Anti-atherosclerotic effects of genistein in preventing ox-low-density lipoprotein-induced smooth muscle-derived foam cell formation via inhibiting SRC expression and L-Ca channel currents

Wei Zhang¹, Liming Zhang¹, Xiaosi Zhang²

¹Department of Neurology, The First Affiliated Hospital of Harbin Medical University, Harbin, China; ²Metro-Medic Clinic, Montreal, Quebec, Canada

Contributions: (I) Conception and design: W Zhang; (II) Administrative support: L Zhang; (III) Provision of study materials or patients: L Zhang; (IV) Collection and assembly of data: W Zhang; (V) Data analysis and interpretation: W Zhang, X Zhang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Liming Zhang. Department of Neurology, The First Affiliated Hospital of Harbin Medical University, 23 Youzheng Street, Nangang District, Harbin 150001, Heilongjiang, China. Email: zhangfx0001@sina.com; Xiaosi Zhang. Metro-Medic Clinic, Suite 100, 1538 Sherbrooke West Street, Montreal, Quebec, Canada. Email: zxiaosi686@163.com.

Background: Atherosclerosis (AS) is associated with inflammation and abnormal proliferation and migration of vascular smooth muscle cells (VSMCs). Genistein may curtail the migration of VSMCs. Therefore, explorations are required to determine the molecular mechanism of genistein in AS. In this context, animal and cell models were developed to ascertain mechanisms of genistein in AS by modulating VSMC activities.

Methods: Genistein treatment and ectopic expression of lectin-like oxidized LDL receptor-1 (LOX-1) were conducted in high-fat diet-induced AS rats, followed by analyses of atherosclerotic plaque lesion areas and lipid deposition using Oil Red O and hematoxylin and eosin (HE) staining. The isolated VSMCs were stimulated with oxidized low-density lipoprotein (ox-LDL). Following genistein treatment combined with gain- and loss-of-function experiments in ox-LDL-exposed VSMCs, viability, migration, intracellular lipid deposition, intracellular cholesterol content, and L-type calcium (L-Ca) channel currents were assessed using Cell Counting Kit-8 (CCK-8), scratch test, Oil Red O staining, enzymatic colorimetry, and patch-clamp experiments, respectively. Western blot analysis was performed to evaluate the protein expression of SRC proto-oncogene, non-receptor tyrosine kinase (SRC), calcium voltage-gated channel subunit alpha1 C (CACNA1C), and LOX-1.

Results: Genistein treatment counteracted upregulated SRC phosphorylation, CACNA1C and LOX-1 expression, and L-Ca channel currents in aortic tissues of AS rats and ox-LDL-exposed VSMCs. Genistein deceased L-Ca channel currents by downregulating SRC, and SRC augmented LOX-1 expression by acting on the L-Ca channel subunit CACNA1C. The ox-LDL-induced VSMC proliferation, migration, and foaming of VSMCs were reduced by genistein treatment, silencing SRC, CACNA1C, or LOX-1, or suppressing L-Ca channels. Genistein treatment diminished atherosclerotic plaque lesion formation and lipid deposition, and these results were annulled by upregulating LOX-1.

Conclusions: Collectively, genistein might block the SRC/CACNA1C/LOX-1 axis to impede ox-LDL-induced VSMC proliferation, migration, and foaming and alleviate AS formation.

Keywords: Atherosclerosis; vascular smooth muscle cell; genistein; SRC; L-type calcium channels

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Introduction

As a chronic inflammatory disorder of large and medium-sized arteries, atherosclerosis (AS) contributes to stroke, peripheral vascular disease, and ischemic heart disease, collectively named cardiovascular diseases (CVDs) (1). The profile of risks carried by low-density lipoprotein (LDL) cholesterol, blood pressure, and smoking has been shifting to triglyceride-rich lipoproteins and LDL as contributors in AS (2). The oxidized low-density lipoprotein (ox-LDL) is abundant in atherosclerotic lesions showing proinflammatory and immune-stimulatory properties, which contributes to development of AS, plaque rupture, and CVDs (3). Atherosclerotic lesions are majorly characterized by lipid deposition in some arteries, accompanied by smooth muscle cell (SMC) and fibrous matrix proliferation, which gradually progresses to atherosclerotic plaque formation (4). Foam cells have been implicated in all stages of atherosclerotic lesion development, a large part of which originate from vascular SMCs (VSMCs) (5). Moreover, the rapid deposition of oxidized lipids, including ox-LDL, in the circulation results in migration and foaming necrosis of a large amount of VSMCs, thus facilitating AS formation (6,7). Of note, the involvement of L-type calcium (L-Ca) channels has been revealed in VSMC migration (8). Therefore, there is an ongoing urgent need to ascertain the molecular mechanism behind L-Ca channel currents and VSMC proliferation, migration, and foaming in AS for the exploration of promising targets for AS treatment.

