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Hydrogen Sulphide Release via the Angiotensin Converting Enzyme Inhibitor Zofenopril Prevents Intimal Hyperplasia in Human Vein Segments and in a Mouse Model of Carotid Artery Stenosis

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WHAT THIS PAPER ADDS

The current strategies to reduce intimal hyperplasia (IH) rely principally on local drug delivery, in an endovascular approach. The oral angiotensin converting enzyme inhibitor (ACEi) zofenopril has additional effects to other non-sulfyhydrated ACEi to prevent intimal hyperplasia and re-stenosis. Given the number of patients treated with ACEi worldwide, these findings call for further prospective clinical trials to test the benefits of sulfhydrated ACEi over classic ACEi for the prevention of re-stenosis in hypertensive patients.

Objective: Hypertension is a major risk factor for intimal hyperplasia (IH) and re-stenosis following vascular and endovascular interventions. Preclinical studies suggest that hydrogen sulphide (H_2S), an endogenous gasotransmitter, limits re-stenosis. While there is no clinically available pure H_2S releasing compound, the sulfhydryl containing angiotensin converting enzyme inhibitor zofenopril is a source of H_2S. Here, it was hypothesised that zofenopril, due to H_2S release, would be superior to other non-sulfhydryl containing angiotensin converting enzyme inhibitors (ACEi) in reducing intimal hyperplasia.

Methods: Spontaneously hypertensive male Cx40 deleted mice ($Cx40^{-/-}$) or wild type (WT) littermates were randomly treated with enalapril 20 mg or zofenopril 30 mg. Discarded human vein segments and primary human smooth muscle cells (SMCs) were treated with the active compound enalaprilat or zofenoprilat. IH was evaluated in mice 28 days after focal carotid artery stenosis surgery and in human vein segments cultured for seven days *ex vivo*. Human primary smooth muscle cell (SMC) proliferation and migration were studied *in vitro*.

Results: Compared with control animals (intima/media thickness 2.3 $\pm$ 0.33 μm), enalapril reduced IH in $Cx40^{-/-}$ hypertensive mice by 30% (1.7 $\pm$ 0.35 μm; $p = .037$), while zofenopril abrogated IH (0.4 $\pm$ 0.16 μm; $p < .002$ *vs.* control and $p > .99$ *vs.* sham operated $Cx40^{-/-}$ mice). In WT normotensive mice, enalapril had no effect (0.9665 $\pm$ 0.2 μm in control *vs.* 1.140 $\pm$ 0.27 μm; $p > .99$), while zofenopril also abrogated IH (0.1623 $\pm$ 0.07 μm; $p < .008$ *vs.* control and $p > .99$ *vs.* sham operated WT mice). Zofenoprilat, but not enalaprilat, also prevented IH in human vein segments *ex vivo*. The effect of zofenopril on carotid and SMCs correlated with reduced SMC proliferation and migration. Zofenoprilat inhibited the mitogen activated protein kinase and mammalian target of rapamycin pathways in SMCs and human vein segments.

Conclusion: Zofenopril provides extra beneficial effects compared with non-sulfhydryl ACEi in reducing SMC proliferation and re-stenosis, even in normotensive animals. These findings may hold broad clinical implications for patients suffering from vascular occlusive diseases and hypertension.

Keywords: ACE inhibitor, Hydrogen sulphide, Hypertension, Intimal hyperplasia, Proliferation, Smooth muscle cells, Restenosis, Zofenopril
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INTRODUCTION

Intimal hyperplasia (IH) remains the major cause of re-stenosis following vascular surgery, leading to potential limb loss and death. IH develops in response to vessel injury, leading to inflammation, vascular smooth muscle cell (VSMC) dedifferentiation, migration, and proliferation, and secretion of extracellular matrix. Despite decades of research, there is no effective medication to prevent re-stenosis.[1] The only validated therapy against IH is the local drug delivery strategy, used especially in the endovascular approach. However, this strategy seems to be limited;[2] other complementary oral treatments target either steps involved in IH, such as SMC proliferation, or risk factors for re-stenosis such as hypertension.

Hydrogen sulphide (H_2S) is an endogenously produced gasotransmitter.[3] Preclinical studies have shown that H_2S has cardiovascular protective properties,[4] including reduction of IH,[5–7] possibly via decreased VSMC proliferation.[6,8] However, there is currently no clinically approved H_2S donor.[9]

Hypertension is a known risk factor for re-stenosis and bypass failure.[10] Current guidelines recommend angiotensin converting enzyme inhibitors (ACEi) as the first line therapy for the treatment of essential hypertension.[11] Although various ACEi reduce re-stenosis in rodent models,[12] prospective clinical trials failed to prove efficacy of the ACEi quinapril or cilazapril for the prevention of re-stenosis at six months after coronary angioplasty.[13–15] Several *in vitro* studies suggest that the ACEi zofenopril, owing to a sulfhydryl moiety in its structure, releases H_2S.[16–18] The therapeutic potential of sulfhydryl ACEi zofenopril has never been tested in the context of re-stenosis.

The purpose of this study was to test whether zofenopril, owing to its H_2S releasing properties, is superior to non-sulfhydryl ACEi in limiting IH in a surgical mouse model of IH *in vivo* and in an *ex vivo* model of IH in human vein culture. Zofenopril was systematically compared with the non-sulfhydrated ACEi enalapril.

MATERIALS AND METHODS

Materials

Drugs and reagents are described in Supplementary Table S1. Datasets are available at https://doi.org/10.5281/zenodo.5017874

Experimental group design

All experiments were performed using 8 — 10 week old male Cx40 deleted mice ($Cx40^{-/-}$)[19] and wild type (WT) littermate mice on a C57BL/6J genetic background. Mice randomly assigned to the experimental groups were treated with the various ACEi at 10 mg/kg/day via a water bottle.

Blood pressure experiments. WT ($n = 22$) or $Cx40^{-/-}$ ($n = 18$) mice were randomly divided into three groups: control, enalapril, and zofenopril. Basal systolic blood pressure (SBP) was measured for four days then treatments were initiated and SBP was measured for 10 more days. WT groups were done in parallel ($n = 22$) with $Cx40^{-/-}$ ($n = 6$) untreated mice. $Cx40^{-/-}$ groups ($n = 18$) were done in parallel with WT untreated mice ($n = 6$).

WT mice ($n = 12$) were randomly divided into three groups: control ($n = 4$), quinapril ($n = 4$), and lisinopril ($n = 4$). Basal SBP was measured for four days and then treatments were initiated and SBP was measured for 10 more days.

