

Hydrogen sulfide accounts for the peripheral vascular effects of zofenopril independently of ACE inhibition

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Aims Therapeutic use of sulfhydrylated inhibitor S-zofenopril has raised different hypotheses regarding the role played by its thiol group in the beneficial clinical effects exerted compared with other angiotensin-converting enzyme (ACE) inhibitors. Here, we investigated hydrogen sulfide (H₂S) pathway as accountable for extra-beneficial effects in vascular function.

Methods and results Spontaneously hypertensive rat (SHRs) and control Wistar Kyoto (WKY) rats were treated with either S-zofenopril or enalapril *in vivo*. Aorta and carotid were harvested and *ex vivo* vascular reactivity to acetylcholine (ACh) and L-cysteine (L-cys) assessed. Cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptosulfur-transferase (3MST) expression, as well as H₂S levels, were evaluated in both vascular tissues. The vascular response to ACh in both carotid and aorta was impaired in SHR (~30%, *P* < 0.001). S-zofenopril, but not enalapril, restored this response, while L-cys-induced relaxation was enhanced. CSE expression in vessels and tissue/plasma H₂S levels were restored to WKY values in SHRs receiving S-zofenopril. In contrast, CBS and 3MST expression were not modified by treatments. S-zofenoprilat, an active metabolite of S-zofenopril, releases H₂S in a 'cell-free' assay and it directly relaxed vessels *in vitro* in a concentration-dependent manner (*P* < 0.001). *In vivo* administration of R-zofenoprilat diastereoisomer, which does not inhibit ACE, did not modify blood pressure; nonetheless, it retained the beneficial effect on SHR vascular function as well as restored plasma/tissue H₂S levels.

Conclusion Our findings establish that S-zofenopril improves vascular function by potentiating the H₂S pathway in a model of spontaneous hypertension. This novel mechanism, unrelated to ACE inhibition and based on H₂S release, could explain the beneficial effects of sulfhydrylated ACE inhibitors reported in the clinical literature.

Keywords Hydrogen sulfide • ACE inhibitors • Hypertension

1. Introduction

Angiotensin-converting enzyme (ACE) activation, with consequent release of angiotensin II, is not only involved in controlling blood pressure, but also contributes to cardiovascular disease by directly acting on tissues, including remodelling and inflammation.¹ Tissue ACE represents an important therapeutic target and its inhibition can restore endothelial function and/or prevent endothelial dysfunction.² Indeed, though ACE can be isolated from plasma, it is also located on the endothelium,

where it is anchored to the plasma membrane and is referred to as tissue ACE.³ ACE inhibitors (ACEis) are usually classified into three main groups: sulfhydryl-, dicarboxylate-, and phosphonate-containing agents.^{4,5} Within the class of sulfhydryl-containing agents, there are only two drugs, captopril and zofenopril. Over the last decade, several clinical papers have shown that sulfhydrylated ACEi zofenopril has additional beneficial actions compared with dicarboxylate-containing agents. For instance, zofenopril improves the clinical outcomes of patients with different cardiovascular diseases, including acute myocardial infarction and

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congestive heart failure.^{6–8} Furthermore, the beneficial effects of zofenopril in endothelial dysfunction following heart failure were more evident compared with the non-sulfhydrylated ACEi lisinopril.⁹ Interestingly, in a clinical comparative study, it has been demonstrated that zofenopril, but not ramipril or atenolol, improves flow-mediated dilation.¹⁰ However, the antioxidant actions of sulfhydrylated ACEi, often ascribed to the presence of a thiol group,¹¹ *per se*, does not provide a full explanation for the additional beneficial effects associated with its use in therapy.¹² It is important to bear in mind that zofenopril is a pro-drug since, *in vivo*, it is converted, within the tissue, to zofenoprilat, the active form.⁷ In addition, it has been demonstrated that tissue ACE has more impact in the antihypertensive effect of ACEi^{13–16} compared with serum enzyme isoform. Thus, all these findings confirm that, at tissue level, a local tuning of angiotensin II production exists, as well as regulation of blood pressure and vascular reactivity. It is important to underline that the interaction between ACE and *S*-zofenoprilat is stereospecific. Indeed, ACE inhibition only occurs with *S*-zofenoprilat, while *R*-zofenoprilat fails to block ACE activity¹⁷ (see Supplementary material online, Figure S1). For this reason, *S*-zofenopril is the only isomer used in clinic. The chemical structure of *S*-zofenoprilat with a free thiol (Figure 1) leads us to hypothesize that it might act as an hydrogen sulfide (H₂S) donor *in vivo* following the metabolism of the parent drug zofenopril.

H₂S has emerged as a novel gaseous mediator involved in vascular homeostasis.^{18,19} It is endogenously formed in mammalian cells from L-cysteine (L-cys) through the action of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), both pyridoxal-phosphate-dependent enzymes. Alternatively, these enzymes can also utilize L-methionine and/or homocysteine as substrates to produce H₂S through different pathways.²⁰ CBS and CSE are widely distributed and, at the present

stage, CBS-derived H₂S acts mainly as a neuromodulator. Conversely, CSE-derived H₂S is mostly involved in vascular homeostasis.^{21,22} H₂S can also act indirectly at vascular level by activating the eNOS/cGMP pathway potentiating the effects of NO through different mechanisms.^{23–25}

Recent generation of mice lacking CSE gene has further highlighted the role of H₂S in vascular homeostasis, since its genetic deletion results in hypertension.²⁶

Here, we assessed whether the H₂S pathway is involved in the additional beneficial properties exhibited by zofenopril. In addition, we have tested the effect of active metabolite of zofenopril, e.g. *S*-zofenoprilat, and compared it with its inactive diastereoisomer *R*-zofenoprilat.

2. Materials and methods

2.1 Drugs and reagents

All reagents for Krebs' solution preparation were purchased from Carlo Erba Reagents (Milano, Italy). Phenylephrine (PE), acetylcholine (ACh), L-cys, Tris-HCl, Triton, sodium deoxycholate, sodium chloride, EDTA, phenylmethanesulfonylfluoride, aprotinin, leupeptin, sodium fluoride, and pyridoxal-5'-phosphate were purchased from Sigma-Aldrich (Milano, Italy). Zofenopril was used as calcium salt, the active metabolite diastereoisomer, e.g. *S,S,S*-zofenoprilat and its inactive metabolite diastereoisomer *S,S,R*-zofenoprilat, were both used as sodium salt (Figure 1). For brevity, *S,S,S*-zofenoprilat and *S,S,R*-zofenoprilat will be referred to as *S*-zofenoprilat and *R*-zofenoprilat, respectively, throughout the manuscript. Enalapril and its active metabolite enalaprilat (Figure 1) were used as a non-sulfhydrylated ACEi control. All ACEi were supplied by the Menarini Research Group (Firenze, Italy).

