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"Role of L-cysteine/hydrogen sulfide (H_2S) pathway in cardiovascular system in pathophysiological conditions"

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List of Abbreviations

Ach	Acetilcholine
ACE	Angiotensin Converting Enzyme
ACEi	ACE-inhibitor
ADMA	Asymmetric Dimethyl arginine
AngII	Angiotensin II
ANOVA	Analysis of variance
AOAA	Aminooxyacetic acid
AT	Angiotensin receptor
ATL	Atlastin
ATP	Adenosine Triphosphate
BK	Bradykinin
BP	Blood Pressure
CaM	Calmodulin
cAMP	cyclic Adenosine 3',5'-Monophosphate
CAT	Cysteine Aminotransferase
Cav-1	Caveolin-1
CBS	Cystathionine β-synthase
CDO	Cysteine dioxygenase
CEM	Caveolin-1 enriched domain
cGMP	cyclic Guanosine 3',5'-Monophosphate
CGRP	Calcitonin gene related protein
CO	Carbon Monoxide

CSE	Cystathionine γ-liase
CSFs	Colony-stimulating factors
DBP	Diastolic blood pressure
DPD	N,N-dimethylphenylendiamine sulphate
ECM	Extracellular Matrix
ECs	Endothelial Cells
EDCF	Endothelium-contracting factor
EDHF	Endothelium-derived Hyperpolarizing factor
EDRF	Endothelium-derived Relaxing factor
eNOS or NOSIII	Endothelial Nitric Oxide Synthase
ENA	Enalapril
EPC	Endothelial progenitor cells
ER	Endoplasmic reticulum
ET	Endothelin
FGF	Fibroblast Growth Factor
G-CSF	Granulocyte CSF
GDP	Guanosine 5'-diphosphate
GM-CSF	Granulocyte-Macrophage CSF
GTP	Guanosine 5'-triphosphate
H_2S	Hydrogen Sulphide
H_4B	Tetrahydrobiopterin
HBSS	Hank's Balanced Salt Solution
HIF-1	Hypoxia-inducible factor-1
IBMX	3-isobutyl-1- methylxanthine

ICAM-1	Intercellular Adhesion Molecule-1
IL	Interleukin
ILK	Integrin-linked kinase
IP3	Inositol Trisphosphate
IRAG	Inositol trisphosphate (IP_3)-receptor-associated G-kinase
K_{ATP}	ATP-sensitive K^+ channel
K_{Ca}	Ca^{2+}-dependent K^+ channel
KO	Knock-out
L-Cys	L-Cysteine
LDL	Low Density Lipoprotein
L-NAME	N-nitro-L-arginine methyl ester
LR	Lipid raft
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage CSF
MLC	Myosin light chain
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
MRP-1	Multidrug resistance-related protein-1
MST	3-mercaptopyruvate sulfurtansferase
MTs	Microtubules
NaHS	Sodium Hydrosulfide
NgR	Nogo receptor
NO	Nitric Oxide

PAG	Propargylglycine
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PE	Phenylephrine
PGI_2	Prostacyclin
PIP3K	Phosphatidylinositol 3-Kinase
PKA	Protein kinase cAMP-dependent
PKC	Protein kinase C
PKG	Protein kinase cGMP-dependent
PLC	Phospholipase C
PLP	Pyridoxal 5'-phosphate
PRA	Plasma renin activity
PVDF	Polyvinylidene fluoride membrane
RAS	Renin Angiotensin System
RER	Rough endoplasmic reticulum
RHD	Reticulon homology domain
ROCK	Rho kinase
ROS	Reactive Oxygen Species
RTN	Reticulon
R-ZAT	R-zofenoprilat
SAM	S-adenosyl-L-methione
SBP	Systolic blood pressure
SER	Smooth endoplasmic reticulum
sGC	Soluble Guanylyl Cyclase

SHR	Spontaneously Hypertensive Rat
SNAP	S-nitroso-N-acetyl-DL-Penicillamine
SOD	Superoxide Dismutase
SUR	Sulphonylurea receptor
S-zat	S-zofenoprilat
TAC	Tip attachment complex
TCA	Trichloroacetic acid
TEM	Transmission Electron Microscope
TGF	Transforming Growth Factor
TNF	Tumor necrosis factor
TOD	Target organ disease
TPR	Total peripheral resistance
TSMT	Thiol S-methyltransferase
VASP	Vasodilator Stimulated Protein
VCAM-1	Vascular Cell Adhesion Molecule-1
VLDL	Very Low density Lipoprotein
VSMC	Vascular Smooth Muscle Cell
VVG	Verhoeff's Van Gieson
vWF	von Willebrand Factor
WT	Wild-type
WKY	Wistar Kyoto
ZIPK	Zipper-interacting protein kinase
ZOF	Zofenopril

Chapter I

INTRODUCTION

The cardiovascular system

The cardiovascular system is a transport system that carries blood and lymph to and from tissues of the body. The constitutive elements of these fluids include cells, nutrients, waste products, hormones, and antibodies. The cardiovascular system consists of a pump represented by the heart and blood vessels, which provide the route by which blood circulates to and from all parts of the body. The heart pumps the blood through the arterial system under significant pressure; blood is returned to the heart under low pressure with the assistance of negative pressure in the thoracic cavity during inspiration and compression of the veins by skeletal muscle. The blood vessels are arranged so that the blood delivered from the heart quickly reaches a network of narrow, thin-walled vessels, the blood capillaries, within or in proximity to the tissue in every part of the body (Fig. 1.1).

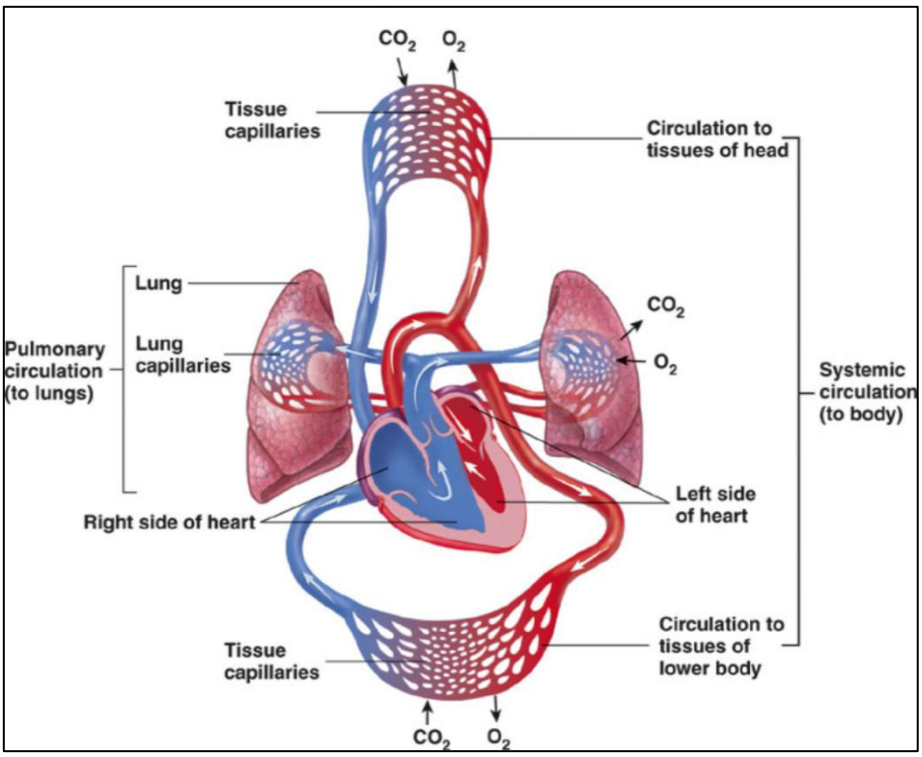


Fig. 1.1: *The cardiovascular system.*

In the capillaries, a two-directional exchange of fluid occurs between the blood and tissues. The fluid, called blood filtrate, carries oxygen and metabolites and passes through the capillary wall. In the tissues, these molecules are exchanged for carbon dioxide and waste products. Most of the fluids re-enters the distal or venous end of the blood capillaries. The remaining fluid enters lymphatic capillaries as lymph and is ultimately returned to the bloodstream through a system of lymphatic vessels. Two pathway of circulation are formed by the blood vessels and heart:

- Pulmonary circulation conveys blood from the heart to the lungs and from lungs to the heart.
- Systemic circulation conveys blood from the heart to other tissues of the body to the heart.

The blood vessels are classified into arteries, veins and capillaries. Arteries are the vessels that deliver blood to the capillaries. The smallest arteries, called arterioles, are functionally associated with networks of capillaries into which they deliver blood. The arterioles regulate the amount of blood that enters these capillary networks. Together, the arterioles associated with capillary network, and postcapillary venules form a functional unit called the microcirculatory or microvascular bed of that tissue. Veins, beginning with the postcapillary venule, collect blood from the microvascular bed and carry it away. Because an artery conducts blood from the heart, its wall must be sufficiently strong to withstand the sudden thrust imposed on it with every heart beat. The walls of the artery are, therefore, thicker and stronger than those of veins of similar diameter. All blood vessels are composed by three layers, from the lumen outward (Fig. 1.2):

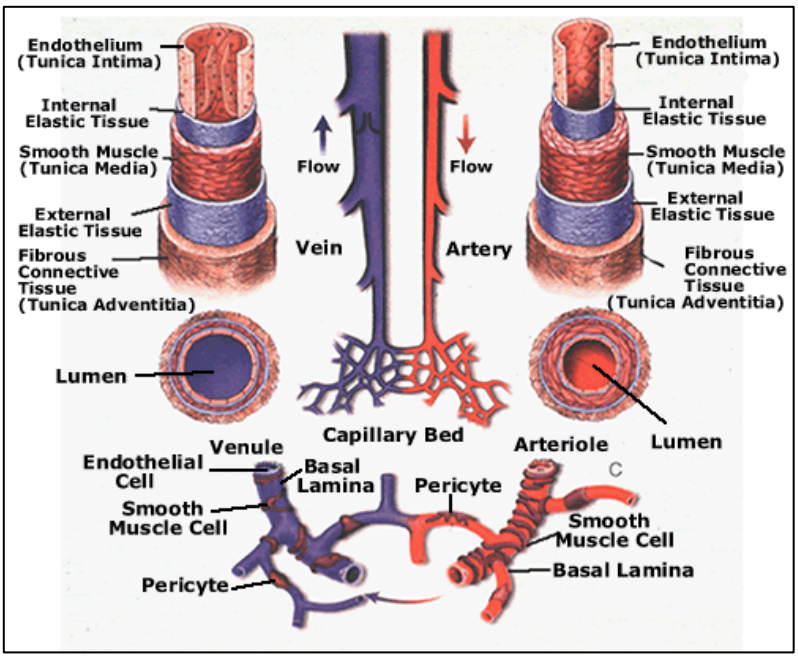


Fig. 1.2: *Schematic diagram of the major structural features of blood vessels.*

- The tunica intima, the innermost layer of the vessel, consists of three components: (a) a single layer of squamous epithelial cells, the endothelium; (b) the basal lamina of the endothelial cells (a thin extracellular layer composed chiefly of collagen, proteoglicans, and glycoprotein); (c) the subendothelial layer, consisting of loose connective tissue. The subendothelial layer of the intima contains a sheetlike layer or lamella of fenestrated elastic material called internal elastic membrane. Fenestrations enable substances to diffuse readily through the layer and reach cells deep within the wall of the vessel.