SBP was monitored daily by the non-invasive plethysmography tail cuff method (BP-2000; Visitech Systems, Apex, NC, USA) on conscious mice.[20]

Mouse carotid artery stenosis model. WT mice ($n = 26$) were divided into three groups: control (ctrl; $n = 9$), enalapril ($n = 9$), and zofenopril ($n = 8$). $Cx40^{-/-}$ mice ($n = 24$) were divided into three groups: ctrl ($n = 11$), enalapril ($n = 6$), and zofenopril ($n = 7$). Seven days post-treatment, IH was induced via a carotid stenosis.

Carotid artery stenosis (CAS) was performed as previously published.[21] For surgery, mice were anaesthetised with ketamine 80 mg/kg and xylazine 15 mg/kg. The left carotid artery was located and separated from the jugular vein and vagus nerve. Then, a 7.0 PERMA silk (Johnson & Johnson AG, Ethicon, Neuchâtel, Switzerland) thread was looped under the artery and tightened around the carotid in the presence of a 35 G needle. The needle was removed, thereby restoring blood flow, albeit leaving a significant stenosis.[21] Buprenorphine 0.05 mg/kg was provided as post-operative analgesia every 12 hours for 48 hours. Treatment with the ACEi of choice was continued for 28 days post-operatively until organ collection. In another set of procedures, WT mice ($n = 17$) were randomly divided into three groups: control ($n = 6$), quinapril ($n = 6$) and lisinopril ($n = 5$). Seven days post-treatment, IH was induced via a carotid stenosis.

All mice were euthanised 28 days post-operatively under general anaesthesia by cervical dislocation and exsanguination, perfused with phosphate buffered saline (PBS) followed by buffered formalin 4% through the left ventricle, and the carotids were taken for IH measurements.

All animal experimentation conformed to the National Research Council: Guide for the Care and Use of Laboratory Animals.[22] All animal care, surgery, and euthanasia procedures were approved by the Centre Hospitalier Universitaire Vaudois (CHUV) and the Cantonal Veterinary Office (Service de la Consommation et des Affaires Vétérinaires SCAV-EXPANIM, authorisation number 3258).

Ex vivo static human vein culture and smooth muscle cell culture

Human vein segments were retrieved from discarded tissue obtained during lower limb bypass surgery. Each native vein was cut into 7 mm segments randomly distributed between conditions (day [D]0; D7, ctrl; D7, enalaprilat; D7, zofenoprilat). One segment (D0) was immediately flash frozen in liquid nitrogen or optimal cutting temperature (OCT) compound and the others were maintained in culture for

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seven days in RPMI-1640 Glutamax supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic solution (10 000 U/mL penicillin G, 10 000 U/mL streptomycin sulphate) at 37°C and 5% CO_2, as described previously.[6] The cell culture medium was changed every 48 hours with fresh ACEi. Six different veins/patients were included in this study.

Human VSMCs were prepared and cultured from human saphenous vein segments as described previously.[6,19] The study protocols for organ collection and use were reviewed and approved by CHUV and the Cantonal Human Research Ethics Committee (http://www.cer-vd.ch/, no Institutional Review Board number, protocol number 170/02), and were in accordance with the principles outlined in the Declaration of Helsinki of 1975, as revised in 1983 for the use of human tissues. Six different veins/patients were used in this study to generate VSMCs.

Histomorphometry

Ligated left carotids were isolated and embedded in paraffin. Six 6 µm cross sections were collected every 100 µm and up to 2 mm from the ligature and stained with Van Gieson Elastic Lamina (VGEL) staining. For intimal and medial thickness, 72 (12 measurements/cross section on six cross sections) measurements were performed.[19] To account for the gradient of IH inrelation to the distance from the ligature, the intima thickness was plotted against the distance to calculate the area under the curve of intima thickness. Mean intima and media thickness over the 2 mm distance were also calculated.

For human vein segments, after seven days in culture, or immediately upon vein isolation (D0), segments were fixed in buffered formalin, embedded in paraffin and cut into 6 µm sections, and stained with VGEL as described previously.[6] For intimal and medial thickness, 96 (four