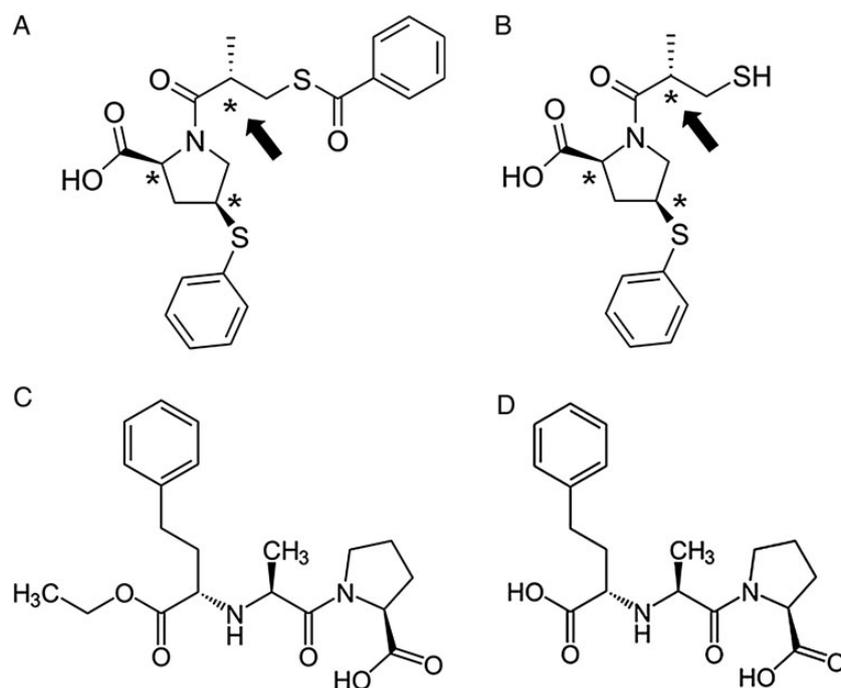


Figure 1 The structures of (A) zofenopril, (B) zofenoprilat, (C) enalapril, and (D) enalaprilat are reported. The *S,S,S*-zofenopril is the only diastereoisomer currently used in clinic. In A and B, the asterisks denote the *S* configurations at the chiral centres. The arrow denotes the only carbon atom whose configuration leads to *S,S,S*-zofenopril(at) or *S,S,R*-zofenoprilat. *S,S,S*-zofenopril(at) and *S,S,R*-zofenoprilat are referred to as *S*-zofenopril(at) and *R*-zofenoprilat, respectively.

2.2 Systolic blood pressure measurement and drug treatment *in vivo* in SHR and WKY rats

Male spontaneously hypertensive rats (SHRs) and control Wistar Kyoto (WKY) rats (8 weeks) were purchased from Charles-River and kept in an animal care facility under controlled temperature, humidity, and light/dark cycle and with food and water *ad libitum*. Rat systolic blood pressure (SBP) was monitored in conscious animals twice a week by using a BP Recorder 58500 (Ugo Basile, Milano, Italy) in an *ad hoc* pre-warmed room within the animal care facility. All animal procedures were performed according to the ARRIVE guidelines and authorized by Centro Servizi Veterinari Università degli Studi di Napoli 'Federico II'.

SBP was monitored for 4–6 weeks until hypertension developed in SHR, and a statistical difference ($P < 0.05$) in pressure values was reached between SHR and WKY. Rats were divided into six different treatment groups ($n = 10$ for each) and organized as follows:

- SHR: (i) vehicle, (ii) *S*-zofenopril, and (iii) enalapril.
- WKY: (i) vehicle, (ii) *S*-zofenopril, and (iii) enalapril.

Rats received zofenopril or enalapril at doses of 10 or 6 mg/kg, respectively, administered daily for 2 weeks in 0.2% carboxymethylcellulose, per gavage. SBP was monitored during the treatment. After 2 weeks, animals were anaesthetized with enflurane (5%) and then euthanized in a CO₂ chamber (70%). Carotid and aorta were harvested and *ex vivo* vascular reactivity assessed. Furthermore, blood samples were collected by cardiac puncture in order to measure H₂S levels and ACE activity (see Supplementary material online).

In another set of experiments, we evaluated blood pressure changes following treatment *in vivo* daily for 14 days with either *S*-zofenoprilat (10 mg/kg) or its inactive epimer *R*-zofenoprilat (10 mg/kg).¹⁷ At the end of the experimental period, rats were sacrificed and aorta and carotid harvested as described above and vascular reactivity assessed. Blood samples were collected by cardiac puncture in order to measure H₂S levels and ACE activity (see Supplementary material online).

2.3 *In vitro* study

SHRs and WKY rats were sacrificed, and aorta tissue was harvested to assess vascular response to direct administration of *S*-zofenoprilat or enalaprilat to organ bath and concentration response curves for *S*-zofenoprilat or enalaprilat were performed within the range 100 nmol/L–1 mmol/L.

2.4 Vascular reactivity assessment

Rings from aorta (~3 mm length) and carotid (~1.5 mm length) arteries were mounted in an isolated organ bath system filled with Krebs' solution (mmol/L: 115.3 NaCl, 4.9 KCl, 1.46 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 11.1 glucose) at 37°C and gassed with O₂/CO₂ mixture (95/5%). Organ baths were equipped with a force transducer (FORT10, Ugo Basile) connected to a PowerLab acquisition data system (ADInstruments, Colorado Springs, CO, USA). The rings were stretched until resting tension was reached and allowed to equilibrate for at least 40 min; over this period, tension was adjusted, as necessary, to the resting tension and bathing solution was periodically changed. In each experiment, rings were first challenged with PE (1 μM) until the responses were reproducible. The rings were then washed and contracted with PE (1 μM) and, once a plateau was reached, a cumulative concentration–response curve of Ach (0.1 nmol/L–30 μmol/L) or L-cys (0.1 μmol/L–10 mmol/L) was performed.

