PEP-1-MsrA ameliorates inflammation and reduces atherosclerosis in apolipoprotein E deficient mice

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Abstract

Background: Methionine sulfoxide reductase A (MsrA) is a potent intracellular oxidoreductase and serves as an essential factor that protects cells against oxidative damage. However, therapeutic use of exogenous MsrA in oxidative stress-induced diseases is limited, because it cannot enter the cells. The aim of this study is to investigate whether MsrA with PEP-1, a cell penetrating peptide, fused to its N-terminus can protect against oxidative stress in macrophages and can attenuate atherosclerosis in apolipoprotein E deficient (apoE−/−) mice.

Methods: MsrA and the fusion protein PEP-1-MsrA were expressed and purified using a pET28a expression system. Transduction of the fusion protein into macrophages was confirmed by Western blot and immunofluorescence staining. Intracellular reactive oxygen species (ROS) and apoptosis levels were measured by flow cytometry. In in vivo study, MsrA or PEP-1-MsrA proteins were intraperitoneally injected into apoE−/− mice fed a Western diet for 12 weeks. Plasma lipids levels, inflammatory gene expression, and paraoxonase-1 (PON1) and superoxide dismutase (SOD) activities were assessed. Atherosclerotic lesions were analyzed by Oil Red O staining and immunohistochemistry.

Results: PEP-1-MsrA could penetrate the cells and significantly reduced intracellular ROS levels and apoptosis in H2O2-treated macrophages. It also decreased TNFα and IL-1β mRNA levels and increased the IL-10 mRNA level in lipopolysaccharide-treated macrophages. In in vivo study, PEP-1-MsrA injection significantly increased plasma PON1 and SOD activities and decreased plasma monocyte chemotactant protein 1 (MCP-1) level compared to the injection of vehicle control or MsrA. In PEP-1-MsrA injected mice, hepatic PON1 levels were increased, while the expression of TNFα and IL-6 mRNA in the liver was suppressed. Although plasma total cholesterol and triglyceride levels did not change, the aortic atherosclerosis in PEP-1-MsrA treated mice was significantly reduced. This was accompanied by a reduction of total and apoptotic macrophages in the lesions.

Conclusion: Our study provides evidence that PEP-1-MsrA may be a potential therapeutic agent for atherosclerosis-related cardiovascular diseases.

Keywords: Methionine sulfoxide reductase A, Atherosclerosis, PEP-1, ROS, Inflammation, Macrophages, Penetrating fusion protein

Background

Cardiovascular disease (CVD), primarily induced by atherosclerosis, is the most common cause of morbidity and disability worldwide. Atherosclerosis is now considered a chronic inflammatory disease associated with oxidative stress and dyslipidemia [1–3]. Numerous studies have documented that among the multifactorial causes of atherosclerosis, reactive oxygen species (ROS) play important roles in its pathogenesis by stimulating the oxidation and modification of lipids and proteins, which can lead to foam cell formation and atherosclerotic plaque progression in the blood vessel wall [4].
ROS, including superoxide anion, hydroxyl radical and hydrogen peroxide (H₂O₂), are constantly generated by cells as byproducts of normal metabolism and highly localized within the cells [5, 6]. Cells contain a series of antioxidant defenses such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase that directly detoxify ROS and reduce their effects. The imbalance between ROS generation and the antioxidant system in vascular cells occurs due to a change in the overall redox balance and in the modification of target molecules, resulting in inflammation and atherogenesis. There is increasing evidence that a therapeutic approach targeting antioxidant enzymes could decrease the progression of atherosclerosis [7].

Sulfur-containing methionine (Met), either free or in proteins, is an essential amino acid and a sensitive target for oxidants. Met is easily oxidized into methionine-S-sulfoxide (MetSO) which can be reduced back to Met by a ubiquitous intracellular enzyme named methionine sulfoxide reductase A (MsrA). In turn, oxidized MsrA can be reduced by the cellular thioredoxin (Trx), thioredoxin reductase and NADPH system [8]. Recent studies have demonstrated that MsrA is involved in oxidative stress and age associated diseases [9], and enhanced MsrA might be an important approach for the prevention of many oxidative stress-induced diseases, such as hypoxia/reoxygenation and ischemia/reperfusion injury [10, 11], cataracts [12], diabetes mellitus [13], Alzheimer’s disease [14, 15] and atherosclerosis related coronary artery disease [16, 17].

