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Perspective

H$_2$S dependent and independent anti-inflammatory activity of zofenoprilat in cells of the vascular wall

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ABSTRACT

Cardiovascular diseases as atherosclerosis are associated to an inflammatory state of the vessel wall which is accompanied by endothelial dysfunction, and adherence and activation of circulating inflammatory cells. Hydrogen sulfide, a novel cardiovascular protective gaseous mediator, has been reported to exert anti-inflammatory activity. We have recently demonstrated that the SH containing ACE inhibitor zofenoprilat, the active metabolite of zofenopril, controls the angiogenic features of vascular endothelium through H$_2$S enzymatic production by cystathionine gamma lyase (CSE). Based on H$_2$S donor/generator property of zofenoprilat, the objective of this study was to evaluate whether zofenoprilat exerts anti-inflammatory activity in vascular cells through its ability to increase H$_2$S availability.

Here we found that zofenoprilat, in a CSE/H$_2$S-mediated manner, abolished all the inflammatory features induced by interlukin-1beta (IL-1β) in human umbilical vein endothelial cells (HUVEC), especially the NF-κB/cyclooxygenase-2 (COX-2)/prostanoid biochemical pathway. The pre-incubation with zofenoprilat/CSE dependent H$_2$S prevented IL-1β induced paracellular hyperpermeability through the control of expression and localization of cell-cell junctional markers ZO-1 and VE-cadherin. Moreover, zofenoprilat/CSE dependent H$_2$S reduced the expression of the endothelial markers CD40 and CD31, involved in the recruitment of circulating mononuclear cells and platelets. Interestingly, this anti-inflammatory activity was also confirmed in vascular smooth muscle cells and fibroblasts as zofenoprilat reduced, in both cell lines, proliferation, migration and COX-2 expression induced by IL-1β, but independently from the SH moiety and H$_2$S availability.

These in vitro data document the anti-inflammatory activity of zofenoprilat on vascular cells, reinforcing the cardiovascular protective effect of this multitasking drug.

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1. Introduction

The opinion that atherosclerosis is an inflammatory disease has become increasingly prevalent in the past twenty years. It is a chronic, progressive, multifocal arterial wall disease caused by local and systemic inflammation, responsible for major cardiovascular complications such as myocardial infarction and stroke.

Atherosclerosis is one of the major cause of illness and death in industrialized nations and is rapidly increasing in developing nations [1,2]. Atherosclerosis is linked to a variety of risk factors including high LDL cholesterol and triglyceride levels, diabetes, smoking, hypertension, sedentary lifestyle, and upregulated inflammatory mediators.

Hypertension exerts pro-inflammatory effects on the artery, stimulating genes involved in the recruitment of inflammatory cells into the arterial wall. Clinical trials using cholesterol-lowering or anti-hypertensive therapies have shown a decrease in cardiovascular events that may have resulted from withdrawal of inflammatory features of the arterial wall [3]. Angiotensin-converting enzyme inhibitors (ACEI) decrease the rate of myocardial infarction in patients with overt congestive heart failure or left ventricular dysfunction. These drugs probably affect several mechanisms related to the inhibition of angiotensin production and the potentiation of bradykinin and resultant enhancement of nitric oxide and prostacyclin, although other mechanisms cannot be excluded [4,5].

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Hydrogen sulfide (H_2S) is a physiologic messenger molecule involved in cardiovascular health. H_2S is generated in the periphery by cystathionine γ-lyase (cystathionase; CSE), while in the brain its biosynthesis may involve cystathionine β-synthase (CBS) [6,7]. CSE knockout (CSE$^{-/-}$) mice display hypertension and a major decrease in endothelial-derived relaxing factor activity, establishing H_2S as an important vasorelaxant molecule [8,9].

We previously demonstrated that zofenoprilat, the active metabolite of the SH containing ACEI zofenopril, controls the angiogenic features of vascular endothelium through H_2S enzymatic production by CSE upregulation [10]. Zofenoprilat, but not other ACEI bearing or not a SH moiety, was the most effective to favour endothelial cell survival and to prolong their lifespan, restoring endothelial cell functions after vascular damage and promoting angiogenesis [11,12].

The H_2S donor/generator property of zofenoprilat, demonstrated by Bucci and our group [7,13] and further strengthened in the present paper, suggested us to investigate the potential protective role of H_2S by zofenoprilat in vascular endothelium and smooth muscle cells (SMC), mimicking the inflammatory environment of atherosclerosis, and the molecular mechanisms involved.

In the present study, we show that H_2S, derived from the ACEI zofenoprilat through CSE activity and up-regulation, abolished all the inflammatory features induced by interlukin-1beta (IL-1β) in HUVEC, especially the NF-κB/cyclooxygenase-2 (COX-2)/prostanoid biochemical pathway activation, permeability, expression and localization of cell-cell junctional markers zonula occludens-1 (ZO-1) and vascular endothelial (VE)-cadherin. Moreover, it reduced the expression of markers, as CD40 and CD31, involved in the recruitment of mononuclear cells and platelets. In experiments carried out in human vascular smooth muscle cells, zofenoprilat reduced cell proliferation, migration and COX-2 expression induced IL-1β, seemingly in a H_2S independent manner.

