

1 **Hydrogen sulfide release via the ACE inhibitor Zofenopril prevents intimal hyperplasia in human**
2 **vein segments and in a mouse model of carotid artery stenosis**

3 Diane MACABREY^{a,b,§}, Céline DESLARZES-DUBUIS^{a,b,§}, Alban LONGCHAMP^{a,b}, Martine LAMBELET^{a,b},
4 Charles K. OZAKI^c, Jean-Marc CORPATAUX^{a,b}, Florent ALLAGNAT^{a,b,#} and Sébastien DÉGLISE^{a,b,#}

5

6 ^aDepartment of Vascular Surgery, Lausanne University Hospital, Switzerland

7 ^bDepartment of Biomedical Sciences, University of Lausanne, Switzerland

8 ^cDepartment of Surgery and the Heart and Vascular Center, Brigham and Women's Hospital and
9 Harvard Medical School, Boston, MA, USA.

10 [§] *These authors have equally contributed as first authors*

11 [#] *These authors have equally contributed as senior authors*

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19 Corresponding Author:

20 Florent Allagnat

21 CHUV-Service de chirurgie vasculaire

22 Département des Sciences Biomédicales

23 Bugnon 7A, 1005 Lausanne, Suisse

24 +41216925582

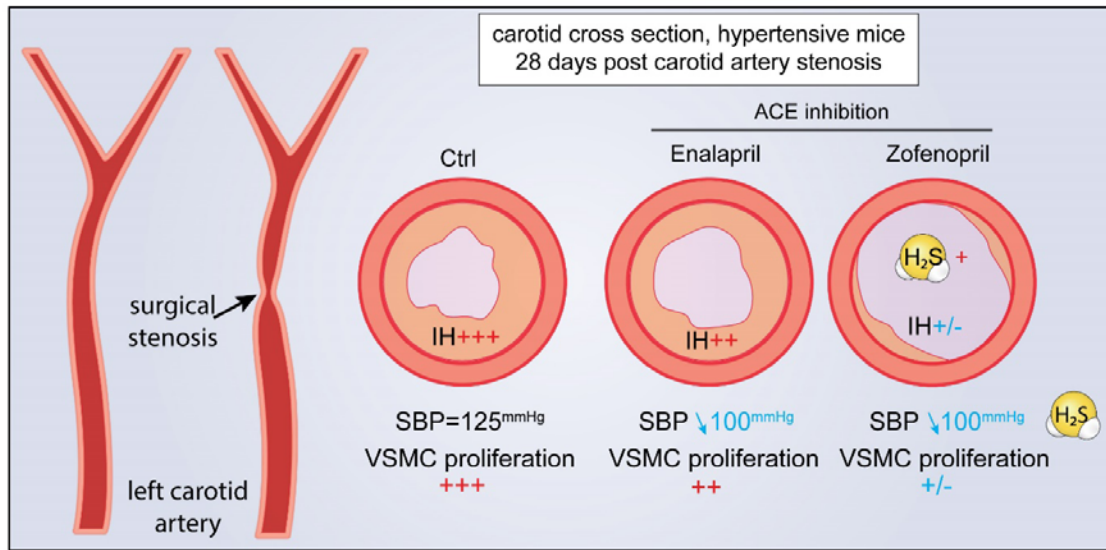
25 Florent.allagnat@chuv.ch

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29 **Graphical Abstract**



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33 What this paper adds

34 The current strategies to reduce intimal hyperplasia (IH) principally rely on local drug delivery, in
35 endovascular approach. The oral angiotensin converting enzyme inhibitor (ACEi) Zofenopril has
36 additional effects compared to other non-sulphydrated ACEi to prevent intimal hyperplasia and
37 restenosis. Given the number of patients treated with ACEi worldwide, these findings call for further
38 prospective clinical trials to test the benefits of sulphydrated ACEi over classic ACEi for the
39 prevention of restenosis in hypertensive patients.

40

41 **Abstract**

42 **Objectives**

43 Hypertension is a major risk factor for intimal hyperplasia (IH) and restenosis following
44 vascular and endovascular interventions. Pre-clinical studies suggest that hydrogen sulfide (H₂S), an
45 endogenous gasotransmitter, limits restenosis. While there is no clinically available pure H₂S
46 releasing compound, the sulfhydryl-containing angiotensin-converting enzyme inhibitor Zofenopril is
47 a source of H₂S. Here, we hypothesized that Zofenopril, due to H₂S release, would be superior to
48 other non-sulphydryl containing angiotensin converting enzyme inhibitor (ACEi), in reducing intimal
49 hyperplasia.

50 **Materials**

51 Spontaneously hypertensive male Cx40 deleted mice (Cx40^{-/-}) or WT littermates were
52 randomly treated with Enalapril 20 mg (Mepha Pharma) or Zofenopril 30 mg (Mylan SA). Discarded
53 human vein segments and primary human smooth muscle cells (SMC) were treated with the active
54 compound Enalaprilat or Zofenoprilat.

55 **Methods**

56 IH was evaluated in mice 28 days after focal carotid artery stenosis surgery and in human
57 vein segments cultured for 7 days *ex vivo*. Human primary smooth muscle cell (SMC) proliferation
58 and migration were studied *in vitro*.

59 **Results**

60 Compared to control animals (intima/media thickness=2.3±0.33), Enalapril reduced IH in Cx40^{-/-}
61 hypertensive mice by 30% (1.7±0.35; p=0.037), while Zofenopril abrogated IH (0.4±0.16; p<.0015 vs.
62 Ctrl and p>0.99 vs. sham-operated Cx40^{-/-} mice). In WT normotensive mice, enalapril had no effect
63 (0.9665±0.2 in control vs 1.140±0.27; p>.99), while Zofenopril also abrogated IH (0.1623±0.07,
64 p<.008 vs. Ctrl and p>0.99 vs. sham-operated WT mice). Zofenoprilat, but not Enalaprilat, also
65 prevented intimal hyperplasia in human veins segments *ex vivo*. The effect of Zofenopril on carotid
66 and SMC correlated with reduced SMC proliferation and migration. Zofenoprilat inhibited the MAPK
67 and mTOR pathways in SMC and human vein segments.

68 **Conclusion**

69 Zofenopril provides extra beneficial effects compared to non-sulphydryl ACEi to reduce SMC
70 proliferation and restenosis, even in normotensive animals. These findings may hold broad clinical
71 implications for patients suffering from vascular occlusive diseases and hypertension.

72 **Keywords:** Intimal hyperplasia; smooth muscle cells; proliferation; hydrogen sulfide; zofenopril;
73 hypertension; ACE inhibitor; restenosis

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76

77 **Abbreviations**

78 ACE: angiotensin converting enzyme

79 CAS: carotid artery stenosis

80 Cx40: connexin 40

81 H₂S: hydrogen sulfide

82 IH: intimal hyperplasia

83 PCNA: proliferating cell nuclear antigen

84 SBP: systolic blood pressure

85 VSMC: vascular smooth muscle cells

86 VGEL: Van Gieson elastic lamina

87

88 INTRODUCTION

89 Intimal hyperplasia (IH) remains the major cause of restenosis following vascular surgery,
90 leading to potential limb loss and death. IH develops in response to vessel injury, leading to
91 inflammation, vascular smooth muscle cells (SMCs) dedifferentiation, migration, proliferation and
92 secretion of extra-cellular matrix. Despite decades of research, there is no effective medication to
93 prevent restenosis¹. The only validated therapy against intimal hyperplasia is the local drug-delivery
94 strategy, especially used in endovascular approach. However, this strategy seems to be limited in
95 time², speaking for other complementary oral treatment, targeting either steps involved in IH, such
96 as SMC proliferation, or risk factors for restenosis such as hypertension.

