BJA

British Journal of Anaesthesia, 125 (6): 1034–1044 (2020)

doi: 10.1016/j.bja.2020.07.054
Advance Access Publication Date: 14 September 2020
Neuroscience and Neuroanaesthesia

NEUROSCIENCE AND NEUROANAESTHESIA

Sevoflurane prevents vulnerable plaque disruption in apolipoprotein E-knockout mice by increasing collagen deposition and inhibiting inflammation

Yonghao Hou[1,2], Xiaowen Lin[3], Zhen Lei[1], Meng Zhao[1], Shengqiang Li[1], Meng Zhang[2], Cheng Zhang[2], Jingui Yu[1,*] and Tao Meng[1,2,**]

[1]Department of Anesthesiology, Qilu Hospital of Shandong University, Jinan, China, [2]The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education, Chinese National Health Commission and Chinese Academy of Medical Sciences, The State and Shandong Province Joint Key Laboratory of Translational Cardiovascular Medicine, Qilu Hospital of Shandong University, Jinan, China and [3]Department of Pain Management, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, China

**Corresponding author. E-mails: yujingui1109@126.com, mengtao@sdu.edu.cn

Abstract

Background: Sevoflurane may reduce the occurrence of major adverse cardiovascular events (MACCEs) in surgical patients, although the mechanisms are poorly understood. We hypothesised that sevoflurane stabilises atherosclerotic plaques by inhibiting inflammation and enhancing prolyl-4-hydroxylase α1 (P4Hα1), the rate-limiting subunit for the P4H enzyme essential for collagen synthesis.

Methods: We established a vulnerable arterial plaque model in apolipoprotein E-knockout mice ($ApoE^{-/-}$) fed a high-fat diet that underwent daily restraint/noise stress, with/without a single prior exposure to sevoflurane for 6 h (1–3%; n=30 per group). *In vitro*, smooth muscle cells (SMCs) were incubated with tumour necrosis factor-alpha in the presence/absence of sevoflurane. Immunohistochemistry, immunoblots, and mRNA concentrations were used to quantify the effect of sevoflurane on plaque formation, expression of inflammatory cytokines, P4Hα1, and collagen subtypes in atherosclerotic plaques or isolated SMCs.

Results: In $ApoE^{-/-}$ mice, inhalation of sevoflurane 1–3% for 6 h reduced the aortic plaque size by 8–29% in a dose-dependent manner, compared with control mice that underwent restraint stress alone (P<0.05); this was associated with reduced macrophage infiltration and lower lipid concentrations in plaques. Sevoflurane reduced gene transcription and protein expression levels of pro-inflammatory cytokines (≥69–75%; P<0.05) and matrix metalloproteinases (≥39–65%; P<0.05) at both gene transcription and protein levels, compared with controls. Sevoflurane dose dependently increased Types I and III collagen deposition through enhanced protein expression of P4Hα1, both *in vivo* and *in vitro* (0.7–3.3-fold change; P<0.05).

Conclusions: Sevoflurane dose dependently promotes plaque stabilisation and decreases the incidence of plaque disruption in $ApoE^{-/-}$ mice by increasing collagen deposition and inhibiting inflammation. These mechanisms may contribute to sevoflurane reducing MACCE.

Keywords: atherosclerosis; collagen metabolism; inflammation; sevoflurane; vulnerable plaque

Received: March 30, 2020; **Accepted:** 21 July 2020

Editor's key points

- Several anaesthetic agents appear to be cardioprotective through multiple mechanisms.
- The authors explored whether sevoflurane stabilises atherosclerotic in apolipoprotein E-knockout mice, which develop unstable atherosclerotic plaques when fed high-fat diets and exposed to chronic stress.
- Sevoflurane dose dependently promoted plaque stabilisation apolipoprotein E-knockout mice by increasing collagen deposition and inhibiting inflammation.
- These data add another mechanism through which anaesthetic agents confer cardioprotection.

Atherosclerotic plaques are characterised by a thin, highly inflamed, and collagen-poor fibrous cap. The formation of atherosclerotic plaques is promoted by an imbalance between extracellular matrix (ECM) synthesis and degradation.[1,2] The rupture of vulnerable atherosclerotic plaques likely contributes to some instances of perioperative myocardial infarction and stroke.[3,4] Plaque stabilisation mostly depends on the relative amounts of smooth muscle cells (SMCs) and collagen.[5,6] Inflammation and collagen metabolism in atherosclerotic plaques chiefly determine plaque stability. Inflammation markedly increases the expression and activation of matrix metalloproteinases (MMPs), which can weaken plaque caps and promote rupture.[7] Prolyl-4-hydroxylase α1 (P4Hα1), the rate-limiting subunit for the P4H enzyme, is essential for all known types of collagen synthesis, maturation, and secretion.[8] During collagen post-translational processing, P4Hα1 assembles procollagen polypeptide chains into stable triple-helical molecules.[9] Inhibition of P4Hα1 activity leads to collagen degradation and atherosclerotic plaque instability.[10] We have demonstrated that the inflammatory cytokine tumour necrosis factor-alpha (TNF-α) suppresses P4Hα1 expression *via* the apoptosis signal-regulated kinase 1–c-Jun N-terminal kinase–non-POU-domain-containing octamer-binding protein (ASK1–JNK–NonO) pathway.[11]

Inhalational anaesthetics, including sevoflurane, have been associated with a lower incidence of perioperative myocardial infarction, 1 yr mortality,[12,13] troponin release, and higher postoperative cardiac index.[14] Laboratory and clinical studies suggest that sevoflurane inhibits inflammation during the perioperative period. However, these studies have not examined whether sevoflurane may limit inflammation in vulnerable atherosclerotic plaques, perhaps through the JNK pathway.[15–17] We hypothesised that sevoflurane administration may dose dependently stabilise atherosclerotic plaques by inhibiting the expression of inflammatory cytokines and MMPs through enhancing P4Hα1 expression in atherosclerotic lesions.

Methods

Full details on methods are provided in the Supplementary material.

Animals

The experimental animal protocol was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Ethics Committee on Animal Experiment of Shandong University Qilu Hospital (Jinan, China; approval no. DWLL-2019-022). Male ApoE$^{-/-}$ mice (C57BL/6 J background) (8 weeks of age) were purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd (Beijing, China). Demonstration of adherence to Animal Research: Reporting of In Vivo Experiments guidelines is provided in Supplementary data.

Experimental protocol: *in vivo* experiments

Ten weeks after high-fat diet feeding (cholesterol 0.25% and cocoa butter 15%), 120 male ApoE$^{-/-}$ mice were randomly divided into four groups (n=30 per group). The control group received no treatment; the other three groups continuously inhaled sevoflurane 1%, 2%, or 3% for 6 h, respectively. These treatments were repeated 4 weeks later. All mice underwent stress stimulation 1 day after the initial inhalation of sevoflurane using a modified standardised method.[18,19] The restraint stress and noise stimulation lasted for 6 h day^{-1} for 4 weeks.[20,21] After 14 weeks, ApoE$^{-/-}$ mice were euthanised by inhalation of carbon dioxide, and the aorta and blood from the inferior vena cava were collected for further analysis.