MsrA plays protective roles by maintaining intracellular redox homeostasis; however, therapeutic use of exogenous MsrA is limited, because this enzyme is poorly taken up by cells [18]. Cell penetrating peptides (CPPs) or protein transduction domains (PTDs) were designed to deliver large proteins into mammalian cells [19]. A 21-amino acid peptide, PEP-1, is a new type of CPP [20], and has been reported to be able to successfully transfer a wide range of therapeutic proteins such as SOD [21], CAT [22], paraoxonase-1 (PON1) [23] and peroxiredoxin2 [24] into cells and thus to achieve their intracellular biological activities [25]. Although the precise mechanism is as yet unclear, PEP-1 fusion proteins have been used in research to protect against many diseases including neuronal disease, myocardial ischemic/reperfusion injury and skin inflammation [26–28].

The goal of this study is to investigate whether the fusion protein PEP-1-MsrA can efficiently enter cells to protect against oxidative stress and to attenuate atherosclerosis in apolipoprotein E deficient (apoE−/−) mice, providing evidence that PEP-1-MsrA may be a potential therapeutic agent for atherosclerosis-related cardiovascular diseases.

**Methods**

**Expression and purification of MsrA and PEP-1-MsrA fusion protein in E. coli**

The plasmid containing the human MsrA cDNA sequence without a signal peptide was provided by Prof. Stefan H. Heinemann (Jena Friedrich Schiller University, Germany) and the pET15b/PEP-1 plasmid was provided by Prof. Jia-Ning Wang (Hubei University of Medicine, China). The pET28a vectors were purchased from Novagen (Merck Millipore, Germany). To generate the recombinant pET28a/PEP-1-MsrA vector, a 642-bp cDNA fragment of human MsrA, digested at BamHI and HindIII sites, from pQE30/MsrA was inserted into a pET15b/PEP-1 plasmid. Then the PEP-1-MsrA fragment was digested at Ndel and HindIII sites and inserted into a pET28a vector. A pET28a/MsrA vector was also constructed as control. The recombinant vectors were verified by DNA sequencing.

To produce PEP-1-MsrA or MsrA proteins, plasmids were transformed into *E. coli* BL21 (DE3). The transformed cells were grown in LB medium containing 10 μg/ml kanamycin at 37 °C to an A₆₀₀ value of 0.6–0.8 and induced with 1 mM isopropyl β-d-1-thiogalactoside (IPTG) at 30 °C for 4–6 h. Then the bacteria were harvested and lysed by a low temperature ultra-high pressure continuous flow cell disrupter (JNIO, China) in lysis buffer (pH 8.0, 50 mM NaH₂PO₄, 400 mM NaCl, 10 mM imidazole including with 3 μg/ml lysozyme, 1 μg/ml DNase I and 5 μM PMSF). The proteins were purified by affinity chromatography using Ni-NTA agarose (Qia-gen, Germany) according to the manufacturer’s instructions. The purity was analyzed by 10 % SDS-PAGE with Coomassie Blue Staining. Concentrations of the purified proteins were determined by the Lowry method using a DC protein assay kit (Bio-Rad, USA).

**Circular dichroism measurements**

Circular dichroism (CD) spectra of proteins were determined using a Jasco J-810 spectropolarimeter (Jasco Corp., Japan) with a 1 mm light-path quartz cell. The far-UV CD spectra were recorded from 190 to 250 nm. PEP-1-MsrA or MsrA protein solutions were prepared at a final concentration of 15 μM in 100 mM phosphate buffer (PB, pH 7.5). The averaged spectra of several scans were corrected relative to the buffer blank. Measurements were made at room temperature.

**MsrA activity assay**

MsrA activity was measured using methyl sulfoxides (DMSO, Sigma-Aldrich, USA) as a substrate as described by Wu [29]. The reaction system (pH 8.0) contained 500 μM DMSO, 10 mM MgCl₂, 30 mM KCl and 50 μM DTT in 25 mM Tris–HCl to which 3.6 μM
of the heat-inactivated or active Msra or PEP-1-Msra was added. To terminate the reaction, 100 μl of reaction mixture and 100 μl of 4 M 5,5'-dithiobis (DTNB, Sigma-Aldrich, USA) were added and the mixture was incubated for 10 min at 37 °C. The A412 value was then recorded at 0 and 10 min after the end of the incubation period. The decrease in A412 value was calculated (ΔA = A412min − A41210min). The heat-inactivated Msra (control) was defined as ΔAControl while active Msra was named ΔATotal. Msra activity was calculated using the formula: ΔAMsra = ΔATotal − ΔAControl. PEP-1-Msra activity was normalized with Msra.