97 Hydrogen Sulfide (H₂S) is an endogenously produced gasotransmitter³. Pre-clinical studies
98 have shown that H₂S has cardiovascular protective properties⁴, including reduction of IH⁵⁻⁷, possibly
99 via decreased VSMC proliferation^{6, 8}. However, there is currently no clinically approved H₂S donor⁹.

100 Hypertension is a known risk factor for restenosis and bypass failure¹⁰. Current guidelines
101 recommend angiotensin converting enzyme inhibitors (ACEi) as first line therapy for the treatment
102 of essential hypertension¹¹. Although various ACEi reduce restenosis in rodent models¹², prospective
103 clinical trials failed to prove efficacy of the ACEi Quinapril¹³ or Cilazapril^{14, 15} for the prevention of
104 restenosis at 6 months following coronary angioplasty. Several *in vitro* studies suggest that the ACEi
105 Zofenopril, due to a sulfhydryl moiety in its structure, releases H₂S¹⁶⁻¹⁸. The therapeutic potential of
106 sulfhydryl ACEi Zofenopril has never been tested in the context of restenosis.

107 The purpose of this study was to test whether Zofenopril, due to its H₂S-releasing properties,
108 is superior to non-sulfhydryl ACEi in limiting IH in a surgical mouse model of IH *in vivo*, and in an *ex*
109 *vivo* model of IH in human vein culture. Zofenopril was systematically compared to the non-
110 sulfhydrated ACEi Enalapril.

111

112 MATERIALS and METHODS

Materials

113 Drugs and reagents are described in supplemental table S1. Datasets are available at
114 <https://doi.org/10.5281/zenodo.5017874>

Experimental group design

115 All experiments were performed using 8 to 10 weeks old male Cx40-deleted mice (Cx40^{-/-})¹⁹
116 and wild-type littermates (WT) mice on a C57BL/6J genetic background. Mice randomly assigned to
117 the experimental groups were treated with the various ACEi at 10mg/Kg/day via the water bottle.

Blood pressure experiments

118 WT (n=22) or Cx40^{-/-} (n=18) mice were randomly divided into 3 groups: control, Enalapril and
119 Zofenopril. Basal systolic blood pressure was measured for 4 days then treatments were initiated
120 and SBP was measured for 10 more days. WT groups were done in parallel (n=22) with Cx40^{-/-} (n=6)
121 untreated mice. Cx40^{-/-} groups (n=18) were done in parallel with WT untreated mice (n=6).

Wild type mice (n=12) were randomly divided into 3 groups: control (n=4), Quinapril (n=4) and Lisinopril (n=4). Basal systolic blood pressure was measured for 4 days then treatments were initiated and SBP was measured for 10 more days.

122 Systolic blood pressure (SBP) was monitored daily by non-invasive plethysmography tail cuff
123 method (BP-2000, Visitech Systems Inc.) on conscious mice²⁰.

Mouse carotid artery stenosis model

WT mice (n=26) were divided into 3 groups: ctrl (n=9), Enalapril (n=9) and zofenopril (n=8). Cx40^{-/-} mice (n=24) were divided into 3 groups: ctrl (n=11), Enalapril (n=6) and zofenopril (n=7). Seven days post-treatment, intimal hyperplasia was induced via a carotid stenosis.

124 The carotid artery stenosis (CAS) was performed as previously published²¹. For surgery, mice
125 were anesthetized with Ketamine 80mg/kg and Xylazine 15 mg/kg. The left carotid artery was
126 located and separated from the jugular vein and vagus nerve. Then, a 7.0 PERMA silk (Johnson &
127 Johnson AG, Ethicon, Switzerland) thread was looped under the artery and tightened around the
128 carotid in presence of a 35-gauge needle. The needle was removed, thereby restoring blood flow,
129 albeit leaving a significant stenosis²¹. Buprenorphine 0.05 mg/kg was provided as post-operative
130 analgesic every 12h for 48 hours. Treatment with the ACEi of choice was continued for 28 days post-
131 surgery until organ collection. In another set of surgeries, WT mice (n=17) were randomly divided
132 into 3 groups: control (n=6), quinapril (n=6) and Lisinopril (n=5). Seven days post-treatment, intimal
133 hyperplasia was induced via a carotid stenosis.

134 All mice were euthanized 28 days post-surgery under general anesthesia by cervical
135 dislocation and exsanguination, perfused with PBS followed by buffered formalin 4% through the left
136 ventricle, and carotids were taken for IH measurements.

137 All animal experimentation conformed to the *National Research Council: Guide for the Care
138 and Use of Laboratory Animals*²². All animal care, surgery, and euthanasia procedures were
139 approved by the Centre Hospitalier Universitaire Vaudois (CHUV) and the Cantonal Veterinary Office
140 (Service de la Consommation et des Affaires Vétérinaires SCAV-EXPANIM, authorization number
141 3258).

Ex vivo static human vein culture and SMC culture

142 Human veins segments were discarded tissue obtained during lower limb bypass surgery.
143 Each native vein was cut into 7mm segments randomly distributed between conditions (D0, D7-Ctrl,
144 D7-enalaprilat or D7-zofenoprilat). One segment (D0) was immediately flash frozen in liquid nitrogen
145 or OCT compound and the other were maintained in culture for 7 days in RPMI-1640 Glutamax
146 supplemented with 10 % FBS and 1% antibiotic solution (10,000 U/mL penicillin G, 10,000 U/mL
147 streptomycin sulphate) at 37°C and 5% CO₂, as previously described⁶. The cell culture medium was
148 changed every 48 h with fresh ACEi. 6 different veins/patients were included in this study.

149 Human vascular smooth muscle cells (VSMC) were prepared and cultured from human
150 saphenous vein segments as previously described^{6,19}. The study protocols for organ collection and
151 use were reviewed and approved by the Centre Hospitalier Universitaire Vaudois (CHUV) and the
152 Cantonal Human Research Ethics Committee (<http://www.cer-vd.ch/>, no IRB number, Protocol
153 Number 170/02), and are in accordance with the principles outlined in the Declaration of Helsinki of

154 1975, as revised in 1983 for the use of human tissues. 6 different veins/patients were used in this
155 study to generate VSMC.

Histomorphometry

156 Left ligated carotids were isolated and paraffin-embedded. Six 6 μm cross sections were
157 collected every 100 μm and up to 2 mm from the ligature, and stained with Van Gieson Elastic
158 Lamina (VGEL) staining. For intimal and medial thickness, 72 (12 measurements/cross section on six
159 cross sections) measurements were performed¹⁹. To account for the gradient of IH in relation to the
160 distance from the ligature, the intima thickness was plotted against the distance to calculate the
161 area under the curve of intima thickness. Mean intima and media thickness over the 2mm distance
162 were calculated as well.

163 For human vein segments, after 7 days in culture, or immediately upon vein isolation (D0),
164 segments were fixed in buffered formalin, embedded in paraffin and cut into 6 μm sections, and
165 stained with VGEL as previously described⁶. For intimal and medial thickness, 96 (4
166 measurements/photos and 4 photos per cross section on six cross sections) measurements were
167 performed¹⁹. Two independent researchers blinded to the conditions did the morphometric
168 measurements using the Olympus Stream Start 2.3 software (Olympus, Switzerland)^{6, 19}.

Immunohistochemistry

169 PCNA (proliferating cell nuclear antigen) immunohistochemistry was performed on paraffin
170 sections as previously described⁶ after antigen retrieval using TRIS-EDTA buffer, pH 9, 17 min in a
171 microwave at 500 watts. Immunostaining was performed using the EnVision +/HRP, DAB+ system
172 according to manufacturer's instructions (Dako, Switzerland), and counterstained with hematoxylin.
173 One slide per series was assessed and 3 images per section were taken at 200x magnification. Two
174 independent observers unaware of the conditions manually counted the PCNA and hematoxylin
175 positive nuclei.

