT1-dependent pathways in HUVEC. **a** Scrambled siRNA, HO-1, or SIRT1siRNA-transfected cells were treated with LPS (200 ng/ml) or LPS plus salsalate (5 mM) for 24 h and co-cultured with fluorescently labeled (green) THP-1 cells for 20 min. **b** Each of siRNAs (scrambled siRNA, HO-1, and SIRT1 siRNA)-transfected cells were treated with LPS or LPS plus salsalate. After 24 h, expression levels of ICAM-1, VCAM-1, and E-selectin in HUVEC were determined by Western blot. Experiments were performed three times. Statistics: one-way ANOVA ($P<0.001$) and Tukey's t test: $^{***}P<0.001$ when compared to control. $^{!!!}P<0.001$ and $^{!!}P<0.01$ when compared to LPS treatment. $^{\#\#}P<0.01$ and $^{\#}P<0.05$ when compared to LPS plus salsalate treatment (color figure online)

reduced the expression of ICAM-1, VCAM-1, and E-selectin in HUVEC (Fig. 2b). These effects were reversed by using siRNAs against HO-1 or SIRT1 (Fig. 2).

Salsalate reduces LPS-induced proinflammatory cytokine secretion

To confirm that salsalate reduces inflammation via HO-1 and SIRT1-dependent pathways, we studied the release of the proinflammatory cytokines TNFα and MCP-1 into the culture media. These cytokines were markedly increased in the media of LPS-treated HUVEC (Fig. 3a) and THP-1 cells (Fig. 3b). Salsalate treatment attenuated LPS-induced secretion of TNFα and MCP-1; these effects were mitigated by treatment of cells with siRNAs against HO-1 or SIRT1 (Fig. 3). Furthermore, more precisely, inhibitors were used to confirm the role of HO-1 and SIRT1. Treatment of HUVEC and THP-1 cells with Z9, an HO-1 inhibitor or ex527, an SIRT1 inhibitor more dramatically abrogated the effects of salsalate on LPS-induced MCP-1 release than ELISA using siRNAs (Fig. 3c, d).

Salsalate reduces LPS-induced ER stress-associated apoptosis via HO-1/SIRT1-mediated signaling

Endothelial endoplasmic reticulum (ER) stress [20] and apoptosis [21] contribute to atherosclerosis development. Thus, we hypothesized that salsalate suppresses ER stress-induced apoptosis. LPS-induced loss of HUVEC viability was ameliorated by salsalate treatment (Fig. 4a). The activity of caspase 3, a marker of apoptosis, was significantly reduced following salsalate treatment (Fig. 4b). In addition, salsalate treatment of HUVEC attenuated LPS-induced expression of ER stress markers IRE-1, eIF2α, and CCAAT/enhancer-binding protein-homologous protein (CHOP)