2.5 Western blot analysis

Vascular tissues harvested from both WKY rats and SHRs following *in vivo* treatment with enalapril and zofenopril were homogenized in RIPA buffer (50 mmol/L Tris–HCl, pH 7.4, 1% v/v Triton, 0.25% w/v sodium deoxycholate, 150 mmol/L sodium chloride, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L phenylmethanesulfonyl fluoride, 10 mg/mL aprotinin, 20 mmol/L leupeptin, and 50 mmol/L sodium fluoride). Denatured proteins (40 μg) were separated on 10% sodium dodecylsulfate polyacrylamide

gels and transferred to the polyvinylidene fluoride membrane. Membranes were blocked in phosphate-buffered saline containing 0.1% v/v Tween-20 (PBST) and 3% w/v non-fat dry milk for 30 min, followed by overnight incubation at 4°C with rabbit polyclonal CBS (1 : 1000), or mouse monoclonal CSE (1 : 500) antibody (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). Rabbit polyclonal 3MST (1 : 500) antibody was purchased from Abnova (Heidelberg, Germany). Mouse anti-ACE antibody was also tested (1 : 500, Santa Cruz Biotechnology, Inc.). Membranes were extensively washed in PBST prior to incubation with horseradish peroxidase-conjugated secondary antibody for 2 h. Following incubation, membranes were washed and developed using ImageQuant-400 (GE Healthcare, USA). The target protein band intensity was normalized over the intensity of the housekeeping protein β-actin (1 : 5000, Sigma-Aldrich).

2.6 H₂S quantification assays

2.6.1 Methylene blue assay

Tissue H₂S production rate was measured using Stipanuk and Beck based assay with modifications.^{18,27,28} Briefly, vessels were homogenized in lysis buffer containing potassium phosphate buffer (100 mmol/L, pH 7.4; 10 mM sodium orthovanadate, and protease inhibitors). Protein concentration was determined by using the Bradford assay (Bio-Rad Laboratories, Milano, Italy). Homogenates were added to a reaction mixture (total volume 500 μL) containing pyridoxal-5'-phosphate (2 mmol/L, 20 μL), L-cys (10 mmol/L, 20 μL), and saline (30 μL). The reaction was performed in sealed eppendorf tubes and initiated by transferring tubes from ice to a water bath at 37°C. After 40 min incubation, zinc acetate (1% w/v, 250 μL) was added followed by trichloroacetic acid (10% w/v, 250 μL). Subsequently, *N,N*-dimethylphenylendiamine sulfate (20 mmol/L, 133 μL) in 7.2 mol/L HCl and FeCl₃ (30 mmol/L, 133 μL) in 1.2 mol/L HCl were added, and solution optical absorbance was measured after 20 min at a wavelength of 650 nm. All samples were assayed in duplicate and the H₂S concentration was calculated against a calibration curve of NaHS (3.12–250 μmol/L).

Plasma H₂S levels were determined in the absence of L-cys in the reaction mixture. Results were expressed as μmol/L concentration. H₂S determination in plasma samples was performed as follows: each sample (100 μL) was added to eppendorf tubes containing trichloroacetic acid (10% w/v, 300 μL), in order to allow protein precipitation. Supernatant was collected after centrifugation and zinc acetate (1% w/v 150 μL) was then added. Subsequently, *N,N*-dimethylphenylendiamine sulfate (20 mmol/L, 100 μL) in 7.2 mol/L HCl and FeCl₃ (30 mmol/L, 133 μL) in 1.2 mol/L HCl were added to each tube and absorbance measured after 20 min at a wavelength of 650 nm. All samples were assayed in duplicate, and the H₂S concentration was calculated against a calibration curve of NaHS (3.12–250 μmol/L). All chemicals used in this assay were purchased at Sigma-Aldrich.

2.6.2 *In vitro* fluorimetric assay for H₂S release

Fluorimetric determination of H₂S released by *S*-zofenoprilat was performed in a cell-free assay according to a method previously described²⁹ with modifications. Briefly, vehicle or the indicated concentration of *S*-zofenoprilat was placed in a black 96-well microplate. Hank's Balanced Salt Solution (HBSS, Cat. No. 14025, Invitrogen, at pH 7.4) containing dibromobimane (250 μmol/L) was then added into the well, and fluorescence was measured immediately in a plate reader at 360/465 nm excitation/emission (GENios, Tecan) every 1 min over a 30-min period. The amount of H₂S (measured as thiobimane) was expressed as relative fluorescence unit (RFU). In experiments designed to quench H₂S, haemoglobin (10 μmol/L) was mixed with *S*-zofenoprilat before adding dibromobimane. All reactions were performed in triplicate.

2.6.3 *In vitro* amperometric assay for H₂S release

The amperometric assay was performed in a cell-free assay by using an Apollo-4000 Free Radical Analyzer detector (VPI) and 2 mm H₂S-selective minielectrodes.³⁰ Assay buffer was prepared according to the manufacturer's

instructions. The minielectrode was equilibrated in 10 mL of assay buffer, until a stable baseline was achieved. Then, 100 μ L of an aqueous solution of S-zofenoprilat was added (final concentration 1 mmol/L). The H₂S generation was monitored for 20 min. L-Cys (4 mmol/L) was added prior to S-zofenoprilat, according to experimental protocol requirements. H₂S concentration was determined against a calibration curve obtained plotting amperometric currents (recorded in nA) vs. increasing concentrations of NaHS (1, 3, 5, and 10 μ mol/L) at pH 4.0.

2.7 Statistical analysis

All data were expressed as mean \pm SEM. Statistical analysis was performed by using one-way analysis of variance (ANOVA) and Dunnett as post-test, or two-way ANOVA and Bonferroni as post-test where appropriate (Graph-Pad software, San Diego, CA, USA). Differences were considered statistically significant with a *P*-value of <0.05.

3. Results

3.1 Blood pressure changes and Ach-induced response following ACE inhibition

Animals were treated daily by oral gavage administration (vehicle, S-zofenopril, and enalapril), and SBP was monitored. SHRs treated with vehicle showed a significant increase in SBP compared with WKY rats (Figure 2A, *P* < 0.001). Both S-zofenopril and enalapril significantly lowered SBP in SHRs compared with the vehicle group (Figure 2A, *P* < 0.001). S-zofenopril was significantly more effective when

compared with enalapril (167.5 \pm 2.5 vs. 178.8 \pm 4.6 mmHg, *P* < 0.05, *n* = 10). Both drugs produced over 90% inhibition of ACE activity (see Supplementary material online, Figure S1). Hypertensive animals show an increased ACE expression in both aorta and carotid. Both S-zofenopril and enalapril mitigated the increase in tissue ACE expression in aorta (Figure 2B, *P* < 0.01), but only S-zofenopril was effective on ACE expression in carotid homogenates harvested from hypertensive animals (Figure 2C, *P* < 0.05). Experiments were performed by using aorta and carotid arteries harvested from rats at the end of treatments, and their response to Ach was assessed in the isolated organ bath system. Ach-induced relaxation was significantly impaired in SHRs compared with WKY rats in both aorta (Figure 3A, *P* < 0.001) and carotid (Figure 3C, *P* < 0.001). S-zofenopril treatment restored the Ach-induced vasodilatation in both vessels (Figure 3B, *P* < 0.001 and Figure 3D, *P* < 0.001). Enalapril did not significantly modify the vascular response to Ach in both vessel types.