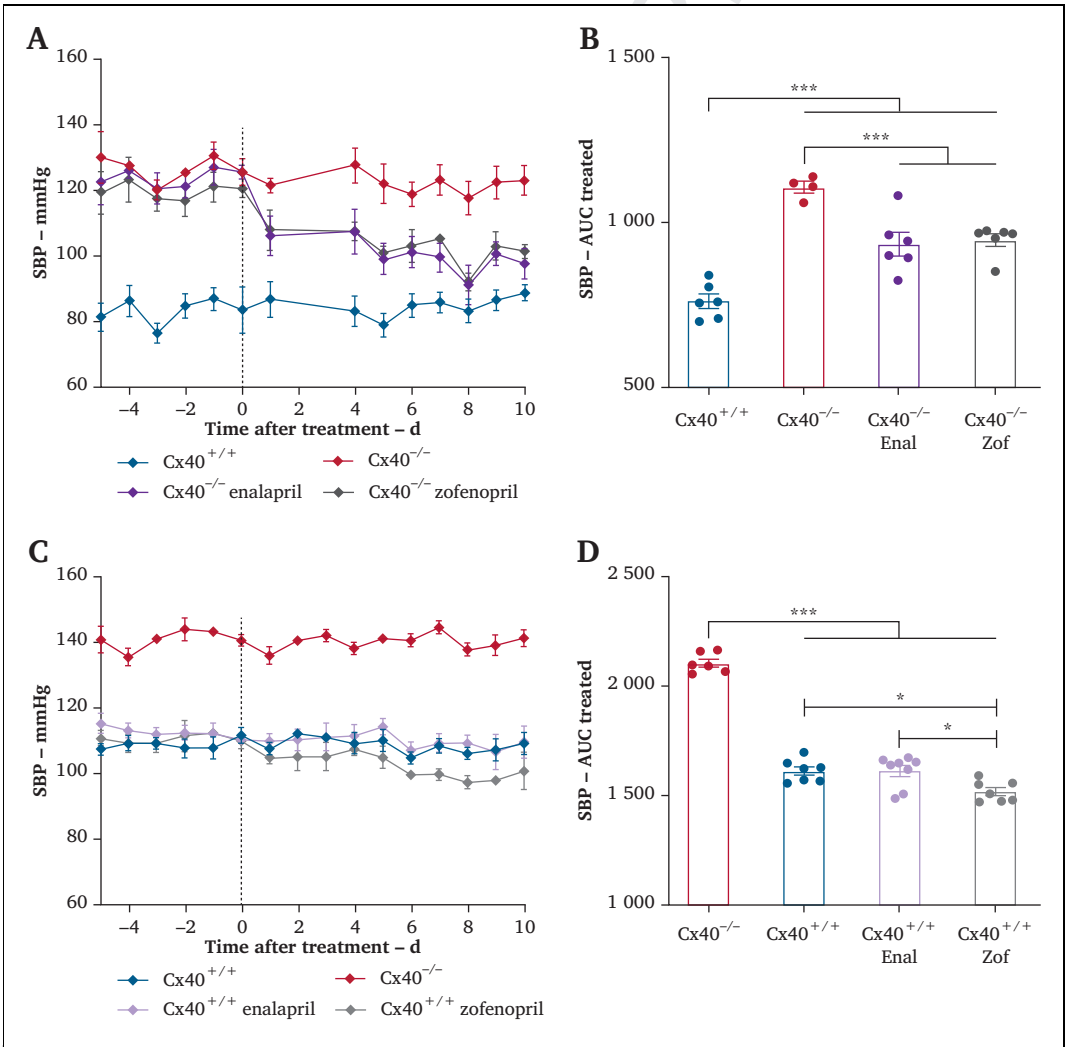

Figure 1. Zofenopril and enalapril similarly lower systolic blood pressure (SBP) in hypertensive $Cx40^{-/-}$ mice. (A) Daily systolic blood pressure (SBP) values (mean ± standard error of mean [SEM]) in wild type (WT; $Cx40^{+/+}$) ($n = 6$) *vs.* $Cx40^{-/-}$ mice treated or not ($n = 5$) with 10 mg/kg zofenopril ($n = 6$) and 10 mg/kg enalapril ($n = 6$) for the indicated time. (B) Area under the curve (AUC) of SBP from day 0 to 10. (C) Daily SBP values (mean ± SEM) in $Cx40^{-/-}$ ($n = 5$) *vs.* WT mice treated or not ($n = 6$) with zofenopril ($n = 8$) and enalapril ($n = 8$) for the indicated time. (D) AUC of SBP between day 0 to 10. *$p < .050$; **$p < .010$; ***$p < .001$ as indicated by one way analysis of variance with Tukey's correction of multiple comparisons.

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measurements/photos and four photos per cross section on six cross sections) measurements were performed.[19] Two independent researchers blinded to the conditions did the morphometric measurements using the Olympus Stream Start 2.3 software (Olympus, Wallisellen, Switzerland).[6,19]

Immunohistochemistry

Proliferating cell nuclear antigen (PCNA) immunohistochemistry was performed on paraffin sections as described previously after antigen retrieval using TRIS—ethylenediaminetetraacetic acid (EDTA) buffer (pH 9) for 17 minutes in a microwave at 500 watts.[6] Immunostaining was performed using the EnVision +/HRP, DAB+ system, according to manufacturer's instructions (Dako, Lausanne, Switzerland), and counterstained with haematoxylin. One slide per series was assessed and three images per section were taken at ×200 magnification. Two independent observers unaware of the conditions manually counted the PCNA and haematoxylin positive nuclei.

Live cell hydrogen sulphide measurement

Free sulphide was measured in cells using a 5 μM SF_7-AM fluorescent probe as described previously.[6] Fluorescence intensity (λ_{ex} = 495 nm; λ_{em} = 520 nm) was measured continuously in a Synergy Mx fluorescent plate reader (Biotek, Basel, Switzerland) at 37°C before and after addition of various compounds, as indicated.

Persulfidation protocol

A persulfidation protocol was performed using a dimedone based probe, as described previously.[23] Flash frozen liver was ground into powder and 20 mg powder was homogenised in 300 μL HEN buffer (i.e., 100 mM HEPES, 1 mM EDTA, 100 μM neocuproin, 1 vol. % NP-40, 1 wt. % sodium dodecyl sulphate [SDS], and proteases inhibitors) supplemented with 5 mM 4-chloro-7-nitrobenzofurazan. Proteins were extracted by methanol/chloroform/water protein precipitation and the pellet was resuspended in 200 μL 50 mM HEPES-2 wt. % SDS. Protein content was measured using a Pierce BCA protein assay kit (Pierce, Rockford, IL, USA), and 75 μg proteins were incubated with 25 μM final Daz-2-biotin for one hour in the dark at 37°C. Daz-2-biotin was prepared with 1 mM Daz-2, 1 mM alkynyl biotin, 2 mM copper(II)-tris(benzyltriazolylmethyl)amine, and 4 mM ascorbic acid with overnight incubation at room temperature, followed by quenching with 20 mM EDTA. Proteins were then extracted by methanol/chloroform/water protein precipitation and the pellets resuspended in 150 μL SDS lysis buffer. Protein concentration was measured using the detergent compatible (DC) protein assay, 10 μg was loaded onto SDS polyacrylamide gel electrophoresis (SDS-PAGE) and the biotin signal was measured by Western blot analyses using a streptavidin—horseradish peroxidase antibody. Protein abundance was normalised to total protein staining using a Pierce Reversible Protein Stain Kit for polyvinylidene fluoride (PVDF) membranes.

BrdU bromodeoxyuridine/5-bromo-2'-deoxyuridine staining for vascular smooth muscle cell proliferation

VSMCs were grown at 80% confluence on glass coverslips in a 24 well plate and starved overnight in serum free medium. Then, VSMCs were either treated or not (ctrl) with the ACEi of choice for 24 hours in full medium (RPMI 10% FBS) in presence of 10 μM BrdU. All conditions were tested in parallel. All cells were fixed in 100% ice cold methanol after 24 hours of incubation and immunostained for BrdU. Images were acquired using a Nikon Eclipse 90i microscope. BrdU positive nuclei and total 4',6-diamidino-2-phenylindole (DAPI) positive nuclei were automatically detected using ImageJ software.[6]

Wound healing assay vascular smooth muscle cell migration

VSMCs were grown at confluence in a 12 well plate and starved overnight in serum free medium. Then, a scratch wound was created using a sterile p200 pipette tip and medium was changed to full medium (RPMI 10% FBS). Repopulation of the wounded areas was recorded by phase contrast microscopy over 24 hours in a Nikon Ti2-E live cell microscope. The area of the denuded area was measured at 0 hours and 10 hours after the wound, using ImageJ software, by two independent observers blind to the conditions.

Western blotting

Human vein segments were washed twice in ice cold PBS, flash frozen in liquid nitrogen, ground to powder, and resuspended in SDS lysis buffer (62.5 mM TRIS pH 6.8, 5% SDS, 10 mM EDTA).