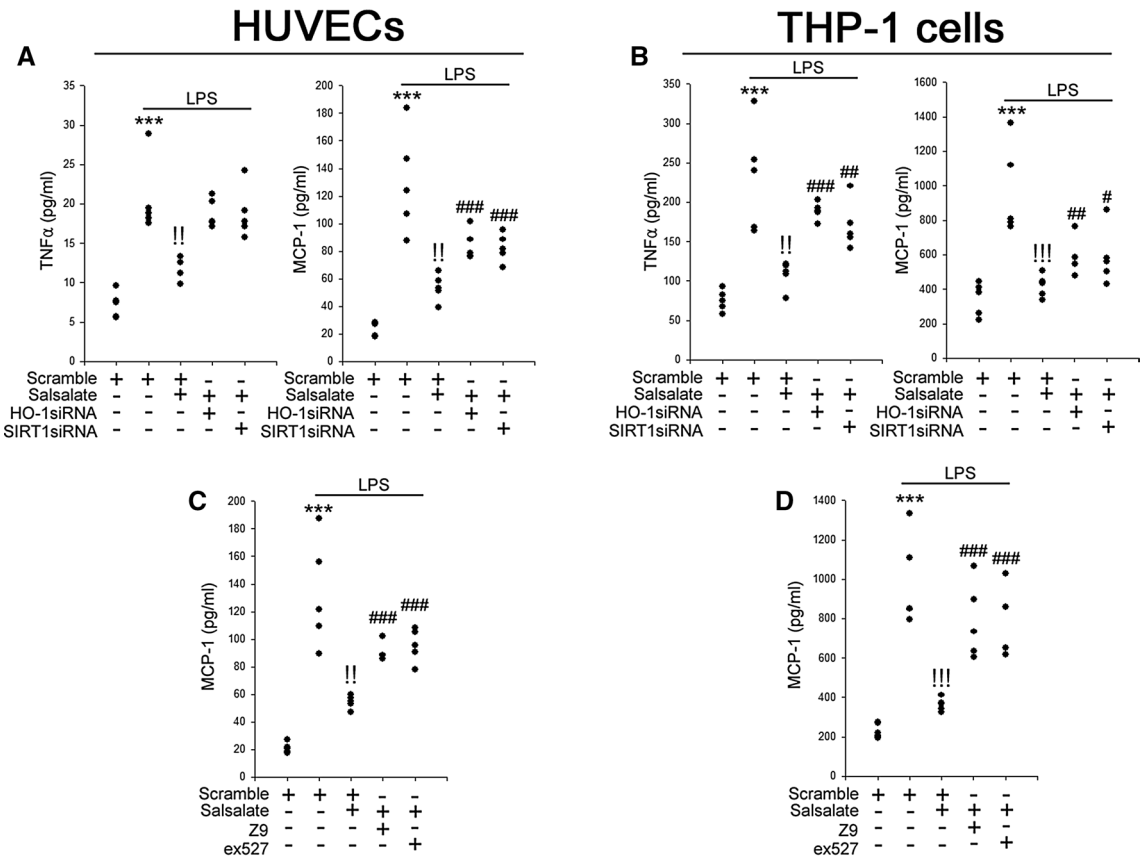

Fig. 3 Salsalate attenuates LPS-induced secretion of proinflammatory cytokines via HO-1- and SIRT1-dependent signaling in HUVEC and THP-1 cells. Each of siRNAs (scrambled siRNA, HO-1, and SIRT1 siRNA)-transfected cells were treated with LPS (200 ng/ml) or LPS plus salsalate (5 mM). After 24 h, TNFα and MCP-1 release in HUVEC (**a**) or THP-1 cells (**b**) were determined by ELISA. Cells were treated with LPS, LPS plus salsalate, LPS plus salsalate plus zinc protoporphyrin 9 (Z9) (1 μM), or LPS plus salsalate plus ex527 (10 μM). After 24 h, MCP-1 release in HUVEC (**c**) or THP-1 cells (**d**) were determined by ELISA. Experiments were performed three times. Statistics: one-way ANOVA ($P<0.001$) and Tukey's *t* test: $^{***}P<0.001$ when compared to control. $^{!!!}P<0.001$ and $^{!!}P<0.01$ when compared to LPS treatment. $^{###}P<0.001$, $^{##}P<0.05$, and $^{#}P<0.05$ when compared to LPS plus salsalate treatment. Student's *t* test or one-way ANOVA were used for statistical analysis

(Fig. 4c). Treatment with siRNA against HO-1 or SIRT1 significantly restored these changes (Fig. 4a–c). Furthermore, siRNA-mediated suppression of HO-1 or SIRT1 also markedly abrogated the effects of salsalate on LPS-induced ER stress in THP-1 cells (Fig. 4d).

HO-1 contributes to salsalate-mediated augmentation of SIRT1 expression

Macrophage HO-1 has been reported to be regulated by SIRT1 in hepatocytes [16]. Thus, we investigated whether inhibition of HO-1 affects SIRT1 expression and vice versa in HUVECs. As shown in Fig. 5, siRNA-mediated suppression of HO-1 significantly abrogated the effects of salsalate on SIRT1 expression (Fig. 5a). Conversely, SIRT1 siRNA did not affect salsalate-induced HO-1 expression (Fig. 5b). These results reveal that salsalate augments SIRT1 expression in an HO-1 expression-dependent manner.

Discussion

Salsalate, a prodrug of salicylate, is a nonsteroidal anti-inflammatory drug produced by plants and functions as part of the immune system to protect against infection. Synthetic compounds that degrade into salicylate in vivo, including aspirin and salsalate, have largely replaced salicylate due to their reduced side effects [22]. Because salsalate and salicylate have strong anti-inflammatory properties, they have been used for several decades to treat pain and inflammation due to rheumatoid arthritis [10, 23]. In the current study, we investigated the effects of salsalate on LPS-induced atherosclerotic responses in HUVEC. We found that salsalate