Immunohistochemistry of aortic tissue

Atherosclerotic plaque formation in whole aortas, including thoracic and abdominal aortas, and the aortic roots were removed and fixed in paraformaldehyde 4% overnight. The thoracic and abdominal aortas were collected for histological analysis of atherosclerosis by Oil Red O staining for lipid deposition. Successive transverse cryosections of 5 μm in thickness of the aortic root were obtained and stained with haematoxylin and eosin, Oil Red O, and Sirius Red.[22] Cryosections of 5 μm in thickness of the aortic root were used for staining of macrophages, SMCs, matrix metalloproteinase (MMP)-2, MMP-9, monocyte chemoattractant protein-1 (MCP-1), TNF-α, and interleukin-6 (IL-6).[20] Full details on primary and secondary antibodies used are provided in Supplementary material. The integration optical density values of positive staining were calculated using Image-Pro Plus software (Media Cybernetics, Cambridge, MA, USA; Research Resource Identifier: SCR_007369). The relative content of macrophages, SMCs, lipids, collagen, and inflammatory cytokines (MMP-2, MMP-9, MCP-1, TNF-α, and IL-6) was quantitated as the ratio of the positive staining area to the cross-sectional area of the aortic root plaques.[1,23] Sections reacted with non-immune immunoglobulin G, secondary antibody only, and no primary and secondary antibodies were used as negative controls. The relative content of macrophages, lipid, SMCs and collagen was quantified as the ratio of the positive staining area to total plaque area. The vulnerability index was calculated as follows: (macrophage staining % + lipid staining %)/(SMC staining % + collagen staining %).[2,24]

Ex vivo experiments

Isolation, culture, and stimulation of vascular SMCs

The primary mouse vascular SMCs were isolated from the media of mouse aortas of ApoE$^{-/-}$ mice as described previously.[25] Smooth muscle cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS) 20% (Invitrogen, Carlsbad, CA, USA) and penicillin and streptomycin 1%, and placed in an incubator with CO_2 5% at 37°C. Vascular SMC phenotype was determined

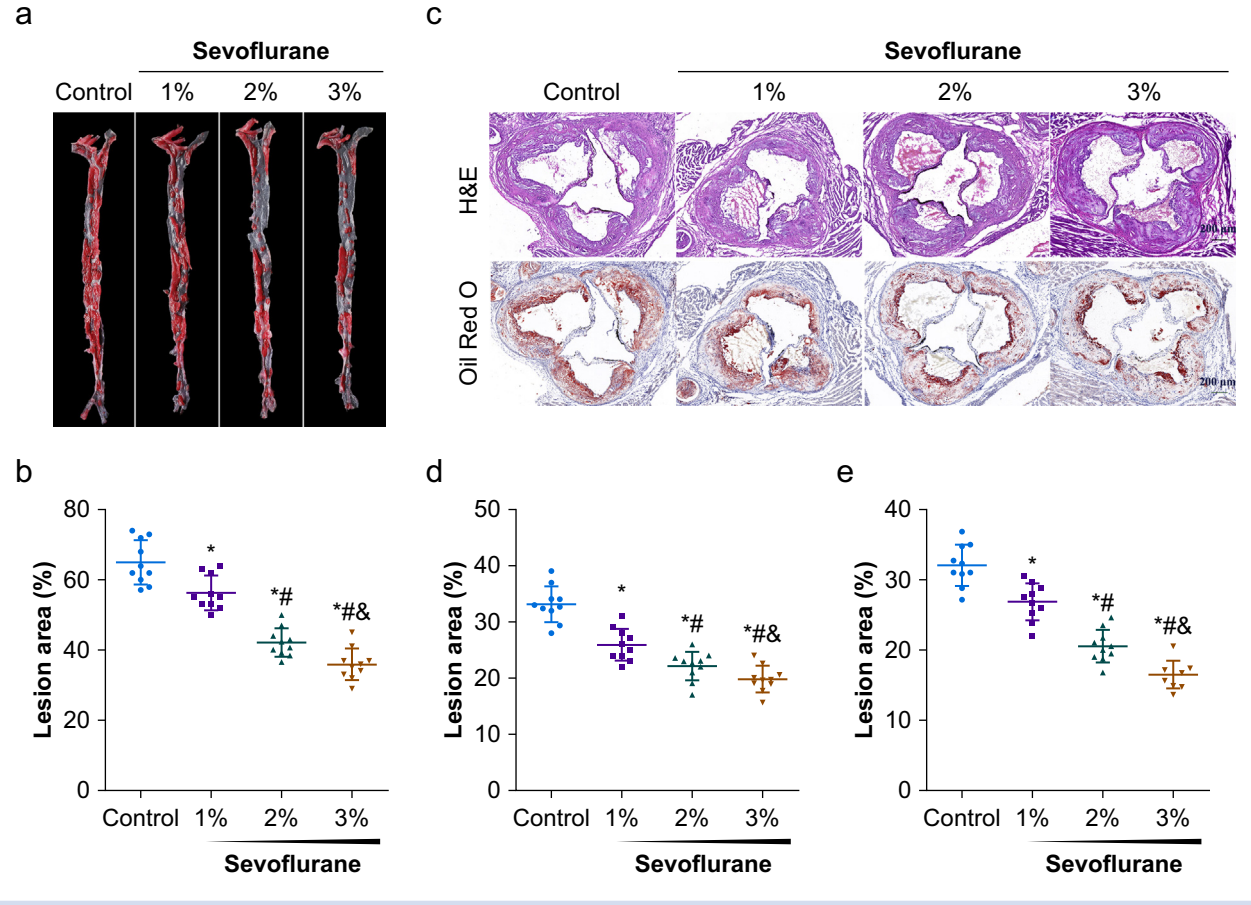

Fig 1. Effects of sevoflurane on aortic atherosclerotic plaque growth in ApoE$^{-/-}$ mice. (a) Representative Oil Red O staining in the whole aortas, including thoracic and abdominal aortas. (b) Quantitative data of atherosclerotic lesions in the whole aortas (n=10 per group). (c) Representative haematoxylin and eosin (H&E) and Oil Red O staining in the aortic root plaques. (d) Quantitative analysis of atherosclerotic lesion size in aortic roots by H&E staining (n=10 per group). (e) Quantitative analysis of atherosclerotic lesion size in aortic roots by Oil Red O staining (n=10 per group). Control (no sevoflurane). *P<0.05 *vs* control group; #P<0.05 *vs* sevoflurane 1% group; &P<0.05 *vs* sevoflurane 2% group. Adjusted P-values (Tukey's *post hoc* test) are reported for multiple comparisons. Scale bar: 200 μm.

by immunostaining cells using a rabbit anti-α smooth muscle actin antibody (ab5694; Abcam, Cambridge, MA, USA).

Ex vivo incubation of SMC with sevoflurane

A fresh solution of sevoflurane (Maruishi Pharmaceutical Co., Ltd, Osaka, Japan) was prepared on each experimental day.[26] Liquid sevoflurane was added to DMEM supplemented with FBS 20%, and the solution was stored in a tightly sealed glass container at room temperature (22–24°C) for at least 2 h. Under these conditions, the serum-containing medium, saturated with sevoflurane, formed an upper layer, whilst liquid sevoflurane stayed below the medium. All test solutions containing sevoflurane were stored in sealed glass containers until use.

The sevoflurane-saturated medium was quickly diluted in the regular medium at the following dilutions: 1:33, 1:10, and 1:3.[27,28] Smooth muscle cells were cultured in a serum-containing medium without sevoflurane (control group and TNF-α group) or with sevoflurane at three different concentrations (1:33, 1:10, and 1:3) for 12 h. To ensure the relative stability of sevoflurane content in the culture medium, the culture medium of each group was changed every 4 h. After 4 h of treatment with sevoflurane, SMCs (except the control group) were treated with human recombinant TNF-α (100 ng ml^{-1}) for an additional 8 h.