Live-cell hydrogen sulfide measurement

176 Free sulfide was measured in cells using 5 μM SF₇-AM fluorescent probe as previously
177 described⁶. Fluorescence intensity ($\lambda_{\text{ex}} = 495 \text{ nm}$; $\lambda_{\text{em}} = 520 \text{ nm}$) was measured continuously in a
178 Synergy Mx fluorescent plate reader (Biotek AG, Switzerland) at 37 °C before and after addition of
179 various compounds, as indicated.

Persulfidation protocol

180 Persulfidation protocol was performed using a dimedone-based probe as recently
181 described²³. Flash-frozen liver was grinded into powder and 20mg of powder was homogenized in
182 300 μl of HEN buffer (100mM HEPES, 1mM EDTA, 100 μM neocuproin, 1 vol. % NP-40, 1 wt. % SDS,
183 proteases inhibitors) supplemented with 5mM 4-Chloro-7-nitrobenzofurazan. Proteins were
184 extracted by methanol/chloroform/water protein precipitation and the pellet was resuspended in
185 200 μl of 50mM HEPES-2 wt. % SDS. Protein content was measured using Pierce BCA protein assay kit,
186 and 75 μg of proteins were incubated with 25 μM final Daz-2-biotin for 1h in the dark at 37°C. Daz-2-
187 biotin was prepared with 1mM Daz-2, 1mM alkynyl biotin, 2mM copper(II)-TBTA, 4mM ascorbic acid
188 with overnight incubation at RT, followed by quenching with 20mM EDTA. Proteins were then
189 extracted by Methanol/Chloroform/Water protein precipitation and the pellets resuspended in

190 150µl SDS lysis buffer. Protein concentration was measured using the DC protein assay, 10 µg were
191 loaded on SDS-PAGE and the Biotin signal was measured by WB analyses using a streptavidin-HRP
192 antibody. Protein abundance was normalized to total protein staining using Pierce™ Reversible
193 Protein Stain Kit for PVDF Membranes.

BrdU staining (VSMC proliferation)

194 VSMC were grown at 80% confluence on glass coverslips in a 24-well plate and starved
195 overnight in serum-free medium. Then, VSMC were either treated or not (ctrl) with the ACEi of
196 choice for 24h in full medium (RPMI 10% FBS) in presence of 10µM BrdU. All conditions were tested
197 in parallel. All cells were fixed in ice-cold methanol 100% after 24h of incubation and immunostained
198 for BrdU. Images were acquired using a Nikon Eclipse 90i microscope. BrdU-positive nuclei and total
199 DAPI-positive nuclei were automatically detected using the ImageJ software⁶.

Wound healing assay (VSMC migration)

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

Western blotting

206 Human vein segments were washed twice in ice-cold PBS, flash-frozen in liquid nitrogen,
207 grinded to power and resuspended in SDS lysis buffer (62.5 mM TRIS pH6.8, 5% SDS, 10 mM EDTA).

208 Vascular smooth muscle cells were kept in serum free media overnight. The next morning,
209 complete media was added with the ACEinhibitors. Five hours post-treatment, cells were washed
210 once with ice-cold PBS and directly lysed with Laemmli buffer. Lysates were resolved by SDS-PAGE
211 and transferred to a PVDF membrane (Immobilon-P, Millipore AG, Switzerland). Immunoblot
212 analyses were performed as previously described⁶ using the antibodies described in the
213 **Supplemental Table S1**. Blots were revealed by enhanced chemiluminescence (Immobilon, Millipore)
214 using the ChemiDoc™ XRS+ System and analysed using the Image Lab (BETA2) software, version
215 3.0.01 (Bio-Rad Laboratories, Switzerland).

Statistical analyses

216 All experiments were quantitatively analysed using GraphPad Prism® 8, and results are
217 shown as mean ± SEM. Statistical test details are indicated in the Figure legends.

218

219 RESULTS

Zofenopril and enalapril similarly lower systolic blood pressure of hypertensive mice

220 Spontaneously hypertensive Cx40 deleted mice (Cx40^{-/-}) and wild-type littermates (WT) were
221 given either 10 mg/kg Zofenopril or 6 mg/kg Enalapril in the drinking water to achieve similar blood
222 lowering effects on hypertensive Cx40^{-/-} mice (**Figure 1A**). Zofenopril also lowered by 6mmHg SBP in

223 normotensive WT mice (**Figure 1B**). The ACEi Enalapril (**Figure 1B**), Quinapril (10 mg/kg) and
224 Lisinopril (10 mg/kg) had no effect on SBP in WT mice (**Figure S1**).

Zofenopril is superior to other ACEi in reducing IH in a mouse model of carotid artery stenosis

225 As expected, the hypertensive mice developed twice more IH than their normotensive
226 littermates following the carotid artery stenosis (CAS) model²¹. Enalapril had a non-specific tendency
227 to reduce IH in Cx40^{-/-} mice (I/M p>.99), while Zofenopril suppressed IH by 90% (I/M p=.0006; **Figure**
228 **2, table S2**). Enalapril had no effect in normotensive WT mice (I/M p>.99), whereas Zofenopril also
229 suppressed IH in those mice (I/M p=.008; **Figure 2, table S3**). The ACEi Quinapril and Lisinopril did
230 not affect IH in WT mice (**Figure S2, table S4**).

Zofenoprilat prevented the development of IH in human saphenous vein segments

231 We next tested the effect of Zofenoprilat and Enalaprilat, the active compounds derived
232 from pro-drugs Zofenopril and Enalapril, in our model of IH in *ex vivo* static vein culture⁶. Continuous
233 treatment with 100 μ M Zofenoprilat, but not with Enalaprilat, fully blocked the development of IH
234 observed in veins maintained for 7 days in culture in absence of blood flow (D7), compared to initial
235 values in freshly isolated veins (D0) (**Figure 3, table S5**).

Zofenoprilat released H₂S.

236 Besides its ACEi activity, Zofenopril has been proposed to work as an H₂S donor¹⁶⁻¹⁸. *In vitro*
237 time-lapse recording of the H₂S-selective probe SF₇-AM revealed that Zofenoprilat, but not
238 Enalaprilat, slowly released H₂S in RPMI medium, compared to the fast-releasing NaHS salt (**Figure**
239 **4A**). Similar experiments in presence of live VSMC (**Figure 4B**) confirmed that Zofenoprilat, but not
240 Enalaprilat, increased the SF₇-AM signal.

241 The biological activity of H₂S is mediated by post-translational modification of reactive
242 cysteine residues by persulfidation, which modulates protein structure and/or function^{9, 23}. We
243 assessed protein persulfidation using a dimedone-based probe as recently described²³. Zofenopril
244 significantly increased protein persulfidation (PSSH) in liver extracts from mice treated with Enalapril
245 or Zofenopril for two weeks (**Figure 4C**).

Zofenopril decreased VSMC proliferation and migration

246 As various H₂S donors decrease VSMC proliferation in the context of IH^{6, 8}, we next tested
247 the effect of Zofenopril on VSMC. In the CAS model, Zofenopril, but not Enalapril, lowered cell
248 proliferation in the carotid wall as assessed by PCNA staining (**Figure 5A-B**). Zofenoprilat further
249 inhibited the proliferation and migration of primary human VSMC *in vitro*, while Enalaprilat had no
250 effect on proliferation (**Figure 5C-D**) and reduced migration by 20% (**Figure 5E-F**). ACEi Lisinopril and
251 Quinaprilat did not affect VSMC proliferation (**Figure S3**).

