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RESEARCH

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 H$_2$O$_2$-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 chemoattractant 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].

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ROS, including superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2), 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 *Bam*HI and *Hind*III sites, from pQE30/MsrA was inserted into a pET15b/PEP-1 plasmid. Then the PEP-1-MsrA fragment was digested at *Nde*I and *Hind*III 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 A_{600} 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 (JNBIO, China) in lysis buffer (pH 8.0, 50 mM NaH_2PO_4, 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 (Qiagen, 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 $MgCl_2$, 30 mM KCl and 50 μM DTT in 25 mM Tris–HCl to which 3.6 μM

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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 A_{412} value was then recorded at 0 and 10 min after the end of the incubation period. The decrease in A_{412} value was calculated ($\Delta A = \Delta A_{0min} - \Delta A_{10min}$). The heat-inactivated MsrA (control) was defined as $\Delta A_{Control}$, while active MsrA was named ΔA_{Total}. MsrA activity was calculated using the formula: $\Delta A_{MsrA} = \Delta A_{Total} - \Delta A_{Control}$. 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 strepto-mycin, 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 (1:200) for 2 h at room temperature in the dark. 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 H_2O_2 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™ III 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 H_2O_2 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 % proteinase 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′

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CGCGGTTCTATTTTGTTGGT 3′ (forward) and 5′ AG
TCGGCATCGTTTATGGTC 3′ (reverse); mouse IL-1β:
5′ GCCCATCCTCTGTGACTCAT 3′ (forward) and 5′
AGGCCACAGGTATTTTGTCG 3′ (reverse); mouse
TNFα: 5′ CGTCAGCCGATTTGCTATCT 3′ (forward)
and 5′ CGGACTCCGCAAAGTCTAAG 3′ (reverse);
mouse IL-10: 5′ AGCCTTATCGGAAATGATCCAGT 3′
(forward) and 5′ GGCCTTGTAGACACCTTGGT 3′
(reverse); mouse PON1: 5′ TGGTGGTAAACCATCCAG
ACTC 3′ (forward) and 5′ TGTGATGGTTTTCAGATG
CAAG 3′ (reverse); and mouse IL-6: 5′ AGTTGCCTTC
TTGGGACTGA 3′ (forward) and 5′ TCCACGATTTCCC
AGAGAAC 3′ (reverse).

Mice

ApoE$^{-/-}$ mice on a C57BL/6 background were pur-
chased 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 pro-
cedures were performed under the regulations of the
Institutional Animal Care and the Ethics Committee for
Animal Experiments of Wuhan University, in accord-
ance with the Guidelines for the Care and Use of Labora-
tory Animals of the Chinese Animal Welfare Committee.
Thirty male apoE$^{-/-}$ mice at 21 weeks of age were ran-
domly 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 cen-
trifugation at 10,000×*g* for 10 min at 4 °C. Total choles-
terol (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 immunosorb-
ent assay (ELISA) kits (eBioscience, USA) according to
the manufacturer's instructions. Plasma PON1 activ-
ity was measured using paraoxon (Sigma-Aldrich, USA)
as a substrate as described previously [31]. Plasma SOD
activity was measured using SOD Detection Kit (Nan-
jing Jiancheng Bioengineering Institute, China). Frozen