As a biologically active isoflavone existing in soy, genistein orchestrates multiple pathophysiologic pathways linked to cancer, metabolic syndrome, and obesity (9). A prior study elaborated the atheroprotective effects of genistein in apolipoprotein E-deficient rats (10). Genistein has also been reported to decrease VSMC proliferation caused by ox-LDL (11). Of note, the study by Ono et al. manifested that genistein restrained SRC proto-oncogene, non-receptor tyrosine kinase (SRC)-induced cell proliferation in human gallbladder carcinoma (12). The SRC protein belongs to the SRC family of protein tyrosine kinases that are present in all mammalian cells and involved in numerous cell processes like differentiation, proliferation, and migration (13). The implication of SRC has been displayed in foam cell formation in macrophages and lesion development in AS (14). Furthermore, SRC could activate L-Ca channels in immature mouse cardiomyocytes (15). Meanwhile, calcium voltage-gated channel subunit alpha1 C (CACNA1C) encodes Ca1.2 of L-Ca channels, which participates in the process of AS (16,17). A study uncovered that CACNA1C was related to the upregulation of lectin-like oxidized LDL receptor-1 (LOX-1) during the differentiation and activation of macrophages (18). Notably, a recent study showed that LOX-1 was upregulated in AS rats, which accelerated ox-LDL uptake in the aorta of the rats (19). Still, the molecular causes leading to VSMC proliferation, migration, and foaming are poorly understood, and effective treatments for AS are slow to emerge. There remain many unanswered questions in regards to the downstream mechanistic actions of genistein and the involvement of L-Ca channel currents in the biological characteristics of VSMCs. These previously uncharacterized questions are considered in the present study.

In this context, we speculated that genistein might control AS progression via the SRC/CACNA1C/LOX-1 axis. Therefore, an AS rat model was established to identify SRC, CACNA1C, and LOX-1 expression after genistein treatment, and a cell model was developed on VSMCs using ox-LDL to further dissect their role in AS by measuring the proliferation, migration, and foaming of VSMCs. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2113/rc).

Methods

AS rat model establishment

A total of 56 12-week-old specific-pathogen-free (SPF) Sprague-Dawley (SD) rats were purchased from the Laboratory Animal Center, Harbin Medical University (Heilongjiang, China). Rats were individually housed in an SPF animal laboratory with 60–65% humidity at 22–25 °C. The experimentations were started after 1-week of acclimatization. The health status of rats was observed prior to the experiments. Animal experiments were performed under a project license (No. 2021086) granted by Animal Ethical Care Committee of the First Affiliated Hospital of Harbin Medical University, in compliance with institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration.

We randomly selected 8 rats from the 56 to undergo sham-operation, and fed them with conventional chow for 12 weeks. The remaining rats were fed with a high-fat diet (HFD; D12108C, Biopike, Beijing, China) containing...
1.25% cholesterol, 10% fat, and 0.1% propylthiouracil for 12 weeks to induce the AS model.

The untreated AS rats served as the AS group, and the remaining AS rats were treated with dimethyl sulfoxide (DMSO), genistein, DMSO+ overexpression (oe)-negative control (NC), genistein + oe-NC, and genistein + oe-LOX-1 (8 rats/group). For the genistein treatment, rats were fed with HFD and gavaged daily with 45 mg/kg genistein or an equivalent volume of DMSO (abs47048069; Absin Bioscience Inc., Shanghai, China) as control. Besides, lentivirus-based LOX-1 overexpression vectors were constructed with a titer of 1x10⁸ TU/mL. While being fed with the HFD, rats were injected with 0.1 mL lentiviruses via the tail vein every 3 days. After 12 weeks, rats were euthanized using the carbon dioxide asphyxiation method, followed by collection of aortic tissues for tissue staining to observe atherosclerotic plaques, lesion areas, and lipid deposition.

**Oil Red O staining**

Following fixation of slides of cells, aorta, or aortic root using 4% formaldehyde, they were stained with Oil Red O solution (01391, Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes. Samples were then rinsed with 75% ethanol for 5 minutes and with phosphate-buffered saline (PBS), followed by immediate imaging. Oil Red O-positive cells were photographed using an optical microscope (Nikon, Tokyo, Japan).

**Hematoxylin and eosin staining**

Aortic tissues were attained at week 12 after modeling, fixed in 4% formaldehyde, and dehydrated in gradient alcohol. Following 2 xylene washes, aortic tissue sections were dewaxed, and hydrated, followed by hematoxylin and eosin (HE) staining according to the manuals of an HE staining kit (PT001; Bogoo, Shanghai, China). In detail, after 10 minutes of staining in hematoxylin at room temperature, the sections were hydrolyzed in 1% hydrochloric acid solution for 20 seconds, and then treated in 1% ammonia solution for 30 seconds. Subsequent to 3 minutes of eosin staining of the sections, the morphology of aortic atherosclerotic lesions was observed under the optical microscope.