- The tunica media, or middle layer, consists primarily of circumferentially arranged layers of smooth muscle cells. This layer is relatively thick and extends from the internal elastic membrane to the external elastic membrane. The external elastic membrane is a layer of elastin that separates the tunica media from the tunica adventitia. Variable amounts of elastin, reticular fibers, and proteoglycans are interposed between the smooth muscle cells of the tunica media. The sheets or lamellae of elastin are fenestrated and arranged in circular concentric layers. All of the extracellular components of the tunica media are produced by the smooth muscle cells.
- The tunica adventitia, or outermost connective tissue layer, is composed primarily of longitudinally arranged collagenous tissue and a few elastic fibers. These connective tissue elements gradually merge with the loose connective tissue surrounding the vessels. The tunica adventitia of large arteries and veins contains a system of vessels called vasa vasorum that supplies blood to the vascular walls themselves, as well as a network of autonomic

nerves called nervi vascularis that control contraction of the smooth muscle in the vessel walls.

The relative contributions of different components of the vessels wall vary throughout the circulation, but an endothelial monolayer is present in all vessels. The endothelial cells lie parallel to the direction of blood flow which allows them to sense changes in shear stress. There are extensive gap junctions between endothelial cells allowing direct passage of small molecules. In contrast, the smooth muscles of the media layer are perpendicular/circumferential allowing them to control vessel radius. There is normally only a small distance, containing the internal elastic lamina, between endothelium and smooth muscle layer which facilitates diffusion of substances between the two. Arterioles only contain a single layer of smooth muscle cells, whereas large may have up to 20 layers.

The structural differences among arteries and veins are primarily the degree of development of the media and adventitia, especially the former. For any given size, arteries tend to have a much stronger and ticker media, veins are more poorly developed one. Also the walls of arteries contain fairly prominent elastic tissue.

1.1 Arteries: general features

Arteries are generally divisible into elastic and muscular types, although the media of most arteries contains some of both types of tissue. Elastic tissue allows the wall of an artery to be distended by the sudden thrust of blood from the heart and then to contract again, which helps force the blood forward, with no initiation of energy by the wall of the vessel. The large arteries near the heart typically contain a great deal more elastic tissue than do the smaller, more distal ones; in general, the greater the pressure in an artery, the more elastic tissue there is. For example, the pressure in the great trunk (pulmonary trunk) going to the lungs is much less than that in the great trunk (aorta) carrying blood to the body as a whole; therefore the wall of the aorta contains far more elastic tissue than the pulmonary trunk. Elastic tissue serves the double purpose of cushioning the sudden rise of pressure induced by the heart beat and of smoothing, by its automatic recoil, what would, in an elastic system, be a sudden drop in pressure. In the largely arteries, particularly the aorta, most of the strength of the wall is provided by elastic tissue. If this undergoes degenerative changes, or is destroyed by disease, the arterial wall may

bulge, either in one spot or all around. As branches of the aorta are traced distally, the relative amount of elastic tissue becomes less and relative amount of smooth muscle more. The elastic tissue of the peripheral arteries tends to smooth the pressure and lessen the velocity of the blood. The smooth muscle, controlled by the autonomic nervous system, can contract or relax to vary the caliber of the vessel and, thereby, the blood flow through it. Even rather large vessels, for instance, the chief arteries of the arm and thigh, contain sufficient smooth muscle to reduce to a dangerous level the blood flow through them if they are incited to maximal contraction. The smallest arteries, arterioles (lumen diameter <300μm) have a media composed almost entirely of smooth muscle and are particularly contractile. They are responsible for blood pressure (BP) control and regional distribution of blood flow, through effects on vascular resistance [180]. Based on Poiseuille's law, vessel resistance is inversely proportional to the radius (lumen diameter) to the fourth power (r^4) and accordingly small changes in lumen size result marked changes in resistance [181]. The lumen diameter of resistance arteries is a function of vasomotor tone (vasoconstriction/vasodilation) and the structural characteristics of the

vessel. Vasomotor control underlies acute rapid adaptation of vessel diameter, due mainly to vasoconstriction exerted by the active contraction of vascular smooth muscle cells (VSMC) in the vessel media, whereas alterations in structure constitute a dynamic process occurring in response to chronic hemodynamic variations. Initially structural changes are adaptive, but subsequently become maladaptive resulting in alterations in media thickness and lumen diameter [181,182]. This process, called vascular remodeling, contributes to the pathophysiology of vascular diseases, including hypertension [183, 184].

There are both intrinsic and extrinsic mechanisms regulating vessel diameter. Circulating vasoactive mediators such as angiotensin II and cathecholamines, as well as locally released mediators from the autonomic nervous system, confer extrinsic regulation of smooth muscle contraction. Intrinsic regulation of vessel diameter is mainly mediated by two mechanosensitive mechanisms. One is the sensing of shear stress on the endothelium caused by an increase in blood flow. This leads to a release of vasodilators from the endothelium, which relaxes the smooth muscle cells. The other mechanism involves

stretch sensing by the smooth muscle cells themselves, resulting in narrowing of the vessel lumen. The latter mechanism is referred to as myogenic tone.

1.2 The vascular endothelium

In the adult human body, a circulatory system consists of about 60,000miles of different-sized vessels that are lined by a simple squamous epithelium called endothelium. The endothelium is formed by a continuous layer of flattened, elongated and polygonally shaped endothelial cells that are aligned with their long axes in the direction of the blood flow. The endothelium has long been viewed as an inert cellophane-like membrane lining the circulatory system with its primary essential function being the maintenance of vessel wall permeability [196], but subsequent studies led to the current view of the endothelium as a dynamic, heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic, and immunologic functions [196]. At the luminal surface, they express a variety of surface adhesion molecules and receptors, so endothelial cells play an important role in blood homeostasis. The functional properties of these cells change in response to various stimuli. This process, known

as endothelial activation, is also responsible for the pathogenesis of many vascular diseases.

Endothelial cells are active participants in a variety of interactions between the blood and underlying connective tissue and are responsible for many properties of the vessels. These properties include the following:

- The maintenance of a selective permeability barrier, which allows selective movement of small and large molecules from the blood to the tissues and vice versa. This movement is related to the size and charge of the molecules. The endothelium is permeable to small hydrophobic (lipid-soluble) molecules (e.g. oxygen, carbon dioxide) that readily pass through the lipid bilayer of the endothelial cell membrane (a process called simple diffusion). However, water and hydrophilic (water-soluble) molecules (e.g., glucose, amino acids, electrolytes) cannot diffuse across the endothelial cell membrane. These molecules and solutes must be either actively transported across the plasma membrane and released into the extracellular space (transcellular pathway) or transported across

the zonula occludens between two epithelial cells (paracellular pathway). The former uses numerous small pinocytic vesicles (a clathrin-independent form of endocytosis) to transport bulk material from the blood into the cell. In addition, some specific molecules (e.g. LDL, cholesterol, transferrin) are transported via receptor-mediated endocytosis (a clathrin-dependent process), which uses endothelial-specific surface receptors. In some blood vessels, larger molecules are transported through fenestrations within the endothelial cells visible in transmission electron microscope (TEM) preparations.

- The maintenance of a nonthrombogenic barrier between blood platelets and subendothelial tissue is done by producing anticoagulants (agents that prevent coagulation such as thrombomodulin and others) and antithrombogenic substances (agents that prevent or interfere with platelet aggregation and release of factors that cause formation of clots, or thrombi, such as prostacyclin [PGI_2] and tissue plasminogen activator). Normal endothelium does not support the adherence of platelets or the formation of thrombi on its surface; conversely, damage

to endothelial cells causes them to release prothrombogenic agents (agents that promote thrombi formation) such as von Willebrand factor or plasminogen-activator inhibitor.

- The modulation of blood flow and vascular resistance, that is achieved by the secretion of vasoconstrictors (endothelin, angiotensin-converting enzyme [ACE], prostaglandin H_2, thromboxane A_2) and vasodilators (nitric oxide [NO], prostacyclin).
- The regulation and modulation of immune responses is done by controlling the interaction of lymphocytes with the endothelial surface, which is mainly achieved through the expression of adhesion molecules and their receptors on the endothelial-free surface as well as by secretion of three classes of interleukins (IL-1, IL-6 and IL-8).
- Hormonal synthesis and other metabolic activities are done by the synthesis and secretion of various growth factors, for example, hemopoietic colony-stimulating factors (CSFs) such as granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF) and macrophage CSF (M-CSF); fibroblast growth

factor (FGF); and platelet-derived growth factor (PDGF). Endothelial cells also synthesize growth inhibitors such as heparin and transforming growth factor β (TGF-β). Endothelial cells function in the conversion of angiotensin I to angiotensin II in the renin-angiotensin system that controls blood pressure, as well as in the inactivation or conversion of a several compounds conveyed in the blood (norepinephrine, thrombin, prostaglandins, bradykinin, and serotonin) to inactive forms.