3.2 H₂S pathway modulation in aorta and carotid following *in vivo* S-zofenopril treatment

Reduction in H₂S levels has been associated with the development of hypertension in this rat strain, as reported by others.^{31,32} Therefore, we questioned whether prolonged ACE inhibition with enalapril or S-zofenopril could affect the H₂S pathway. We first assessed CSE expression by western blot analysis in aorta and carotid samples harvested from treated animals. We found, as already reported in the literature,^{31,32} that

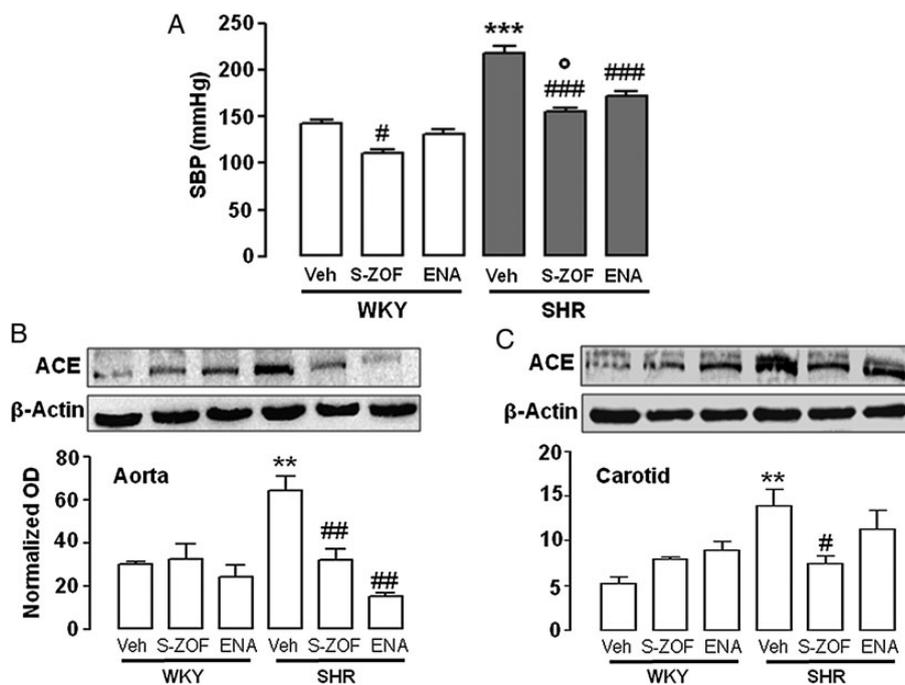


Figure 2 Blood pressure and ACE expression following ACEi treatment. (A) Blood pressure values (SBP, mmHg) following 2-weeks treatment with S-zofenopril (S-ZOF) or enalapril (ENA) in SHRs and WKY rats. Data are expressed as mean \pm SEM for *n* = 10. * vs. WKY vehicle, *P* < 0.001; # vs. SHR vehicle, *P* < 0.001; ° vs. SHR ENA, *P* < 0.05. (B) Expression of ACE in aortic tissue harvested from WKY rats or SHRs, following ACEi treatment. * vs. WKY vehicle, *P* < 0.01; # vs. SHR vehicle, *P* < 0.01. (C) Expression of ACE in carotid tissue harvested from WKY rats or SHRs, following ACEi treatment. * vs. WKY vehicle, *P* < 0.01; # vs. SHR vehicle, *P* < 0.05. Forty microgram of total protein was loaded per each lane, and the target protein band intensity was normalized with the housekeeping protein β -actin. Blots are representative of three different experiments. Optical density is expressed as arbitrary units (mean \pm SEM).

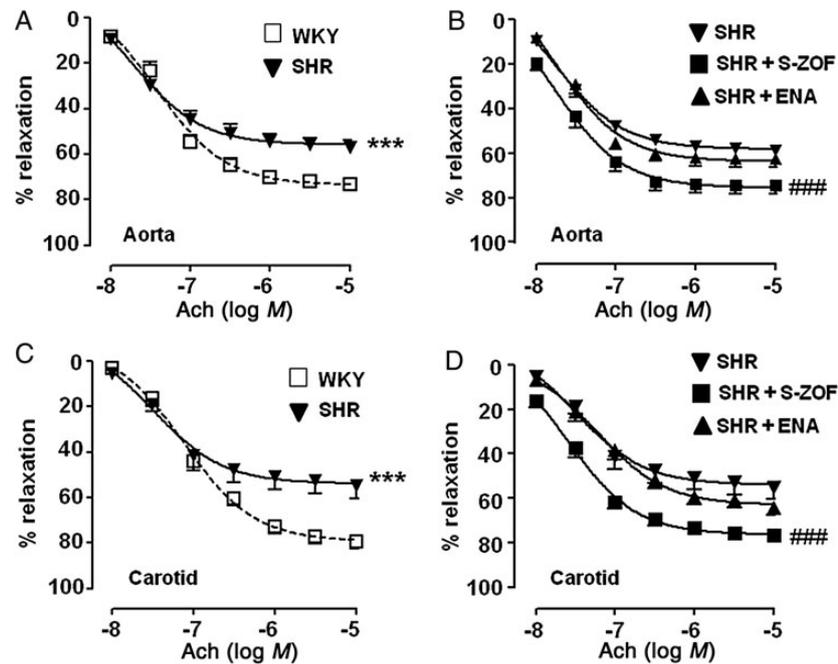


Figure 3 Functional reactivity in vascular tissue harvested from WKY rats treated with vehicle (open square, dashed line) and SHRs treated with vehicle (inverted triangle), *S*-zofenopril (closed square, *S*-ZOF), or enalapril (triangle, ENA). (A) Ach-induced vasorelaxation in aortic rings is reduced in SHRs compared with WKY, $P < 0.001$. (B) Ach-induced vasorelaxation is restored after *S*-zofenopril, but not enalapril, treatment, $P < 0.001$. (C) Ach-induced vasorelaxation in carotid rings is reduced in SHRs compared with WKY rats, $P < 0.001$. (D) Ach-induced vasorelaxation is restored after *S*-zofenopril, but not enalapril, treatment, $P < 0.001$. Ach-induced response is expressed as % of relaxation (mean \pm SEM for $n = 6$). * vs. WKY vehicle; # vs. SHR vehicle.