**Cell culture**

Murine macrophage cell line Raw 264.7 cells and human HeLa cells were obtained from the Animal Biosafety Level 3 Laboratory (ABSL-III) at Wuhan University, China. Mouse peritoneal macrophages were harvested by peritoneal lavage 3–4 days after intraperitoneal injection of 3 ml 3 % thioglycollate [30]. The cells were cultured in Dulbecco's modified Eagle medium (DMEM, ThermoFisher, USA) containing 10 % fetal bovine serum (FBS, Gibco, USA) and antibiotics (100 mg/ml streptomycin, 100 U/ml penicillin, Beyotime, China) at 37 °C. Cells were seeded in plates and incubated with serum-free DMEM at 37 °C overnight before further treatment.

**Transduction of PEP-1-Msra protein into cells**

MTT method was performed to test the cytotoxicity of the proteins. HeLa cells were treated with Msra or PEP-1-Msra (0–18 μM) for 72 h. To determine the transduction efficiency of PEP-1-Msra, peritoneal macrophages were treated with various concentrations of PEP-1-Msra or Msra protein (0.5–8 μM) for 1 h. Then cells were washed with PBS and harvested for Western blot analysis. The cells were also treated with 1 μM of proteins for various times (5 min–24 h) to examine the stability of PEP-1-Msra by Western blot analysis.

We further detected the intracellular distribution of transduced protein using an immunofluorescence assay. Briefly, peritoneal macrophages were seeded on coverslips and treated with selected concentrations of PEP-1-Msra or Msra protein (1–4 μM) for 1 h, and then washed with PBS at least three times and fixed with 4 % paraformaldehyde for 10 min at 37 °C. The cells were incubated with the primary antibody (anti-Msra antibody, Abcam, USA) overnight at 4 °C, then with FITC-conjugated goat anti-rabbit IgG (1:10,000). Nuclei were stained with 300 nM DAPI (Sigma-Aldrich, USA) for 15 min at 37 °C. The intracellular localization of Msra protein was analyzed with confocal microscopy (Olympus Fluoview FV1000, Japan).

**Determination of intracellular ROS levels**

Intracellular ROS levels were detected by the sensitive fluorescent dye 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich, USA) as described in previous studies [23, 28]. RAW264.7 cells were pre-incubated with PEP-1-Msra or Msra protein (1 μM) for 1 h, and then exposed to 1 mM H2O2 for 1 h. After washing with PBS, cells were incubated with 20 μM DCFH-DA for 30 min at 37 °C. The DCF fluorescence intensity was measured by flow cytometry (FACS Aria™ system, BD, USA).

**Cell death assays**

Cell death was detected by Annexin V and propidium iodide (PI) binding assay. Peritoneal primary macrophages were pre-treated with PEP-1-Msra and Msra protein (1 μM) for 1 h before incubated with 1 mM H2O2 for 1 h. Cells were resuspended in Annexin-V binding buffer and incubated with Annexin V-FITC and PI for 15 min at room temperature, according to the procedure for the Annexin-V-fluorescein (Annexin-V-FITC) Apoptosis Detection Kit (Bestbio, China). Total cell death was analyzed by flow cytometry.

**Western blot analysis**

Harvested cells or frozen tissues were lysed by RIPA (Beyotime Institute of Biotechnology, China) with 1 % protease inhibitors (Roche, Germany) for Western blot analysis. Appropriate amounts of proteins were loaded and separated by 10 % SDS-PAGE and transferred onto a nitrocellulose membrane. Protein expression was detected using primary antibodies, anti-Msra antibody or anti-PON1 antibody (Abcam, USA) (1:1000), followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, USA) (1:10,000). Signal was detected using an enhanced chemiluminescence kit (ECL, GE Healthcare, USA). The band densitometry was analyzed with Image J software (NIH, USA).