- Modification of lipoproteins occurs by oxidation. Lipoproteins, mainly LDLs with high cholesterol content and very low-density lipoproteins (VLDLs), are oxidized by free radical produced by endothelial cells. Modifies LDLs, in turn, are rapidly endocytosed by macrophages to form foam cells, which are a characteristic feature in the formation of atheromatous plaques.

1.2.1 Regulation of vascular tone by the endothelium

The endothelium not only provides a structural barrier between the circulation and surrounding tissue, but ECs also secrete mediators that influence vascular hemodynamics in the physiologic state. Thus, ECs contribute to the regulation of blood pressure and blood flow by releasing both vasodilator and vasoconstrictors agents, that are collectively called endothelium-derived autacoids, because they are generally short-lived and locally acting (Fig. 1.3 and 1.4).

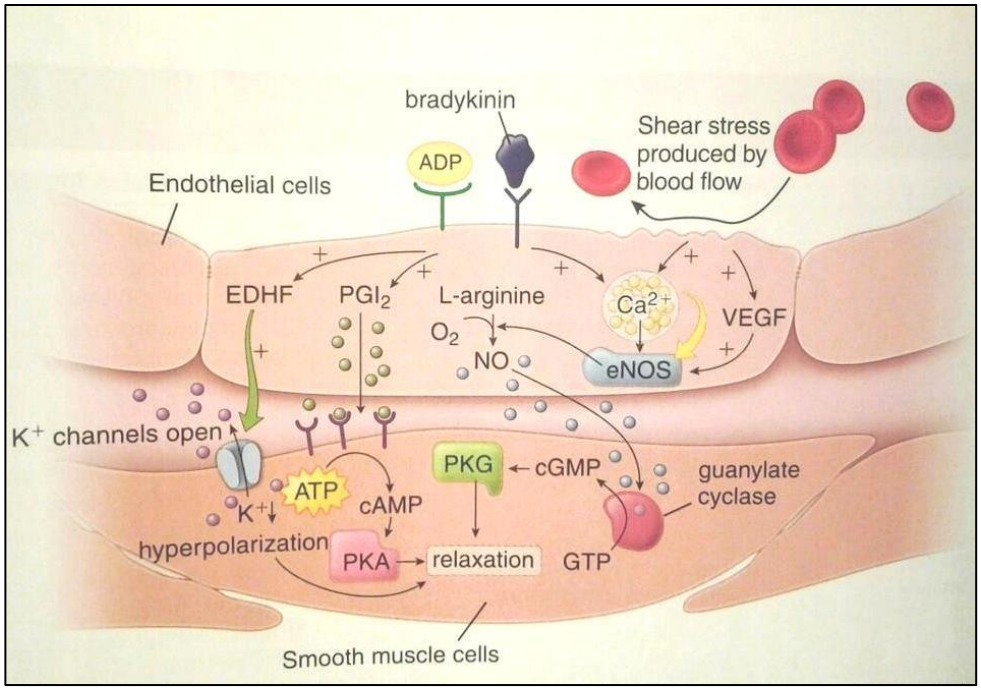


Fig. 1.3: *Molecular mechanisms of blood vessel vasodilation.*

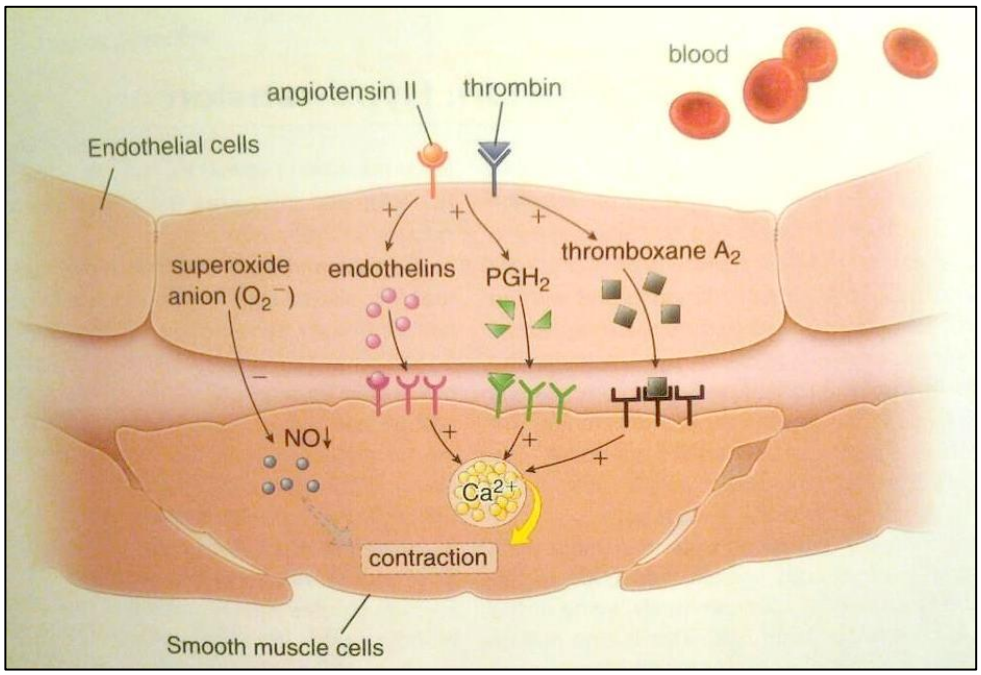


Fig. 1.4: *Molecular mechanisms of blood vessel vasoconstriction.*

The best characterized endothelial-derived autacoids are the vasodilators nitric oxide (NO) and prostacyclin (PGI_2), and the vasoconstrictor peptide endothelin 1 (ET-1). More recently, endothelium-derived hyperpolarizing factor (EDHF) and reactive oxygen species (ROS) showed vasorelaxant effect as well. Both NO and PGI_2 contributes to vessel dilation with two different mechanisms: NO induces smooth muscle relaxation by activating soluble guanylyl cyclase (sGC). This increases intracellular concentration of cyclic guanosine monophosphate (cGMP), which in turn, activates the G kinase and reduces the intracellular

concentration of Ca^{2+} ($[Ca^{2+}]_i$) in smooth muscle and results in relaxation. PGI_2 binds to receptors on the smooth muscles; stimulates cAMP-activated protein kinase A (PKA), which in turn phosphorylates myosin light chain kinase (MLCK); and prevents activation of the calcium-calmodulin complex. In addition, EDHF acts on Ca^{2+}-dependent potassium channels causing hyperpolarization of smooth muscle cells and their relaxation. On the other hand, ET-1, and other members of endothelin family (ET-2 and ET-3), is the most potent vasoconstrictors, which interacts with its ETA receptor on vascular smooth muscles. The vasoconstriction reduces the luminal diameter of these vessels and increases vascular resistance; consequently systemic blood pressure is also increased.

1.2.2 The L-arginine/NO/cGMP/PKG pathway in vascular tissue

The endothelial-derived relaxing factor (EDRF) was historically one of the early compounds discovered in the endothelial cells, that was causing relaxation of blood vessels. For years, researchers had difficulty characterizing EDRF chemically. It is now known that

most of the vascular effects of EDRF can be attributed to nitric oxide (NO) and its related compounds, which are released by endothelial cells in arteries, blood capillaries, and even lymphatic capillaries. Since then, it has been shown that NO is a gaseous signal molecule of key importance for the cardiovascular system and the brain [197]. NO, which is unstable and decomposes within ten seconds, regulates a variety of cellular functions by diffusion from its originating cell to surrounding cells. It regulates the blood vessel diameter, because it acts as endogenous vasodilatatory gas continuously synthesized in ECs by endothelial nitric oxide synthase (eNOS or NOS III). In response to receptor-dependent agonists (bradykinin, acetylcholine, ATP) and physicochemical stimuli (shear, stretch), nitric oxide synthase catalyzes the oxidation of one of the terminal guanidine groups of L-arginine to produce NO, with the stoichiometric formation of L-citrulline (fig. 1.3) [198]. Molecular cloning and sequence analysis has led to the identification of three broad categories of NOS isoenzymes, which are: neuronal NOS (nNOS or NOS I), inducible (iNOS or NOS II), and endothelial (eNOS or NOS III). The nNOS isoform has a

widespread distribution in both the central and peripheral nervous system, as well as in a number of non-neuronal cell types. The iNOS isoform was initially identified in activated macrophages, but has since been found to be expressed by virtually all nucleated cells if they are subjected to the appropriate stimuli. The endothelial NOS isoform was first identified in vascular endothelial cells, and as such has a ubiquitous distribution, although its expression has also been demonstrated in several non-endothelial cell types.

Endothelial-derived NO diffuses to the adjacent smooth muscle, where it interacts with different receptor molecules, of which the soluble guanylyl cyclase (sGC) is the best characterized and presumably most important one with regard to control of vessel tone and smooth muscle proliferation. sGC contains heme iron in the ferrous (II) state, which is required for the binding and interaction with NO to cause enzyme activation. Upon binding with NO, cyclic guanosine monophosphate (cGMP) formation increases substantially. cGMP activates the cGMP-dependent kinase I which in turn increases the open probability of Ca^{2+}-

activated K^+ (B_K)-channels, thereby inducing a hyperpolarization of the smooth muscle cells and inhibition of agonist-induced Ca^{2+} influx. In addition, activated cGK-Iβ phosphorylates the inositol trisphosphate (IP_3)-receptor-associated G-kinase substrate (IRAG), thereby inhibiting agonist-induced Ca^{2+} release and smooth muscle contraction. Another cGK-I substrate found in many cell types is the 46/50 kDa vasodilator-stimulated phosphoprotein (VASP). cGK-I phosphorylates VASP specifically at serine 239, and this reaction can be exploited as a biochemical monitor for the integrity and activity of the NO-cGMP pathway [199].