VSMCs were kept in serum free media overnight. The next morning, complete media was added with the ACEi. Five hours post-treatment, cells were washed once with ice cold PBS and directly lysed with Laemmli buffer. Lysates were resolved by SDS-PAGE and transferred to a PVDF membrane (Immobilon-P; Millipore, Schaffhausen, Switzerland). Immunoblot analyses were performed as described previously,[6] using the antibodies described in the Supplementary Table S1. Blots were revealed by enhanced chemiluminescence (Immobilon; Millipore) using the ChemiDoc XRS+ System and analysed using Image Lab (BETA2) software, version 3.0.01 (Bio-Rad Laboratories, Fribourg, Switzerland).

Statistical analyses

All experiments were analysed quantitatively using GraphPad Prism 8 (GraphPad Inc., La Jolla, CA, USA), and results are shown as mean ± standard error of the mean. Statistical test details are indicated in the figure legends.

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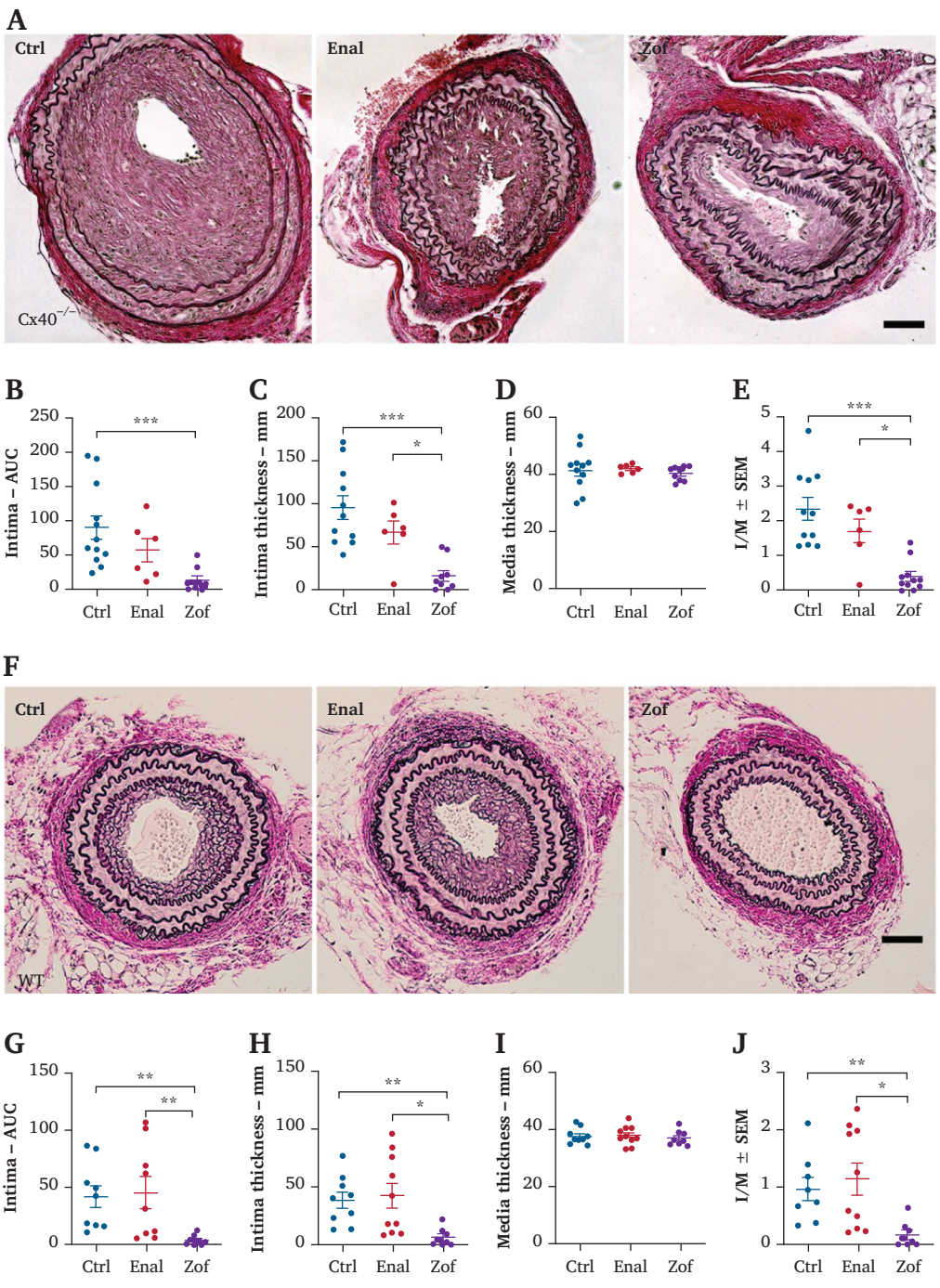

Figure 2. Zofenopril treatment reduces intimal hyperplasia in a mouse model of carotid artery stenosis. (A–E) Cx40$^{-/-}$ or (F–J) wild type (WT) mice, treated or not (control; Ctrl) with zofenopril (Zof) and enalapril (Enal), were submitted to carotid artery stenosis. (A, F) Representative images of left carotid cross sections stained with Van Gieson Elastic Lamina 28 days post-operatively in (A) Cx40$^{-/-}$ or (F) WT mice. Scale bar represents 40 μm. (B–E, G–I) Morphometric measurements of area under the curve (AUC) of (B, G) intima thickness, (C, H) intima thickness, (D, I) media thickness, and (E, J) intima over media (I/M) ratio. Data are presented as scatter plots of 9 − 12 animals per group, with mean ± standard error of mean (SEM). *p <.050; **p <.010; ***p < .001 as indicated by a Kruskal–Wallis test followed by Dunn's multiple comparisons tests.