mouse liver samples were lysed by RIPA with 1 % protein-
ase inhibitors for Western blot analysis. Liver homogen-
ates 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 visu-
alized 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-medi-
ated dUTP nickend-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
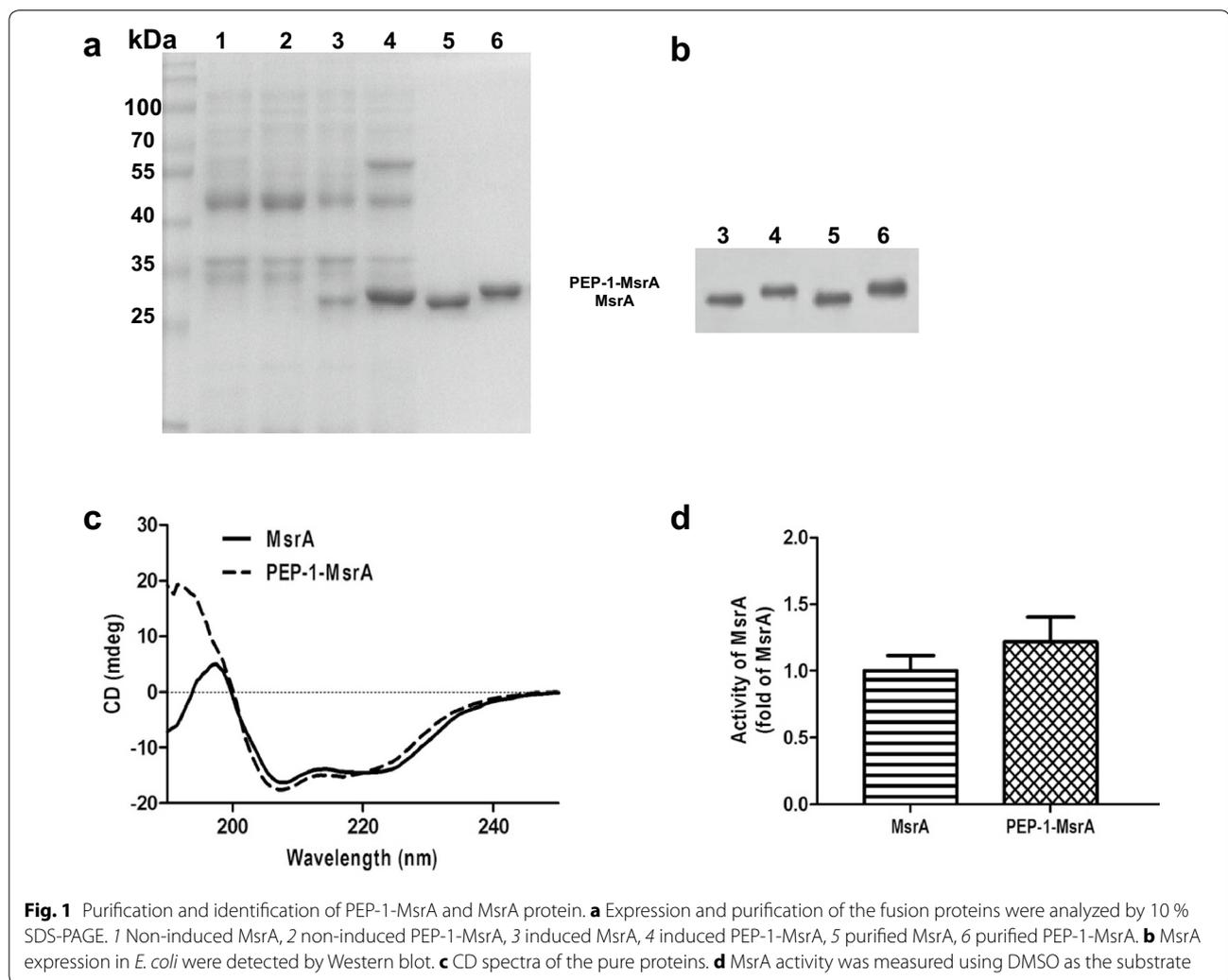apoptosis were analyzed with Image-Pro Plus 6.0.

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 con-
sidered 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 chromatog-
raphy. 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

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Fig. 1 Purification and identification of PEP-1-MsrA and MsrA protein. **a** Expression and purification of the fusion proteins were analyzed by 10 % SDS-PAGE. *1* Non-induced MsrA, *2* non-induced PEP-1-MsrA, *3* induced MsrA, *4* induced PEP-1-MsrA, *5* purified MsrA, *6* purified PEP-1-MsrA. **b** MsrA expression in *E. coli* were detected by Western blot. **c** CD spectra of the pure proteins. **d** MsrA activity was measured using DMSO as the substrate

structural features of PEP-1-MsrA and MsrA by far-UV CD. Both PEP-1-MsrA and MsrA adopt a predominantly α-helix conformation with double minima at 208 and 222 nm and have similar α-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 μM, neither purified MsrA nor PEP-1-MsrA protein reduced cell viability (Additional file 2: Figure S2). We selected 0.5–8 μM 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 μM 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.