1.2.3 Role of PKG in the regulation vascular tone

During the past few years, it has become clear that cGMP kinases regulate some switches that control essential cellular circuits. The cGMP-dependent protein kinases (cGKs) belong to the family of serine/threonine kinases and are present in a variety of eukaryotes ranging from the unicellular organism *Paramecium* to *Homo sapiens* [266, 267]. Mammals have two cGK genes, *prkg1* and *prkg2*, that encode cGKI and cGKII. The N terminus (the first 90–100 residues) of cGKI is encoded by two alternatively spliced

exons that produce the isoforms cGKIα and cGKIβ. The enzymes have a rod-like structure and are activated at submicromolar to micromolar concentrations of cGMP [268, 269]. They are composed of three functional domains: an N-terminal (A) domain, a regulatory (R) domain, and a catalytic (C) domain. The regulatory domain contains two tandem cGMP-binding sites that interact allosterically and bind cGMP with high and low affinity. Occupation of both binding sites induces a large change in secondary structure (270) to yield a more elongated molecule (271, 272). The catalytic domain contains the MgATP- and peptide-binding pockets. This latter domain catalyses the transfer of the γ phosphate from ATP to a serine/threonine residue of the target protein. The N terminus has three functions: (1) dimerization: cGKs are homodimers that are held together by a leucine zipper present in the N terminus; (2) activation/inhibition: binding of cGMP to both sites in the R domain induces a conformational change that releases the inhibition of the catalytic center by the N terminus and allows phosphorylation of substrate proteins. Activation of heterophosphorylation may be preceded by

autophosphorylation of the N terminus. Autophosphorylation increases the spontaneous activity of cGKI and cGKII [273–276] and is initiated by the binding of low cGMP concentrations to the high affinity site of cGKI [277, 278]; (3) targeting: the enzymes are targeted to different subcellular localizations by their N termini. Together with the Iα isozyme, the Iβ isozyme is highly expressed in smooth muscle, including uterus, vessels, intestine, and trachea [279]. Platelets, hippocampal neurons, and olfactory bulb neurons contain mainly the Iβ isozyme [279]. The Iα and Iβ cGKs are soluble enzymes and interact with different proteins through their distinct N termini. The main role of cGKI is the control of smooth muscle tone. NO and other NO-generating organic nitrates stimulate the soluble guanylyl cyclase, increase cGMP levels, and thereby relax vascular and other smooth muscles. The relaxing effect of these compounds involves the activation of cGKI as shown by deleting the *cGKI* gene in mice [280]. cGKI-deficient mice are hypertensive at 4-6 weeks. In these mice, precontracted smooth muscle strips are not relaxed by acetylcholine or NO, although both compounds increase smooth

muscle cGMP levels. Deletion of cGKI did not affect cAMP-induced relaxation of vascular smooth muscle supporting the conclusion that cAMP and cGMP use different signal pathways in smooth muscle.

Smooth muscle tone is regulated by the rise and fall of $[Ca^{2+}]_i$. Contraction is initiated by receptor-mediated generation of IP_3 that releases Ca^{2+} from intracellular stores followed by an influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels (281, 282). The rise in $[Ca^{2+}]_i$ initiates contraction by activation of the Ca^{2+}/calmodulin-dependent MLCK, which phosphorylates RLC and, consequently, activates myosin ATPase. A decrease in $[Ca^{2+}]_i$ inactivates MLCK and induces dephosphorylation of RLC by MLCP. Smooth muscle contraction is modulated also at constant $[Ca^{2+}]_i$ by changing the sensitivity of contraction to $[Ca^{2+}]$ [283]. cGKI interferes both with the increase in $[Ca^{2+}]_i$ and with the Ca^{2+} sensitivity at several levels (Fig. 1.5).

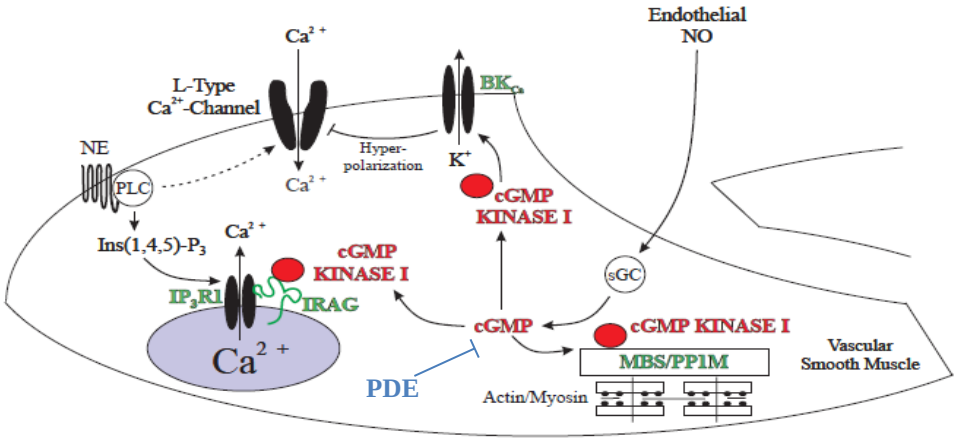


Fig. 1.5: *Mechanisms leading to relaxation of smooth muscle by cGMP kinase-I.*

Some evidence indicates that cGKIα inhibits the generation of IP_3 in smooth muscle and Chinese hamster ovary cells [289] by interfering with the inactivation of $G\alpha_q$ [290] or the activation of phospholipase Cβ3 [291]. However, the biological significance of these findings remains unclear because a direct correlation between the cGMP-dependent phosphorylation of a target protein and decreased IP_3 synthesis has not been established. An additional mechanism that lowers $[Ca^{2+}]_i$ is the direct phosphorylation of Ca^{2+}-activated maxi-K^+ (BK_{Ca}) channels by cGKI. Phosphorylation increased channel opening at constant $[Ca^{2+}]_i$ [284, 285]. The cGKI isozyme that phosphorylates BK_{Ca} channels is not known. Opening of BK_{Ca} channels hyperpolarizes the membrane and

closes a number of channels, including L-type calcium channels, thereby reducing Ca^{2+} influx. This mechanism contributes to the regulation of vascular tone, as shown in wild type and cGKI deficient mice [292]. The cGKI-dependent regulation of BK_{Ca} channels depended on specific splice variants [286] and may be mediated indirectly by cGKI-dependent activation of an associated protein phosphatase 2A [287, 288].

1.2.4 Regulation of NO production

The nitric oxide synthase (NOS) present in vascular endothelial cells is a multi-domain enzyme consisting of an N-terminal oxygenase domain (amino acids 1–491) that contains binding sites for heme, L-arginine (Glu361) [395] and tetrahydrobiopterin (H_4 B), and a reductase domain (amino acids 492–1205) containing binding sites for FMN, FAD, NADPH and calmodulin (CaM) [396].

The eNOS is constitutively expressed and is generally regulated by Ca^{2+}/CaM as well as by phosphorylation [397], which activates eNOS. Conversely, the protein Caveolin-1 (Cav1) inhibits enzyme

activity by antagonizing CaM binding. Although eNOS is constitutive enzyme, numerous physical (receptor-independent agonists) and chemical (receptor-independent agonists) stimuli affect eNOS levels *in vitro* and *in vivo*. Application of a receptor-dependent agonist to endothelial cells is followed by a burst of NO production (15–20 times the basal production) that peaks after 30–60 seconds of stimulation and, generally, returns to baseline within a few minutes, despite the continuous presence of the agonist. Such responses are accompanied by similar changes in $[Ca^{2+}]_i$ and are abolished by either the removal or chelation of extracellular Ca^{2+}. By contrast, following the application of fluid shear stress to endothelial cells, NO production is enhanced twofold over basal values and is maintained as long as the stimulus is applied. Although the acute application of shear stress to endothelial cells maintained under static conditions does increase $[Ca^{2+}]_i$, this response is transient and generally lasts only a few seconds [200]. Such a short pulse of Ca^{2+} is unlikely to be the intracellular stimulus for the maintained generation of endothelium-derived NO. However, other signal transduction cascades are activated by

the mechanical stimulation of endothelial cells. Perhaps, the most important of these for the regulation of NO production results in activation of phosphatidylinositol 3-kinase and, subsequently, serine kinases Akt and protein kinase A, which phosphorylate a serine residue in the reductase domain of eNOS (Ser1177 of human eNOS and Ser1179 of bovine eNOS) and increase eNOS activity [201, 202]. Although this process, which also involves the formation of an eNOS signaling complex and the association of heat shock protein 90 with eNOS [203], has been referred to as the 'Ca^{2+}-independent activation of eNOS', chelation of intracellular Ca^{2+} also abolishes the increase in eNOS activity induced by shear stress. This indicates that the increase in NO production is still, strictly speaking, Ca^{2+}-dependent but that eNOS can be activated at resting $[Ca^{2+}]_i$ [201]. Several additional eNOS phosphorylation sites (Ser114, Thr495 and Ser633) might regulate eNOS activity and/or localization but it is unclear whether mechanical stimulation of endothelial cells affects phosphorylation of these residues. Wall shear stress not only increases the activity of eNOS but also modulates its expression by enhancing transcription and

prolonging mRNA stability [204]. In general, ‘arterial levels’ of laminar shear stress (4–20 dynes cm^2) are reported to increase expression of eNOS whereas either low or oscillatory shear stresses do the opposite [205, 206]. Unidirectional and oscillatory flow differentially affect eNOS expression; unidirectional shear stress increases expression of eNOS mRNA via a transcriptional mechanism whereas oscillatory shear stress appears to control eNOS expression through posttranscriptional regulatory events [205]. By contrast, cyclic stretch has little or no effect on eNOS expression. Thus, it appears that endothelial cells can discriminate specific hemodynamic forces and translate them into distinct responses.

1.2.5 How the endothelium senses mechanical stimuli

Physiologically the most relevant determinants for the continuous activation of the endothelium are hemodynamic stimuli. Particularly important are the viscous drag that is generated at the luminal surface of endothelial cells by the streaming blood (wall shear stress) and the cyclic strain of the vascular wall that results from the pulsatile changes in blood pressure.