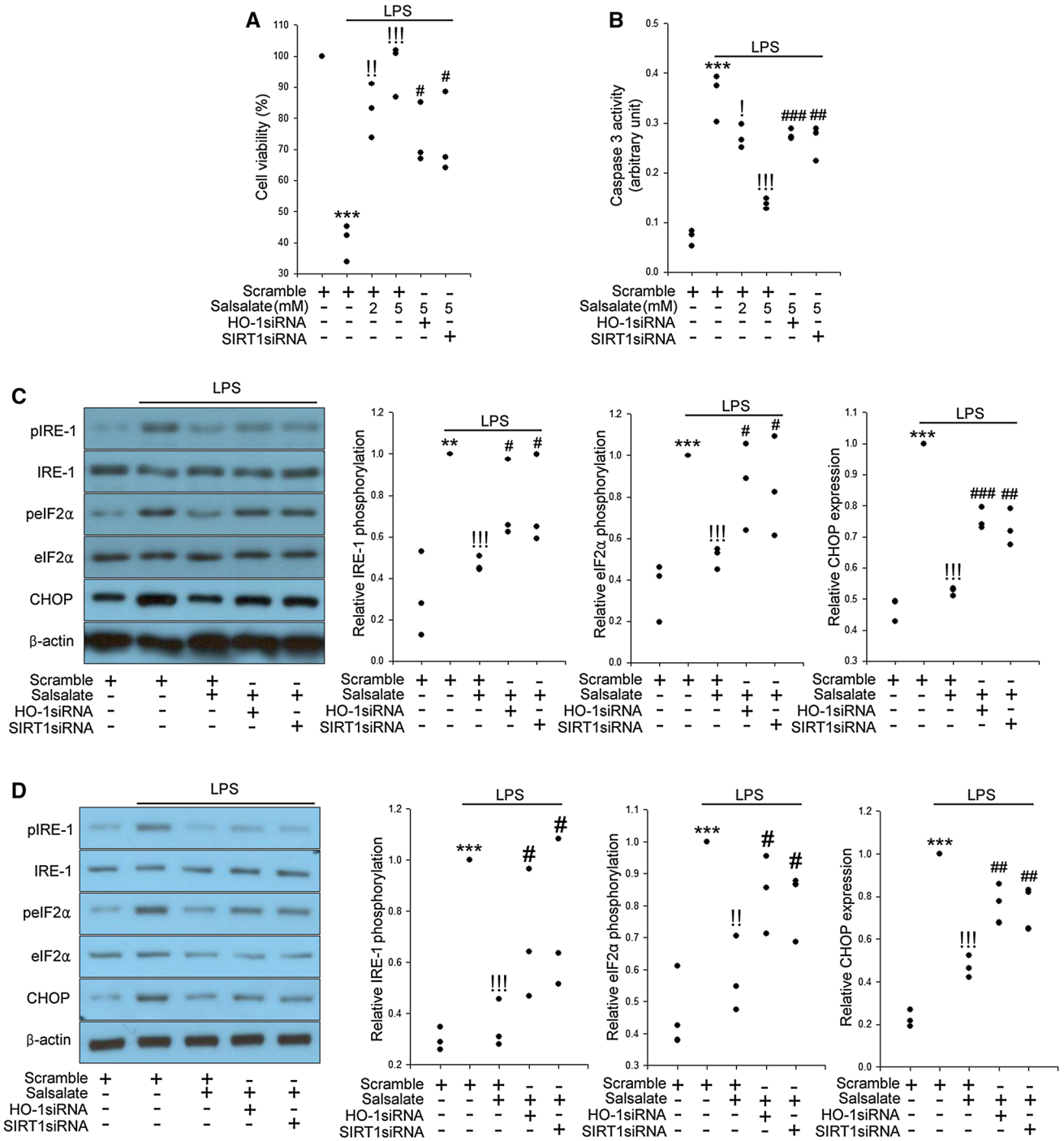

Fig. 4 Salsalate attenuates LPS-induced apoptosis via HO-1- and SIRT1-mediated suppression of ER stress in HUVEC. Each of siRNAs (scrambled siRNA, HO-1, and SIRT1 siRNA)-transfected cells were treated with LPS (200 ng/ml) or LPS plus salsalate (5 mM). After 24 h, cell viability was measured by MTT assay (**a**), caspase 3 activity was measured by colorimetric enzyme assay (**b**), and the level of ER stress was measured by expression of markers **c** in HUVEC and **d** in THP-1 cells. Experiments were performed three times. Statistics: one-way ANOVA ($P<0.001$) and Tukey's t test: $^{***}P<0.001$ and $^{**}P<0.01$ when compared to control. $^{!!!}P<0.001$, $^{!!}P<0.01$ and $^{!}P<0.05$ when compared to LPS treatment. $^{\#\#\#}P<0.001$, $^{\#\#}P<0.01$, and $^{\#}P<0.05$ when compared to LPS plus salsalate treatment. Student's t test or one-way ANOVA were used for statistical analysis