**Isolation and identification of VSMCs**

Aortas were collected, and arterial fat and connective tissue were carefully isolated. The arteries were then cut into blocks of 1 mm in length. These sections were incubated for 20–25 minutes at 37 °C in a solution containing 1.5 mg/mL papain, 1.5 mg/mL threitol, and 1.5 mg/mL bovine serum albumin (BSA), followed by 6–10 minutes of treatment with 1.0 mg/mL collagenase, 1.5 mg/mL hyaluronidase, and 1.5 mg/mL BSA. After that, SMCs were separated by gentle grinding with a pipette. Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) comprising 20% fetal bovine serum (FBS) and 100 U/mL streptomycin/penicillin. Finally, immunofluorescence detection of α-smooth muscle actin (α-SMA) was conducted to identify VSMCs using primary rabbit antibody against α-SMA (1:500, Ab124964; Abcam, Cambridge, UK) and Alexa Fluor® 488-labeled secondary goat anti-mouse immunoglobulin G (IgG) (H+L) antibody (1:800, Ab150081; Abcam, UK). Papain, threitol, BSA, collagenase, hyaluronidase, FBS, and streptomycin/penicillin were purchased from Solarbio (Beijing, China; G8430, D8220, A8010, C8140, H8030, S9030, and P1400, respectively).

**In vitro cell model of ox-LDL-exposed VSMCs**

The isolated VSMCs were stimulated with ox-LDL to develop in vitro cell model. The VSMCs received no treatment and served as controls, whilst ox-LDL-exposed VSMCs were untreated or treated with DMSO, genistein, BayK8644, nifedipine, genistein + BayK8644, short hairpin RNA (sh)-NC, sh-SRC, DMSO + oe-NC, genistein + oe-NC, genistein + oe-SRC, genistein + oe-LOX-1, oe-NC + sh-NC, oe-SRC + sh-NC, oe-SRC + sh-CACNA1C, sh-CACNA1C + oe-NC, or sh-CACNA1C + oe-LOX-1.

For the ox-LDL exposure, VSMCs were stimulated with 75 μg/mL of ox-LDL for 72 hours. The VSMCs were treated with 50 μmol/L genistein, 30 μmol/L BayK8644 (the L-Ca channel activator), and 50 μmol/L nifedipine (the L-Ca channel blocker) (HY-10588 and HY-B0284, respectively; MedChemExpress, Monmouth Junction, NJ, USA) 6 hours before ox-LDL exposure. Cells were grouped by liposome transfection with shRNA primers (sh-SRC, GCAAGATCAGTACGGGGAAT and sh-CACNA1C, CGCAGTCAAGTCTAATGTCTT) and pCMV6-AC-GFP (the overexpression vector, FH1215; Feng Hui Biotechnology Co., Ltd., Hunan, China). Transfection was performed using a lipofectamine 2000 kit (11668019, Thermo Fisher Scientific, Waltham, MA, USA). After 6 hours transfection, the medium was replaced with complete medium for 48 hours incubation. The cells were
harvested to detect the transfection efficiency and utilized for subsequent experiments.

**Western blot analysis**

Total tissue or cellular protein was extracted using radio-immunoprecipitation assay (RIPA) lysis containing phenylmethylsulfonyl fluoride (P0013C; Beyotime, Shanghai, China), followed by 30 minutes incubation on ice and 10 minutes centrifugation at 4 ℃ and 8,000 g. Following the collection of the supernatant, the total protein concentration was measured with a bicinchoninic acid (BCA) kit. After 50 μg proteins were dissolved in 2× sodium dodecyl sulfate (SDS) loading buffer, each sample was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-blotted to polyvinylidene fluoride (PVDF) membranes. Afterward, the membranes underwent 1 hour blocking with 5% skimmed milk powder at room temperature and probed with diluted primary antibodies (Abcam, UK) against SRC (1:1,000, ab133283, rabbit), phosphorylation (p)-SRC (1:5,000, ab185617, rabbit), CACNA1C (1:1,200, ab84814, mouse), LOX-1 (1:1,000, ab214427, rabbit), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab9485, 1:2,500, loading control) overnight at 4 ℃. Next, the membranes were re-probed with horseradish peroxidase (HRP)-tagged secondary antibodies (Abcam, UK) against goat anti-rabbit IgG H&L (ab97051, 1:2,000) and goat anti-mouse IgG (H&L) (ab6789, 1:2,000) for 1 hour. The blots were visualized by enhanced chemiluminescence (ECL) kit (abs920, Absin) and photographed with a Bio-Rad image analysis system (Bio-Rad Laboratories, Hercules, CA, USA), followed by analysis with Quantity One v4.6.2 software (Bio-Rad, USA).