**Quantitative real-time PCR (qPCR)**

Mouse peritoneal macrophages were co-cultured with 25 ng/ml LPS and 1 μM PEP-1-Msra or Msra proteins for 3 h, and then harvested with Trizol reagent (Invitrogen, USA). Mouse liver tissue was also homogenized with Trizol reagent. Total RNA was extracted using an RNeasy kit (Qiagen, Germany) and reverse transcribed into cDNA using a PrimerScript® RT reagent Kit with gDNA Eraser (Takara, Japan). Target mRNA levels were measured by qPCR with the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) according to the manufacturer’s instructions. Mouse genes were normalized with 18s RNA as an endogenous control. Primers used were as follows: mouse 18s RNA: 5′
CGCGGTTCATTACATTTTGGGTTG 3′ (forward) and 5′ AGTGCTTCTCCTGATGTC 3′ (reverse); mouse IL-1β: 5′ GCCATCCGACTGTCGTC 3′ (forward) and 5′ AGGCCACAGGTAATTTTGGC 3′ (reverse); mouse TNFα: 5′ GTCCAGCGATTTGCTATCTG 3′ (forward) and 5′ CGGACTCCGAAGCTTAAG 3′ (reverse); mouse IL-10: 5′ AGCTTTACGAGAATGATCCATG 3′ (forward) and 5′ GGCCTTGTAGACACTTGTTTCAAG 3′ (reverse); and mouse IL-6: 5′ AGTTGCCCTTCGCGTGGT 3′ (forward) and 5′ CTCCCCGCTATTTGCAAG 3′ (reverse).

Mice
ApoE−/− mice on a C57BL/6 background were purchased from Vital River Laboratory Animal Technology Company, China, and housed in microisolator cages in the Wuhan University Animal Center. Mice were fed in a temperature-controlled facility (temperature 22 ± 1 °C, humidity 60 %, 12:12 h dark-light cycle) with free access to food and water. Animal care and experimental procedures were performed under the regulations of the Institutional Animal Care and the Ethics Committee for Animal Experiments of Wuhan University, in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee. Thirty male apoE−/− mice at 21 weeks of age were randomly divided into three groups and intraperitoneally injected with one of the two target proteins (dose of 5.5 nmol per mouse every 36 h) or 10 mM phosphate buffered saline (PBS, pH 7.4), respectively. Mice were fed with AIN76A Western diet (HFK bioscience company, China) for 12 weeks to accelerate the development of atherosclerosis.

Determination of biochemical parameters in plasma and liver
Blood samples were collected from mice after overnight fasting by retro-orbital venous plexus puncture at 4 week intervals. Plasma was immediately separated by centrifugation at 10,000×g for 10 min at 4 °C. Total cholesterol (TC) and triglyceride (TG) levels in fresh plasma were measured by enzymatic colorimetric assay kits (Mind Bioengineering, China). The remaining plasma was stored at −80 °C for other analysis. Plasma MCP-1 levels were determined by enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, USA) according to the manufacturer’s instructions. Plasma PON1 activity was measured using paraaxon (Sigma-Aldrich, USA) as a substrate as described previously [31]. Plasma SOD activity was measured using SOD Detection Kit (Nanjing Jiancheng Bioengineering Institute, China). Frozen mouse liver samples were lysed by RIPA with 1 % proteinase inhibitors for Western blot analysis. Liver homogenates treated with Trizol reagent were used for qPCR analysis of target mRNA levels.

Histochemical and immunocytochemical analyses of atherosclerotic lesions
Mice were intraperitoneally injected with either proteins or PBS at 36 h intervals for 12 weeks, then sacrificed 2 h after the last of these injections. The aortic roots were embedded in OCT (SAKURA, USA) and quickly frozen to −20 °C; then 8-μm serial sections of the aortic root were collected for atherosclerosis analysis. Aortas fixed in 4 % paraformaldehyde were analyzed en face. The extent of atherosclerosis was determined by Oil Red O staining and quantification with Image J software as described previously [32]. The location of MsrA in the aortic root was detected by immunofluorescence staining with anti-MsrA antibody. Rat anti-mouse monocytes/macrophage antibody-2 (MOMA2, Bio-rad, USA) was used for macrophage detection in the aortic root. Immunoreactivity was visualized using the Vectastain ABC kit (Vector Labs, USA) and then reacted with DAB substrate (ZSGB-BIO, China). Analysis of apoptosis in aortic root tissue was performed using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling assay kit (TUNEL, Roche Diagnostics, Germany) according to the manufacturer’s instructions. The TUNEL positive cells were counted and normalized to the cell totals. MOMA-2 staining area and apoptosis were analyzed with Image-Pro Plus 6.0.

Statistical analysis
Data are represented as the mean ± SEM. Statistical analyses were performed using Student’s t test and one-way ANOVA between the groups. Differences were considered to be significant at P < 0.05.

Results
Purified PEP-1-MsrA had similar structure and enzyme activity to MsrA
Recombinant pET28a/MsrA and pET28a/PEP-1-MsrA plasmids were constructed (Additional file 1: Figure S1). PEP-1-MsrA or MsrA proteins were highly expressed in E. coli BL21 (DE3) induced by IPTG and were purified from bacteria extracts using Ni-NTA affinity chromatography. The purified proteins were checked by 10 % SDS-PAGE and shown to be more than 95 % purity (Fig. 1a). MsrA proteins were confirmed by Western blot (Fig. 1b). The molecular weights of MsrA and PEP-1-MsrA are ~27 and ~30 kDa, respectively.