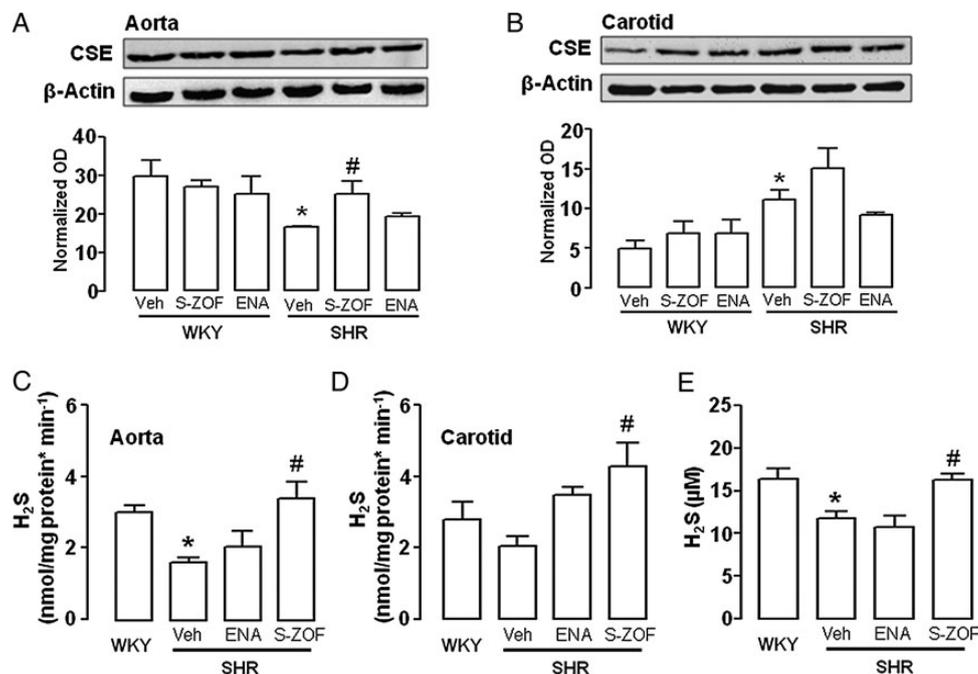


Figure 4 Biochemical analysis of the H_2S pathway following *S*-zofenopril or enalapril treatment. (A) Expression of CSE protein levels in aorta lysates. (B) Expression of CSE protein levels in carotid lysates. Forty microgram of total protein was loaded per each lane, and β -actin has been used as loading control. Blots are representative of three different experiments. Optical density is expressed in arbitrary units (mean \pm SEM) and normalized to β -actin expression levels as the housekeeping gene. * vs. WKY vehicle, $P < 0.05$; # vs. SHR vehicle, $P < 0.05$. (C) H_2S production rate in aorta harvested from WKY rats and SHRs treated with vehicle, *S*-zofenopril, or enalapril, after 40-min incubation in the presence of *L*-cysteine as substrate. (D) H_2S production rate in carotid harvested from WKY rats and SHRs treated with vehicle, *S*-zofenopril, or enalapril, after 40-min incubation in the presence of *L*-cysteine as substrate. (E) H_2S levels determination in plasma samples. Data are expressed as mean \pm SEM for $n = 6$. * vs. WKY vehicle, $P < 0.05$; # vs. SHR vehicle, $P < 0.05$.

expression of CSE in aorta samples was significantly lower in untreated SHRs compared with WKY rats (Figure 4A, $P < 0.05$). Treatment with *S*-zofenopril, but not enalapril, restored CSE expression in SHRs bringing back it to WKY level (Figure 4A, $P < 0.05$). Interestingly, CSE expression in carotid arteries of SHR was significantly higher than in WKY. Neither *S*-zofenopril nor enalapril significantly altered CSE expression in carotid arteries (Figure 4B, $P < 0.05$). Furthermore, CBS and 3MST expression were not modified by treatments (see Supplementary material online, Figures S2 and S3, respectively).

Further analysis provided measurements of H₂S levels in both aorta and carotid using a methylene blue-based assay. As already reported, H₂S levels within the aorta (Figure 4C) and the carotid (Figure 4D) of SHR animals were lower than the content measured in WKY. *S*-zofenopril restored H₂S levels to WKY levels in the aorta, as opposite to enalapril (Figure 4C, $P < 0.05$). There was no significant reduction in H₂S levels between WKY and SHR in the carotid. However, H₂S levels were significantly enhanced in the carotid harvested from SHRs treated with *S*-zofenopril (Figure 4D, $P < 0.05$). Treatment with either *S*-zofenopril or enalapril did not affect H₂S production in WKY rats (see Supplementary material online, Figure S4). Plasma H₂S values observed were significantly lower in SHRs when compared with WKY levels (Figure 4E, $P < 0.05$). *S*-zofenopril treatment restored the H₂S plasma concentration to WKY levels, while enalapril did not modify H₂S plasma levels (Figure 4E, $P < 0.05$).

3.3 Ex vivo functional study: L-cyst-induced vasodilatation

In another set of experiments, a concentration–response curve to L-cys was performed on both the aorta and carotid harvested from WKY rats

and SHRs at the end of treatments. L-Cys induces a concentration-dependent vasodilation (0.1–1 mmol/L). The vasorelaxant effect induced by L-cys on either aorta or carotid (Figure 5A and C, respectively) harvested from SHRs was not significantly different from the response obtained in WKY. However, *S*-zofenopril, but not enalapril treatment, enhanced L-cys-induced vasodilatation in aorta and carotid rings (Figure 5B and D, $P < 0.05$ and < 0.01 , respectively).

3.4 S-zofenoprilat is an H₂S donor

To evaluate whether *S*-zofenoprilat can act as an H₂S donor, we measured the H₂S release in cell-free assays. The evaluation was performed by using two different analytical approaches, a fluorimetric and an amperometric assay, since methylene blue assay was not chemically suitable for the detection of *S*-zofenoprilat-derived H₂S. The fluorimetric assay showed that *S*-zofenoprilat can spontaneously release H₂S and the fluorescent signal is significantly inhibited by haemoglobin (Hb), a H₂S scavenger (Figure 6A). In parallel, we also assessed that H₂S release by *S*-zofenoprilat occurred in a concentration-dependent manner (Figure 6B), and that it occurs at a different rate compared with other H₂S donors (see Supplementary material online, Figure S5D).²⁹ To further confirm these data, we performed measurements using an amperometric assay, confirming that H₂S is released in solution, triggered by addition of a nucleophilic agent, such as L-cys (Figure 6C).