**Patch-clamp experiments**

Electrode solution (mM) comprised CsCl 110, Na2 phosphocreatine 20, egtazic acid (EGTA) 10, Na2ATP 5, MgCl2 5, NaGTP 0.2, and HEPES 10, with solution PH adjusted to 7.3 by CsOH. Extracellular bath solution (mM) was comprised of NaCl 135, BaCl2 10, TEA-Cl 10, HEPES 10, CsCl 5, and Glucose 10, with solution PH adjusted to 7.4 by CsOH. Experiments were carried out at room temperature. The glass microelectrode was made by 3–4 pulls of a microelectrode micropipette puller (Model P-97; Sutter Instrument, Novato, CA, USA) on the day of the experiment which was used with a resistance range of 4–7 MΩ. The cell membrane capacitance was in the range of 12.15±3.41 pF (n=96), with an access resistance ≥2 GΩ. The current at 10 mV was depolarized from −50 to +40 mV with a step of 500 ms, and current was recorded with a sampling frequency of 10 kHz at a holding potential of −70 mV. In this study, the whole-cell L-Ca current was recorded using an Axopatch 200B amplifier (Axon Instruments, Burlingame, CA, USA), Axon pCLAMP9.2 software, and digidata1322A interface (Axon Instruments, USA).

**Cell Counting Kit-8 (CCK-8)**

A CCK-8 kit (CK04; Dojindo Laboratories, Kumamoto, Japan) was applied for measurement of cell viability. Logarithmic growing cells were seeded at 1×10⁴ per well in a 96-well plate for 24 hours. Cells were then grouped, and 10 μL CCK-8 reagents were added at 0, 24, 48, and 72 hours after grouping, and incubated at 37 ℃ for 3 hours. The absorbance values of each well at 450 nm were measured on a microplate reader, and the values were proportional to the number of cells proliferating in the culture medium. The growth curve was plotted.

**Scratch test**

The concentration of the cells was adjusted to 1×10⁶ cells/mL with DMEM, and the cells were routinely cultured in 10% DMEM encompassing FBS until cell monolayers formed. The pipette tip was adopted to scratch along the bottom of the culture plate in a “1” shape, and the relative distance of the scratch area was recorded under a microscope. Then, the cells were washed with serum-free medium, followed by 24 hours of routine incubation. The cells were observed and photographed under an inverted microscope (Nikon, Japan) to assess the relative distance of cell migration to the wounded area. The actual cell migration distance was calculated as per the distance from the original cell injury area.

**Immunofluorescence**

The VSMCs were treated with PBS with the cell concentration adjusted to 1×10⁶ cells/mL. Cells were supplemented with 20 μg/mL 1,1’-dioctadecyl-3,3’,3”-tetramethylindocarbocyanine perchlorate (Dil) cell membrane-labeled ox-LDL (Dil-ox-LDL) fluorescent dye for 4 hours incubation in a 37 ℃ incubator with 5% CO₂ and saturation humidity. Following incubation, the intensity of Dil-ox-LDL fluorescence staining in cells was...
observed under an inverted fluorescence microscope (BX50; Olympus, Japan).

**Enzymatic colorimetry**

The cells were attained, and 1×10^6 cells were extracted with 200 μL cholesterol extract (trichloromethane: isopropanol: NP40 =7:11:0.1), followed by the determination of the cholesterol concentration. The remaining precipitates were lysed with 200 μL RIPA lysis buffer. Afterward, the protein concentration was estimated using the Lowry method, and the amount of cholesterol per mg of protein (μg/mg) was calculated.

**Statistical analysis**

All statistical analyses of the data were implemented using the software SPSS 21.0 (IBM Corp., Armonk, NY, USA). The measurement data were summarized as mean ± standard deviation and tested for normal distribution and homogeneity of variance. If the data conformed to normal distribution and homogeneity of variance, unpaired t-tests were utilized to compare data between the 2 groups. One-way analysis of variance (ANOVA) or repeated measures ANOVA was employed to compare data among multiple groups, followed by Tukey’s post-hoc test. A P value <0.05 indicated that the differences were statistically significant.

**Results**

**Genistein alleviated AS in rats and inhibited ox-LDL-induced proliferation, migration, and foaming of VSMCs in vitro**

To investigate the molecular mechanisms by which genistein affected AS, a rat model of AS was established. The results of Oil Red O staining and HE staining (Figure 1A-1C) showed that the atherosclerotic plaque lesion area and lipid deposition were significantly increased in rats after AS modeling, and that when AS rats were treated with genistein, the atherosclerotic plaque lesion area and lipid deposition were obviously decreased. This indicated that genistein could alleviate AS in rats.