To determine whether fusion with PEP-1 could alter the structure of MsrA, we compared the secondary
structural features of PEP-1-MsrA and MsrA by far-UV CD. Both PEP-1-MsrA and MsrA adopt a predominantly $\alpha$-helix conformation with double minima at 208 and 222 nm and have similar $\alpha$-helix contents (17.1 % for PEP-1-MsrA and 18.1 % for MsrA) (Fig. 1c), suggesting that the fusion with PEP-1 does not change the structure of MsrA.

We also compared the enzyme activity of purified PEP-1-MsrA to that of MsrA. The enzyme activity of PEP-1-MsrA appeared to be slightly higher than that of MsrA, but the difference was not statistically significant (Fig. 1d), indicating that the fusion with PEP-1 does not compromise the function of MsrA.

**Transduction of PEP-1-MsrA into macrophages**

The cytotoxicity of proteins was tested on HeLa cells using the MTT method. We found that even at concentrations up to 18 $\mu$M, neither purified MsrA nor PEP-1-MsrA protein reduced cell viability (Additional file 2: Figure S2). We selected 0.5–8 $\mu$M purified protein to determine the transduction ability and intracellular stability of PEP-1-MsrA protein. As shown in Fig. 2a, PEP-1-MsrA protein could successfully enter the cells in a dose-dependent manner, whereas control MsrA protein did not enter the cells. Also, peritoneal macrophages were treated with 1 $\mu$M PEP-1-MsrA proteins; the presence of PEP-1-MsrA protein was maintained for more than 24 h in the cells (Fig. 2b).

In addition, the intracellular distribution of transduced PEP-1-MsrA was detected by confocal microscopy. As shown in Fig. 2c, PEP-1-MsrA protein was detected in the cytoplasm, and immunofluorescence was increased in a concentration dependent manner, whereas MsrA protein was not detected in macrophages even when incubated with higher concentrations of MsrA protein.
PEP-1-MsrA inhibited H\textsubscript{2}O\textsubscript{2} induced oxidation and cell death of macrophages

To determine the effects of transduced PEP-1-MsrA on oxidative stress, RAW264.7 cells were pre-treated with 1 \( \mu \)M PEP-1-MsrA or MsrA proteins for 1 h followed by treatment with 1 mM \( \text{H}_2\text{O}_2 \). ROS levels were then measured. As shown in Fig. 3a, \( \text{H}_2\text{O}_2 \) treatment resulted in high ROS levels in the cells. ROS production was markedly reduced in the cells pre-treated with PEP-1-MsrA, but was not changed by MsrA pre-treatment. We also examined the impact of PEP-1-MsrA on cell survival under \( \text{H}_2\text{O}_2 \) induced oxidative stress using peritoneal macrophages. Compared to MsrA treated cells, cells treated with PEP-1-MsrA showed a remarkable reduction in apoptosis and necrosis (Fig. 3b), indicating that intracellular PEP-1-MsrA could protect macrophages from \( \text{H}_2\text{O}_2 \)-induced cell death by reducing intracellular ROS levels.

PEP-1-MsrA suppressed LPS induced inflammation in macrophages

To investigate the effects of intracellular MsrA on inflammatory responses, mouse peritoneal macrophages were pre-treated with PEP-1-MsrA or MsrA and then treated with LPS to induce inflammatory responses. In PEP-1-MsrA pre-treated cells, LPS-induced expression of the proinflammatory cytokines IL-1\( \beta \) and TNF\( \alpha \) was significantly inhibited, while the expression of anti-inflammatory cytokine IL-10 was increased compared to the respective levels in MsrA pre-treated cells (Fig. 3c–e),
indicating that intracellular PEP-1-MsrA significantly suppressed the macrophage inflammatory response.

**PEP-1-MsrA ameliorated inflammatory and oxidative stress in apoE−/− mice**

ApoE−/− mice were intraperitoneally injected with MsrA, PEP-1-MsrA proteins or vehicle and fed a Western-diet for 12 weeks. The body weights (at various time-points) and spleen weights (at the end point) of mice showed no difference between all three groups (Additional file 3: Figure S3). Plasma PON1 and SOD activities are the indicators of anti-oxidative status in circulation. We found that plasma PON1 and SOD activities were significantly increased, while the plasma inflammatory factor MCP-1 level was decreased in PEP-1-MsrA injected mice compared to the respective levels in control mice or MsrA injected mice (Fig. 4a–c).