In a separate set of experiment, we tested whether such H₂S release by *S*-zofenoprilat could directly induce vasodilation of aorta rings. *S*-zofenoprilat elicits a concentration-dependent relaxation of the isolated aorta harvested from both WKY rats and SHRs (Figure 6D). This effect was inhibited by H₂S pathway inhibitors (see Supplementary

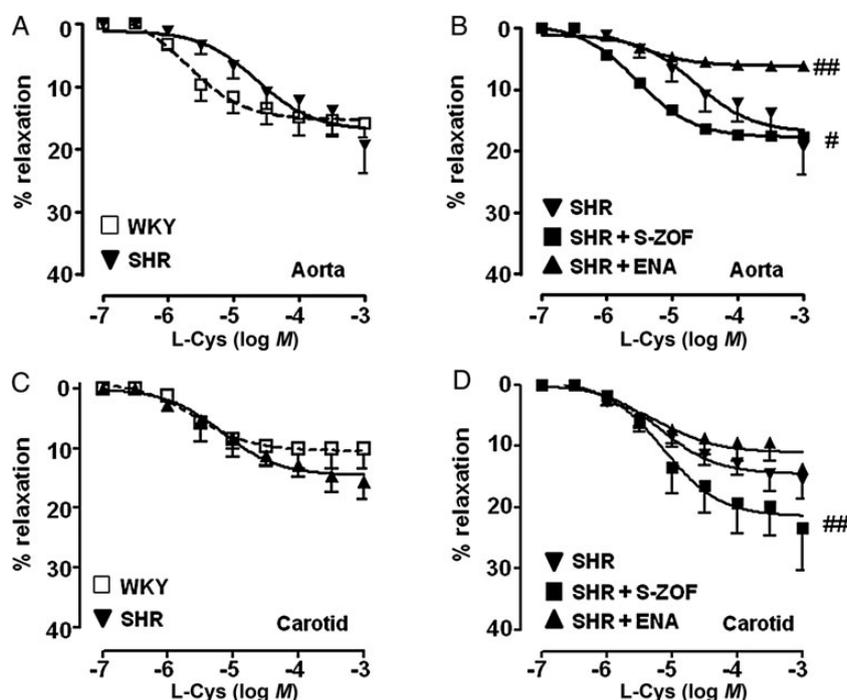


Figure 5 L-Cys-induced vasorelaxation in aortic rings from WKY rats treated with vehicle (open square, dashed line) and SHRs treated with vehicle (inverted triangle), *S*-zofenopril (closed square, *S*-ZOF), or enalapril (triangle, ENA). (A) L-Cys-induced vasorelaxation in aortic rings is slightly, though not significantly, reduced in SHRs compared with WKY. (B) L-Cys-induced vasorelaxation is significantly enhanced in the aorta of *S*-zofenopril-treated animals, $P < 0.05$. (C) L-Cys vasodilation does not significantly change between carotid rings harvested from SHRs compared with WKY rats. (D) *S*-zofenopril treatment significantly increases vasorelaxation induced by L-cys administration in carotid rings, compared with vehicle-treated animals, $P < 0.01$. Data are expressed as mean \pm SEM for $n = 6$. # vs. SHR vehicle.

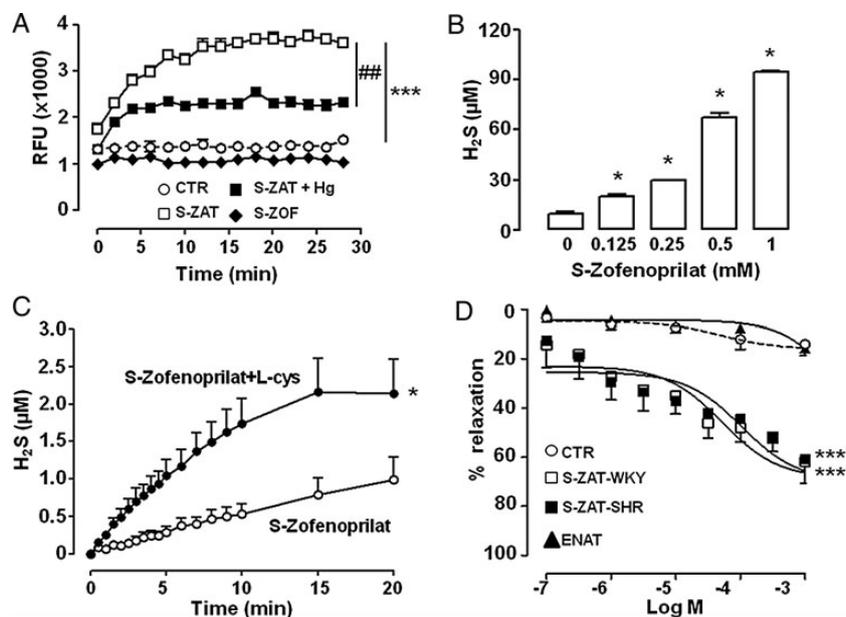


Figure 6 Free H₂S release determination. (A) Time-dependent release of H₂S by S-zofenoprilat (open square, 0.2 mmol/L) was detected using dibromobimane (0.25 mmol/L) and fluorescence was measured every 2 min, $P < 0.001$. S-zofenopril (diamond, S-ZOF, 0.1 mmol/L) shows that no H₂S release and haemoglobin (closed square, Hg, 10 μmol/L) reduces the amount of H₂S detected from S-zofenoprilat (S-ZAT), $P < 0.01$. Data are presented as means \pm SEM for $n = 4$. * vs. control vehicle (open circle, CTR); # vs. S-ZAT. (B) H₂S release from different concentration of S-zofenoprilat in Dulbecco's Modified Eagle Medium (DMEM), quantified again by using dibromobimane fluorimetric determination. (C) Amperometric measurement vs. time of H₂S release in aqueous buffer, $P < 0.05$. * vs. S-zofenoprilat. (D) Vasorelaxant effect of S-zofenoprilat (open square, S-ZAT) vs. enalaprilat (triangle, ENAT) in rat isolated aortic rings harvested from WKY rats. Effect of S-zofenoprilat is also shown on aortic rings harvested from SHR (closed square, S-ZAT). Data are expressed as mean \pm SEM for $n = 6$. * vs. ENA; $P < 0.001$.

material online, Figure S5A–C). Conversely, enalaprilat had no direct vasoactive effect when added *in vitro* to aorta rings (Figure 6D).