Rat VSMCs were isolated to develop an in vitro cell model. The VSMCs were identified by immunofluorescence detection of α-SMA in cells. The results (Figure 1D) displayed that VSMCs had green fluorescence, indicating their successful extraction. Next, VSMCs were stimulated with ox-LDL to develop an in vitro cell model. As reflected by the results of CCK-8 and scratch test (Figure 1E,1F, Figure S1A), ox-LDL strikingly enhanced cell viability and migration in VSMCs, whilst treatment with genistein markedly reduced the viability and migration of ox-LDL-exposed VSMCs. Uptake of large amounts of fat by VSMCs leads to cholesterol accumulation, which foams the cells, causing AS (20). Oil Red O staining (Figure 1G) demonstrated a dramatic increase of intracellular lipid particles in ox-LDL-exposed VSMCs and noticeable decline of intracellular lipid particles in ox-LDL-exposed VSMCs following genistein treatment. Enzymatic colorimetry results (Figure 1H) documented that intracellular cholesterol content was substantially elevated in VSMCs by ox-LDL exposure, and that intracellular cholesterol content was conspicuously diminished in ox-LDL-exposed VSMCs after genistein treatment. These data suggested that genistein could inhibit ox-LDL-induced proliferation, migration, and foaming of VSMCs.

**Genistein restrained ox-LDL-induced proliferation, migration, and foaming of VSMCs by decreasing L-Ca channel currents**

Research has illustrated that genistein curtails L-Ca channels (21). To explore the influence of genistein on L-Ca channels, patch-clamp experiments were performed to detect L-Ca channel currents in VSMCs, which displayed that L-Ca channel currents were evidently augmented in VSMCs by ox-LDL exposure, which was nullified by genistein treatment (Figure 2A). Treatment of VSMCs with the L-Ca channel activator BayK8644 and blocker nifedipine revealed that cellular L-Ca channel currents were clearly increased after treatment with BayK8644 but decreased after treatment with nifedipine in ox-LDL-exposed VSMCs. Moreover, BayK8644 reversed the reduction of L-Ca channel currents by genistein in ox-LDL-exposed VSMCs (Figure 2B). Collectively, genistein suppressed ox-LDL-induced L-Ca channel currents in VSMC-derived foam cells.

We further ascertained the effect of genistein on ox-LDL-exposed VSMC activities through inhibition of L-Ca channel currents. With respect to CCK-8 and scratch test results (Figure 2C,2D, Figure S1B), BayK8644 considerably enhanced but nifedipine prominently reduced ox-LDL-exposed VSMC viability and migration, and then BayK8644 annulled the decline of ox-LDL-exposed VSMC viability and migration induced genistein. As for the results of Oil
Red O staining and enzymatic colorimetry (Figure 2E,2F), BayK8644 significantly increased intracellular lipid particles and cholesterol content in ox-LDL-exposed VSMCs, which was contrary after nifedipine treatment. BayK8644 abrogated the repressive impact of genistein on intracellular lipid particles and cholesterol content in ox-LDL-exposed VSMCs.

Conclusively, genistein could subdue ox-LDL-induced proliferation, migration, and foaming of VSMCs via the reduction of L-Ca channel currents.

**Genistein suppressed ox-LDL-exposed VSMC proliferation, migration, and foaming by diminishing SRC activity**

As tyrosine kinases, SRC family kinases have been indicated to assume a role in the atherosclerotic process (22). Since genistein is a tyrosine kinase inhibitor, we hypothesized that it could impact the activities of ox-LDL-induced VSMCs by repressing SRC activity. Western blot analysis results (Figure 3A,3B) exhibited that SRC phosphorylation level was significantly enhanced in the aortic tissues of rats by AS modeling, which was counteracted by genistein treatment. The SRC phosphorylation level was obviously elevated in VSMCs by ox-LDL exposure, which was negated after genistein treatment. This suggested that SRC was hyperphosphorylated in atherosclerotic tissues and that genistein could restrict SRC activity in AS.

To dissect the influence of genistein on activities of ox-LDL-induced VSMCs by downregulating SRC, ox-LDL-induced VSMCs were treated with genistein, sh-SRC, or oe-SRC. Western blot analysis results (Figure 3C) documented appreciable reduction of SRC phosphorylation level in ox-
Figure 2 Genistein decreases proliferation, migration, and foaming of ox-LDL-induced VSMCs through inhibiting L-Ca channel currents. (A) Patch-clamp experiments to measure L-Ca channel currents in VSMCs after ox-LDL exposure. ox-LDL-induced VSMCs were treated with DMSO, genistein, BayK8644, nifedipine, or genistein + BayK8644. (B) Patch-clamp experiments to determine L-Ca channel currents in VSMCs. (C) CCK-8 detection of viability of VSMCs. (D) Scratch test to examine migration of VSMCs. (E) Oil Red O staining analysis of lipid deposition in VSMCs (all observed at 200 μm). (F) Enzymatic colorimetry assessment of cholesterol content in VSMCs. *P<0.05. The cell experiments were repeated 3 times independently. ox-LDL, oxidized low-density lipoprotein; VSMC, vascular smooth muscle cell; DMSO, dimethyl sulfoxide; CCK-8, Cell Counting Kit-8.
Ligand density lipoprotein; VSMC, vascular smooth muscle cell; DMSO, dimethyl sulfoxide; CCK-8, Cell Counting Kit-8.