The first step in this process is thought to be activation of a mechanoreceptor that senses changes in membrane tension on the luminal surface of the endothelial cells. Endothelial cells contain mechanosensitive ion channels, that respond primarily to changes in membrane tension and renders them appropriate transducers for forces derived from osmotic or hydraulic gradients and shear stress.

The open probability of these, mainly cation-selective, channels might be modulated by membrane potential and various ligands. Stress-activated ion channels might also be activated by virtue of cytoskeletal interconnections, rather than directly through the plasma membrane [207]. There are reports that disruption of the actin cytoskeleton attenuates the generation of NO induced by shear stress [208]. The intermediate filament vimentin might have a central role in this because it is rapidly displaced in response to the application of shear stress [209], and flow-induced dilatation is markedly attenuated in mice that lack the gene that encodes vimentin [210]. These and similar observations indicate that rather than there being a specific mechanoreceptor expressed on

endothelial cells, the entire cytoskeleton might act as a mechanoreceptor. Indeed, endothelial cells contain a complex network of microtubules, intermediate filaments and actin fibers that traverse the cell. These are tacked down to the basement membrane at cell–cell contacts and end in caveolae and characteristic adhesion complexes. Focal adhesion points, caveolae and cell–cell contacts are all signaling ‘hot spots’ that contain a vast number of signaling molecules. Such a construction (tensegrity architecture) within the cell means that there is tension across the entire endothelial cytoskeleton. Shear stress or stretch at the cell surface elicits a redistribution of these forces, which is translated into a chemical signal at the end of a filament/fiber [211]. Signaling molecules, including integrins within caveolae [212], the lateral zone of cell–cell adhesion points [213] and focal adhesion contacts [214] are activated rapidly by fluid shear stress and cyclic stretch. Forces can also be directly transmitted from the cell surface to the nucleus across filaments that interconnect physically, which raises the possibility that mechanical stimuli might be passed on to the nucleus either in the absence of, or

simultaneously with, mechano-chemical signaling processes [215]. There might also be cross-talk between mechanosensitive pathways and growth factor receptors because the application of fluid shear stress to endothelial cells enhances the tyrosine phosphorylation of the receptors for vascular endothelial growth factor [216] and insulin [217]. Shear stress is also reported to induce the release of vasoactive compounds, such as ATP and bradykinin that activate endothelial cells and enhance autacoid production by binding to their respective receptors [218]. Thus, in endothelial cells mechanotransduction does not occur via a single pathway; rather it reflects the integrated response of multiple signaling networks that are spatially organized throughout the cell.

1.3 The vascular smooth muscle cells (VSMCs)

Vascular smooth muscle cells (VSMCs) are one of the major components of blood vessels responsible for the maintenance of vessels structure and functions [219]. VSMCs maintain contractile tone by a highly organized architecture of contractile/cytoskeleton proteins and associated regulatory components within the cell cytoplasm and establish distensibility by synthesis, secretion, and

organization of extracellular matrix (ECM) components with elastic recoil and resilience properties [220]. VSMCs within the vascular continuum have the ability to adapt expression of proteins involved in contraction and ECM synthesis according to intrinsic and extrinsic cues during different developmental stages and in disease or response to injury. This ability is due to a phenomenon known as VSMC phenotypic modulation and is a major feature that distinguishes VSMCs from terminally differentiated cells [221]. Vascular smooth muscle cell phenotypic modulation is the ability to switch phenotypic characteristics from a migratory synthetic phenotype in embryonic tissue patterning to a quiescent, contractile phenotype in maintenance of vascular tone in mature vessels. Importantly, during vascular remodeling in response to injury VSMCs can switch back to a synthetic phenotype characterized by increased VSMC proliferation and ECM synthesis. Although the ability to switch phenotypes may have evolved as an adaptive survival mechanism for VSMCs to adjust physiological responses due to changing hemodynamic demands or to repair damage after vascular injury, phenotypic modulation has

important implications both during development and during vascular disease [221].

Acute regulation of vascular diameter depends on the activation/deactivation of the contractile machinery involving actin-myosin interaction in VSMCs. In fact, vascular smooth muscle contraction is an active process wherein the myosin light chains must be phosphorylated before shortening can occur. This phosphorylation is performed by myosin light chain kinase (MLCK), which is activated by Calcium-Calmodulin (Ca-CAM). Tension maintenance in vascular smooth muscle is a graded phenomenon in that there is no twitch tension and the vessels can generate and maintain tension along their entire length. Vascular smooth muscle has a relatively unique ability to maintain tension with relatively low ATP consumption using a mechanism called Latch [222]. The latch mechanism is where the vessel's cross bridges remain attached under tension but without being phosphorylated. This results in a slowly cycling bridge that consumes about seven fold less ATP to maintain tension at a constant length. Therefore, vascular smooth muscle is very

economical when it maintains tension because of a low cost for maintaining latch bridges. The contractile apparatus and signaling mechanisms in the vascular smooth muscle generally work to produce slow and maintained responses to the signals the vessels receives. Importantly the phosphorylation mechanisms for tension generation are also modulated by thin filament proteins of caldesmon and calponin. These regulatory proteins and mechanisms make it such that tension in vascular smooth muscle is graded and highly controlled by several factors mediating length and rate of shortening. It is when these control mechanisms go awry that vascular pathologies occur.

Normally, arterial SMCs of adults are contractile and are not very responsive to growth factors or growth regulatory molecules on cell migration and proliferation [223]. In response to altered mechanical stress, numerous contractile proteins that exist in normal adult arterial walls gradually disappear, such as smooth muscle myosin heavy chain isoforms, α-actin, h-caldesmon, and calponin, while non-muscle myosin heavy chain, which mainly expresses in fetal vessel walls, re-expresses in activated VSMCs

[223-225]. As a result, this modulation leads to a prominent structural reorganization with loss of myofilaments and formation of an extensive endoplasmic reticulum and a large Golgi complex termed synthetic phenotype. Thus, the cells lose their ability to contract, increase their secretion of proteins, and become more responsive to growth factors produced via autocrine and paracrine of medial SMCs in response to increased mechanical stress (high blood pressure), which, in turn, further stimulates medial SMC hypertrophy and /or intimal hyperplasia of the arterial walls [223, 226, 227], leading to a narrowing of lumen diameter.

Indeed, the lumen diameter of resistance arteries is governed not only by the magnitude of vasoconstriction, but also by the structural characteristics of the vessel wall, influenced by vascular remodeling processes [228]. At the molecular and cellular levels remodeling involves changes in cytoskeletal organization, cell-to-cell connections, and altered growth/apoptosis, senescence, calcification and rearrangement of VSMCs [229, 230]. At the extracellular level, remodeling is influenced by changes in matrix protein composition and reorganization of proteoglycans, collagens

(type I and III), and fibronectin, which provide tensile strength, and elastin, responsible for vascular elasticity [231]. These sub-cellular, cellular, and extracellular matrix (ECM) events manifest as structural changes with modifications in lumen diameter, wall thickness, and media and adventitia cross-sectional areas, processes that define remodeling. With respect to resistance arteries, the most widely used classification of vascular remodeling is that described by Mulvany et al. [232], where changes in the passive luminal diameter may be increased (outward) or decreased (inward) and media mass (cross-sectional area) may be increased, unchanged or reduced (hypertrophic, eutrophic, hypotrophic respectively) [232, 233] (Fig. 1.6).

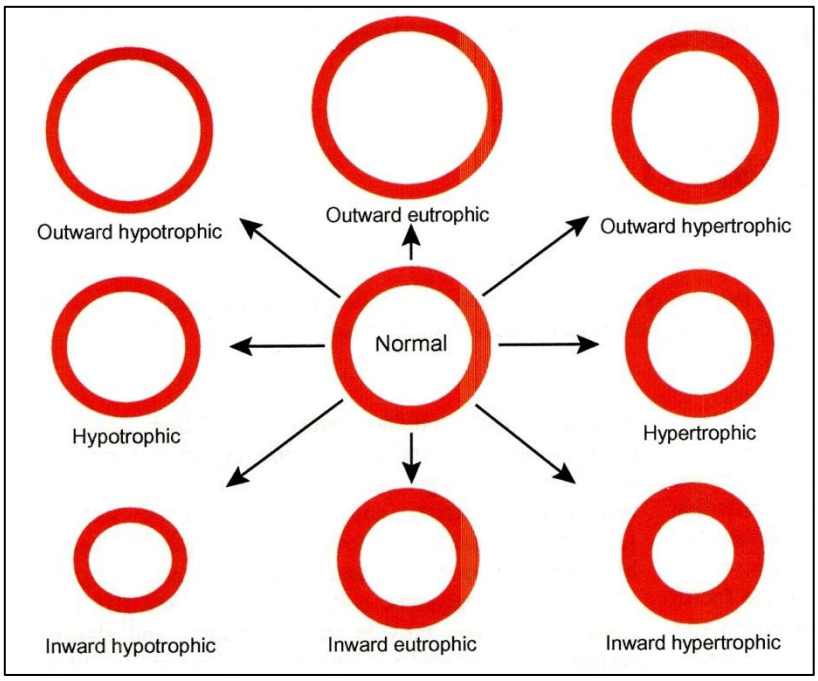


Fig. 1.6: Classification of vessel remodeling: *changes in diameter (inward) and cross-sectional area (outward).*

Whether increased pressure itself or other factors are responsible for the initiation of vascular remodeling remains unclear, but the endothelium probably plays an important role as it a sensor of hemodynamic and humoral factors and is a moderator of signals to underlying VSMCs critically involved in the remodeling process [234]. Remodeling allows arteries to withstand an increased pressure load and under physiological conditions (e.g. aging and exercise) is adaptive. Pathological remodeling occurs when the adaptive process is overwhelmed, resulting in rigid, stiff, and

poorly compliant vessels, typically observed in hypertension. Molecular and cellular mechanisms implicated in changes in vasomotor tone and structural remodeling in hypertension involve multiple cell types, complex signaling pathways, and multiple local and circulating factors.