Immunoblot analysis

Total proteins extracted from frozen tissue samples of abdominal aortas or treated SMCs were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes, which were blocked with non-fat milk 5% and incubated with primary antibodies for no less than 12 h at 4°C and appropriate secondary antibodies for 1 h at room temperature. Full details on primary and secondary antibodies used are provided in the Supplementary material. Transferred blots were developed

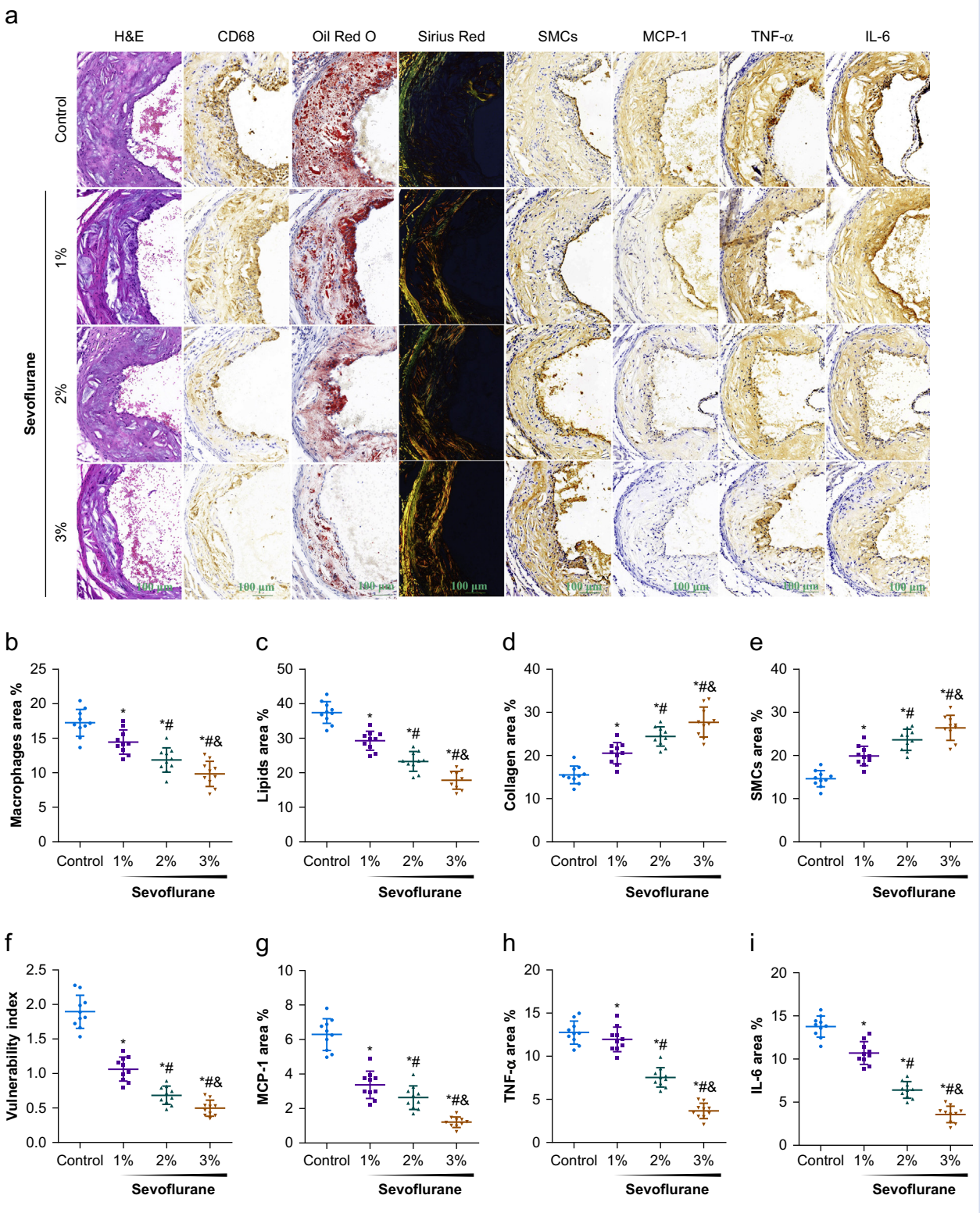

Fig 2. Effects of sevoflurane on the aortic root plaque composition and pro-inflammatory cytokine expression in ApoE$^{-/-}$ mice. (a) Representative haematoxylin and eosin (H&E); CD68; Oil Red O; Sirius Red; and α-smooth muscle actin, monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) staining control and sevoflurane experimental groups. CD68 staining was performed to detect macrophages, Oil Red O staining was for lipids, and Sirius Red staining was for collagen. (b–e) Relative contents of (b) macrophages, (c) lipids, (d) collagen, and (e) smooth muscle cells in the aortic root plaques (n=10 per group). (f) Measurements of plaque vulnerability index in the aortic root plaques (n=10 per group). (g–i) Relative contents of (g) MCP-1, (h) TNF-α, and (i) IL-6 in the aortic root plaques (n=10 per group). *P<0.05 *vs* control group; $^{\#}P$<0.05 *vs* sevoflurane 1% group; $^{\&}P$<0.05 *vs* sevoflurane 2% group. Adjusted P-values (Tukey's *post hoc* test) are reported for multiple comparisons. Scale bar: 100 μm.

with a chemiluminescent reagent (Millipore, Billerica, MA, USA). The protein expression levels of MMP-2, MMP-9, P4Hα1, Type I collagen, Type III collagen, ASK1, JNK, and NonO were normalised to glyceraldehyde-3-phosphate dehydrogenase.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from frozen tissue samples of abdominal aortas or treated SMCs. RNA was reverse transcribed (PrimeScript™ RT reagent kit with gDNA Eraser; Takara, Kusatsu, Shiga, Japan), and the amplification of complementary DNA was measured by use of SYBR Green PCR Master Mix (Takara, Otsu, Shiga, Japan). The threshold cycle value (Ct) and the $2^{-\Delta\Delta CT}$ method were used for quantitative calculation of relative mRNA concentrations. The transcript amount of β-actin was taken as an internal RNA control. The sequences of primers used are listed in Supplementary Table 1.

Statistical analysis

Data were assessed for normality using the Shapiro–Wilk test. Normally distributed data were expressed as mean (standard deviation). Analysis of variance followed by Tukey's *post hoc* test was used for multiple comparisons. $P<0.05$ was considered statistically significant. All experimental procedures were performed at least three times. Statistical analysis was conducted using SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

Sample size estimation

We used PROC POWER procedure in SAS (version 9.4, SAS Institute Inc., Cary, NC) to determine the sample size in each experiment. We set significant level (α) at 0.05 and power (1-β) at 80%. Effect size (expected differences and variances) between groups are based on our previosuly published work.[11]

Results

High-fat diet in ApoE$^{-/-}$ mice

There was no local or systemic adverse effects noted from the high-fat diet in ApoE$^{-/-}$ mice for any experimental group, with similar body weight and serum lipid concentrations throughout the experiment (n=30 per group; Supplementary Tables 2 and 3).

Sevoflurane attenuates atherosclerotic plaque growth in ApoE$^{-/-}$ mice

The relative size of the atherosclerotic lesions in whole aortas were dose dependently reduced per sevoflurane-treated group, compared with the control group (n=10 per group; Fig. 1a and b). Similar reductions in atherosclerotic plaque load after sevoflurane treatment were observed in the aortic roots (Fig. 1c–e).

Sevoflurane increases atherosclerotic plaque stability in ApoE$^{-/-}$ mice

Sevoflurane dose dependently reduced the number of CD68-positive macrophages in atherosclerotic plaques (Fig. 2a and b) and increased the expression of collagen and α-smooth muscle actin, indicators of atherosclerotic plaque stability (Fig. 2b–e). Sevoflurane dose dependently reduced the vulnerability index of atherosclerotic plaques, as reflected by the ratio of relative content of macrophages and lipids to that of SMCs and collagen in atherosclerotic plaques[29] (n=10 per group; Fig. 2f).