In conclusion, genistein could downregulate SRC to restrict ox-LDL-induced VSMC proliferation, migration, and foaming.

Genistein repressed L-Ca channel currents by downregulate SRC in ox-LDL-induced VSMCs

Existing research has documented that SRC activates L-Ca channels (15). The results of patch-clamp experiments (Figure 4A) showed that cellular L-Ca channel currents were substantially decreased in ox-LDL-induced VSMCs after silencing SRC. Therefore, we speculate that genistein reduced L-Ca channel currents via SRC downregulation. After genistein treatment and SRC overexpression, patch-clamp experiments (Figure 4B) manifested conspicuous diminishment of cellular L-Ca channel currents in ox-LDL-induced VSMCs following genistein treatment, which was nullified by further overexpressing SRC.

In summary, genistein could curtail L-Ca channel currents by reducing SRC activity.
Figure 4 Genistein-mediated downregulation of SRC suppresses L-Ca channel currents in ox-LDL-induced VSMCs. (A) L-Ca channel currents in ox-LDL-induced VSMCs after silencing SRC examined by patch-clamp experiments. (B) L-Ca channel currents in ox-LDL-induced VSMCs after treatment with genistein and/or oe-SRC examined by Patch-clamp experiments. *P<0.05. The cell experiments were repeated 3 times independently. ox-LDL, oxidized low-density lipoprotein; VSMC, vascular smooth muscle cell; DMSO, dimethyl sulfoxide.

**SRC facilitated ox-LDL-induced proliferation, migration, and foaming of VSMCs by acting on the L-Ca channel subunit CACNA1C**

As a subunit of the L-Ca channel, CACNA1C has been reported to be involved in the process of AS (17,23). Hence, a hypothesis was proposed that SRC might act on CACNA1C. Based on western blot analysis results (Figure 5A,5B), AS modeling conspicuously augmented CACNA1C protein expression in aortic tissues of rats. In VSMCs, ox-LDL induction evidently increased CACNA1C protein expression, which was neutralized by additional SRC silencing. The aforesaid results indicated that CACNA1C was highly expressed in atherosclerotic tissues of rats, and that silencing SRC could lower CACNA1C protein expression in ox-LDL-induced VSMCs.

To evaluate whether SRC affected the activities of ox-LDL-induced VSMCs via CACNA1C, SRC was overexpressed and CACNA1C was silenced in ox-LDL-
induced VSMCs. As indicated by western blot analysis results (Figure 5C), SRC and CACNA1C protein expression were significantly elevated in ox-LDL-induced VSMCs following overexpression of SRC, and CACNA1C protein expression considerably decreased in ox-LDL-induced VSMCs after silencing CACNA1C in the presence of SRC overexpression. For the results of CCK-8 and scratch test (Figure 5D,5E, Figure S1D), the noticeable increase of cell proliferation and migration was observed in ox-LDL-induced VSMCs subsequent to SRC overexpression, whilst these trends were abolished by further silencing CACNA1C. Oil Red O staining and enzymatic colorimetry results (Figure 5F,5G) demonstrated that SRC overexpression caused the prominent elevation of intracellular lipid particles and
cholesterol content, which was counteracted after further silencing CACNA1C.

Taken together, SRC could accelerate the proliferation, migration, and foaming of ox-LDL-induced VSMCs by acting on the L-Ca channel subunit CACNA1C.

**Silencing CACNA1C restricted ox-LDL-induced proliferation, migration, and foaming of VSMCs through downregulation of LOX-1**

Existing literature has demonstrated that CACNA1C facilitates cellular uptake of ox-LDL by upregulating LOX-1 (18). Western blot analysis (Figure 6A,6B) exhibited that LOX-1 protein expression was evidently elevated in aortic tissues of rats after AS modeling and in VSMCs following ox-LDL induction. In ox-LDL-exposed VSMCs, LOX-1 protein expression considerably diminished after silencing CACNA1C, which was annulled by further overexpression of LOX-1. The data revealed that LOX-1 expression was high in atherosclerotic tissues of rats and that silencing CACNA1C resulted in downregulation of LOX-1.

Immunofluorescence (Figure 6C) manifested that CACNA1C silencing prominently lowered cellular uptake of Dil-ox-LDL by VSMCs, which was negated after additional LOX-1 overexpression. The results of CCK-8 and scratch test (Figure 6D,6E, Figure S1E) displayed the marked reduction of ox-LDL-exposed VSMC proliferation and migration after silencing of CACNA1C, which was counteracted following further overexpression of LOX-1. The results of Oil Red O staining and enzymatic colorimetry (Figure 6F,6G) depicted that CACNA1C silencing appreciably decreased intracellular lipid particles and cholesterol content in ox-LDL-exposed VSMCs and that additional LOX-1 overexpression abrogated these trends.

In summary, CACNA1C silencing reduced the uptake of ox-LDL by VSMCs by downregulating LOX-1, thereby repressing ox-LDL-induced proliferation, migration, and foaming of VSMCs.