Zofenoprilat inhibited the MAPK and mTOR pathways

252 The MAPK and mTOR signalling pathways contribute to VSMC proliferation in the context of
253 IH²⁴. Western blot analyses revealed that Zofenoprilat reduced by 50% the levels of P-ERK1,2, P-p38
254 and P-S6RP in cultured VSMC, while Enalaprilat had no effect (**Figure 6A-F**). Moreover, P-S6RP and P-
255 ERK1,2 levels were also decreased by Zofenoprilat in human vein segments placed in culture for 7
256 days (**Figure 6G-I**).

257

258 DISCUSSION

259 In this study, we hypothesized that Zofenopril, an ACEi with a free thiol moiety acting as an
260 H₂S donor, would be more efficient than other ACEi to inhibit IH in the context of hypertension. Not
261 only Zofenopril is more potent than Enalapril in reducing IH in hypertensive Cx40^{-/-} mice, it also
262 suppresses IH in normotensive condition, where other ACEi have no effect. Furthermore, Zofenopril
263 prevents IH in human saphenous vein segments in absence of blood flow. The effect of Zofenopril on
264 IH correlates with reduced VSMC proliferation and migration and decreased activity of the MAPK
265 and mTOR pathways.

266 Several pre-clinical studies have shown that that SBP-lowering medication such as ACEi
267 reduce IH¹², which prompt the large-scale MERCATOR/MARCATOR^{14, 15} and PARIS clinical trials¹³.
268 Here, we also observed that lowering SBP using the ACEi Enalapril had a non-significant tendency to
269 protect from IH in hypertensive mice. However, Enalapril, Quinapril and Lisinopril, had no effect in
270 normotensive WT mice. The fact that the sulhydrated ACEi Zofenopril almost abrogated IH in
271 hypertensive and normotensive mice strongly supports that this ACEi provides additional effects
272 independent of its ACEi activity, as previously suggested¹⁶⁻¹⁸. Of interest, the SMILE clinical trials
273 concluded that, compared to placebo or Ramipril, Zofenopril reduces the 1-year risk of
274 cardiovascular events after acute myocardial infarction²⁵. These benefits might be related to H₂S
275 release by Zofenopril, as pre-clinical studies consistently show that H₂S supplementation promote
276 recovery after acute myocardial infarction⁴.

277 Zofenopril has been proposed to work as a H₂S donor in several studies¹⁶⁻¹⁸. Here, we
278 confirmed that Zofenoprilat releases detectable amounts of H₂S. H₂S modifies proteins by post-
279 translational persulfidation (S-sulfhydration) of reactive cysteine residues, which modulate protein
280 structure and/or function²³. Here, we further observed that Zofenopril increases overall protein
281 persulfidation *in vivo*, suggesting that Zofenopril generates H₂S *in vivo* as well.

282 We and others previously demonstrated that various H₂S donors inhibit VSMC proliferation⁶,
283 ^{8, 26}. Consistently, we confirmed that Zofenopril inhibits VSMC proliferation and migration *in vitro*
284 and reduces cell proliferation in the carotid wall *in vivo*. Although the exact mechanisms of action of
285 Zofenoprilat and H₂S remain to be elucidated, we demonstrated that Zofenoprilat inhibits the MAPK
286 and mTOR signalling pathways, which contribute to VSMC proliferation and neointima formation²⁴.
287 Overall, our data strongly suggest that Zofenopril acts similarly to other known H₂S donors to limit IH
288 through inhibition of the MAPK and mTOR signalling pathways, leading to decreased VSMC
289 proliferation and migration.

290 Overall, our data suggests that Zofenopril might show benefits against restenosis in patients
291 unlike other ACEi. These findings raise the question as to whether the scientific community was not
292 too quick to discard the whole class of ACEi as a treatment of restenosis based on the disappointing
293 results of the MERCATOR/MARCATOR^{14, 15} and PARIS trials¹³. In the last decade, many efforts have
294 been put on the development of local drug delivery strategy, well adapted to endovascular
295 interventions. However, this strategy seems to bring great improvement in the mid-term but not in
296 the long-term follow-up². Thus, a more chronic approach, sustaining the early effect on cell

297 proliferation and IH inhibition, should be encouraged. Such a strategy relies on oral medication,
298 which is also better adapted to open surgery.

299 Nevertheless, our study carries some limitations.

300 Firstly, numerous oral drugs have been clinically tested over the years to limit restenosis and,
301 in most trials, the pharmacologic treatment of restenosis failed to show positive results, despite
302 promising results obtained in experimental models²⁷. While there is no doubt that pre-clinical
303 models have significantly advanced our understanding of the mechanisms of restenosis formation,
304 none of them fully mimic restenosis in human. The genetic model of renin-dependent hypertension
305 used in that study is also rarely observed in patients, which have complex multifactorial essential
306 hypertension. Additional studies reflecting better the patients' comorbidities (dyslipidemia, renal
307 insufficiency, smoking, atherosclerosis, etc..) with a vein bypass model and larger animal models, or
308 small phase II clinical trial, are required before testing the benefits of Zofenopril in a large, phase III
309 clinical trials.

310 Secondly, although Zofenopril was the only ACEi providing benefits in normotensive
311 condition, we cannot exclude that other ACEi not tested here could work as well. We further
312 acknowledge that pharmacokinetics and pharmacodynamics differences between Zofenopril and
313 other ACEi may contribute to the superiority of Zofenopril. Zofenopril is more lipophilic and may
314 have better tissue penetration than Enalapril or Ramipril, which may have an impact beyond the
315 effect of H₂S liberated by Zofenopril. However, it has been shown that vessel wall penetration of
316 various ACEi is independent of lipophilia and that the endothelium constitutes no specific barrier for
317 the passage of ACE inhibitors²⁸.

318 Finally, our working hypothesis is that Zofenopril inhibits VSMC proliferation via direct
319 release of H₂S at the level of the media of vessel. However, we could not ascertain that H₂S is
320 released at the level of the VSMC. H₂S⁹ and Zofenoprilat^{17, 18} have been shown to promote
321 endothelial cell function, including proliferation and migration. Thus, we cannot exclude that
322 Zofenopril limits IH via a positive effect on endothelial cells. Further studies are required to carefully
323 assess the impact of Zofenopril on the endothelium and quantify H₂S in vascular tissue.

324 **Conclusion**

325 Under the conditions of these experiments, Zofenopril is superior to Enalapril in reducing IH
326 and provides beneficial effect against IH in mice and in a model of IH in human vein segments *ex vivo*.
327 Our data strongly support that Zofenopril limits the development of IH via H₂S release,
328 independently of its ACEi activity. The effects of Zofenopril correlate with reduced MAPK and mTOR
329 pathways activities, leading to decreased VSMC proliferation and migration.

330 Given the number of patients treated with ACEi worldwide, these findings may have broad
331 implications for the treatment of patients suffering from peripheral atherosclerotic disease
332 undergoing revascularization, and beyond. Our results warrant further research to evaluate the
333 benefits of Zofenopril in limiting restenosis and eventually prospective clinical trials to test the
334 superiority of sulfhydrated ACEi on restenosis over other ACEi.