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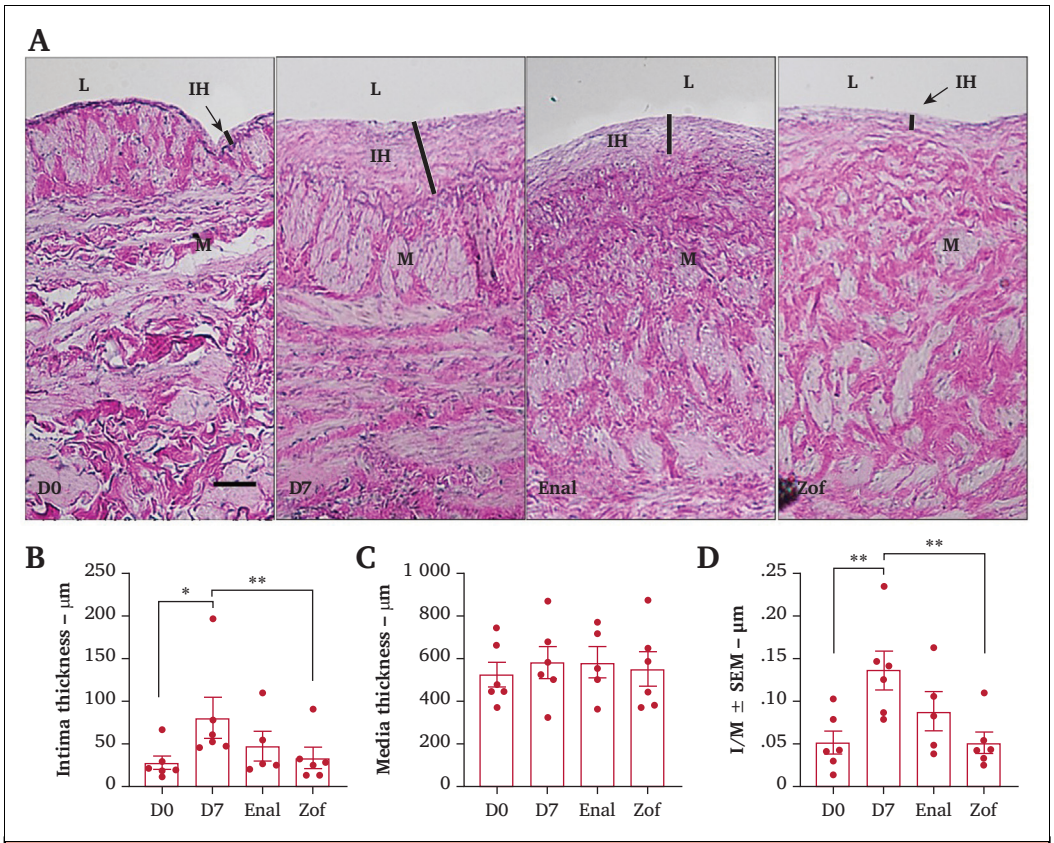

Figure 3. *Ex vivo* treatment with zofenoprilat prevents the development of intimal hyperplasia (IH) in human saphenous vein segments. Human great saphenous vein segments obtained from donors who underwent lower limb bypass surgery were put or not (day [D]0, $n = 6$) in static culture for seven days in the presence or not (D7, $n = 6$) of 100 µM zofenopril (Zof; $n = 6$) or enalapril (Enal; $n = 5$). (A) Representative Van Gieson Elastic Lamina staining presenting IH and media (M). L = lumen. Scale bar represents 50 µm. (B–D) Morphometric measurements of (B) intima thickness, (C) media thickness, and (D) intima over media (I/M) ratio. Data are presented as scatter plots of six different vein/patient with mean ± standard error of mean (SEM). *p <.050 and **p <.010 as indicated from repeated measures one way analysis of variance with post-hoc t test with Dunnet's correction of multiple comparisons.

RESULTS

Zofenopril and enalapril similarly lower systolic blood pressure of hypertensive mice

Spontaneously hypertensive Cx40 deleted mice ($Cx40^{-/-}$) and WT littermates were given either 10 mg/kg zofenopril or 6 mg/kg enalapril in the drinking water to achieve similar blood lowering effects on hypertensive $Cx40^{-/-}$ mice (Fig. 1A). Zofenopril also lowered SBP by 6 mmHg in normotensive WT mice (Fig. 1B). Enalapril (Fig. 1B), quinapril (10 mg/kg), and lisinopril (10 mg/kg) had no effect on SBP in WT mice (Supplementary Fig. S1).

Zofenopril is superior to other angiotensin converting enzyme inhibitors in reducing intimal hyperplasia in a mouse model of carotid artery stenosis

As expected, the hypertensive mice developed twice as much IH as their normotensive littermates following the CAS model.[21] Enalapril had a non-specific tendency to reduce IH in $Cx40^{-/-}$ mice (intima [I]/media [M] $p = 1.0$), while Zofenopril suppressed IH by 90% (I/M $p > .001$; Fig. 2, Supplementary Table S2). Enalapril had no effect in normotensive WT mice (I/M $p = 1.0$), whereas zofenopril also suppressed IH in those mice (I/M $p = .008$; Fig. 2, Supplementary Table S3). Quinapril and lisinopril did not affect IH in WT mice (Supplementary Fig. S2, Supplementary Table S4).

Zofenoprilat prevented the development of intimal hyperplasia in human saphenous vein segments

Next, the effect of zofenoprilat and enalaprilat, the active compounds derived from the prodrugs zofenopril and enalapril, respectively, were tested in the model of IH in *ex vivo* static vein culture.[6] Continuous treatment with 100 µM zofenoprilat, but not with enalaprilat, fully blocked the development of IH observed in veins maintained for seven days in culture in the absence of blood flow (D7), compared with initial values in freshly isolated veins (D0) (Fig. 3, Supplementary Table S5).

Zofenoprilat released H_2S

Besides its ACEi activity, zofenopril has been proposed to work as an H_2S donor.[16–18] *In vitro* time lapse recording of

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the H_2S selective probe SF_7-AM revealed that zofenoprilat, but not enalaprilat, slowly released H_2S in RPMI medium, compared with the fast releasing sodium hydrosulphide salt (Fig. 4A). Similar experiments in the presence of live VSMCs (Fig. 4B) confirmed that zofenoprilat, but not enalaprilat, increased the SF_7-AM signal.

The biological activity of H_2S is mediated by post-translational modification of reactive cysteine residues by persulfidation, which modulates protein structure and/or function.[9,23] Protein persulfidation was assessed using a dimedone based probe, as described previously.[23] Zofenopril significantly increased protein persulfidation in liver extracts from mice treated with enalapril or zofenopril for two weeks (Fig. 4C).

Zofenopril decreased vascular smooth muscle cell proliferation and migration

As various H_2S donors decrease VSMC proliferation in the context of IH,[6,8] the effect of zofenopril on VSMCs was tested next. In the CAS model, zofenopril, but not enalapril, lowered cell proliferation in the carotid wall as assessed by PCNA staining (Fig. 5A, B). Zofenoprilat further inhibited the proliferation and primary human VSMC migration *in vitro*, while enalaprilat had no effect on proliferation (Fig. 5C, D) and reduced migration by 20% (Fig. 5E, F). Lisinopril and quinaprilat did not affect VSMC proliferation (Supplementary Fig. S3).

Zofenoprilat inhibited the mitogen activated protein kinase and mammalian target of rapamycin pathways

The mitogen activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) signalling pathways contribute to VSMC proliferation in the context of IH.[24] Western blot analyses revealed that zofenoprilat reduced by 50% the levels of P-ERK1,2, P-p38, and P-S6RP in cultured VSMCs, while enalaprilat had no effect (Fig. 6A—F). Moreover, P-S6RP and P-ERK1,2 levels were also decreased by zofenoprilat in human vein segments placed in culture for seven days (Fig. 6G—I).