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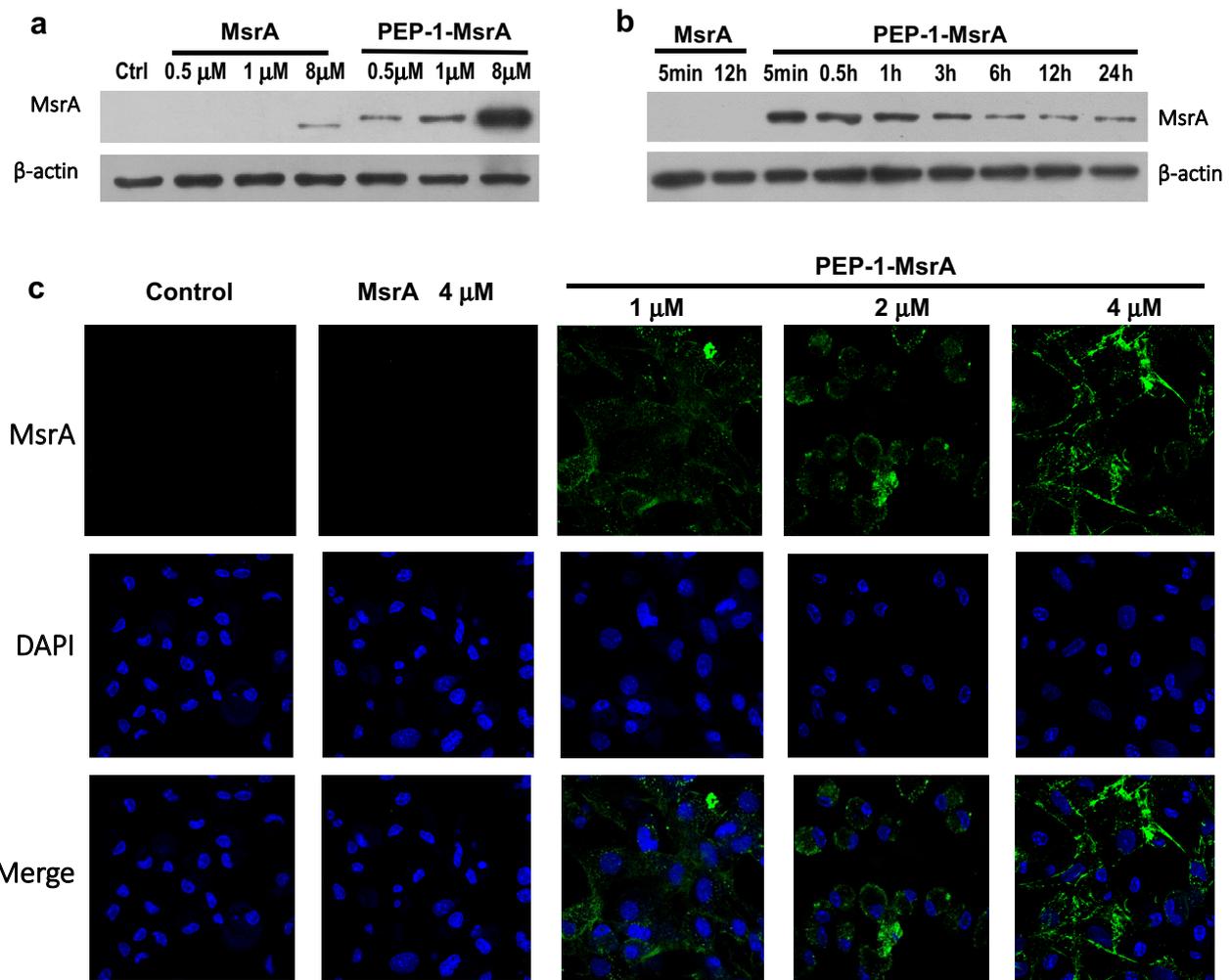
Fig. 2 PEP-1-MsrA was transduced into macrophages. Mouse peritoneal macrophages was incubated with PEP-1-MsrA or MsrA (0.5–8 μM) for 1 h (**a**) and 1 μM protein for various times (5 min–24 h) (**b**), then the intracellular MsrA was analyzed by Western