Sevoflurane reduces inflammatory cytokine expression *in vivo* and *in vitro*

Sevoflurane reduced expression of MCP-1, TNF-α, and IL-6 in the aortic root plaques as assessed by immunohistochemistry (n=10 per group; Fig. 2g–i). In cultured SMCs incubated with TNF-α (Fig. 3a), sevoflurane reduced mRNA expression levels of MCP-1 (Fig. 3b), vascular cell adhesion molecule-1 (VCAM-1) (Fig. 3c), and intercellular adhesion molecule-1 (ICAM-1) (Fig. 3d) compared with SMCs incubated with TNF-α alone (n=6 per group).

Sevoflurane decreases MMP-2 and MMP-9 expression *in vivo* and *in vitro*

Sevoflurane reduced MMP-2 and MMP-9 in the aortic root plaques (Fig. 4a), as assessed by immunoblot (Fig. 4b), quantitative immunohistochemistry (Fig. 4c), and reverse transcription–polymerase chain reaction (n=10 per group; Fig. 4d and e). Similarly, in cultured SMCs, MMP-2 and MMP-9 were reduced in sevoflurane-treated SMCs (n=6 per group), as assessed by immunoblot analysis (Fig. 3e and f), immunofluorescence (Fig. 3g), and mRNA concentrations (Fig. 3h).

Sevoflurane increases P4Hα1 expression and collagen deposition

Sevoflurane reduced protein expression of P4Hα1 dose dependently, compared with the control group, as quantified by immunoblotting (n=10 per group; Fig. 5a and b). In cultured SMCs, mRNA and protein expression of P4Hα1 was increased in sevoflurane-treated SMCs (n=6 per group; Fig. 5c–e) compared with SMCs incubated with TNF-α alone. Sevoflurane also increased protein expression levels of Types I and III collagen in a dose-dependent manner (n=6 per group; Fig. 5f–h).

Sevoflurane reduces ASK1–JNK–NonO expression by enhancing P4Hα1 levels

As our previous study showed that TNF-α suppressed P4Hα1 expression by activating the ASK1–JNK–NonO pathway,[11] the effects of sevoflurane treatment on expression of ASK1–JNK–NonO pathway proteins were further examined *in vitro*. Sevoflurane reduced the protein concentrations of ASK1, NonO (Fig. 6a), and JNK (Fig. 6b) in SMCs compared with concentrations quantified in SMCs incubated with TNF-α alone (n=6 per group; summary data shown in Fig. 6c–e).

To examine whether sevoflurane increased P4Hα1 expression by suppressing the ASK1–JNK–NonO pathway, the ASK1 inhibitor selonsertib (GS-4997; 10 μM) was added 1 h before treating the cells with TNF-α or vehicle for 8 h (Fig. 6f and g). We found that selonsertib inhibited the ASK1–JNK–NonO pathway and prevented the TNF-α-mediated suppression of P4Hα1 (Fig. 6h–j). Treatment with sevoflurane inhibited the TNF-α-mediated activation of the ASK1–JNK–NonO pathway and suppression of P4Hα1 to a similar degree to that observed by selonsertib (Fig. 6f–l).

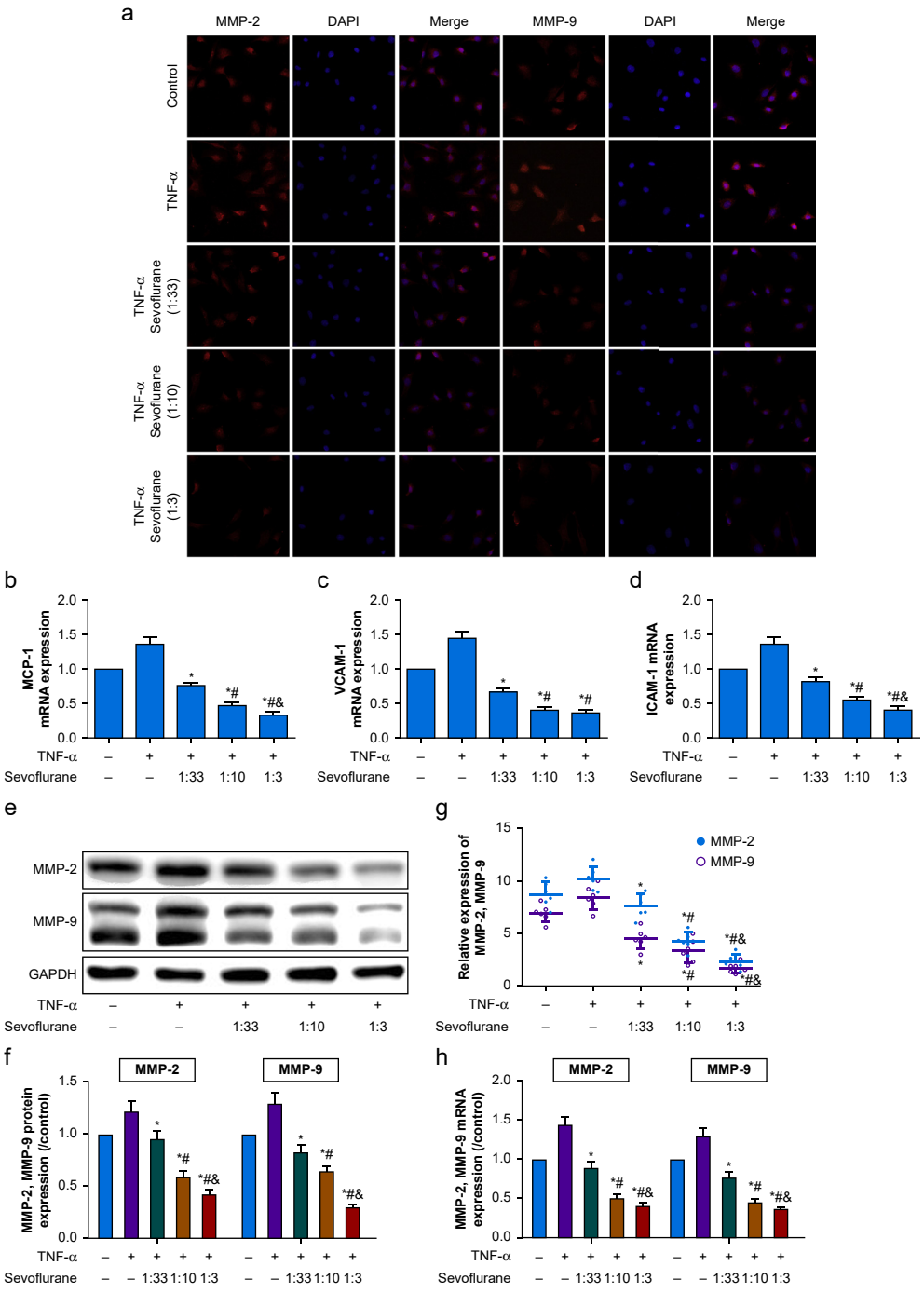

Fig 3. Effects of sevoflurane on tumour necrosis factor-alpha (TNF-α)-induced expression of inflammatory cytokines and matrix metalloproteinases *in vitro*. (a) Representative immunofluorescence assay of protein expression of matrix metallopeptidase (MMP)-2 and MMP-9 in smooth muscle cells (SMCs) incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane. Red colour depicts positive MMP-2 or MMP-9 staining, and blue colour indicates positive 4′,6-diamidino-2-phenylindole (DAPI) staining for cell nuclei. Scale bar: 100 μm. (b–d) Quantitative analysis by reverse transcription–polymerase chain reaction (RT–PCR) of (b) monocyte chemoattractant protein-1 (MCP-1), (c) vascular cell adhesion molecule-1 (VCAM-1), and (d) intercellular adhesion molecule-1 (ICAM-1) mRNA expression in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane (n=6 per group). (e) Representative western blot of MMP-2 and MMP-9 protein expression in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane (n=6 per group). (f) Quantitative analysis of MMP-2 and MMP-9 protein expression in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane (n=6 per group). (g) Quantitative analysis of MMP-2 and MMP-9 protein expression in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane determined by immunofluorescence (n=6 per group). (h) Quantitative analysis of MMP-2 and MMP-9 mRNA expression in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane, as determined by RT–PCR (n=6 per group). $^{*}P<0.05$ *vs* TNF-α group; $^{\#}P<0.05$ *vs* 1:33 group; $^{\&}P<0.05$ *vs* 1:10 group. Adjusted *P*-values (Tukey's *post hoc* test) are reported for multiple comparisons. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