335

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346

347 **Disclosures**

348 None

349

350 **REFERENCES**

351

1. Davies MG, Hagen PO. Reprinted article "Pathophysiology of vein graft failure: a review". *Eur J Vasc Endovasc Surg*. 2011;42 Suppl 1:S19-29. DOI: 10.1016/j.ejvs.2011.06.013.
2. Phillips J. Drug-eluting stents for PAD: what does (all) the data tell us? *J Cardiovasc Surg (Torino)*. 2019;60(4):433-8. DOI: 10.23736/S0021-9509.19.10965-2.
3. Islam KN, Polhemus DJ, Donnarumma E, Brewster LP, Lefer DJ. Hydrogen Sulfide Levels and Nuclear Factor-Erythroid 2-Related Factor 2 (NRF2) Activity Are Attenuated in the Setting of Critical Limb Ischemia (CLI). *J Am Heart Assoc*. 2015;4(5). DOI: 10.1161/JAHA.115.001986.
4. Zhang L, Wang Y, Li Y, Li L, Xu S, Feng X, et al. Hydrogen Sulfide (H₂S)-Releasing Compounds: Therapeutic Potential in Cardiovascular Diseases. *Front Pharmacol*. 2018;9:1066. DOI: 10.3389/fphar.2018.01066.
5. Ma B, Liang G, Zhang F, Chen Y, Zhang H. Effect of hydrogen sulfide on restenosis of peripheral arteries after angioplasty. *Mol Med Rep*. 2012;5(6):1497-502. DOI: 10.3892/mmr.2012.853.
6. Longchamp A, Kaur K, Macabrey D, Dubuis C, Corpataux JM, Deglise S, et al. Hydrogen sulfide-releasing peptide hydrogel limits the development of intimal hyperplasia in human vein segments. *Acta Biomater*. 2019. DOI: 10.1016/j.actbio.2019.07.042.
7. Yang G, Li H, Tang G, Wu L, Zhao K, Cao Q, et al. Increased neointimal formation in cystathionine gamma-lyase deficient mice: role of hydrogen sulfide in alpha5beta1-integrin and matrix metalloproteinase-2 expression in smooth muscle cells. *J Mol Cell Cardiol*. 2012;52(3):677-88. DOI: 10.1016/j.yjmcc.2011.12.004.
8. Yang G, Wu L, Bryan S, Khaper N, Mani S, Wang R. Cystathionine gamma-lyase deficiency and overproliferation of smooth muscle cells. *Cardiovasc Res*. 2010;86(3):487-95. DOI: 10.1093/cvr/cvp420.
9. Li Z, Polhemus DJ, Lefer DJ. Evolution of Hydrogen Sulfide Therapeutics to Treat Cardiovascular Disease. *Circ Res*. 2018;123(5):590-600. DOI: 10.1161/CIRCRESAHA.118.311134.
10. Venermo M, Sprynger M, Desormais I, Bjorck M, Brodmann M, Cohnert T, et al. Editor's Choice - Follow-up of Patients After Revascularisation for Peripheral Arterial Diseases: A Consensus Document From the European Society of Cardiology Working Group on Aorta and Peripheral Vascular Diseases and the European Society for Vascular Surgery. *Eur J Vasc Endovasc Surg*. 2019;58(5):641-53. DOI: 10.1016/j.ejvs.2019.06.017.
11. Flu HC, Tamsma JT, Lindeman JH, Hamming JF, Lardenoye JH. A systematic review of implementation of established recommended secondary prevention measures in patients with PAOD. *Eur J Vasc Endovasc Surg*. 2010;39(1):70-86. DOI: 10.1016/j.ejvs.2009.09.027.
12. Osgood MJ, Harrison DG, Sexton KW, Hocking KM, Voskresensky IV, Komalavilas P, et al. Role of the renin-angiotensin system in the pathogenesis of intimal hyperplasia: therapeutic potential for prevention of vein graft failure? *Ann Vasc Surg*. 2012;26(8):1130-44. DOI: 10.1016/j.avsg.2011.12.001.

13. Meurice T, Bauters C, Hermant X, Codron V, VanBelle E, Mc Fadden EP, et al. Effect of ACE inhibitors on angiographic restenosis after coronary stenting (PARIS): a randomised, double-blind, placebo-controlled trial. *Lancet*. 2001;357(9265):1321-4. DOI: 10.1016/S0140-6736(00)04518-9.
14. Does the new angiotensin converting enzyme inhibitor cilazapril prevent restenosis after percutaneous transluminal coronary angioplasty? Results of the MERCATOR study: a multicenter, randomized, double-blind placebo-controlled trial. Multicenter European Research Trial with Cilazapril after Angioplasty to Prevent Transluminal Coronary Obstruction and Restenosis (MERCATOR) Study Group. *Circulation*. 1992;86(1):100-10. DOI: 10.1161/01.cir.86.1.100.
15. Faxon DP. Effect of high dose angiotensin-converting enzyme inhibition on restenosis: final results of the MARCATOR Study, a multicenter, double-blind, placebo-controlled trial of cilazapril. The Multicenter American Research Trial With Cilazapril After Angioplasty to Prevent Transluminal Coronary Obstruction and Restenosis (MARCATOR) Study Group. *J Am Coll Cardiol*. 1995;25(2):362-9. DOI: 10.1016/0735-1097(94)00368-z.
16. Bucci M, Vellecco V, Cantalupo A, Brancaleone V, Zhou Z, Evangelista S, et al. Hydrogen sulfide accounts for the peripheral vascular effects of zofenopril independently of ACE inhibition. *Cardiovasc Res*. 2014;102(1):138-47. DOI: 10.1093/cvr/cvu026.
17. Monti M, Terzuoli E, Ziche M, Morbidelli L. H₂S dependent and independent anti-inflammatory activity of zofenoprilat in cells of the vascular wall. *Pharmacol Res*. 2016;113(Pt A):426-37. DOI: 10.1016/j.phrs.2016.09.017.
18. Terzuoli E, Monti M, Vellecco V, Bucci M, Cirino G, Ziche M, et al. Characterization of zofenoprilat as an inducer of functional angiogenesis through increased H₂S availability. *Br J Pharmacol*. 2015;172(12):2961-73. DOI: 10.1111/bph.13101.
19. Allagnat F, Haefliger JA, Lambelet M, Longchamp A, Berard X, Mazzolai L, et al. Nitric Oxide Deficit Drives Intimal Hyperplasia in Mouse Models of Hypertension. *Eur J Vasc Endovasc Surg*. 2016;51(5):733-42. DOI: 10.1016/j.ejvs.2016.01.024.
20. Le Gal L, Alonso F, Wagner C, Germain S, Nardelli Haefliger D, Meda P, et al. Restoration of connexin 40 (Cx40) in Renin-producing cells reduces the hypertension of Cx40 null mice. *Hypertension*. 2014;63(6):1198-204. DOI: 10.1161/HYPERTENSIONAHA.113.02976.
21. Tao M, Mauro CR, Yu P, Favreau JT, Nguyen B, Gaudette GR, et al. A simplified murine intimal hyperplasia model founded on a focal carotid stenosis. *Am J Pathol*. 2013;182(1):277-87. DOI: 10.1016/j.ajpath.2012.10.002.
22. National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals., Institute for Laboratory Animal Research (U.S.), National Academies Press (U.S.). Guide for the care and use of laboratory animals. 8th ed. Washington, D.C.: National Academies Press; 2011. xxv, 220 p. p.
23. Zivanovic J, Kouroussis E, Kohl JB, Adhikari B, Bursac B, Schott-Roux S, et al. Selective Persulfide Detection Reveals Evolutionarily Conserved Antiaging Effects of S-Sulfhydration. *Cell Metab*. 2019;30(6):1152-70 e13. DOI: 10.1016/j.cmet.2019.10.007.
24. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*. 2004;84(3):767-801. DOI: 10.1152/physrev.00041.2003.