blot. **c** The intracellular distribution of PEP-1-MsrA or MsrA in macrophages was determined by confocal microscopy. FITC-conjugated antibody was used for immunofluorescence assay and micrographs were captured at ×1000 magnification
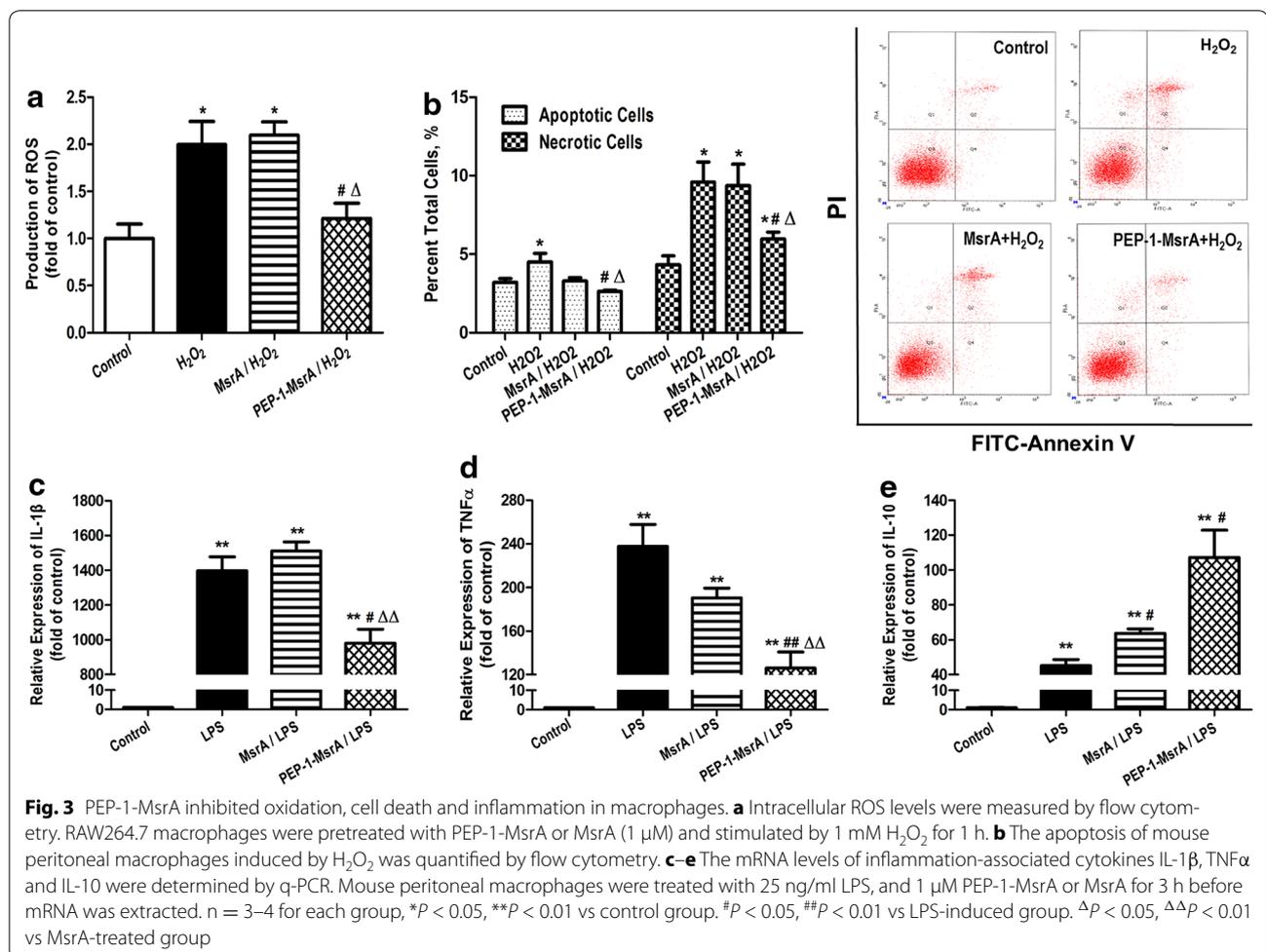
PEP-1-MsrA inhibited H_2O_2 induced oxidation and cell death of macrophages