2. Materials and methods

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVEC) were purchased from Promocell (Heidelberg, Germany), human umbilical artery smooth muscle cells (HUASMs) were from Clonetics (Lonza, Basel Switzerland) and normal human dermal fibroblasts (NHDF) were from Clonetics (Lonza, Basel, Switzerland). All cell lines were grown following manufacturer instructions, and further information are available on Supplemental material.

2.2. Fluorescence measurement of H_2S

The fluorescent dye WSP-1 (Cayman Chemical, Ann Arbor, MI, USA) was used to assess H_2S levels directly released by zofenoprilat or NaHS, and by cells exposed to drugs [ACEI (10 μM), NaHS (10 μM), IL-1β (10 ng/ml), in the absence/presence of propargylglycine (PAG) (3 mM)] for 6 h. Through a reaction-based fluorescent turn-on strategy, WSP-1 selectively and rapidly reacts with H_2S to generate benzodithiolone and a fluorophore with excitation and emission maxima of 465 and 515 nm, respectively [14]. Fluorescence data were collected by means of Tecan (Thermo Fisher Scientific, Waltham, USA). Cell related fluorescence was revealed also by confocal microscopy (Zeiss LSM500, Milan, Italy) within the cells grown on glass coverslips and then fixed in cold acetone.

2.3. Western blot

Cells (3×10^5/6 cm plate), at 90% of confluence, were treated with IL-1β (10 ng/ml, indicated time) with or without the pretreatment with ACEI (10 μM) or PAG (3 mM) or IKK inhibitor VII (0.2 μM) for 30 min. CSE, COX-2, microsomal prostaglandin E2 synthase (mPGES-1) and prostaglandin I2 (prostacyclin) synthase (PTGIS) expression or IKKα/β phosphorylation were evaluated by western blot [15].

To evaluate p65 translocation, nuclear and cytosolic fractions were prepared following the protocol below [15]. To prepare the nuclear fractions, HUVEC (8×10^5 cells) were plated in 10 cm diameter dishes and, after treatment, cells were suspended in extraction buffer containing (in mM) 10 HEPES, 1 DTT, 10 KCl, 50 NaF, 0.1 EDTA, 0.1 EGTA, 1 Na3VO4, 0.5 PMSF and 0.1 NP-40 at 4 °C, homogenized, and centrifuged at 1,000g for 10 min to separate the nuclei. The supernatant was centrifuged at 13,000g for 15 min three times to yield the cytosolic fraction. The nuclear fraction was lysed in buffer containing (in mM) 20 HEPES, 1 EDTA, 1 EGTA and 0.5 PMSF and stored at −80 °C before use.

Proteins from cell extracts were electrophoresed in SDS/4-12% polyacrylamide gels (Life Technologies, Paisley, UK). Proteins were then blotted onto activated nitrocellulose membranes, incubated overnight with the indicated antibodies and antigen-antibody complexes were detected with enhanced chemiluminescence kit (Bio-Rad, Milan, Italy). Band intensity was measured by scanning densitometry and the results were normalized to total β-actin or laminin and tubulin.

2.4. Prostaglandin E2 (PGE-2) and 6-keto prostaglandin F1α (PGF1α) measurements

PGE-2 and 6-keto PGF1α levels were measured by EIA kits (ENZO Lifetech, New York, NY, USA) following manufacturer instructions. Cells were exposed to IL-1β (10 ng/ml, 8 h) in presence/absence of zofenoprilat (10 μM) or PAG (3 mM) and treated with 10 μM arachidonic acid (AA). Cell culture supernatants were assayed at a final dilution of 1[ratio]10. PGE-2 was expressed as [pg/ml] [15], while 6-keto PGF1α was expressed as fold of increase vs. 0.1% FCS.

2.5. Immunofluorescence analysis

Nuclear translocation of p65 or VE-Cadherin, ZO-1, CD31 and CD40 protein, expressed at cell surface, were visualized by confocal analysis. 5×10^4 cells were seeded on 1-cm-round glass coverslips. After 24 h, cells were washed and treated with the indicated stimuli. Cells were fixed in 4% paraformaldehyde/PBS without permeabilization. Unspecific binding sites were blocked in 3% bovine serum albumin (BSA) and then cells were incubated for 2 h with anti-VE-Cadherin, anti-ZO-1, anti-CD31 or anti-CD40 diluted 1:50 in PBS containing 0.5% BSA. Cells were then washed, incubated for 1 h with a rodhamine- or fluorescenin-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:50 in PBS with 0.5% BSA. Coverslips were mounted in Moviol 4–88 (Calbiochem, La Jolla, CA) and pictures of stained cells were taken using a confocal microscope (Zeiss LSM500).

2.6. Proliferation

HUVEC, HUASM or NHDF (1.5×10^3 cells/well in 96 multiplates), after adherence, were treated with zofenoprilat, its R-Isomer, other ACEI (10 μM), or NaHS (10 μM) in presence/absence of IL-1β (10 ng/ml, 5 days) or PAG (3 mM) or ODQ (10 μM), where indicated. Cells were kept in culture for 5 consecutive days, and ACEI were freshly added every 2 days. Cells were then fixed, stained and randomly counted at 20× original magnification in 5 fields, as previously reported [10].