25. Borghi C, Omboni S, Novo S, Vinereanu D, Ambrosio G, Ambrosioni E. Efficacy and Safety of Zofenopril Versus Ramipril in the Treatment of Myocardial Infarction and Heart Failure: A Review of the Published and Unpublished Data of the Randomized Double-Blind SMILE-4 Study. *Adv Ther.* 2018;35(5):604-18. DOI: 10.1007/s12325-018-0697-x.
26. Wang Y, Wang X, Liang X, Wu J, Dong S, Li H, et al. Inhibition of hydrogen sulfide on the proliferation of vascular smooth muscle cells involved in the modulation of calcium sensing receptor in high homocysteine. *Exp Cell Res.* 2016;347(1):184-91. DOI: 10.1016/j.yexcr.2016.08.004.
27. Seedial SM, Ghosh S, Saunders RS, Suwanabol PA, Shi X, Liu B, et al. Local drug delivery to prevent restenosis. *J Vasc Surg.* 2013;57(5):1403-14. DOI: 10.1016/j.jvs.2012.12.069.
28. Raasch W, Dendorfer A, Ball B, Dominiak P. The lipophilic properties of angiotensin I-converting enzyme inhibitors do not influence their diffusion through cultured endothelium. *Jpn J Pharmacol.* 1999;81(4):346-52. DOI: 10.1254/jjp.81.346.

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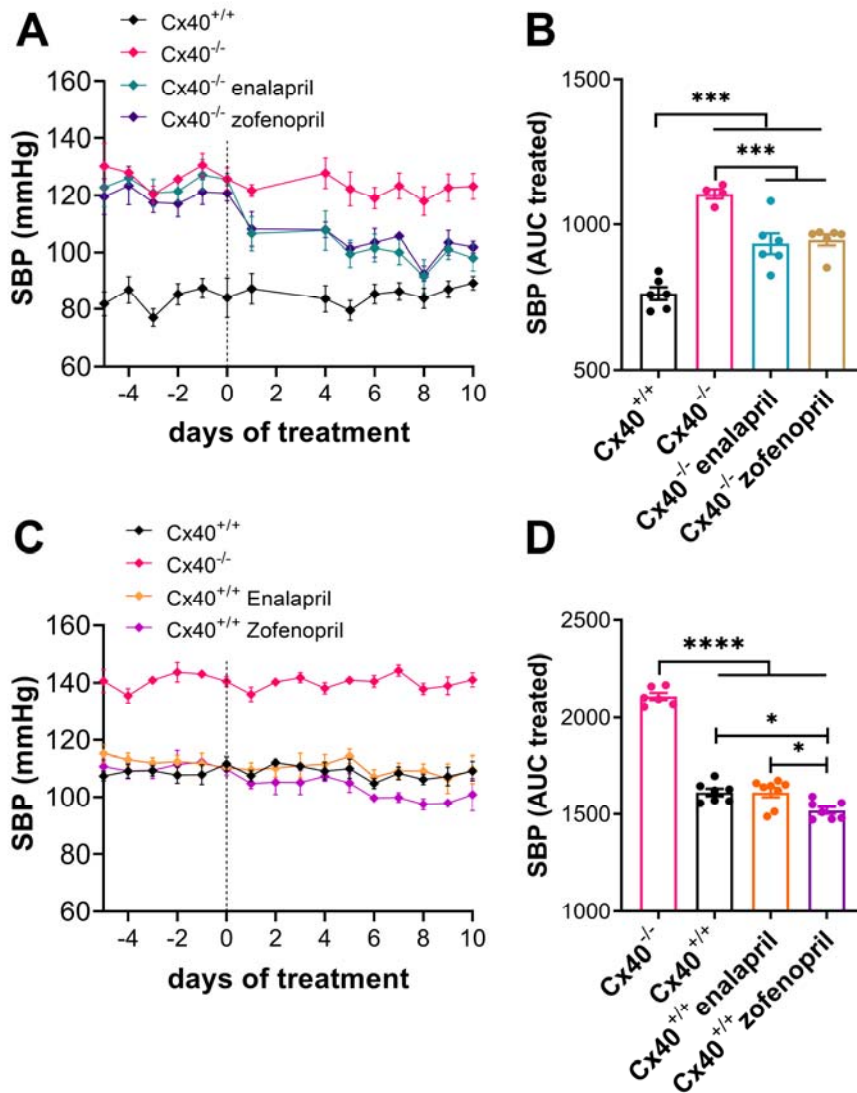


Figure 1: Zofenopril and Enalapril similarly lower systolic blood pressure in hypertensive Cx40^{-/-} mice

A) Daily systolic blood pressure (SBP) values (mean±SEM) in WT (n=6) vs. Cx40^{-/-} mice treated or not (n=5) with 10mg/kg Zofenopril (n=6) and 10mg/kg Enalapril (n=6) for the indicated time. **B)** Area under the curve (AUC) of SBP from day 0 to 10. **C)** Daily systolic blood pressure 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)** Area under the curve (AUC) of SBP between day 0 to 10. *p<.05; **p<.01; ***p<.001 as indicated from one-way ANOVA with Tukey's correction of multiple comparisons.

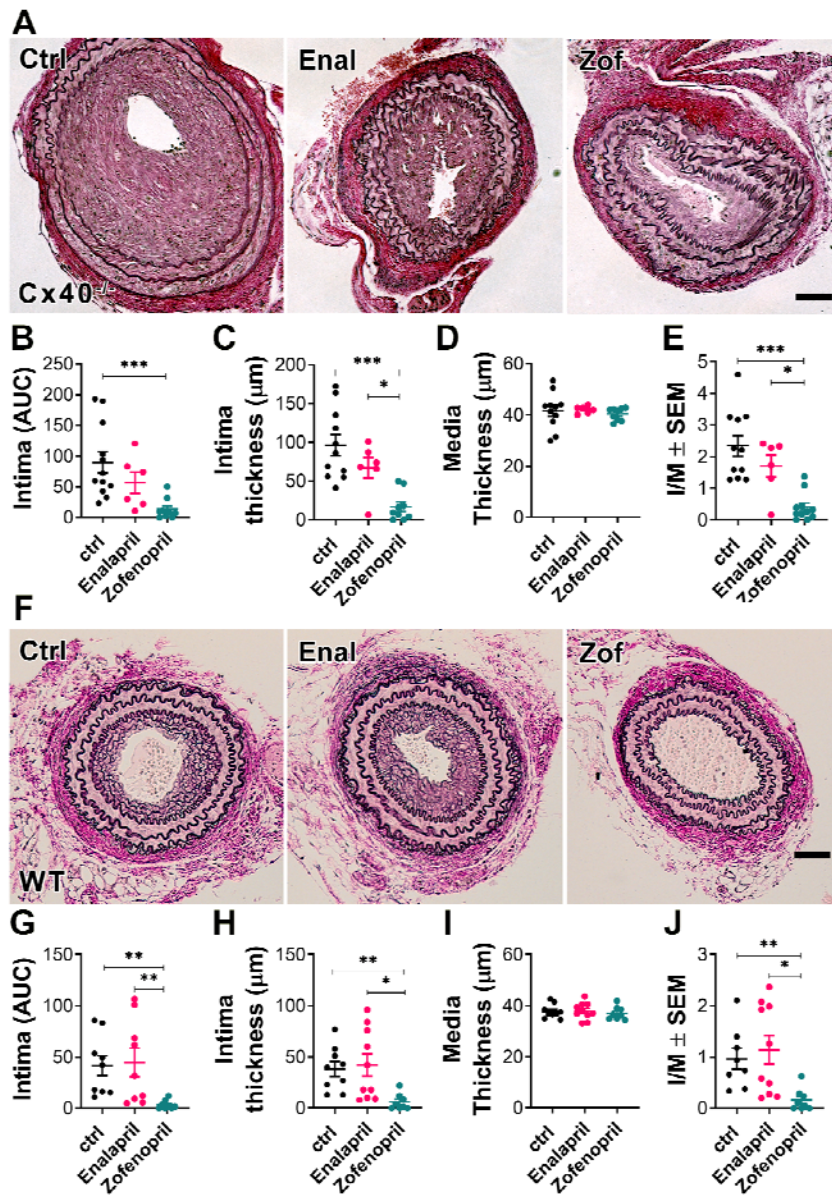


Figure 2. Zofenopril treatment reduces IH in a mouse model of carotid artery stenosis