To determine the effects of transduced PEP-1-MsrA on oxidative stress, RAW264.7 cells were pre-treated with 1 μM PEP-1-MsrA or MsrA proteins for 1 h followed by treatment with 1 mM H_2O_2. ROS levels were then measured. As shown in Fig. 3a, H_2O_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 H_2O_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 H_2O_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-1β and TNFα 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),

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Fig. 3 PEP-1-MsrA inhibited oxidation, cell death and inflammation in macrophages. **a** Intracellular ROS levels were measured by flow cytometry. RAW264.7 macrophages were pretreated with PEP-1-MsrA or MsrA (1 μM) and stimulated by 1 mM H_2O_2 for 1 h. **b** The apoptosis of mouse peritoneal macrophages induced by H_2O_2 was quantified by flow cytometry. **c–e** The mRNA levels of inflammation-associated cytokines IL-1β, TNFα and IL-10 were determined by q-PCR. Mouse peritoneal macrophages were treated with 25 ng/ml LPS, and 1 μM PEP-1-MsrA or MsrA for 3 h before mRNA was extracted. n = 3–4 for each group, *$P < 0.05$, **$P < 0.01$ vs control group. #$P < 0.05$, ##$P < 0.01$ vs LPS-induced group. $^\Delta P < 0.05$, $^{\Delta\Delta} P < 0.01$ vs MsrA-treated group

indicating that intracellular PEP-1-MsrA significantly suppressed the macrophage inflammatory response.