The liver is an important organ for the regulation of oxidative and inflammatory status. PON1 is an HDL-associated antioxidase that is mainly synthesized in liver. We found that mRNA and protein levels of PON1 in liver tissue were significantly increased in PEP-1-MsrA injected mice compared to those of MsrA injected or control mice. This is consistent with the high level of MsrA in the livers of PEP-1-MsrA injected mice (Fig. 4d, e). Meanwhile, the mRNA levels of inflammatory cytokines TNFα and IL-6 were significantly decreased in PEP-1-MsrA treated mice (Fig. 4f). These data indicate that when PEP-1-MsrA enters the cells in the liver, it plays an anti-oxidative and anti-inflammatory role through upregulating PON1 expression and inhibiting the expression of inflammatory cytokines. These data demonstrated that transduced PEP-1-MsrA improved circulation and hepatic anti-oxidative and anti-inflammatory status in apoE−/− mice.

**PEP-1-MsrA attenuated atherosclerosis in apoE−/− mice**

During the treatment with purified proteins and Western-diet feeding, the plasma TC and TG levels were not significantly different between the three groups (Table 1). The impact of PEP-1-MsrA injection on the development of atherosclerosis in apoE−/− mice was
assessed. Representative atherosclerotic lesions in cross-sections of aortic roots and en face aorta images stained with Oil Red O are shown in Fig. 5a, b. The lipid staining area in the lesion of PEP-1-MsrA injected mice (0.32 ± 0.02 mm²) was significantly smaller than that in control mice (0.40 ± 0.02 mm²) or MsrA injected mice (0.38 ± 0.03 mm²), reduced by 20 and 16 % respectively (P < 0.01 or P < 0.05, Fig. 5c). En face analysis of pinned-out aortas revealed that the percent area of atherosclerotic lesion in PEP-1-MsrA injected mice was also significantly reduced compared to MsrA injected mice and control mice (P < 0.01, Fig. 5d).

We found that higher levels of MsrA were detected in the arterial walls of PEP-1-MsrA injected mice compared to those in the control and MsrA groups, indicating that PEP-1-MsrA successfully entered the cells of aorta (Fig. 6a). We further stained macrophages in aortic root lesions using a mouse macrophage specific antibody, MOMA2, and found the PEP-1-MsrA injected mice displayed 28 % smaller macrophage areas compared to control mice (0.28 ± 0.01 vs 0.39 ± 0.01 mm²) and 20 % smaller macrophage areas compared with MsrA injected mice (0.35 ± 0.02 mm²) (Fig. 6b). In addition, there were 46 % fewer apoptotic cells in the aortic root cross-sections of PEP-1-MsrA injected mice than in those of control mice and 48 % fewer than in MsrA injected mice (Fig. 6c). These data show that PEP-1-MsrA attenuates the development of atherosclerosis in apoE−/− mice by reducing the accumulation of macrophages, especially apoptotic macrophages, in the lesions.

**Discussion**

Intracellular redox status is tightly regulated by oxidant and antioxidant systems. An imbalance between these systems causes ROS accumulation which leads to oxidative stress and inflammation. It has been shown that ROS-induced oxidation is a well-known cause of atherogenesis and is involved in every step of atherosclerosis development, including vascular endothelial cell injury, macrophage chemotaxis, foam cell formation...
and smooth muscle cell migration and proliferation [33]. Many research efforts have been made without much success to develop a therapeutic approach for atherosclerosis through regulating the redox status by improving the efficacy of antioxidant enzymes.

MsrA, one of the antioxidant defenses in cells, is important in the maintenance of redox homeostasis and in the prevention of oxidative stress-related disease. MsrA is distributed in the cytoplasm, mitochondria and nuclei and is involved in regulating the redox state and the function of numerous proteins, for examples, calmodulin, apolipoprotein AI, IL-6, interferon and cytochrome c oxidase [34]. Studies have shown that MsrA activity was significantly reduced in the brains of Alzheimer’s disease