3.5 ACE involvement in H₂S pathway activation

Zofenopril(at) is a chiral molecule and *S,S,S*-zofenoprilat (Figure 1) is the only active diastereoisomer used in therapy, inhibiting ACE with an IC₅₀ of 10.9 nM. Conversely, *S,S,R*-zofenoprilat (IC₅₀ = 1700 nM)¹⁷ is not used in clinic. In order to further define the net contribution of the thiol group without affecting ACE activity, we treated SHR *in vivo* with either *S*- or *R*-zofenoprilat. As expected, only *S*-zofenoprilat significantly lowered blood pressure (Figure 7A). Nevertheless, *R*-zofenoprilat, despite the lack of an effect on blood pressure, was as active as *S*-zofenoprilat in restoring the vascular response to Ach in both the aorta (Figure 7B) and carotid (Figure 7C). The treatment with either *R*- or *S*-zofenoprilat restored H₂S levels in both the aorta (Figure 7D) and carotid (Figure 7E), as well as in plasma samples (Figure 7F).

4. Discussion

Clinical evidence has accumulated demonstrating that the sulfhydrylated ACEi zofenopril exerts additional beneficial effects beyond ACE inhibition.^{33–36} Zofenopril is a highly lipophilic ACEi characterized by long-acting tissue penetration and cardioprotective properties.⁸ Zofenopril is considered a pro-drug, since its action is due to the active metabolite *S*-zofenoprilat. It has a free thiol and, therefore, we tested the hypothesis that the modulation of the H₂S pathway could be involved.

The study has been performed *in vivo* by comparing zofenopril with a non-sulfhydrylated ACEi also used in clinic, e.g. enalapril. Similar to zofenopril, enalapril is a pro-drug that following oral administration is metabolized *in vivo* to enalaprilat, its active form. To define the specific contribution of H₂S in zofenopril action, we used SHR as a model of hypertension. SHR and their matched control WKY were treated with zofenopril and enalapril at doses already known to cause a maximal reduction in blood pressure and almost complete inhibition of ACE in this animal model.³⁷ As expected, both ACEis reduced blood pressure as well as ACE expression in both aorta and carotid in SHR.³⁸ Nevertheless, *S*-zofenopril was significantly more effective than enalapril in lowering blood pressure *in vivo*, despite the same extent of ACE inhibition (see Supplementary material online, Figure S1).^{37,39} To investigate the involvement of the H₂S pathway, we harvested carotid and aorta and assessed their ability to respond to acetylcholine *ex vivo*. Ach-induced vasorelaxation was significantly reduced in both isolated aorta and carotid from SHR compared with WKY rats following treatment with the vehicle. This impaired response was rescued by zofenopril treatment *in vivo*, while enalapril was ineffective. Therefore, both drugs inhibit ACE, lower blood pressure but only zofenopril exerts a peripheral beneficial vascular effect. Nevertheless, even though the dose used of enalapril causes the same reduction in ACE activity, it has to be stated that the dose of enalapril used did not exert the same blood pressure lowering effect. This could be a possible explanation why enalapril, which has been shown in other experimental setting to rescue vascular reactivity, did not reach statistical significance in our experimental conditions. This result implies that the vascular effect exerted by zofenopril treatment involves an additional mechanism(s)