PEP-1-MsrA ameliorated inflammatory and oxidative stress in apoE$^{-/-}$ mice

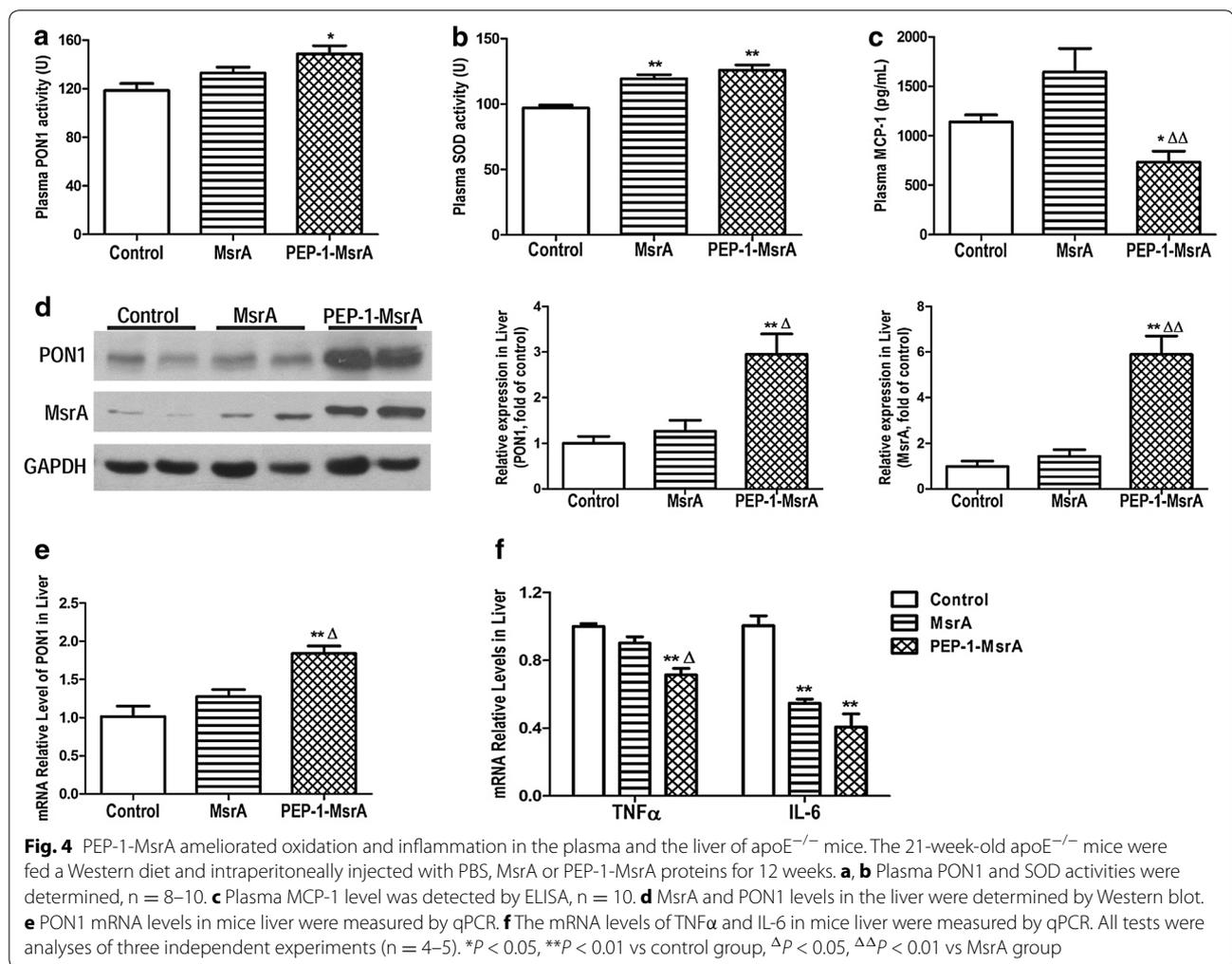ApoE$^{-/-}$ mice were intraperitoneally injected with MsrA, PEP-1-MsrA proteins or vehicle and fed a Western-diet for 12 weeks. The body weights (at various time-points) and spleen weights (at the end point) of mice showed no difference between all three groups (Additional file 3: Figure S3). Plasma 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

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Fig. 4 PEP-1-MsrA ameliorated oxidation and inflammation in the plasma and the liver of apoE$^{-/-}$ mice. The 21-week-old apoE$^{-/-}$ mice were fed a Western diet and intraperitoneally injected with PBS, MsrA or PEP-1-MsrA proteins for 12 weeks. **a**, **b** Plasma PON1 and SOD activities were determined, n = 8–10. **c** Plasma MCP-1 level was detected by ELISA, n = 10. **d** MsrA and PON1 levels in the liver were determined by Western blot. **e** PON1 mRNA levels in mice liver were measured by qPCR. **f** The mRNA levels of TNFα and IL-6 in mice liver were measured by qPCR. All tests were analyses of three independent experiments (n = 4–5). *$P < 0.05$, **$P < 0.01$ vs control group, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs MsrA group