### Table 1 Plasma lipids levels in apoE−/− mice

| Time (weeks) | Control   | MsrA      | PEP-1-MsrA |
|--------------|-----------|-----------|------------|
| TC (mg/dL)   |           |           |            |
| 0            | 522.9 ± 89.1 | 561.6 ± 76.6 | 528.0 ± 28.1 |
| 4            | 696.7 ± 116.9 | 727.1 ± 92.8 | 699.7 ± 83.7 |
| 8            | 824.6 ± 67.5 | 809.1 ± 126.9 | 863.8 ± 143.1 |
| 12           | 888.6 ± 75.7 | 929.9 ± 91.7 | 867.3 ± 119.1 |
| TG (mg/dL)   |           |           |            |
| 0            | 127.4 ± 19.7 | 139.3 ± 25.4 | 137.7 ± 21.3 |
| 4            | 170.4 ± 20.7 | 164.9 ± 22.0 | 168.3 ± 16.1 |
| 8            | 188.0 ± 30.7 | 206.2 ± 40.4 | 192.6 ± 35.7 |
| 12           | 191.5 ± 41.5 | 185.3 ± 30.0 | 194.3 ± 30.6 |

Data are given as the mean ± SEM, n = 10. The statistical analysis was performed using a two-tailed Student’s t test. Mice were fed a Western-type diet and intraperitoneally injected with PBS, MsrA or PEP-1-MsrA proteins for 12 weeks

TC total cholesterol, TG triglycerides

![Fig. 5](image-url) PEP-1-MsrA reduced atherosclerotic lesions in apoE−/− mice. The atherosclerotic lesions was examined using Oil Red O-stained cross-sections of the aorta root (8-µm serial sections) and by en face analysis of the aorta. a, b Representative lesions in cross-sections of the aorta root and en face aortas. c, d The atherosclerotic lesion areas were quantified with Image J software.
patients and in the cardiomyocytes in cardiac ischemia models [35, 36]. E. coli and yeast with MsrA mutations or deficiency were particularly sensitive to oxidative damage [37, 38]. MsrA knockout mice are highly sensitive to oxidative stress and show nerve damage and shortened life spans [39]. Whereas MsrA transgenic Drosophila show significantly enhanced anti-oxidation and anti-aging characteristics [40]. However, whether or not MsrA could be used as a therapeutic agent for atherosclerosis has not been investigated.

Due to their size and low permeability, it is usually difficult for foreign proteins to enter cells and perform intracellular functions [18]. A recent report showed that the 21-residue PEP-1 peptide is a new type of amphipathic CPP [20]. Several PEP-1 fusion proteins, such as PEP-1-SOD, PEP-1-CAT, PEP-1-SIRT2 and PEP-1-PON1, have been designed; and studies have demonstrated that PEP-1 is a promising tool that may help in the development of potential protein therapeutic agents against oxidative stress [21–23, 28]. We constructed a PEP-1-MsrA fusion protein in order to deliver MsrA into cells and allow it to regenerate its reductase activity using the intrinsic cellular Trx reduction system. We found that MsrA, fused with a PEP-1 peptide, was able to efficiently enter macrophages with regular incubation. Lee et al. recently reported that a similar fusion protein of MsrA could rapidly enter keratinocytes and play a protective role against oxidative stress and ultraviolet radiation-induced cell death [41]. Interestingly, they found that their fusion protein was truncated in the N-terminal region of MsrA between Lys-27 and Val-28 during expression in E. coli and purification. MsrA in our study was designed to cleave off N-terminal domain of mitochondrial targeting signal peptide, so we did not observe the phenomenon of MsrA protein truncation in vitro. Our study showed that PEP-1-MsrA entered into macrophages and exerted the anti-oxidative and anti-inflammatory functions through decreasing intracellular ROS levels. Also

![Figure 6](image-url)
of note, PEP-1-MsrA suppressed LPS-induced expression of the proinflammatory cytokines TNFα and IL-1β and increased expression of the anti-inflammatory cytokine IL-10 [42], suggesting that intracellular MsrA could inhibit M1 polarization of macrophages under pro-inflammatory stimulations.