**Overexpression of LOX-1 counterweighed the ameliorative impacts of genistein on AS in rats and ox-LDL on VSMCs**

To further elucidate the mechanism by which genistein alleviated AS in vivo, AS rats were treated with genistein and oe-LOX-1. Western blot analysis (Figure 7A) manifested that genistein treatment of AS rats strikingly lowered SRC phosphorylation level and CACNA1C and LOX-1 protein expression in aortic tissues. Meanwhile, LOX-1 overexpression reversed the decrease of LOX-1 protein expression triggered by genistein without impacts on SRC phosphorylation level and CACNA1C protein expression. As illustrated by the results Oil Red O staining and HE staining (Figure 7B-7D), observably decreased atherosclerotic plaque lesion areas and lipid deposition were found in AS rats after genistein treatment, which was neutralized by additional overexpression of LOX-1.

The ox-LDL-exposed VSMCs were treated with genistein and oe-LOX-1. Western blot analysis (Figure 7E) showed that genistein treatment dramatically lowered SRC phosphorylation level and CACNA1C and LOX-1 protein expression in ox-LDL-exposed VSMCs. In the presence of genistein, overexpression of LOX-1 augmented LOX-1 protein expression but did not affect SRC phosphorylation level and CACNA1C protein expression in ox-LDL-exposed VSMCs. For immunofluorescence results (Figure 7F), cellular uptake of Dil-ox-LDL by VSMCs was observably reduced after genistein treatment, which was noticeably nullified through further overexpression of LOX-1. The CCK-8 and scratch test results demonstrated that ox-LDL-exposed VSMC proliferation and migration were markedly diminished following genistein treatment, which was abrogated after additional LOX-1 overexpression (Figure 7G,7H, Figure S1F). Oil Red O staining and enzymatic colorimetry showed the dimishment in intracellular lipid particles and cholesterol content inox-LDL-exposed VSMC after genistein treatment and that these results were negated by further overexpressing LOX-1 (Figure 7I,7J).

Together, LOX-1 upregulation abolished the alleviative impacts of genistein on AS in rats and ox-LDL on VSMCs.

**Discussion**

Genistein supplementation is an attractive alternative for cardiovascular protection based on previous epidemiological and animal model studies (24). The study of Thangavel et al. has highlighted inhibitory effect of genistein on incidence of cardiovascular disease, osteoporosis, and the postmenopausal symptoms (25). Some ideas have been presented by Si et al. demonstrating the possible mechanisms of the genistein action on vascular protective effects, where genistein may attenuate pro-inflammatory factor-induced vascular endothelial barrier dysfunction, inactivate the cAMP/PKA cascade, activate peroxisome proliferator-activated receptors, or reduce production of reactive oxygen.
Figure 6 CACNA1C silencing diminishes LOX-1 expression to restrain the proliferation, migration, and foaming of ox-LDL-exposed VSMCs. (A) Western blot analysis to measure LOX-1 protein expression in aortic tissue of rats after AS modeling (8 rats per group). (B) Western blot analysis of LOX-1 protein expression in ox-LDL-exposed VSMCs after CACNA1C silencing and LOX-1 overexpression. ox-LDL-induced VSMCs were treated with sh-NC + oe-NC, sh-CACNA1C + oe-NC, or sh-CACNA1C + oe-LOX-1. (C) Immunofluorescence to assess the uptake of Dil-ox-LDL by VSMCs (all observed at 100 μm). (D) VSMC viability determined by CCK-8. (E) VSMC migration examined by scratch test. (F) Lipid deposition in VSMCs analyzed by Oil Red O staining (all observed at 200 μm). (G) Cholesterol content in VSMCs observed by enzymatic colorimetry. *P<0.05. The cell experiments were repeated 3 times independently. LOX-1, lectin-like oxidized LDL receptor-1; ox-LDL, oxidized low-density lipoprotein; AS, atherosclerosis; VSMC, vascular smooth muscle cell; DMSO, dimethyl sulfoxide; CCK-8, Cell Counting Kit-8.
species-producing enzymes (26). However, few studies exist to provide a clear mechanistic understanding of the molecular mechanistic basis of genistein in AS. Based on this, this research set out to ascertain the mechanism of genistein in AS, and the obtained data revealed that genistein might reduce the uptake of ox-LDL by VSMCs through inhibiting SRC action on the L-Ca channel subunit CACNA1C to downregulate LOX-1, thereby suppressing ox-LDL-induced proliferation, migration, and foaming of VSMCs and preventing the formation of AS (Figure 8).