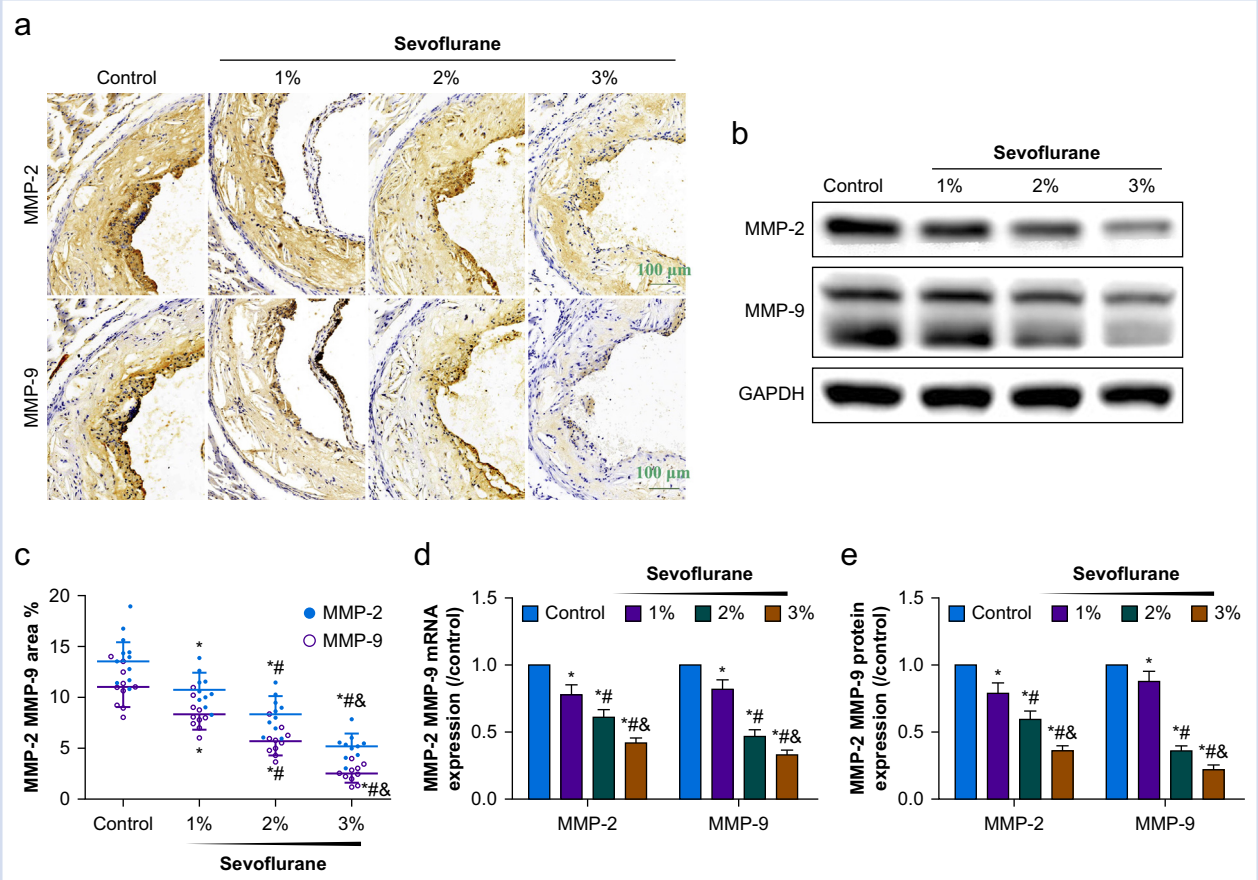

Fig 4. Effects of sevoflurane on the mRNA and protein expression of matrix metallopeptidase (MMP)-2 and MMP-9 in ApoE$^{-/-}$ mice. (a) Representative MMP-2 and MMP-9 staining in the aortic root plaques in control *vs* sevoflurane-treated mice. (b) Representative western blot analysis of MMP-2 and MMP-9 protein expression in the whole aorta plaques in control *vs* sevoflurane-treated mice. (c) Relative content of MMP-2 and MMP-9 in control *vs* sevoflurane-treated mice (n=10 per group). (d) Quantitative analysis of MMP-2 and MMP-9 mRNA expression by reverse transcription–polymerase chain reaction in control *vs* sevoflurane-treated mice (n=10 per group). (e) Quantitative analysis of MMP-2 and MMP-9 protein expression by western blot in control *vs* sevoflurane-treated mice (n=10 per group). For quantitative data panels, $^{*}P<0.05$ *vs* control group; $^{\#}P<0.05$ *vs* sevoflurane 1% group; $^{\&}P<0.05$ *vs* sevoflurane 2% group. Adjusted P-values (Tukey's *post hoc* test) are reported for multiple comparisons. Scale bar: 100 μm. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

Our main finding in this translational murine study was that sevoflurane dose dependently lowered the incidence of plaque disruption by attenuating the growth of atherosclerotic plaques and changing plaque composition to a stable plaque phenotype. The underlying mechanisms involved promoting the maturation and secretion of collagen and decreasing expression of pro-inflammatory cytokines. This study is the first to provide evidence that sevoflurane dose dependently prevented vulnerable plaques from disruption in ApoE$^{-/-}$ mice, and showed a theoretical mechanism for the protective effect of sevoflurane.

Atherosclerotic plaques that are prone to rupture are characterised by a thin and collagen-poor fibrous cap, a large lipid core, a low content of SMCs, and infiltration of macrophages.[1,2] Lipid accumulation increases the size of the necrotic core and macrophage infiltration activates the inflammation response in plaques. Smooth muscle cells and collagen-rich ECM are the major components of the fibrous cap to maintain the strength of plaque.[30] In the present study, we measured the relative size of the atherosclerotic lesion and the relative content of plaque components, and calculated the vulnerability index (a histological marker for plaque instability) in the aortic root plaques of ApoE$^{-/-}$ mice. Compared with the control group, sevoflurane reduced the relative size of the atherosclerotic lesion, decreased the content of macrophages and lipids, but increased that of SMCs and collagen, leading to a more than 50% reduction in vulnerability index in the sevoflurane 2% and 3% groups. In cultured SMCs, the protein expression levels of Types I and III collagen increased after sevoflurane treatment. These results suggest that sevoflurane may stabilise vulnerable plaques to become more stable.