1.4 Regulation of vascular function and structure in arterial hypertension

Smooth muscle cells constitute the bulk of the arterial wall, playing a key function in vascular resistance and blood flow. Basal vascular tone and contractility are increased in hypertension. The contractile machinery of vascular smooth muscle, actin and myosin, is activated in a Ca^{2+}-dependent and Ca^{2+}-independent manner. The key event in VSMC excitation-contraction coupling is an increase in intracellular free $[Ca^{2+}]_i$ in response to agonist-induced activation of receptors coupled to phospholipase C (PLC), responses that are up-regulated in experimental and human hypertension [228, 234-236]. In addition to VSMC-derived factors regulating tone, endothelium-derived factors, such as prostacyclin,

PGH_2, and thromboxane A_2 act as endothelium-derived contracting factors (EDCF) that counteract the vasodilator effect of NO [237]. Increased generation of EDCFs has been demonstrated in human essential hypertension [238, 239] and in spontaneously hypertensive rats (SHR) [237], and further contributes to increased vascular tone in hypertension.

Smooth muscle myosin can also be phosphorylated in a Ca^{2+}-independent manner by additional kinase like Rho kinase (ROCK) [240], integrin-linked kinase (ILK) [241] and zipper-interacting protein kinase (ZIPK) [242]. These Ca^{2+}-independent processes influence contraction by increasing Ca^{2+} sensitization and by actin filament remodeling. Rho kinase (ROCK1 and ROCK2) are serine/threonine kinases and are down-stream effectors of the small GTPase RhoA. RhoA is abundantly expressed in VSMC and is rapidly activated by vascoconstrictors, such as Ang II via the $G_{12/13}$ family of G proteins and G_q [243]. RhoGEFs, which catalyzes exchange of GDP for GTP on RhoA, are sensitive to $G_{12/13}$ such as Arhgef11 (PDZ-RhoGEF), and hence play an important role in RhoA activation [244]. Increased RhoA/Rho

kinase activity leads to decreased MLCP activation and consequent sustained vasoconstriction and blood pressure elevation.

Ang II-induced hypertension in rodents exhibits increased vascular RhoA/Rho kinase activation, without changes in expression [245]. This is associated with increased activity of Arhgef1, implicated to be important in RhoA hyperactivation, vasoconstriction, and hypertension [246].

In addition to the Ca^{2+}-dependent and –independent modulation of MLC20 phosphorylation, changes in organization of actin filaments, intermediate filaments, and microtubules play an important role in the acute phase of VSMC contraction. Increased polymerization of actin, tyrosine phosphorylation of paxillin, activation of small GTP-binding proteins Rho and Cdc42 and conformational changes in focal adhesion sites result in stiffening and reorganization of the cytoskeleton [230]. This dynamic arrangement of the actin cytoskeleton is key in maintaining vascular tone and plasticity, especially important in the regulation of vascular diameter related to pressure-dependent myogenic tone.

In hypertension, resistance arteries undergo vascular remodeling characterized by reduced vascular lumen with increased media thickness [229, 232, 247]. Myogenic tone, the intrinsic ability of vessels to constrict in response to increased intraluminal pressure, contributes to structural alterations within the arterial wall. On the other hand, structural narrowing of the lumen may amplify vasoconstriction. Eutrophic vascular remodeling, characterized by reduced outer diameter and lumen with no change in media mass and cross-sectional area [247, 248], is observed in patients with mild-moderate hypertension. With chronic vasoconstriction and longstanding or severe hypertension, blood vessels develop hypertrophy in response to increased wall stress. Chronic vasoconstriction associated with deposition of extracellular matrix which characterizes hypertensive vascular remodeling may lead to a smaller lumen as the constricted state becomes embedded in the newly deposited extracellular matrix [248]. Changes in the interaction between extracellular matrix components and integrins on the cell membrane of smooth muscle cells may also contribute to the remodeling of arteries in hypertension [249]. Other

mechanisms that may participate include inward growth encroaching on the lumen associated with apoptosis of cells in the periphery of the vessel wall. Some studies have implicated tissue transglutaminases that participate in the interactions of fibrillar components in extracellular matrix (ECM) that attach smooth muscle cells and play a role in signal transduction. VSMC growth may predominate over apoptosis, and in this scenario, remodeling may be hypertrophic [247-250]. This type of vascular remodeling is characterized by an increased media cross section and media to lumen ratio (M/L). The process of remodeling is dynamic and eutrophic and hypertrophic remodeling may occur simultaneously in different vascular beds. Hypertrophic remodeling of resistance arteries is more common in renovascular hypertension, diabetes, acromegaly, and hyperaldosteronism [251-253]. Inflammation and matrix deposition are critically involved in these processes and activation of the RAS also plays a role in the development of hypertension-induced vascular remodeling [254].

1.4.1 Endothelial dysfunction in hypertension

By definition, endothelial dysfunction is a functional and reversible alteration of endothelial cells, resulting from impairment in nitric oxide availability. Endothelial cells normally regulate vascular tone by releasing relaxing and constricting factors such as NO, arachidonic acid metabolites, ROS, and vasoactive agents. They also produce EDHF that induce endothelium-dependent relaxation through hyperpolarization of underlying VSMCs independently of NO. EDHF-mediated responses are important in hypertension, where they provide a vasorelaxation reserve for endothelial dysfunction due to decreased NO bioavibility [237]. Endothelial dysfunction is a hallmark of hypertension and may reflect the premature aging of the intima exposed to chronic blood pressure increase. It is characterized by impaired vasomotor responses, VSMC proliferation and migration, ECM, protein deposition, platelet activation, vascular permeability, and a pro-inflammatory and prothrombotic phenotype [254-256]. Of the many factors important in the protection of the endothelium are endothelial progenitor cells (EPCs). EPCs are bone-marrow-

derived cells capable of developing into mature endothelial cells [257]. They contribute to vascular homeostasis through direct cell-to-cell contact and through autocrine and paracrine actions. EPCs mobilize out of bone marrow in response to peripheral tissue hypoxia and injury and release EPC-activation factors, such as hypoxia-inducible factor-1 (HIF-1), VEGF, erythropoietin and NO to facilitate endothelial healing, regeneration and reendothelialization after vascular damage [258]. The multidrug resistance-related protein-1 (MRP1) has been identified as a negative regulator of EPC function and survival and MRP1 inhibition has been suggested as a novel strategy to increase EPC survival [259]. The number of circulating EPCs may reflect endothelial function since decreased numbers are associated with reduced arterial elasticity, and decreased endothelial integrity [260]. Circulating EPCs are reduced in hypertension [259, 260]. Studied in cultured EPCs suggest that ROS are involved in Ang II-mediated EPC senescence [261]. In addition to EPCs, microparticles have been considered as biomarkers of vascular status. Microparticles are tiny fragments of cellular membranes

that are generated from activated or apoptotic cells [262]. Microparticles circulate in healthy individuals and their levels increase in cardiovascular and athero-thrombotic diseases [262]. In patients with hypertension plasma levels of microparticles correlated with systolic and diastolic blood pressure [263]. In patients with diabetes, endothelial microparticle levels are a strong predictor of myocardial infarction and correlate with arterial stiffness and endothelium-mediated vasodilation [264]. They also relate to the extent and severity of coronary stenosis in patients with coronary syndromes [264]. *In vitro* endothelial microparticles are released in response to inflammatory stimuli such as TNF-α, thrombin, uremic toxins, ROS, and PAI-1, which are increased in hypertension. Although the precise mechanism leading to *in vivo* generation of microparticles is not fully understood, there is evidence that eNOS uncoupling and low shear stress [265], which are features of endothelial dysfunction in hypertension, enhance their production. They in turn may contribute to some extent to endothelial dysfunction through ROS production [263-265]. The exact role of microparticles in vascular remodeling awaits

clarification, but it is possible that they may be more than biomarkers of endothelial dysfunction. They interfere with target cell responses by transferring chemokines and adhesion molecules to endothelial cells leading to monocyte adhesion and hence have the potential to directly contribute to vascular injury in hypertension. Endothelial dysfunction is also characterized by a phenotypic switch and increased adhesiveness for vascular cells [169]. In this respect, there are now several studies demonstrating the release of adhesion molecules from activated endothelium in hypertension. Examples are von Willebrand factor (vWF), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin [170]. Among them, vWF is probably the most extensively studied endothelial product. Pedrinelli et al. demonstrated the presence of increased vWF levels in patients with essential hypertension and microalbuminuria, suggesting that a dysfunctional glomerular endothelium may cause reduced renal function [171], as also indicated by the demonstration of the importance of NO in long term regulation of renal hemodynamics and renal excretory

function [172]. Furthermore, vWF levels correlate well with flow-mediated dilatation (FMD) and both methods of assessment of endothelial function correlate with 10-year cardiovascular risk using the Framingham equation [173]. While vWF is an established marker of endothelial damage/dysfunction, other markers may reflect distinct functions of the endothelium. ICAM-1, for example, reflects a disruption of the equilibrium between pro- and anti-inflammatory mechanisms, and it has been suggested that sICAM-1 and FMD are both related to the estimated risk of coronary heart disease, independently of each other [174]. Thus, biochemical and hemodynamic tests of endothelial function may give complementary information on the risk prediction of hypertensive patients. Plasma levels of soluble adhesion molecules (E-selectin, ICAM-1 and VCAM-1) were also shown to be elevated in hypertensive patients in comparison to normotensive controls [175-177], and even mild elevation of blood pressure may be sufficient to activate their expression [178]. In addition, Goonasekera et al. demonstrated that an isolated increase in plasma VCAM-1 in hypertensives, associated with raised levels of

the endogenous NO inhibitor asymmetric dimethyl arginine (ADMA), may signify a selective ‘‘non-inflammatory’’ endothelial activation triggered by endothelial NO synthase (NOS) inhibition [179]. E-selectin has been also proposed as a surrogate marker of endothelial dysfunction as elevated levels can be found in patients with hypertension [5]. However, no conclusive data on its predictive value are available at present.