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 mm^2) was significantly smaller than that in control mice (0.40 ± 0.02 mm^2) or MsrA injected mice (0.38 ± 0.03 mm^2), 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 mm^2) and 20 % smaller macrophage areas compared with MsrA injected mice (0.35 ± 0.02 mm^2) (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.
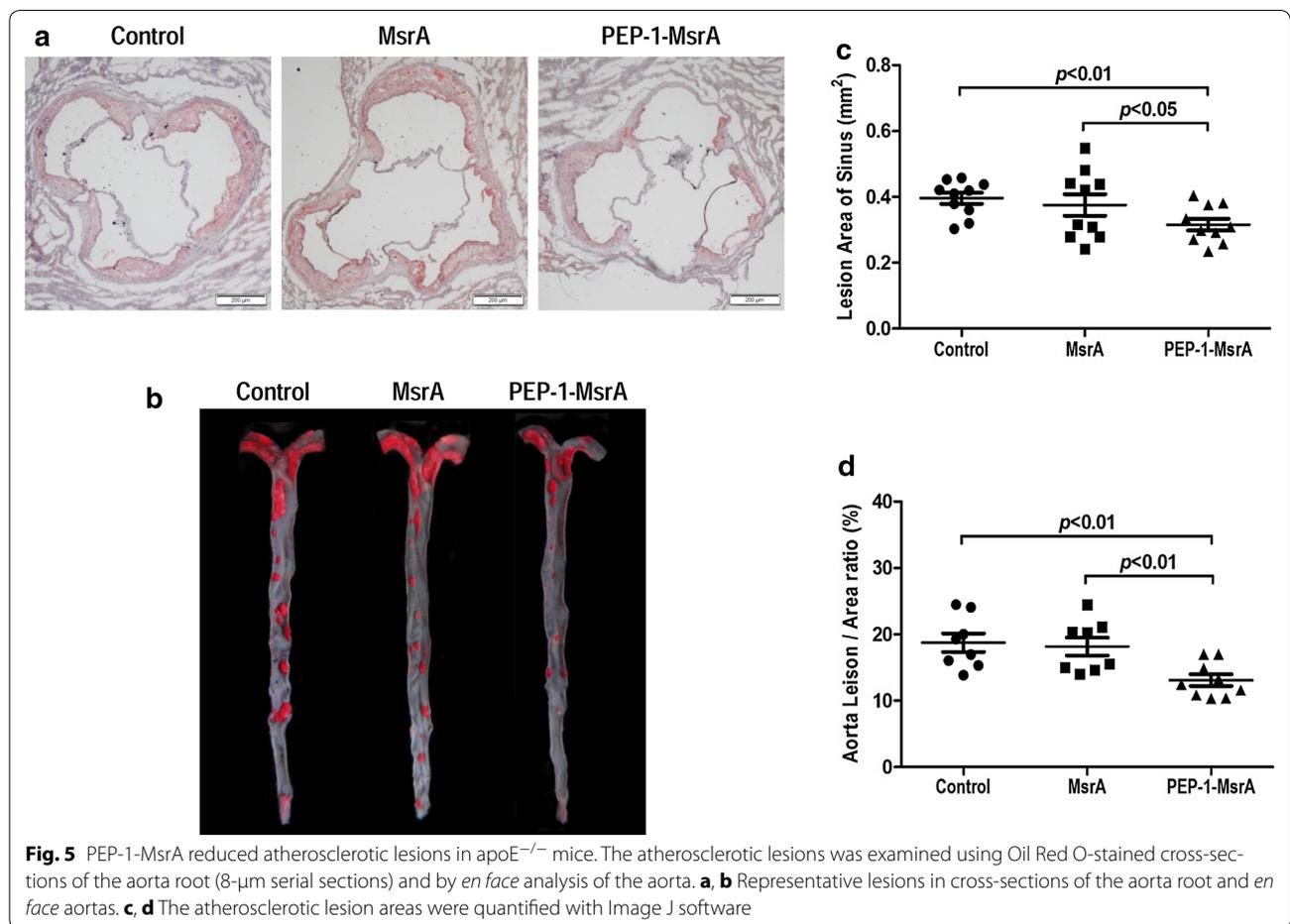
Discussion

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

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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 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

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

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Fig. 6 PEP-1-MsrA reduced macrophage infiltration and cell apoptosis in the lesions. **a** The MsrA level in the aortic lesions was detected by immunofluorescence assay. **b** The macrophages in aortic root lesions were visualized by Immunostaining for MOMA-2 and MOMA-2 staining area was analyzed with Image-Pro Plus 6.0. **c** Apoptosis in lesions was detected by TUNEL assay. The apoptotic cells were counted and normalized to the total number of cells in the lesion area

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 lifespans [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]. Interesting, 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

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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 proinflammatory 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.

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