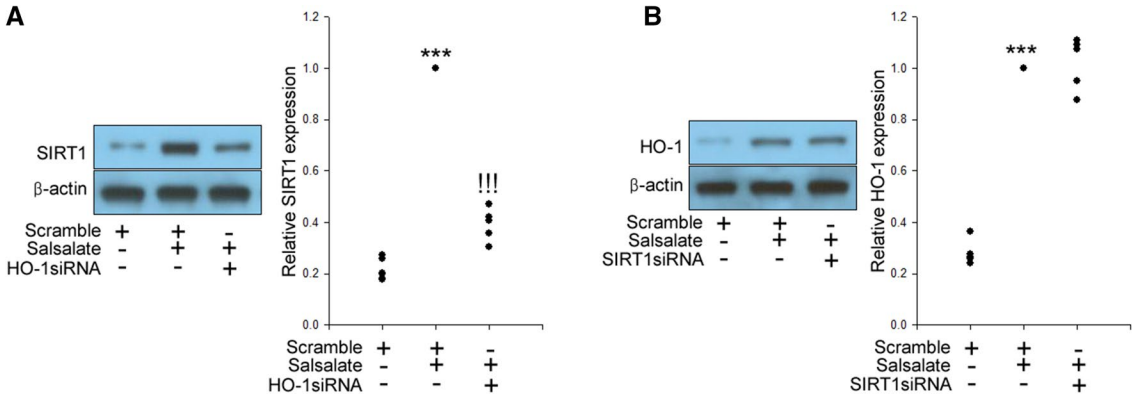

Fig. 5 Salsalate induces SIRT1 expression through the up-regulation of HO-1 expression. Each of siRNAs (scrambled siRNA, HO-1, and SIRT1 siRNA)-transfected cells were treated with salsalate. After 24 h, expression levels of **a** SIRT1 or **b** HO-1 expression in HUVEC were determined by Western blot. Experiments were performed three times. Statistics: one-way ANOVA ($P<0.001$) and Tukey's t test: $^{***}P<0.001$ when compared to control. $^{!!!}P<0.001$ when compared to salsalate treatment

suppresses LPS-induced inflammatory marker expression, monocyte adhesion to HUVEC, and ER stress and apoptosis.

The ER serves as an important site for protein maturation and lipid synthesis in eukaryotic cells [24]. Accumulation of unfolded or misfolded proteins in the ER stimulates three stress-inducible sensors: inositol-requiring enzyme (IRE) 1α, RNA-dependent protein kinase-like endoplasmic reticulum kinase (PERK), and activating transcription factor (ATF) 6. These sensors regulate gene-associated protein synthesis to maintain ER homeostasis. Altered protein folding and aggregation occur in the ER during pathogenic conditions such as hypoxia, oxidative stress, and calcium depletion—collectively called ER stress [25]. ER stress plays a causative role in atherosclerosis development [26]. Several inducers of ER stress exacerbate features of atherosclerosis, including endothelial apoptosis, accumulation of cholesterol, and NFκB-mediated proinflammatory pathways [27]. Here, we report for the first time that salsalate attenuates LPS-induced NFκB phosphorylation and expression of ER stress markers (e.g., IRE-1, eIF2α, and CHOP) in HUVEC. These results suggest that salsalate ameliorates atherogenic responses via suppression of inflammation as well as ER stress.

Based on these results, we next investigated mechanisms through which salsalate attenuates LPS-induced inflammation and ER stress. HO-1, a ubiquitous stress-inducible cellular protein, is the rate-limiting enzyme in oxidative catabolism of heme, followed by generation of biliverdin, free ion, and carbon monoxide (CO) [28]. HO-1 expression increases in response to ER stress in smooth muscle cells (SMC) [29]. Treatment of SMC with CO reduces ER stress-induced apoptosis. HO-1/CO signaling attenuates ER stress-induced apoptosis through p38-mediated suppression of CHOP expression [30]. The anti-inflammatory role of HO-1 has been observed in HO-1-knockout mice [31], in which up-regulation of proinflammatory cytokines occurs in peritoneal macrophages [32]. Several natural phytochemicals suppress inflammation through induction of HO-1 expression [33], suggesting that HO-1 may be a useful therapeutic agent for treating inflammatory diseases including atherosclerosis. In this study, salsalate treatment increased HO-1 expression in HUVEC and THP-1 cells, while treatment with siRNA for HO-1 significantly abrogated the effects of salsalate on LPS-induced inflammation, monocyte adhesion to HUVEC, and ER stress. These results indicate that HO-1 is important in salsalate-mediated attenuation of atherogenic responses through suppression of inflammation and ER stress.