Cx40^{-/-} (A-E) or WT mice (F-J), treated or not (Ctrl) with Zofenopril (Zof) and Enalapril (Enal), were submitted to carotid artery stenosis. **A, F**) Representative images of left carotid cross sections stained with VGEL 28 days post-surgery in Cx40^{-/-} (A) or WT (F) mice. Scale bar represents 40 µm. **B-E, G-I**) Morphometric measurements of area under the curve (AUC) of intima thickness (**B, G**) intima thickness (**C, H**), media thickness (**D, I**) and intima over media ratio (**E, J**). Data are presented as scatter plots of 9 to 12 animals per group, with mean±SEM. *p<.05; **p<.01; ***p<.001 as indicated from Kruskal-Wallis test followed by Dunn's multiple comparisons tests.

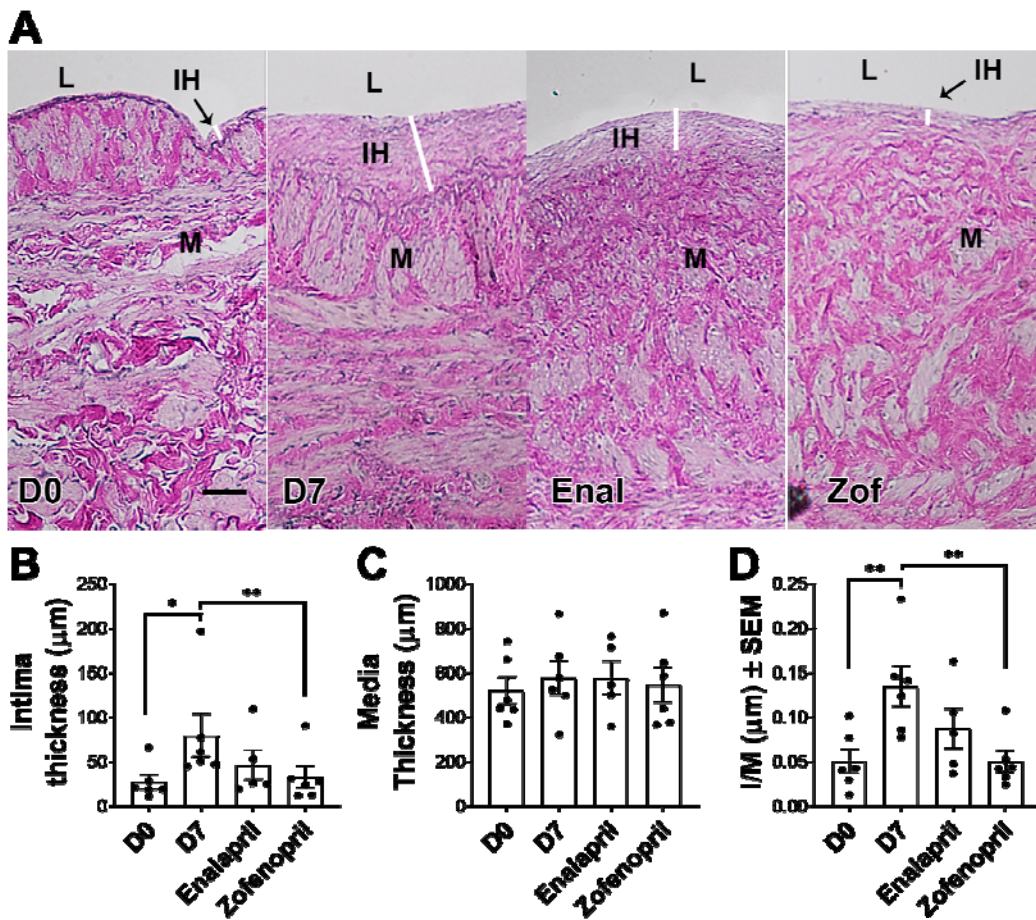


Figure 3: Ex vivo treatment with Zofenoprilat prevents the development of IH in human saphenous vein segments

Human great saphenous vein segments obtained from donors who underwent lower limb bypass surgery were put or not (D0, n=6) in static culture for 7 days in presence or not (D7, n=6) of 100 μM of Zofenoprilat (n=6) or Enalaprilat (n=5). **A**) Representative VGEL staining. Scale bar= 50 μm . **B-D**) Morphometric measurements of intima thickness (**B**), media thickness (**C**) and intima over media ratio (**D**). Data are presented as scatter plots of 6 different vein/patient with mean \pm SEM. *p<.05; **p<.01 as indicated from repeated measures one-way ANOVA with post-hoc t-test with Dunnet's correction of multiple comparisons.

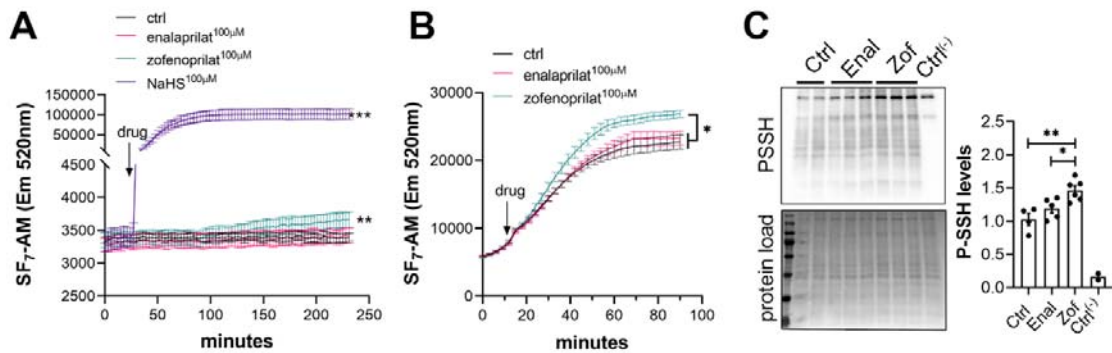


Figure 4. Zofenoprilat release H₂S as measured by SF₇-AM fluorescent probe

A-B) SF₇-AM fluorescent signal (mean ± SEM) in a cell free assay in RPMI medium (**A**), in live primary VSMC (**B**) exposed or not (Ctrl) to 100 μM NaHS, 100 μM Zofenoprilat or 100 μM Enalaprilat for the indicated time. *p<.05; **p<.01; ***p<.001 vs. respective ctrl as determined by repeated measures two-way ANOVA with post-hoc t-test with Tukey's correction for multiple comparisons. Data are representative of 3 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 to 6 animals/group with mean±SEM with *p<.05, **p<.01, as determined by one-way ANOVA with post-hoc t-test with Tukey's correction for multiple comparisons.