Chapter II

The gasotransmitter role of H_2S

The past two decades have seen an increasing interest in the biology of endogenous gases. It is becoming clear that nitric oxide (NO) is not the only such biologically active gas, and that molecules such as carbon monoxide (CO) and hydrogen sulfide (H_2S), and perhaps others, are also important. Cells are constantly enveloped in an atmosphere containing many different gases, and it is not surprising that at least some of those gases have evolved over the ages into potent regulators of cell function. The latest addition to the list of biologically relevant gases is H_2S, once viewed solely as an environmental pollutant but now seen more and more as a biologically significant molecule in its own right. Compared with NO and CO, the study of the biology of H_2S is still in its infancy. Nevertheless, much research has already been devoted to a better understanding of the physiological and pathophysiological significance of this gas and the way in which it interacts with NO and CO. Here, I describe recent advances in the

understanding of the biology of H_2S, especially its involvement in the homeostasis of the cardiovascular system.

2.1 The concept of "gasotransmitter"

Signaling molecules come in all sizes and chemical dispositions, ranging from relatively large proteins, lipids, and peptides through biogenic amines and amino acids, to gaseous molecules. Variations in the properties of signaling molecules that depend upon their chemical nature are best exemplified among neurotransmitters [1]. Amine, amino acid, and peptide neurotransmitters share a number of features. All are stored in synaptic vesicles, so that with each nerve impulse only a small proportion of the stores is released, leaving a large safety net of reserve pools. Release involves exocytosis, in which the vesicle fuses with the plasma membrane to expel its contents. These neurotransmitters bind to receptor proteins on the external surface of adjacent membranes. Inactivation occurs by reuptake of neurotransmitters into the releasing nerve terminal or adjacent glia, by enzymatic degradation, or by simple diffusion away from the synapse.

The conventional concepts of intercellular communication has been overturned by the discovery small gaseous molecules are involved in any signaling process. For instance, a gaseous substance is not readily stored in vesicular structures and so must be resynthesized as needed. This implies that, rather than supply depending on regulation of exocytosis, the biosynthetic enzymes must be subject to extraordinarily nuanced regulatory mechanisms. Vehicles for intercellular communication are either electrical signals via gap junction or chemicals. The conventional doctrine about chemical-mediated communication claims that the binding of neurotransmitters or humoral factors to receptors located on the plasma membrane is the essential triggering event. The ligand–receptor interaction generates intracellular second messengers that relay and direct the extracellular signals to different intracellular destinations, resulting in modulated cellular activity. Instead of binding to plasma membrane receptors, gasotransmitters diffuse into adjacent cells to interact with their targets.

The Nobel Prize-winning discovery of NO in the 1990s sets the stage for recognizing a membrane receptor-independent signaling

mechanism, emphasizing the necessity to modify the conventional doctrine about cellular signal transduction. The resurgence of CO and the discovery of H_2S as important endogenous signaling gases strengthen and endorse the physiological importance of this group of gas molecules. To distinguish NO, CO, and H_2S from classical neurotransmitters and humoral factors while acknowledging their common natures, these endogenous gaseous transmitters have been defined as "gasotransmitters," gauged by the following criteria [2]:

1. They are small molecules of gas.
2. They are freely permeable to membranes. As such, their effects do not rely on the cognate membrane receptors, and they can have endocrine, paracrine, and autocrine effects.
3. They are endogenously and enzymatically generated and regulated.
4. They have well defined and specific functions at physiologically relevant concentrations.
5. Their cellular effects may or may not be mediated by second messengers, but should have specific cellular and molecular targets.

2.2 Toxicological and chemical features of H_2S gas molecule

Hydrogen sulfide (H_2S), a colorless gas with a strong odor of rotten eggs, was known for many decades only as a toxic environmental pollutant [5]. Since the first description of H_2S toxicity in 1713, most studies about this gas have been devoted to its toxic effects with little attention paid to its physiological function. In the literature several massive intoxications are reported and in the non-scientific literature poisoning by this gas is referred to as "sulphurated hydrogen" intoxications. These fatal episodes often occurred in sewers and swamps as the main biological source is represented by anaerobic bacterial digestion of organic substrates [7] but it is also produced through inorganic reactions in volcanic gases, natural gas and well waters [8]. Chemical and enzymatic transformation of the sulfur compounds of foodstuff such as mushrooms, garlic and onions are also responsible for H_2S production in human gut [9].

Early studies in dogs [185] and other mammals [186, 187], and more recently anecdotal information from human cases [188] have shown that H_2S toxicity is closely related with its concentration. For example, strong H_2S odor can be tolerated at 27 ppm, but eye irritation would already occur with 10 ppm H_2S. Acute exposure to 50-100 ppm leads to neurological disorders, skin symptoms, behavior changes, general deficits, respiratory symptoms and cardiovascular abnormalities. When ambient concentration H_2S concentration reaches 100-150ppm, the olfactory nerve is paralyzed after a few inhalations; furthermore it can leads to pulmonary edema with possibility of death up to 320-530ppm of H_2S. The molecular mechanisms underlying the toxicological effects of H_2S are mostly attributed to mitochondrial poisoning [11, 14]. Cytochrome c oxidase is the terminal enzyme in the electron transport chain in mitochondria that catalyzes the oxidation of ferrocytochrome c by molecular oxygen. By inhibiting cytochrome C oxidase, H_2S uncouples oxidative phosphorylation, and the production of ATP is subsequently decreased.

Chemically, H_2S is the sulfur analog to the water molecule and can be oxidized in a series of reactions to form sulfur dioxide (SO_2), sulfates such as sulfuric acid, and elemental sulfur [6]. In water or plasma, H_2S is a weak acid that dissociates as follows: $H_2S \leftrightarrow HS^- + H^+ \leftrightarrow S^{2-} + H^+$ [5]. In body fluids H_2S exists in three free states, H_2S gas, HS^-, and a trace of S^{2-}. It has been suggested that at pH 7.4 and at a mammalian body temperature of 37°C, the pK_{a1} for H_2S is 6.76 [12]; therefore when either sodium hydrosulfide (NaHS, commonly used as an H_2S donor since it dissociates to Na^+ and $HS^–$) or H_2S is dissolved in physiological solution (pH 7.4, 37.8°C), it will form approximately 18.5% H_2S and 81.5% hydrosulfide anion (HS^-), as predicted by the Henderson–Hasselbach equation [12]. There will be only a trace of S^{2-}. Because pk_{a2} is greater than 12, thus S^{2-} does not occur *in vivo* at substantial amounts [13]. Since all three species of sulfide are always present in aqueous solution, it has not been possible to determine which of these species is biologically active. Thus, the terminology of "the H_2S concentration" usually refers to the sum of H_2S, HS^-, and S^{2-}.

One important property of the H_2S gas is that it is a highly lipophilic molecule (its solubility in lipophilic solvents is fivefold greater than in water): it easily partitions into the hydrophobic core of the cell membrane similar to O_2, CO_2, NO and CO, and, thus rapidly diffuses into or out of cells [18]. Since the H_2S gas is also very volatile, it rapidly diffuses out of the blood into the lungs [19] or out of organ baths or cell culture media into the air. So, this aspect should be kept in mind for performing *in vitro* experiments. Recent interest in H_2S as an endogenously-produced biological mediator has broadened research in this field considerably beyond its toxicology [10]. In fact, as endogenous levels of H_2S were detected in the rat brain [15] and normal human post-mortem tissues at the end of the 1980s [16], a physiologic role for H_2S in mammalian tissues has been proposed. The physiological importance of H_2S became recognized in the last one and half decades; starting when Abe and Kimura reported in 1996 that hydrogen sulfide acts as a novel neuromodulator, by describing for the first time H_2S production in the hippocampus by an enzyme-regulated pathway and its ability to influence hippocampal long-

term potentiation [17]. Since these descriptions, H_2S has received increasing interest and is now regarded along with CO and NO as an endogenous gaseous mediator produced by regulated pathways and exerting physiological actions [10].

2.3 Endogenous biosynthesis of H_2S

The idea that H_2S could be of some physiological importance arose when it became clear that it was naturally produced in several organisms by constitutively expressed enzymes and that it is present in mammalian blood at nanomolar concentration [57]. The normal blood level of H_2S is reported to be between ~10 μM in Wistar [75] and ~50 μM in Sprague–Dawley rats [42]; in human, 10-100 μM H_2S in blood was reported [77]. Aside from circulating H_2S, a significant amount of H_2S is produced in various tissues. For instance, the physiological concentration of H_2S in brain tissue has been reported to be 50–160 μM [17, 41] and significant amounts of H_2S are generated from the vascular system as well [42]. Hydrogen sulfide is synthesized in mammalian cells by both endogenous enzymes and by non-enzymatic pathways. The latter

consists of reduction of elemental sulfur to H_2S using reducing equivalents obtained from the oxidation of glucose [20] (Fig. 2.1).