The initial finding in our research was that genistein alleviated AS in rats. Similarly, mounting evidence has illustrated the atheroprotective impacts of genistein. For instance, it has been displayed that genistein can repress tumor necrosis factor-α (TNF-α)-triggered adhesion of monocytes to human umbilical vein endothelial cells, a crucial event in AS pathogenesis (27). In addition, a prior work had indicated that a genistein derivative, 7-Difluoromethyl-5,4’-dimethoxygenistein, can lower lipid plasma levels to curtail AS development (28). On the grounds of ‘inflammation’ hypotheses of AS formation, anti-inflammatory agents hold remarkable potential for AS management (29). Of interest, genistein has been suggested to present anti-inflammatory properties in VSMCs of atherosclerosis lesions, which were realized by regulating the ER-p38/NF-κB-CRP/MMP-9 axis (30).
Moreover, another study revealed that genistein causes significant declines in the formation of atherosclerotic plaques in LDL receptor-/- rats by downregulating inflammatory NF-kB and VCAM-1 (31). It should be noted that VSMCs are a primary cell type existing at all stages of atherosclerotic plaques (32).

In the subsequent assays, we observed that genistein diminished VSMC proliferation, migration, and foaming caused by ox-LDL. Consistent with our results, the research performed by Bai et al. elucidated that genistein restricted the proliferation of ox-LDL-induced VSMCs (11). Besides, a prior work unraveled that genistein resulted in the decrease in VSMC proliferation and migration during neointima formation (33). Genistein could suppress VSMC-derived foam cell formation through the downregulation of LOX-1 by blocking tyrosine kinase pathway (34).

Furthermore, our work exhibited that inhibition of L-Ca channels participated in the suppressive impacts of genistein on ox-LDL-induced VSMC proliferation, migration, and foaming. Concordant with our findings, it was noted in a previous study that genistein restrained L-Ca channels in gastrointestinal smooth muscle (35). Of note, the L-Ca channel blocker, diltiazem, has been documented to reduce VSMC migration during AS (36). Also, it was previously reported that another L-Ca channel blocker, nifedipine, exerted anti-proliferative effects on rat aortic VSMCs (37), which is in line with our results. Hence, genistein suppressed L-Ca channels to diminish ox-LDL-induced VSMC proliferation, migration, and foaming.

Another finding of our study was that genistein restricted ox-LDL-induced VSMC proliferation, migration, and foaming by inhibiting SRC activity. This finding is partially supported by a report by Lee et al. that genistein obviously decreased SRC phosphorylation in prostate cancer cells (38). A recent report unveiled that repressing SRC activation attenuated ox-LDL-induced lipid accumulation and foam cell formation in VSMCs (39). Concordantly, Lang et al. demonstrated that the downregulation of SRC correlated to the reduced proliferation and migration of hydrogen peroxide-exposed VSMCs (40). Notably, another study manifested that c-SRC could induce the migration of VSMCs through activating L-Ca channels (8), which is in parallel with our results that genistein suppressed L-Ca channels by reducing SRC activity.

Besides, we observed that SRC accelerated ox-LDL-triggered proliferation, migration, and foaming of VSMCs via LOX-1 downregulation by acting on CACNA1C. As an L-Ca channel subunit (23), CACNA1C has been implicated in AS formation (17). Moreover, the same relationship between CACNA1C and LOX-1 was illustrated in a previous study (18). More importantly, the anti-atherosclerotic activity of LOX-1 has been found by the research of Li et al. (41). Concurrently, a recent work exhibited that LOX-1 overexpression was associated with VSMC proliferation caused by ox-LDL (42). As previously reported, LOX-1 downregulation culminated in restricted ox-LDL-exposed AS model.

Figure 8 The mechanism graph of genistein in AS. Genistein inhibits the uptake of ox-LDL by VSMCs by restricting SRC action on the L-Ca channel subunit CACNA1C to downregulate LOX-1, thus decreasing ox-LDL-induced proliferation, migration, and foaming of VSMCs and preventing the formation of AS. AS, atherosclerosis; ox-LDL, oxidized low-density lipoprotein; VSMC, vascular smooth muscle cell; LOX-1, lectin-like oxidized LDL receptor-1.
foam cell formation in rat aortic VSMCs, thus exerting an ameliorating effect in AS formation (43,44). In alignment with our findings, LOX-1 upregulation was involved in ox-LDL-triggered VSMC proliferation, migration, and foam cell formation (45).

Taken together, our study adds to the growing evidence for the atheroprotective effects of genistein. Specifically, genistein protected VSMCs against ox-LDL-exposed proliferation, migration, and foam cell formation through LOX-1 downregulation by blocking the action of SRC on the L-Ca channel subunit CACNA1C, which alleviated AS. Investigation of genistein and the SRC/CACNA1C/LOX-1 axis in VSMCs and AS yields a better understanding of their in vivo mechanisms and may have potentially critical therapeutic implications in the treatment of AS.

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**Footnote**

**Reporting Checklist:** The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2113/rc

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**Conflicts of Interest:** All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2113/coif). The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 2021086) granted by Animal Ethical Care Committee of the First Affiliated Hospital of Harbin Medical University, in compliance with institutional guidelines for the care and use of animals.

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