In the in vivo study, we injected purified PEP-1-MsrA proteins into apoE−/− mice fed a Western diet. ApoE−/− mice have been widely used as an atherosclerotic mice model due to the development of spontaneous hypercholesterolemia and atherosclerosis, which can be accelerated by high fat diet feeding. We found that injection of PEP-1-MsrA could significantly reduce the size of atherosclerotic lesions without altering plasma lipid levels, but injection of MsrA did not affect atherosclerosis. These results suggest that the anti-oxidative and anti-inflammatory effects of cell penetrating PEP-1-MsrA confer anti-atherogenic benefit to the hyperlipidemic mice through lipid-independent mechanisms. In fact, our results showed that PEP-1-MsrA injection increased mouse plasma PON1 and SOD activities and decreased plasma MCP-1 levels. PON1 is mainly synthesized in the liver and secreted to the plasma where it is mainly associated with HDL particles. Our previous study indicated the negative correlation between the activity of plasma PON1 and the extent of atherosclerosis [31]. SOD is another important protein that maintains redox homeostasis. A recent study reported that Met/MetSO modified liver homeostasis and altered the redox state by increasing the activity of SOD [43]. Our results further demonstrated that accumulation of PEP-1-MsrA in the liver led to an increase in hepatic PON1 mRNA and protein levels, indicating that PEP-1-MsrA may enter hepatocytes and up-regulate PON1 expression. In the meantime, the expression of inflammatory cytokines TNFα and IL-6 was also reduced in the liver of the PEP-1-MsrA injected mice. Interestingly, the injection of MsrA, although largely ineffective, indeed also increased plasma SOD activity and reduced hepatic IL-6 expression. These data indicate the extracellular MsrA may exert mild anti-oxidative and anti-inflammatory function in the liver, but not robust enough to reduce atherosclerosis.

Another important mechanism by which PEP-1-MsrA reduces atherosclerosis, however, may lie in its effects on macrophages. First, our in vitro data clearly showed that PEP-1-MsrA could effectively enter macrophages and exert anti-oxidative and anti-inflammatory function as mentioned earlier. Second, PEP-1-MsrA injection reduced plasma MCP-1 levels. Although we do not know in what organs and cell types MCP-1 production was inhibited by PEP-1-MsrA, the reduced plasma MCP-1 levels would lead to reduced monocyte/macrophage infiltration to the arterial wall [44]. Third, in fact, macrophage content in the lesions of PEP-1-MsrA injected mice was significantly reduced. Fourth, more interestingly, the apoptotic macrophages were even more reduced in lesions of PEP-1-MsrA injected mice than total macrophages, suggesting the apoptosis-preventive function of PEP-1-MsrA shown in vitro also operated in the atherosclerotic lesions; and actually PEP-1-MsrA protein was detected enriched in the lesions.

**Conclusions**

MsrA, as a specific intracellular MetSO reductase, plays an important role in redox homeostasis. Our studies indicate that PEP-1-MsrA effectively enter cells and plays a protective role against oxidative stress and inflammation. In Western diet-fed apoE−/− mice model, administration of this protein could significantly halt atherogenesis through increasing the anti-oxidative and anti-inflammatory capacity in the liver and reducing macrophage accumulation, inflammatory responses, and apoptosis in the lesions. Therefore, PEP-1-MsrA may be developed as a potent therapeutic agent for reducing atherosclerosis-related cardiovascular diseases.

**Additional files**

**Additional file 1: Figure S1.** Vector diagrams of pET28a/MsrA and pET28a/PEP-1-MsrA. A 642-bp cDNA fragment of human MsrA was inserted into pET28a vector, PEP-1 was inserted at the N-terminus of MsrA.

**Additional file 2: Figure S2.** Cell viability analysis of MsrA and PEP-1-MsrA on HeLa cells. Different concentrations of MsrA and PEP-1-MsrA proteins were incubated with HeLa cells for 72 h. Cell viability was analyzed by MTT method, n = 3.

**Additional file 3: Figure S3.** Effects of MsrA or PEP-1-MsrA on body weight, spleen weight of apoE−/− mice. The 21-week-old apoE−/− mice were fed with a Western-type diet and intraperitoneally injected with PBS, MsrA or PEP-1-MsrA proteins for 12 weeks and then sacrificed. The body weights at various time-points (A) and spleen weights at the end point (B) were measured, n = 10.

**Abbreviations**

CAT: catalase; CD: circular dichroism; CPPs: cell penetrating peptides; CVD: cardiovascular disease; IC: interleukin; LPS: lipopolysaccharide; MCP-1: monocyte chemotactrant protein 1; MetSO: methionine sulfone; MsrA: methane sulfoxide reductase A; PBS: phosphate-buffered saline; PON1: paraoxonase-1; PTDs: protein transduction domains; ROS: reactive oxygen species; SOD: superoxide dismutase; TC: total cholesterol; TG: triglycerides; Trx: thioredoxin.

**Authors’ contributions**

YW performed the experiments, analyzed the data, and drafted the manuscript. GX carried out q-PCR. YX carried out Western blotting. LM and CT participated in animal experiments. DF participated in the design of the fusion protein. FD and HY conceived the study, analyzed the results. DF, FD and HY wrote the manuscript. All authors read and approved the final manuscript.

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