Inflammation plays an indispensable role in the pathogenesis of plaque destabilisation and disruption.[21] Sevoflurane inhibits the release of inflammatory factors.[31,32] In our study, we detected the expression level of a number of pro-inflammatory cytokines *in vivo* and *in vitro*. We found that sevoflurane administration decreased the protein expression

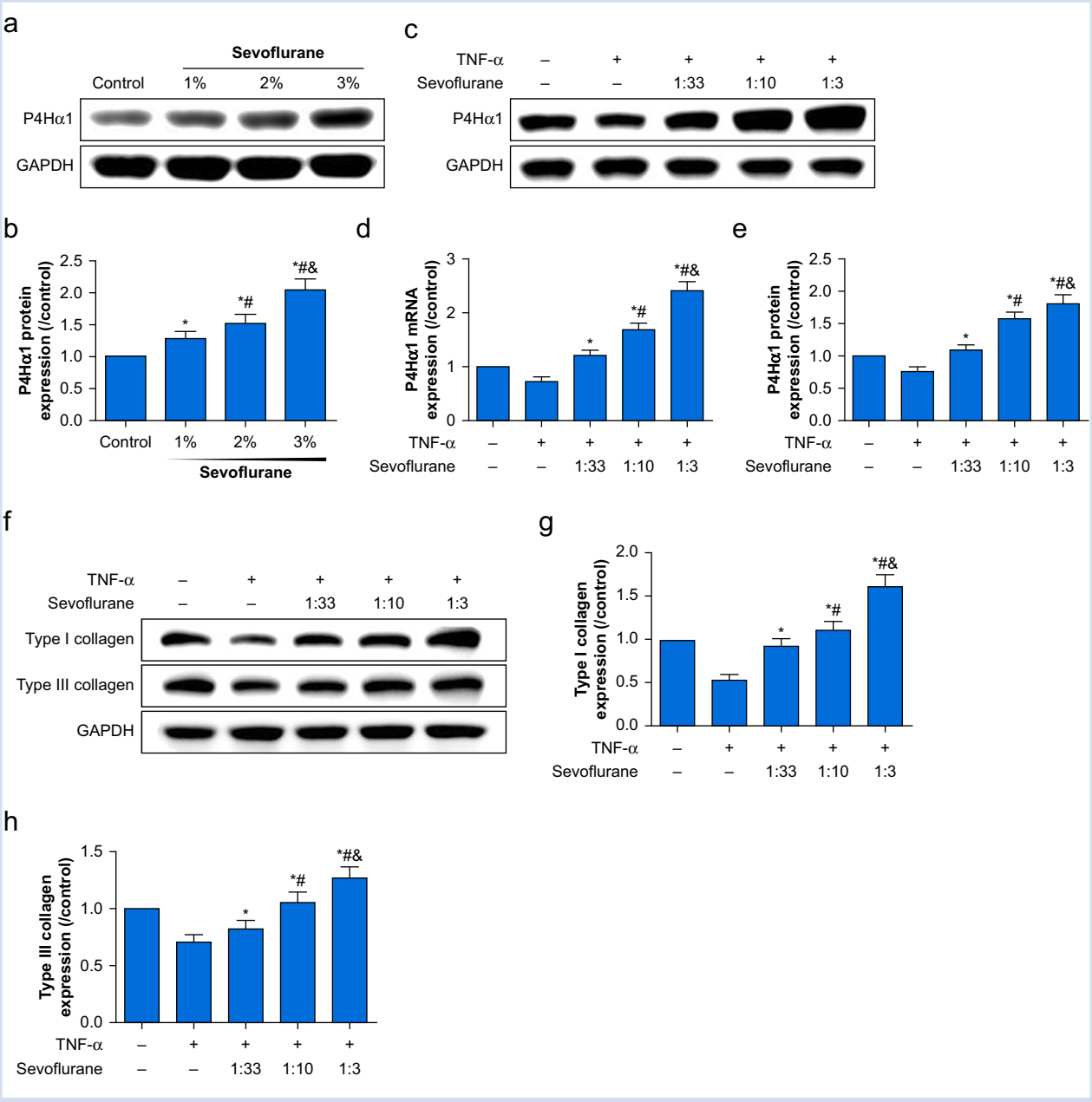

Fig 5. Effects of sevoflurane on prolyl-4-hydroxylase α1 (P4Hα1) and collagen expression. (a) Representative western blot of P4Hα1 protein expression in the whole aorta plaques of ApoE$^{-/-}$ mice treated with sevoflurane 1–3%. (b) Quantitative analysis of P4Hα1 protein expression in the whole aorta plaques of control and sevoflurane-treated ApoE$^{-/-}$ mice (n=10 per group). *P<0.05 *vs* control group; $^{\#}$P<0.05 *vs* sevoflurane 1% group; $^{\&}$P<0.05 *vs* sevoflurane 2% group. (c) Representative immunoblot showing P4Hα1 protein expression in smooth muscle cells (SMCs) incubated with tumour necrosis factor-alpha (TNF-α) in the absence, or presence, of increasing doses of sevoflurane. (d) Quantitative summary data of P4Hα1 mRNA expression in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane (n=6 per group). (e) Quantitative summary data for protein expression detected by immunoblot in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane (n=6 per group). (f) Representative immunoblot of Type I and Type III collagen protein expression in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane. (g) Quantitative summary data for Type I collagen protein expression detected by immunoblot in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane (n=6 per group). (h) Quantitative summary data for Type III collagen protein expression detected by immunoblot in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane (n=6 per group). (c–h) *P<0.05 *vs* TNF-α group; $^{\#}$P<0.05 *vs* 1:33 group; $^{\&}$P<0.05 *vs* 1:10 group. Adjusted *P*-values (Tukey's *post hoc* test) are reported for multiple comparisons. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