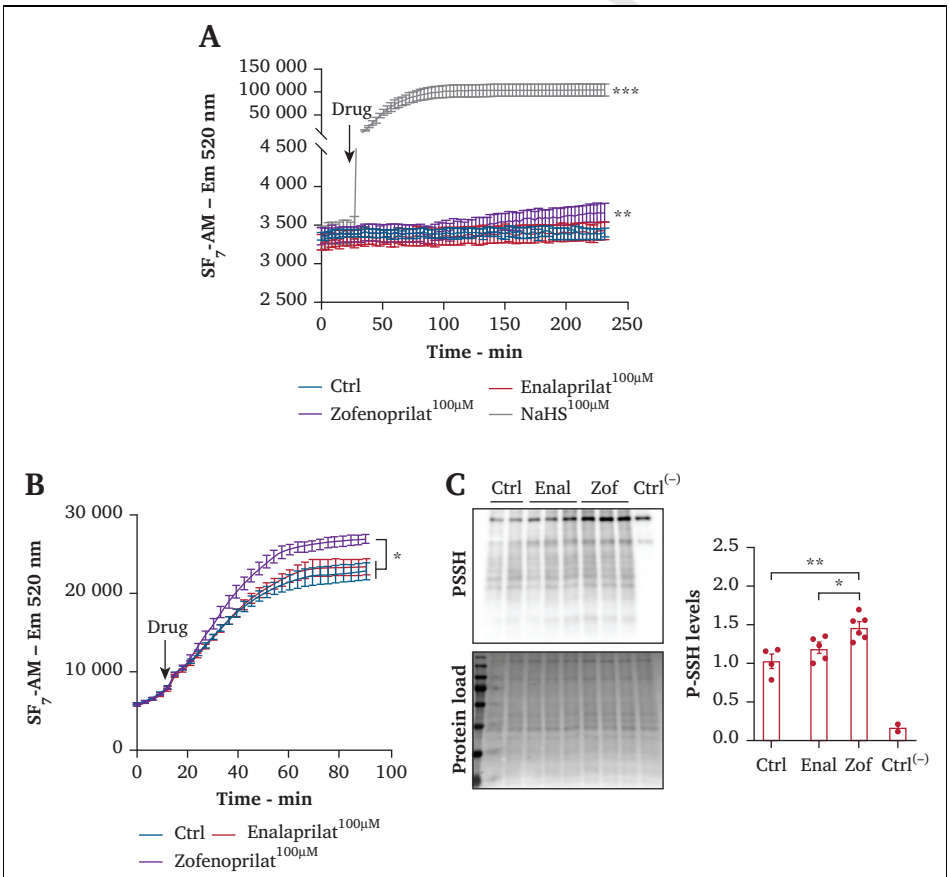

Figure 4. Zofenoprilat H_2S release as measured by a SF_7-AM fluorescent probe. (A, B) SF_7-AM fluorescent signal (mean ± standard error of mean) in (A) a cell free assay in RPMI medium, (B) in live primary vascular smooth muscle cells (VSMC) exposed or not (control; Ctrl) to 100 µM NaHS, 100 µM zofenoprilat, or 100 µM enalaprilat for the indicated time. *p <.050, **p <.010, and ***p <.001 *vs.* respective Ctrl as determined by repeated measures two way analysis of variance (ANOVA) with post hoc t test with Tukey's correction for multiple comparisons. Data are representative of three individual experiments. (C) Global protein persulfidation (PSSH; labelled with DAz-2:Biotin as a switching agent) over total proteins in liver extracts from C57BL/6J male mice treated for two weeks with enalapril or zofenopril. Data are presented as scatter plots of 5 − 6 animals/group with mean ± SEM with *p <.050 and **p <.010, as determined by one way ANOVA with post hoc t test with Tukey's correction for multiple comparisons.

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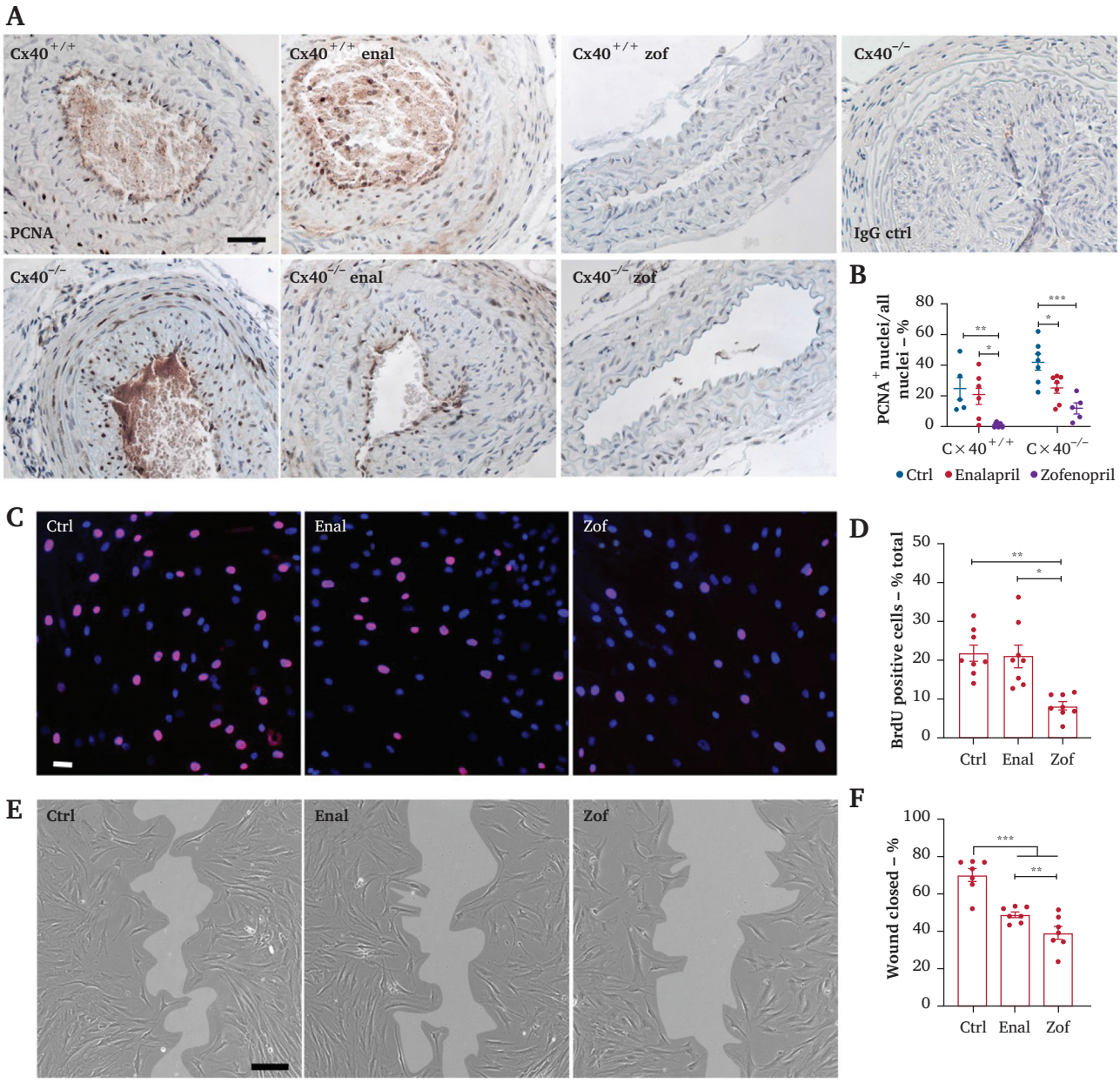