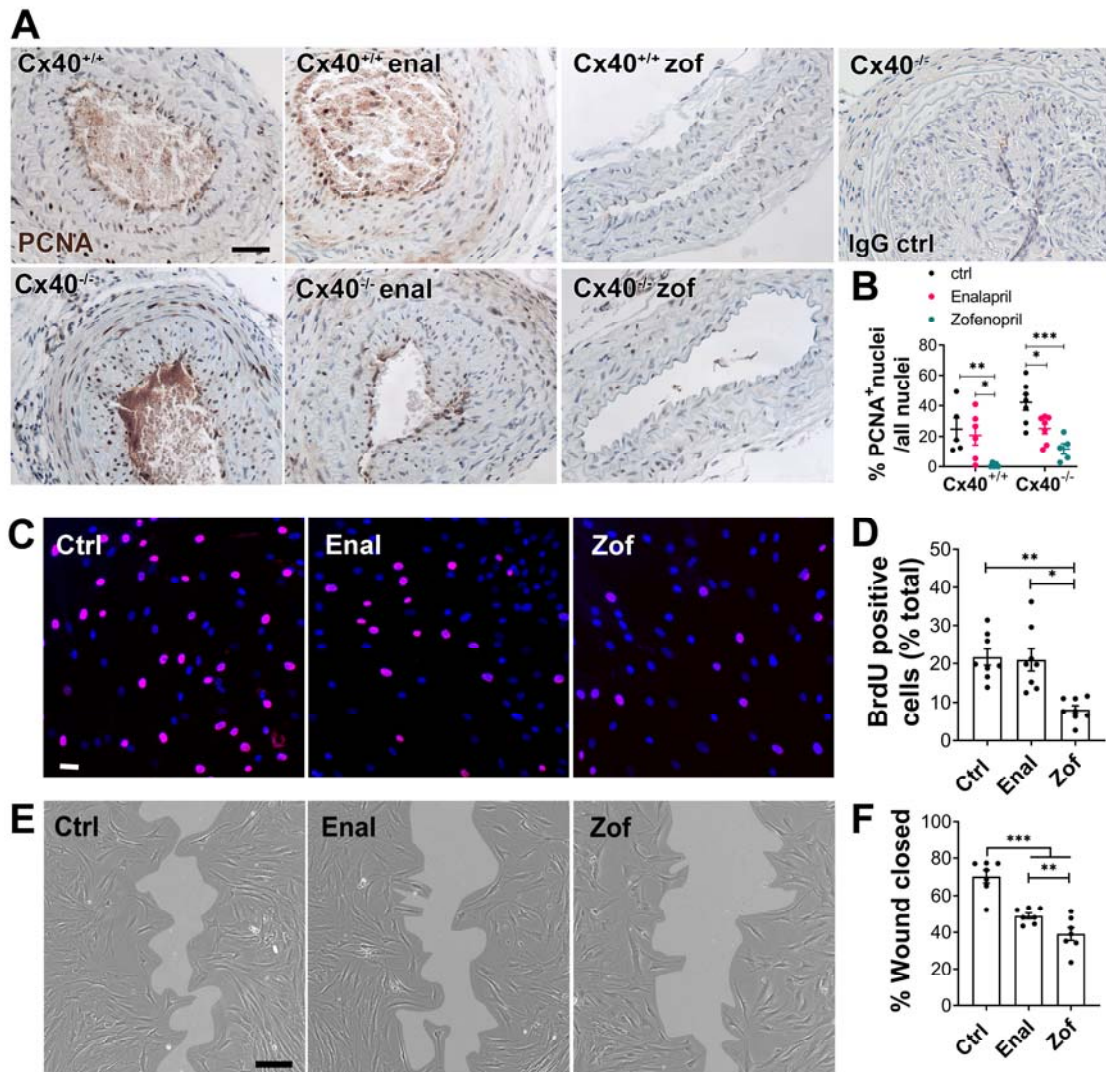


Figure 5. Zofenopril treatment reduces cell proliferation in a mouse model of carotid artery stenosis

A-B) PCNA immunostaining 28 days post carotid artery stenosis in 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 μ m. **B)** Quantitative assessment of PCNA positive cells over total cells of 5 to 8 animals/group with mean \pm SEM. *p<.05; **p<.01; ***p<.001, as determined by two-way ANOVA with post-hoc t-test with Sidak's correction of multiple comparisons. **C-D)** Primary human vascular smooth muscle cells (VSMC) were exposed or not (Ctrl) to 100 μ M Zofenoprilat or Enalaprilat for 24 h in presence of BrdU. **C)** Representative images of BrdU-positive nuclei (pink) and DAPI-stained nuclei (blue). Bar scale represents 10 μ m. **D)** Proliferation was calculated as the percentage of BrdU-positive nuclei over total nuclei. Data are scatter plots of 8 independent experiments with mean \pm SEM with *p<.05, **p<.01, as determined by repeated measures one-way ANOVA with post-hoc t-test with Dunnett's correction for multiple comparisons. **E-F)** Wound healing assay with VSMC exposed or not (Ctrl) to

100 μ M Zofenoprilat or Enalaprilat. E) Representative images of VSMC in brightfield 10 hours post wound. Scale bar represents 50 μ m. F) Data are scatter plots of 7 independent experiments with mean \pm SEM of wound area after 10h, expressed as a percentage of the initial wound area. ** p <.01, *** p <.001, as determined by repeated measures one-way ANOVA with post-hoc t-test with Dunnet's correction for multiple comparisons.

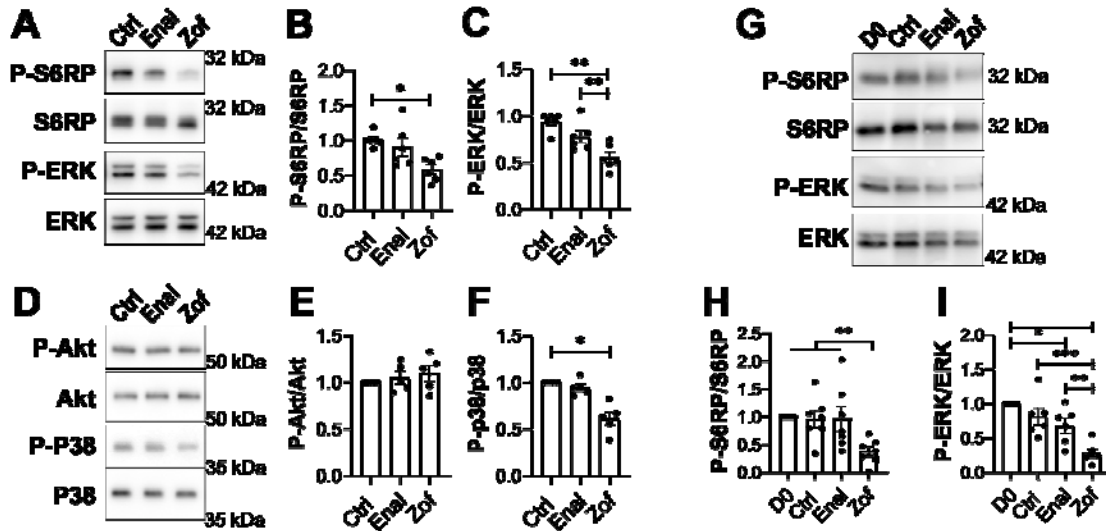


Figure 6: Zofenoprilat inhibits ERK and S6RP phosphorylation

A-F) Western Blot analyses from VSMC exposed or not (Ctrl) to 100 μ M Zofenoprilat or Enalaprilat for 5h. 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 6 independent experiments, normalized to their respective ctrl condition, with mean \pm SEM. * p <.05, ** p <.01, as determined by repeated measures one-way 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 μ M Zofenoprilat or Enalaprilat for 7 days. G) Representative Western Blot for P-S6RP and total S6RP, P-ERK and total ERK. H-I) Quantitative assessment of 7 different veins, normalized to their respective ctrl condition, with mean \pm SEM. * p <.05, ** p <.01, *** p <.001, as determined by repeated measures one-way ANOVA with post-hoc t-test with Dunnet's correction for multiple comparisons.