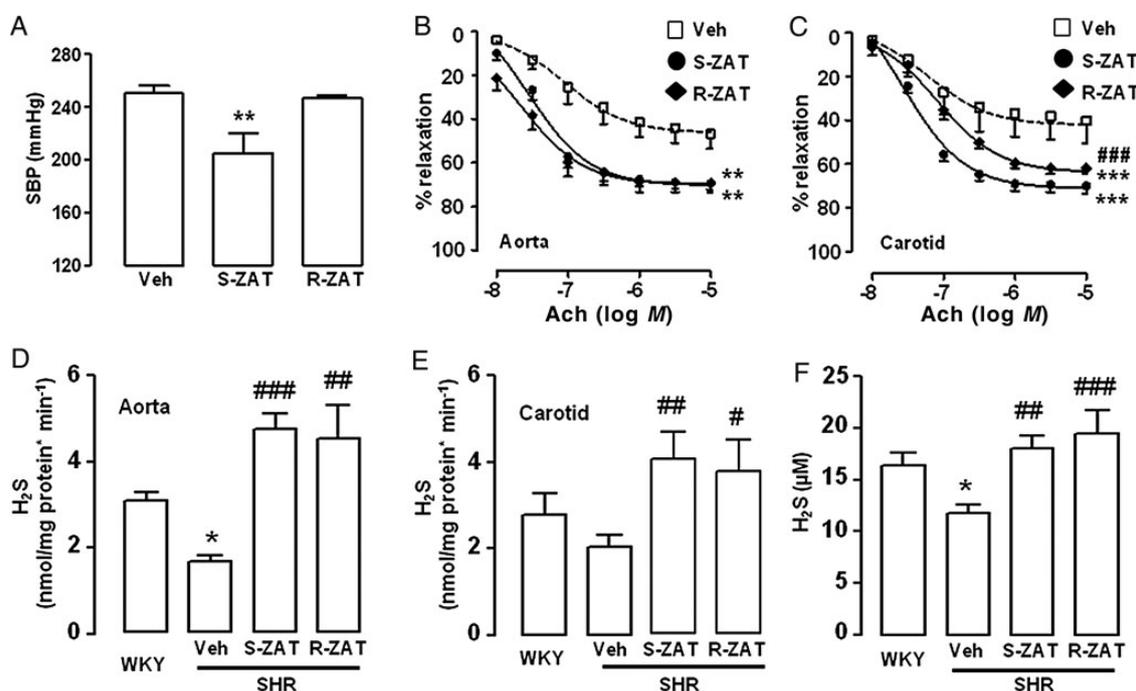


Figure 7 Changes in blood pressure, Ach-induced response, and biochemical analysis of H₂S pathway following R- or S-zofenoprilat treatment. (A) Blood pressure values (SBP, mmHg) following 2-weeks treatment with S-zofenoprilat (S-ZAT) or R-zofenoprilat (R-ZAT) in SHRs. Data are expressed as mean \pm SEM for $n = 10$. * vs. SHR vehicle, $P < 0.01$. (B) Functional reactivity in vascular tissues harvested from SHRs treated with vehicle (open square, dashed line), S-zofenoprilat (closed circle, S-ZAT), or R-zofenoprilat (diamond, R-ZAT). Reduced Ach-induced vasorelaxation observed in aortic rings from SHRs was restored following S-zofenoprilat or R-zofenoprilat treatment, $P < 0.01$. (C) Impaired Ach-induced vasorelaxation shown in carotid rings from SHRs is restored after S- or R-zofenoprilat treatment, $P < 0.001$. Ach-induced response is expressed as % of relaxation (mean \pm SEM for $n = 6$). * vs. SHR vehicle; # vs. SHR S-ZAT. (D) H₂S production rate in aorta harvested from WKY rats and SHRs treated with vehicle, S-zofenoprilat, or R-zofenoprilat, after 40-min incubation in the presence of L-cysteine as substrate. (E) H₂S production rate in carotid harvested from WKY rats and SHRs treated with vehicle, S-zofenoprilat, or R-zofenoprilat, after 40-min incubation in the presence of L-cysteine as substrate. (F) H₂S level determination in plasma samples. Data are expressed as mean \pm SEM for $n = 6$. * vs. WKY vehicle, $P < 0.05$; # vs. SHR vehicle, $P < 0.01$ for S-ZAT and $P < 0.001$ for R-ZAT, respectively.

beyond ACE inhibition. One of the possible mechanisms involved could be the inhibition of bradykinin degradation. This activity promotes generation of endothelial nitric oxide. The contribution of NO through ACE inhibition has also been clinically demonstrated.⁴⁰ However, since this effect is secondary to ACE inhibition and in our conditions both enalapril and zofenopril do block ACE at the same extent, it is clear that zofenopril has additional action(s) not linked to ACE inhibition. It has been already shown that expression of CSE and H₂S plasma levels are reduced in SHRs.^{31,32} In line with these data, we did find a similar pattern in SHRs treated with the vehicle. However, analysis of the tissue harvested from the treated rats showed that, zofenopril, but not enalapril, restored CSE expression and H₂S levels. In addition, H₂S plasma levels were also re-established after zofenopril treatment. These data imply that, following *in vivo* administration of zofenopril, activation of L-cys/H₂S pathway occurs within the vessels, which in turn restores Ach response. This hypothesis is further sustained by the evidence that L-cys-induced vasodilation was significantly enhanced exclusively following zofenopril treatment. In summary, in SHR animals, the *in vivo* treatment with zofenopril restores (i) Ach-induced relaxation, (ii) tissue and plasma H₂S levels, and (iii) CSE expression and enhancement of the response to L-cys in both vessel types analysed.

Therefore, the active metabolite S-zofenoprilat positively modulates the H₂S pathway within the vessel wall. Next, we questioned whether

S-zofenoprilat could *per se* release H₂S. In order to answer this question and to further demonstrate that zofenopril additional effects do involve H₂S, we employed two different cell-free assays. Indeed, the widely used methylene blue-based colorimetric assay could not be employed to measure direct H₂S release since S-zofenoprilat interferes when it is directly added to the assay solution. Therefore, we utilized two different approaches by using both fluorimetric- and amperometric-based assays. Both assays clearly demonstrated that S-zofenoprilat releases H₂S. In the assay performed with the fluorescent probe, S-zofenoprilat, but not zofenopril, released H₂S. The fluorescent signals are significantly inhibited by haemoglobin. The amperometric assay confirmed that S-zofenoprilat slowly releases H₂S in solution and this effect is further increased by adding L-cys. The use of L-cys in the assay did not only stem from the idea that H₂S release from S-zofenoprilat is facilitated by nucleophilic substances; it was also based on the observation that the metabolism of S-zofenoprilat involves cysteine conjugation, as demonstrated by using radiolabelled zofenopril.¹⁷ In order to confirm data obtained in cell-free assays, we tested whether S-zofenoprilat could relax rat aorta *in vitro*. Addition of cumulative concentration of S-zofenoprilat, but not enalaprilat, on isolated aorta rings induced relaxation of vascular tissue in a concentration-dependent manner, further demonstrating that S-zofenoprilat can release hydrogen sulfide and generate H₂S-dependent vasorelaxation (see Supplementary material

online, Figure S5). Therefore, the *in vivo* treatment with zofenopril brings about a tonic pulse of H₂S, e.g. slow release within the cellular environment, where the ACE resides, leading to the beneficial vascular effects described above. The vascular effects observed are in line with the finding that zofenopril exerts a preferential inhibition on tissue ACE rather than on the plasma isoform.⁷ In other words, *S*-zofenoprilat reaches its target, e.g. tissue ACE, and releases H₂S within the vascular cells, thus operating a site-directed local delivery.

Zofenopril has active chiral centres and the isomer used in therapy is the *S*-isomer that, leading to the formation *in vivo* of *S*-zofenoprilat, inhibits ACE with an IC₅₀ 10.9 nM.¹⁷ In order to further strengthen our findings regarding the role of H₂S in the action of zofenopril, we dosed SHR with either the *R*- or *S*-diastereoisomer of zofenoprilat. As expected, *R*-zofenoprilat treatment did not reduce blood pressure in SHR as opposite to *S*-zofenoprilat. However, when we performed the study on rat carotid and aorta from SHR, we found that the Ach-induced vasorelaxation was equally rescued by both *R*- and *S*-zofenoprilat. Therefore, the *R*-form, devoid of ACE inhibitory action, can still act at the vessel level through the H₂S pathway. This hypothesis is further sustained by the finding that H₂S levels found in tissues and plasma of *R*-zofenoprilat-treated animals were identical to the ones observed in the *S*-zofenoprilat-treated group. Thus, the modulation of the H₂S pathway by zofenopril represents an additional beneficial mechanism unrelated to ACE inhibition. On the clinical side, it is important to point out that zofenopril, but not ramipril or atenolol, improves flow-mediated dilation.¹⁰ Interestingly, this action by zofenopril did not involve NO, but was ascribed to a generic antioxidant action linked to the thiol group of *S*-zofenoprilat. On the basis of our data, we can now suggest that such action can be ascribed to stimulation of the H₂S pathway, rather than to an antioxidant property, since it is well known that antioxidant do not work in this clinical setting.¹⁰

In conclusion, we show that the extra-beneficial effects described in the literature for zofenopril over ACE inhibition^{3,7–10,12} involve the L-cys/H₂S pathway. Overall, these findings confirm that H₂S plays an important role in cardiovascular homeostasis and provide proof of concept for the development of H₂S-releasing therapeutic agents in cardiovascular disease therapy.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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