Figure 5. Zofenopril treatment reduces cell proliferation in a mouse model of carotid artery stenosis. (A, B) Proliferating cell nuclei antigen (PCNA) immunostaining 28 days after carotid artery stenosis in wild type (WT; $Cx40^{+/+}$) or $Cx40^{-/-}$ mice treated or not (Ctrl) with zofenopril (Zof) and enalapril (Enal). (A) Representative images of PCNA positive nuclei (brown) and negative nuclei (haematoxylin stained blue nuclei). Scale bar represents 40 μm. (B) Quantitative assessment of PCNA positive cells over total cells of 5 − 8 animals/group with mean ± standard error of mean (SEM). *p <.050, **p <.010, and ***p <.001, as determined by two way analysis of variance (ANOVA) with post-hoc t test with Sidak's correction of multiple comparisons. (C, D) Primary human vascular smooth muscle cells (VSMCs) were exposed or not (Ctrl) to 100 μM zofenoprilat or enalaprilat for 24 hours in the presence of BrdU. (C) Representative images of BrdU positive nuclei (pink) and 4',6-diamidino-2-phenylindole (DAPI) stained nuclei (blue). Scale bar represents 10 μm. (D) Proliferation was calculated as the percentage of BrdU positive nuclei over total nuclei. Data are scatter plots of eight independent experiments with mean ± SEM, with *p <.050 and **p <.010, as determined by repeated measures one way ANOVA with post hoc t test with Dunnet's correction for multiple comparisons. (E, F) Wound healing assay with VSMC exposed or not (Ctrl) to 100 μM zofenoprilat or enalaprilat. (E) Representative images of VSMCs in brightfield 10 hours post-wound. Scale bar represents 50 μm. (F) Data are scatter plots of seven independent experiments with mean ± SEM of wound area after 10 hours, expressed as a percentage of the initial wound area. **p <.010 and ***p <.001, as determined by repeated measures one way ANOVA with post hoc t test with Dunnet's correction for multiple comparisons.

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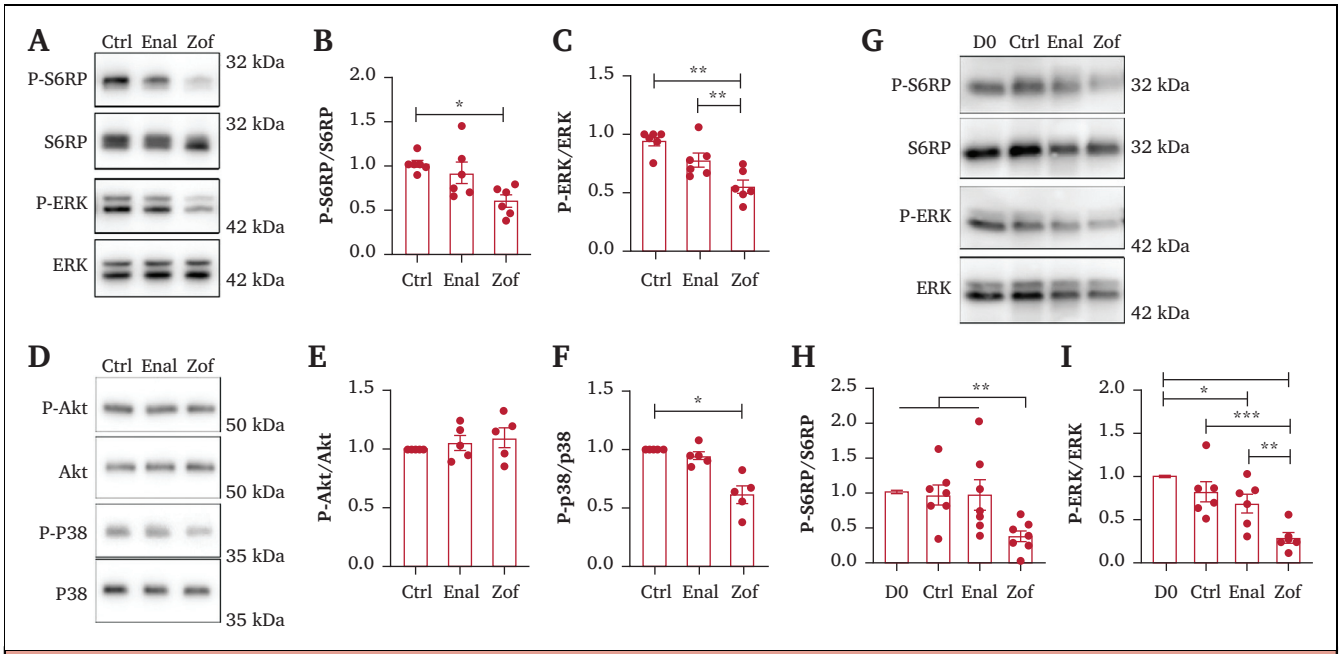

Figure 6. Zofenoprilat inhibits extracellular regulated kinase (ERK) and S6RP phosphorylation. (A–F) Western Blot analyses from vascular smooth muscle cells (VSMCs) exposed or not (Ctrl) to 100 μM zofenoprilat or enalaprilat for five hours. (A, D) Representative Western blot for P-S6RP and total S6RP, P-ERK, and total ERK, P-Akt and total Akt, P-p38 and total p38. (B, C, E, F) Quantitative assessment of six independent experiments, normalised to their respective Ctrl condition, with mean ± standard error of mean (SEM). *p <.050 and **p <.010, as determined by repeated measures one way analysis of variance (ANOVA) with post hoc t test with Dunnet's correction for multiple comparisons. (G–I) Western blot analyses from human vein segments exposed or not (Ctrl) to 100 μM zofenoprilat or enalaprilat for seven days. (G) Representative Western blot for P-S6RP and total S6RP, P-ERK, and total ERK. (H, I) Quantitative assessment of seven different veins, normalised to their respective control condition, with mean ± SEM. *p <.050, **p <.010, and ***p <.001, as determined by repeated measures one way ANOVA with post hoc t test with Dunnet's correction for multiple comparisons.