SIRT1, an NAD^+-dependent lysine deacetylase, is a stress-inducible protein [34]. SIRT1 plays a role in protection from atherosclerosis by regulating NFκB-mediated inflammatory responses and preventing monocyte-derived foam cell formation [15]. Overexpression of SIRT1 in transgenic mice reduces NFκB-dependent signaling caused by a high-fat diet (HFD) and reduces the expression of proinflammatory cytokines [35]. HFD-fed SIRT1-knockout mice demonstrate increased hepatic inflammation [36]. SIRT1 deficiency increases microvascular inflammation, morbidity, and mortality in early sepsis [37], demonstrating its important anti-inflammatory role. SIRT1 ameliorates hepatic ER stress through FOXO1-mediated ORP150 signaling [38], and hepatic overexpression of SIRT1 in mice alleviates insulin resistance in the liver through suppression of ER stress [39]. MicroRNA-204-mediated suppression of SIRT1 promotes ER stress, leading to vascular dysfunction [40]. HO-1/SIRT1 signaling regulates inflammation [16] and reduces ER stress [17]. Therefore, we investigated the effects of salsalate on SIRT1 expression. Treatment of HUVEC and THP-1 cells with salsalate increased SIRT1 expression in a dose-dependent manner. Inhibition of SIRT1 using siRNA significantly abolished

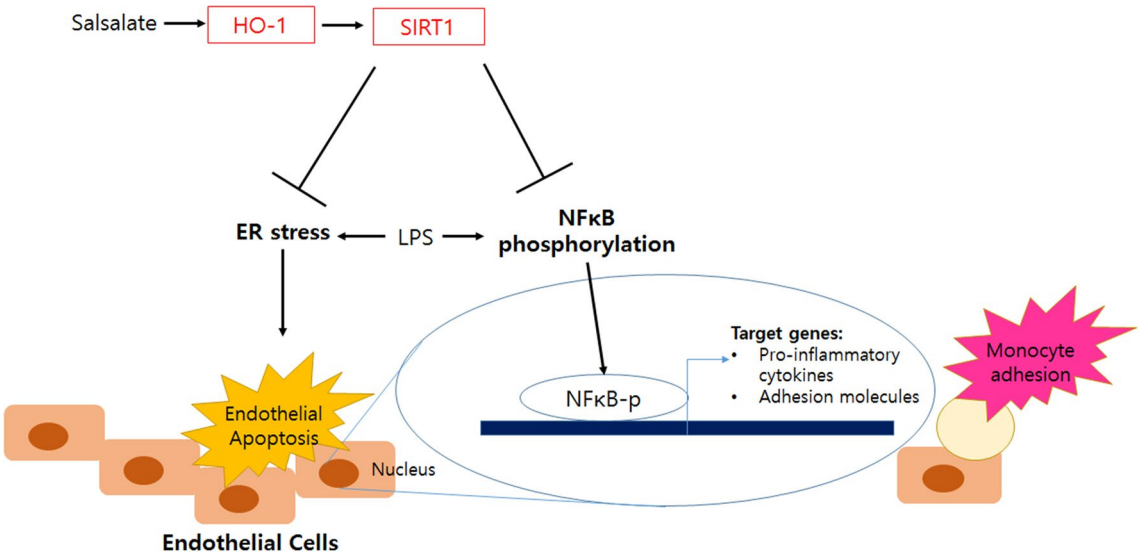

Fig. 6 Schematic diagram of the effects of salsalate on atherosclerotic response in HUVEC

the suppressive effects of salsalate on inflammatory responses, monocyte adhesion to HUVEC, and ER stress. Additionally, we found that salsalate augmented SIRT1 expression via the HO-1-dependent pathway. These results suggest that salsalate ameliorates atherogenic responses through HO-1/SIRT1-dependent signaling.

In conclusion, salsalate suppresses inflammation and ER stress through HO-1/SIRT1-mediated pathways and consequently ameliorates the atherogenic reaction in HUVEC (Fig. 6). These results suggest that regulation of inflammation and ER stress by salsalate may provide an effective therapeutic approach for treatment of atherosclerosis. Moreover, on the basis of the current study, salsalate may be more likely to treat ER stress and inflammation-mediated diseases in additions to atherosclerosis.

Author contributions TWJ, HSP, JHJ, and TSL: substantial contribution to conception and design; TWJ: acquisition of data, analysis, and interpretation of data; JHJ: drafting and revising of the manuscript. All authors approved the final version of the manuscript. TWJ and TSL are responsible for the integrity of the work as a whole.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to report.

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