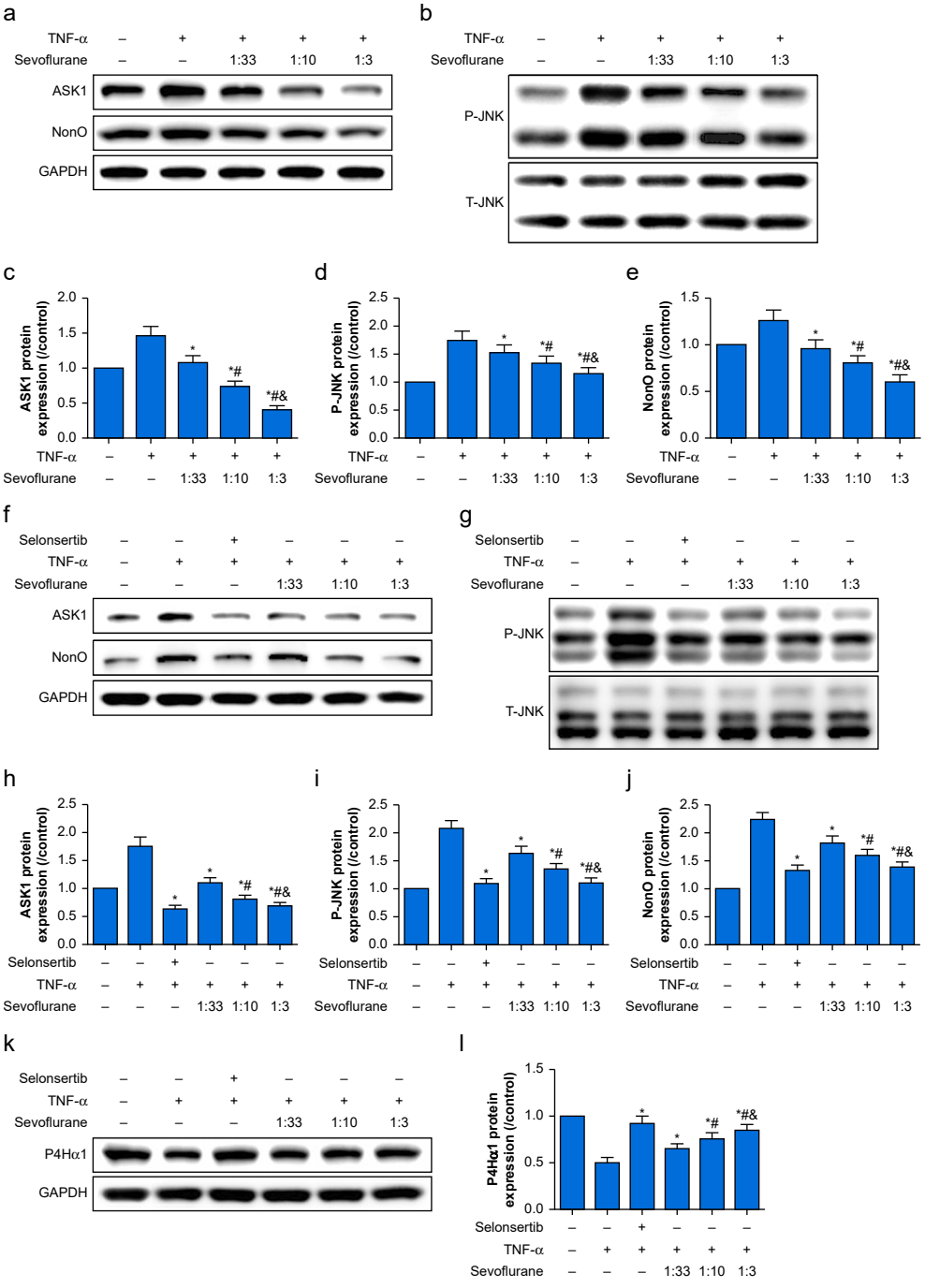

Fig 6. Effects of sevoflurane on apoptosis signal-regulated kinase 1–c-Jun N-terminal kinase–non-POU-domain-containing octamer-binding protein (ASK1–JNK–NonO) expression. (a) Representative immunoblot showing ASK1 and NonO in smooth muscle cells (SMCs) incubated with tumour necrosis factor-alpha (TNF-α) in the absence, or presence, of increasing doses of sevoflurane. (b) Phospho-Jun N-terminal kinase (P-JNK) and total-Jun N-terminal kinase (T-JNK) expression in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane. (c–e) Quantitative analysis of (c) ASK1, (d) JNK, and (e) NonO protein expression in SMCs incubated with TNF-α in the absence, or presence, of increasing doses of sevoflurane (n=6 per group). *P<0.05 *vs* TNF-α group; #P<0.05 *vs* 1:33 group; &P<0.05 *vs* 1:10 group. (f) Representative western blot showing ASK1 and NonO protein expression in SMCs incubated with TNF-α in the absence, or presence, of selonsertib (10 μM) or increasing doses of sevoflurane. (g) Representative western blot showing P-JNK and T-JNK protein expression in SMCs incubated with TNF-α in the absence, or presence, of selonsertib (10 μM) or increasing doses of sevoflurane. (h–j) Quantitative analysis of (h) ASK1, (i) JNK, and (j) NonO expression in SMCs incubated with TNF-α in the absence, or presence, of selonsertib (10 μM) or increasing doses of sevoflurane (n=6 per group). *P<0.05 *vs* TNF-α group; #P<0.05 *vs* 1:33 group; &P<0.05 *vs* 1:10 group. Adjusted P-values (Tukey's *post hoc* test) are reported for multiple comparisons. (k) Representative western blot of prolyl-4-hydroxylase α1 (P4Hα1) protein expression in SMCs incubated with TNF-α in the absence, or presence, of selonsertib (10 μM) or increasing doses of sevoflurane. (l) Quantitative analysis of P4Hα1 protein expression in SMCs incubated with TNF-α in the absence, or presence, of selonsertib (10 μM) or increasing doses of sevoflurane (n=6 per group). *P<0.05 *vs* TNF-α group, #P<0.05 *vs* 1:33 group; &P<0.05 *vs* 1:10 group. Adjusted P-values (Tukey's *post hoc* test) are reported for multiple comparisons. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

levels of MCP-1, TNF-α, and IL-6 in ApoE$^{-/-}$ mice. Similarly, in SMCs treated with TNF-α, the mRNA expression levels of MCP-1, VCAM-1, and ICAM-1 were downregulated by sevoflurane. Matrix metalloproteinases secreted by SMCs and macrophages degrade collagen in ECM. The increasing secretion of MMPs, in particular MMP-2 and MMP-9, leads to an accelerated degradation of collagen, which can weaken fibrous caps and promote rupture.[33] In our study, the expression of MMP-2 and MMP-9 was lowered after sevoflurane administration both *in vivo* and *in vitro*. Taken together, our results suggest that sevoflurane stabilises atherosclerotic plaque by reducing pro-inflammatory cytokine concentrations and MMPs.

The JNK signalling pathway is an important pathway in cardiovascular disease, including myocardial hypertrophy, ventricular remodelling after myocardial infarction, and atherosclerosis.[34–36] Activation of the ASK1–JNK–NonO signalling pathway plays a crucial role in TNF-α-suppressed P4Hα1 expression and impairs collagen synthesis in SMCs.[11,20] We found that sevoflurane administration increased the mRNA and protein concentrations of P4Hα1 both *in vivo* and *in vitro*, but decreased the protein expression levels of ASK1, JNK, and NonO *in vitro*. Furthermore, we found that sevoflurane had a similar effect to the ASK1 inhibitor selonsertib, with both inhibiting the ASK1–JNK–NonO pathway and preventing TNF-α-mediated suppression of P4Hα1. Our results suggest that sevoflurane administration promoted collagen synthesis by suppressing ASK1–JNK–NonO signalling and enhancing the expression of P4Hα1.

This translational study has limitations. First, the major limitation to all studies on plaque instability is the lack of an ideal animal model of vulnerable plaques. However, using the model described herein, we achieved a substantial plaque disruption rate of 53% in the control group.[37] Second, for the *ex vivo* experiments, we saturated the serum-containing medium with liquid sevoflurane, which, because of the volatility of sevoflurane, may not reflect clinically relevant doses used in previous studies.[27,28] Nonetheless, a strength of this study was the dose–response relationship sought in both *in vivo* and *ex vivo* experimental designs.

In conclusion, sevoflurane dose dependently attenuated the growth of atherosclerotic plaques and transformed an unstable plaque to a stable plaque phenotype in ApoE$^{-/-}$ mice by increasing collagen deposition and inhibiting inflammation. These data may explain why sevoflurane has been associated with a lower rate of atherosclerosis-associated perioperative complications, including stroke and myocardial infarction presumably caused by rupture of atherosclerotic plaques.

Authors' contributions

Study conception/design: TM, JY, CZ, YH
Conduct of experiments: YH, XL, SL, M Zhao
Data analysis: YH, XL, ZL
Drafting of paper: YH, TM
Editing/revision of paper: TM, JY, M Zhang, CZ
Reading and approval of the final version of the paper: all authors

Acknowledgements

The authors thank Editage (www.editage.cn) for English language editing.

Declarations of interest

The authors declare that they have no conflicts of interest.

Funding

National Natural Science Foundation of China (81900394 and 81570241); Shandong Province Natural Science Foundation (ZR2017BH022 and ZR2017BH041); China Postdoctoral Science Foundation (2019M652395).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2020.07.054.