DISCUSSION

In this study, it was hypothesised that zofenopril, an ACEi with a free thiol moiety acting as an H_2S donor, would be more efficient than other ACEi in the inhibition of IH in the context of hypertension. Zofenopril is not only more potent than enalapril in reducing IH in hypertensive Cx40$^{-/-}$ mice, but it also suppresses IH in the normotensive condition, where other ACEi have no effect. Furthermore, zofenopril prevents IH in human saphenous vein segments in the absence of blood flow. The effect of zofenopril on IH correlates with reduced VSMC proliferation and migration, and decreased activity of the MAPK and mTOR pathways.

Several preclinical studies have shown that that SBP lowering medication such as ACEi reduce IH,[12] which prompted the large scale MERCATOR (Multicenter European Research Trial with Cilazapril after Angioplasty to prevent Transluminal Coronary Obstruction and Restenosis)/MARCATOR (Multicenter American Research Trial With Cilazapril After Angioplasty to Prevent Restenosis) and PARIS clinical trials.[13–15] Here, it was also observed that lowering SBP with enalapril had a non-significant tendency to protect from IH in hypertensive mice. However, enalapril, quinapril and lisinopril had no effect in normotensive WT mice. The fact that the sulfhydrated ACEi zofenopril almost abrogated IH in hypertensive and normotensive mice strongly supports the hypothesis that this ACEi provides additional effects independent of its ACEi activity, as suggested previously.[16–18] Of interest, the SMILE (Survival of Myocardial Infarction Long-term Evaluation) clinical trials concluded that, compared with placebo or ramipril, zofenopril reduced the one year risk of cardiovascular events after acute myocardial infarction (MI).[25] These benefits might be related to H_2S release by zofenopril, as preclinical studies consistently show that H_2S supplementation promotes recovery after acute MI.[4]

Zofenopril has been proposed in several studies to work as a H_2S donor.[16–18] Here, it was confirmed that zofenoprilat releases detectable amounts of H_2S. H_2S modifies proteins by post-translational persulfidation (S-sulfhydration) of reactive cysteine residues, which modulate protein structure and/or function.[23] Here, it was seen that zofenopril increases overall protein persulfidation *in vivo*, suggesting that zofenopril also generates H_2S *in vivo*.

It has previously been demonstrated that various H_2S donors inhibit VSMC proliferation.[6,8,26] It was consistently confirmed that zofenopril inhibits VSMC proliferation and migration *in vitro* and reduces cell proliferation in the carotid wall *in vivo.* Although the exact mecanisms of action of Zofenoprilat and H_2S remain to be elucidated, it was demonstrated that zofenoprilat inhibits the MAPK and mTOR signalling pathways, which contribute to VSMC proliferation and neointima formation.[24] Overall, the data strongly suggest that zofenopril acts similarly to other known H_2S donors to limit IH through inhibition of the MAPK and mTOR signalling pathways, leading to decreased VSMC proliferation and migration.

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Overall, the data suggest that zofenopril, unlike other ACEi, might show benefits against re-stenosis in patients. These findings raise the question as to whether the scientific community was too quick to discard the whole class of ACEi as a treatment of re-stenosis based on the disappointing results of the MERCATOR/MARCATOR and PARIS trials.[13–15] In the last decade, many efforts have been made in the development of a local drug delivery strategy well adapted to endovascular interventions. However, this strategy seems to bring great improvement in the mid term but not in the long term.[2] Thus, a more chronic approach, sustaining the early effect on cell proliferation and IH inhibition, should be encouraged. Such a strategy relies on oral medication, which is also better adapted to open surgery.

The present study had some limitations. Firstly, numerous oral drugs to limit re-stenosis have been tested clincially over the years, and in most trials the pharmacological treatment of re-stenosis failed to show positive results, despite promising results obtained in experimental models.[27] While there is no doubt that preclinical models have significantly advanced understanding of the mechanisms of re-stenosis formation, none fully mimics re-stenosis in humans. The genetic model of renin dependent hypertension used in that study is rarely observed in patients, which have complex multifactorial essential hypertension. Additional studies that better reflect comorbidities (dyslipidaemia, renal insufficiecy, smoking, atherosclerosis, etc.) with a vein bypass model and larger animal models, or a small phase II clinical trial, are required before testing the benefits of zofenopril in a large, phase III clinical trials.

Secondly, although zofenopril was the only ACEi to provide benefits in the normotensive condition, it cannot be excluded that other ACEi not tested here could work as well. It is further acknowledged that pharmacokinetic and pharmacodynamic differences between zofenopril and other ACEi may contribute to the superiority of zofenopril. Zofenopril is more lipophilic and may have better tissue penetration than enalapril or ramipril, which may have an impact beyond the effect of H_2S liberated by zofenopril. However, it has been shown that vessel wall penetration of various ACEi is independent of lipophilia and that the endothelium constitutes no specific barrier for the passage of ACEi.[28]

Finally, the working hypothesis is that zofenopril inhibits VSMC proliferation via direct release of H_2S at the level of the vessel media. However, it could not be ascertained that H_2S is released at the level of the VSMC. H_2S and zofenoprilat have been shown to promote endothelial cell function,[9,17,18] including proliferation and migration. Thus, it cannot be excluded that zofenopril limits IH via a positive effect on endothelial cells. Further studies are required to assess carefully the impact of zofenopril on the endothelium and quantify H_2S in vascular tissue.

Conclusion

Under the conditions of these experiments, zofenopril was superior to enalapril in reducing IH and providing a beneficial effect against IH in mice and in a model of IH in human vein segments *ex vivo*. The data strongly support the suggestion that zofenopril limits the development of IH via H_2S release, independently of its ACEi activity. The effects of zofenopril correlate with reduced MAPK and mTOR pathway activity, leading to decreased VSMC proliferation and migration.

Given the number of patients treated with ACEi worldwide, these findings may have broad implications for the treatment of patients suffering from peripheral atherosclerotic disease undergoing revascularisation, and beyond. These results warrant further research to evaluate the benefits of zofenopril in limiting re-stenosis and, eventually, prospective clinical trials to test the superiority of sulfhydrated ACEi on re-stenosis over other ACEi.

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CONFLICT OF INTEREST

None.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejvs.2021.09.032.

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