References

1. Virmani R, Burke AP, Farb A, Kolodgie FD. Pathology of the vulnerable plaque. *J Am Coll Cardiol* 2006; **47**: C13–8
2. Cheng C, Noordeloos AM, Jeney V, et al. Heme oxygenase 1 determines atherosclerotic lesion progression into a vulnerable plaque. *Circulation* 2009; **119**: 3017–27
3. Haft JI. Multiple atherosclerotic plaque rupture in acute coronary syndrome. *Circulation* 2003; **107**: e65–6
4. Naghavi M, Libby P, Falk E, et al. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: part II. *Circulation* 2003; **108**: 1772–8
5. Ji QW, Guo M, Zheng JS, et al. Downregulation of T helper cell type 3 in patients with acute coronary syndrome. *Arch Med Res* 2009; **40**: 285–93
6. Hu C, Dandapat A, Sun L, et al. LOX-1 deletion decreases collagen accumulation in atherosclerotic plaque in low-density lipoprotein receptor knockout mice fed a high-cholesterol diet. *Cardiovasc Res* 2008; **79**: 287–93
7. Newby AC. Metalloproteinases and vulnerable atherosclerotic plaques. *Trends Cardiovasc Med* 2007; **17**: 253–8
8. Annunen P, Autio-Harmainen H, Kivirikko KI. The novel type II prolyl 4-hydroxylase is the main enzyme form in chondrocytes and capillary endothelial cells, whereas the type I enzyme predominates in most cells. *J Biol Chem* 1998; **273**: 5989–92
9. Kivirikko KI, Pihlajaniemi T. Collagen hydroxylases and the protein disulfide isomerase subunit of prolyl 4-hydroxylases. *Adv Enzymol Relat Areas Mol Biol* 1998; **72**: 325–98
10. Li L, Cai XJ, Feng M, Rong YY, Zhang Y, Zhang M. Effect of adiponectin overexpression on stability of preexisting plaques by inducing prolyl-4-hydroxylase expression. *Circ J* 2010; **74**: 552–9
11. Zhang C, Zhang MX, Shen YH, et al. TNF-alpha suppresses prolyl-4-hydroxylase alpha 1 expression via the ASK1-JNK-NonO pathway. *Arterioscler Thromb Vasc Biol* 2007; **27**: 1760–7
12. Bignami E, Guarnieri M, Pieri M, et al. Volatile anaesthetics added to cardiopulmonary bypass are associated with reduced cardiac troponin. *Perfusion* 2017; **32**: 547–53
13. Landoni G, Lomivorotov V, Silvetti S, et al. Nonsurgical strategies to reduce mortality in patients undergoing cardiac surgery: an updated consensus process. *J Cardiothorac Vasc Anesth* 2018; **32**: 225–35
14. Bonanni A, Signori A, Alicino C, et al. Volatile anesthetics versus propofol for cardiac surgery with cardiopulmonary

bypass: meta-analysis of randomized trials. *Anesthesiology* 2020; **132**: 1429–46
15. Bi C, Cai Q, Shan Y, et al. Sevoflurane induces neurotoxicity in the developing rat hippocampus by upregulating connexin 43 via the JNK/c-Jun/AP-1 pathway. *Biomed Pharmacother* 2018; **108**: 1469–76
16. Wang LY, Tang ZJ, Han YZ. Neuroprotective effects of caffeic acid phenethyl ester against sevoflurane-induced neuronal degeneration in the hippocampus of neonatal rats involve MAPK and PI3K/Akt signaling pathways. *Mol Med Rep* 2016; **14**: 3403–12
17. Wang WY, Yang R, Hu SF, Wang H, Ma ZW, Lu Y. N-stearoyl-l-tyrosine ameliorates sevoflurane induced neuroapoptosis *via* MEK/ERK1/2 MAPK signaling pathway in the developing brain. *Neurosci Lett* 2013; **541**: 167–72
18. Ni M, Wang Y, Zhang M, et al. Atherosclerotic plaque disruption induced by stress and lipopolysaccharide in apolipoprotein E knockout mice. *Am J Physiol Heart Circ Physiol* 2009; **296**: H1598–606
19. Liu X, Ni M, Ma L, et al. Targeting blood thrombogenicity precipitates atherothrombotic events in a mouse model of plaque destabilization. *Sci Rep* 2015; **5**: 10225
20. Meng X, Li W, Yang J, et al. Regulatory T cells prevent plaque disruption in apolipoprotein E-knockout mice. *Int J Cardiol* 2013; **168**: 2684–92
21. Zhang K, Zhang F, Yang JM, et al. Silencing of non-POU-domain-containing octamer-binding protein stabilizes atherosclerotic plaque in apolipoprotein *E*-knockout mice *via* NF-κB signaling pathway. *Int J Cardiol* 2018; **263**: 96–103
22. Sui W, Li H, Yang Y, et al. Bladder drug mirabegron exacerbates atherosclerosis through activation of brown fat-mediated lipolysis. *Proc Natl Acad Sci U S A* 2019; **116**: 10937–42
23. Chen HZ, Wang F, Gao P, et al. Age-associated sirtuin 1 reduction in vascular smooth muscle links vascular senescence and inflammation to abdominal aortic aneurysm. *Circ Res* 2016; **119**: 1076–88
24. Li H, Li J, Jiang X, et al. Melatonin enhances atherosclerotic plaque stability by inducing prolyl-4-hydroxylase alpha1 expression. *J Hypertens* 2019; **37**: 964–71
25. Xu X, Zhang F, Lu Y, et al. Silencing of NONO inhibits abdominal aortic aneurysm in apolipoprotein E-knockout mice via collagen deposition and inflammatory inhibition. *J Cell Mol Med* 2019; **23**: 7449–61
26. Caraiscos VB, Newell JG, You-Ten KE, et al. Selective enhancement of tonic GABAergic inhibition in murine hippocampal neurons by low concentrations of the volatile anesthetic isoflurane. *J Neurosci* 2004; **24**: 8454–8
27. Meng T, Bu W, Ren X, et al. Molecular mechanism of anesthetic-induced depression of myocardial contraction. *FASEB J* 2016; **30**: 2915–25
28. Meng T, Ren X, Chen X, et al. Anesthetic agents isoflurane and propofol decrease maximal Ca^{2+}-activated force and thus contractility in the failing myocardium. *J Pharmacol Exp Ther* 2019; **371**: 615–23
29. Yang JJ, Li P, Wang F, et al. Activation of activator protein 2 alpha by aspirin alleviates atherosclerotic plaque growth and instability in vivo. *Oncotarget* 2016; **7**: 52729–39
30. Auge N, Maupas-Schwalm F, Elbaz M, et al. Role for matrix metalloproteinase-2 in oxidized low-density lipoprotein-induced activation of the sphingomyelin/ceramide pathway and smooth muscle cell proliferation. *Circulation* 2004; **110**: 571–8
31. Satomoto M, Sun Z, Adachi YU, Kinoshita H, Makita K. Sevoflurane preconditioning ameliorates lipopolysaccharide-induced cognitive impairment in mice. *Exp Anim* 2018; **67**: 193–200
32. Wagner J, Strosing KM, Spassov SG, et al. Sevoflurane posttreatment prevents oxidative and inflammatory injury in ventilator-induced lung injury. *PLoS One* 2018; **13**, e0192896
33. Gough PJ, Gomez IG, Wille PT, Raines EW. Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice. *J Clin Invest* 2006; **116**: 59–69
34. Yang C, Li B, Wang G, Xing Y. The attenuation of myocardial hypertrophy by atorvastatin via the intracellular calcium signal and the p38 MAPK pathway. *Int J Clin Exp Pathol* 2019; **12**: 798–807
35. Zhou QL, Teng F, Zhang YS, Sun Q, Cao YX, Meng GW. FPR1 gene silencing suppresses cardiomyocyte apoptosis and ventricular remodeling in rats with ischemia/reperfusion injury through the inhibition of MAPK signaling pathway. *Exp Cell Res* 2018; **370**: 506–18
36. Geng J, Yang CC, Wang BJ, et al. Trimethylamine N-oxide promotes atherosclerosis via CD36-dependent MAPK/JNK pathway. *Biomed Pharmacother* 2018; **97**: 941–7
37. Ni M, Zhang M, Ding SF, Chen WQ, Zhang Y. Micro-ultrasound imaging assessment of carotid plaque characteristics in apolipoprotein-E knockout mice. *Atherosclerosis* 2008; **197**: 64–71

Handling editor: Gareth Ackland