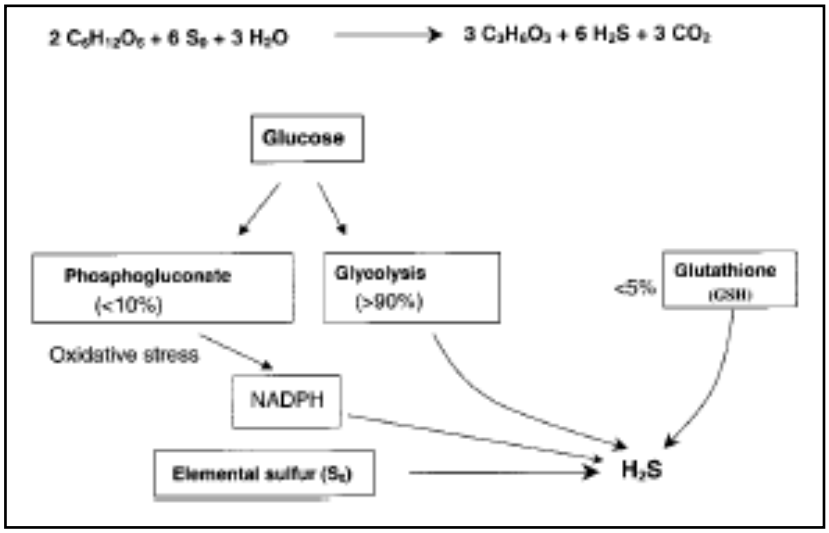


Fig. 2.1: *Endogenous non-enzymatic production of H_2S.*

All essential components of this pathway are present *in vivo*, including the supply of reducible sulfur [2]. However, the non-enzymatic pathway only accounts for a small portion of H_2S production [20]. Conversely, most emphasis has been placed on the enzymatic formation of H_2S. The main substrate for H_2S biosynthesis is L-cysteine, a sulphidrylated amino acid derived from the diet or synthesized from the L-methionine via cystathionine by the reverse transsulfuration pathway [22]. This pathway is believed to be the solely route for cysteine synthesis in

vertebrates and it contributes significantly to the intracellular L-cysteine pool [30]. L-cysteine may be metabolized to H_2S via the action of endogenous enzymes found in many of the body's cells.

Actually, it has been shown that four separate pathways are involved in the enzymatic production of H_2S (Fig. 2.2).

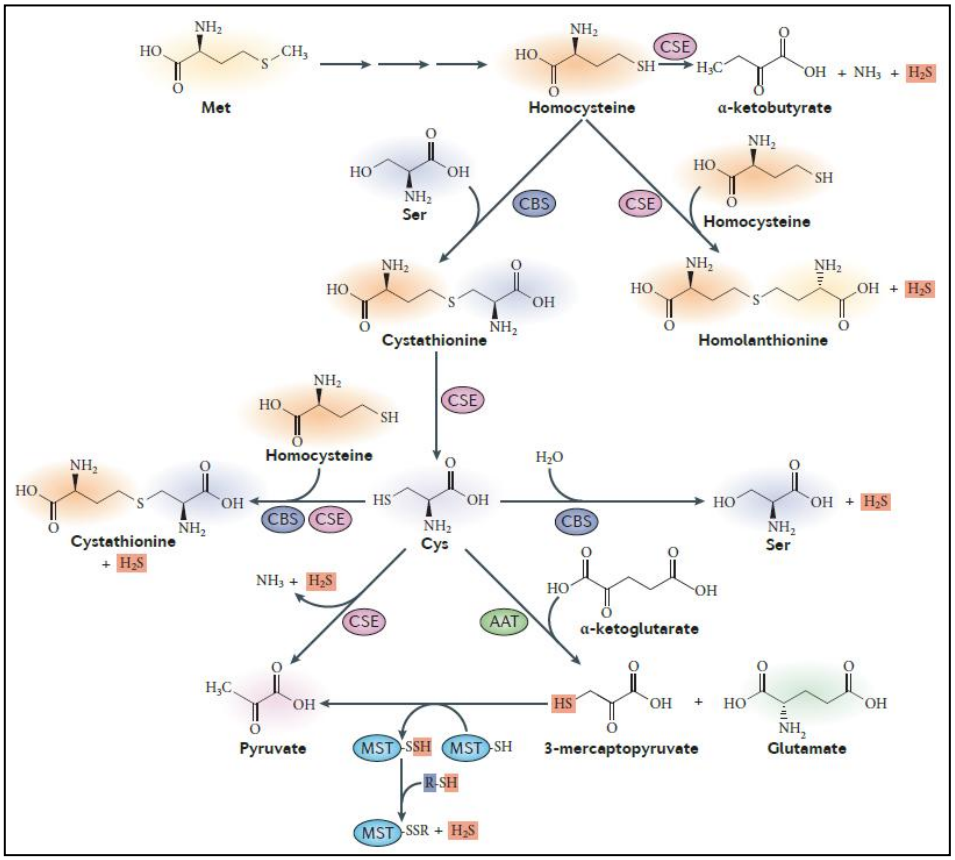


Fig. 2.2: *Endogenous enzymatic production of H_2S.*

In the first pathway, L-cysteine is hydrolyzed by cystathionine β-synthase (CBS) to produce equimolar amounts of H_2S and L-

serine. This reaction is reversible in the presence of low cysteine concentrations or during fetal development, when liver and brain CSE activity appears to be low [23]. The most well-known reaction catalyzed by CBS is the condensation of homocysteine and serine to form cystathionine in an irreversible reaction:

$$\text{L-serine + L-homocysteine} \rightarrow \text{L-cystathionine + } H_2O.$$

This is the first step in the biosynthesis of cysteine from methionine by reverse transsulfuration. CBS also catalyzes the condensation of cysteine with homocysteine to form cystathionine and H_2S [24].

In the second pathway, two L-cysteine molecules undergo dimerization to form L-cystine, which is transformed into thiocysteine, pyruvate and NH_3 by cystathionine γ-liase (CSE). The thiocysteine can then undergo one of two additional reactions to form H_2S: thus CSE may catalyze the reaction of thiocysteine with other thiol (R-SH) compounds (e.g. glutathione or cysteine) to form H_2S and CysSR [25, 26] or alternatively, thiocysteine may form L-cysteine and H_2S, possibly non-enzymatically [27].

In the third pathway, cysteine aminotransferase (CAT), in conjunction with 3-mercaptopyruvate sulfurtansferase (MST), generates H_2S. CAT catalyzes the reaction of L-cysteine with a ketoacid (e.g. α-ketoglutarate) to form 3-mercaptopyruvate and an aminoacid such as L-glutammate. The 3-mercaptopyruvate may then be desulphurated by MST to form H_2S and pyruvate, or, in the presence of sulphite (SO_3^{2-}), to form thiosulphate and pyruvate [29]. In the "thiosulphate cycle", the thiosulphate reacts with reduced glutathione (GSH) to produce H_2S, H_2SO_3 and oxidized glutathione (GSSG).

Recently, also a fourth enzyme, named cysteine lyase (CL), seems to be involved in H_2S production. It converts L-cysteine and sulphite to L-cysteate and H_2S. However, less is known about the real contribution of this enzyme in H_2S biosynthesis.

CBS, CSE and CAT use the cofactor pyridoxal 5'-phosphate (PLP), which is the active form of vitamin B_6, conversely to MST which activity is zinc-dependent. The expression of these enzymes has been detected in a broad variety of cell types, including those from the liver, kidney, heart, vasculature, brain, skin, fibroblasts

and lymphocytes. In some tissues, such as liver kidney, both CBS and CSE contribute to the local generation of H_2S, whereas in others, one enzyme predominates. For example, CSE is the main H_2S-generating enzyme in the cardiovascular system [42], meanwhile CBS is the predominant H_2S synthase in the brain and nervous system [17]. MST, along with CAT, contributes to H_2S formation in both the brain [190] and the vascular endothelium [53]. A recent paper has shown that MST and CAT can produce H_2S only in alkaline conditions and in presence of strong reducing agents [191]. Moreover, it has been demonstrated that a specific subcellular compartmentalization of these enzymes exists in the eukaryotic cells. Indeed, CBS and CSE are located exclusively in the cytosol [189], whereas CAT and MST are found both in mitochondria and in the cytosol [28]. Interestingly, recently it has been showed that CBS and CSE exist also as circulating enzymes. In fact, they are secreted by microvascular endothelial cells and hepatocytes, circulate as members of the plasma proteome, and actively produce hydrogen sulfide from homocysteine in human blood [73].

2.3.1 CBS and CSE: structure, regulation and tissue distribution

Although four different enzymatic pathways lead to H_2S biosynthesis, CBS and CSE appear to be responsible for the majority of the endogenous production of H_2S in mammalian tissues [28].

CBS is a homotetramer consisting of 63-kDa subunits; each subunit comprises 551 amino acid residues [31]. The NH_2 terminal of CBS contains the binding sites for both PLP and heme. It is not clear the functional role of heme domain in the catalytic activity of CBS, but it seems that its deletion renders CBS insensitive to oxidative stress. As such, a redox sensor role is suggested for this heme binding domain [32]. The PLP binding domain is considered to be the catalytic domain, and it is deep in the heme domain. The COOH-terminus of CBS contains a regulatory domain of ~140 residues, playing an autoinhibitory role for the activity of CBS. Binding of S-adenosyl-L-methione (AdoMet or SAM, an endogenous methyl donor) to this domain markedly activates CBS [33]. Deletion of the regulatory domain constitutively activates

CBS [30]. This regulatory domain is important for maintaining the tetrameric state of the protein. Then calmodulin binding consensus sequence (19 aminoacids) has also been identified in CBS at its COOH-terminal domain. After calcium-activated calmodulin binds to this consensus sequence, the catalytic domain of CBS will be opened and the enzyme becomes active [22]. Furthermore, the activity of CBS is regulated, presumably at the transcriptional level, by glucocorticoid and insulin, which stimulate and inhibit gene expression, respectively [34]. Also sex hormones seem to regulate brain H_2S levels. Since male brains contain much more testosterone than female brains at each age, the involvement of testosterone in H_2S production has been suggested. In agreement with this hypothesis, Eto and Kimura reported that the application of testosterone to female mice increases H_2S and SAM in the brain, almost reaching the levels of males [47]. Conversely, castration of male mice decreases testosterone and SAM levels as well as H_2S formation [47]. Binding of NO or CO to the heme pocket of CBS also appears to modulate its catalytic activity [35]. As mentioned above, CBS is mainly localized in the brain as the

primary physiological source of H_2S in the central nervous system, especially in the hippocampus, cerebellum, cerebral cortex and brainstem, and its activity is 30-fold greater than CSE [17]. Abe and Kimura showed that the production of H_2S from L-cysteine by rat brain homogenates is inhibited in the presence of CBS inhibitors such as hydroxylamine or aminooxyacetic acid (AOAA), which are reversible and non-selective inhibitors of CBS, because they are inhibitors of heme-containing protein and aminotransferase respectively [36, 37]. Later, it was showed that CBS is preferentially expressed in astrocytes rather than in neurons [38]. In other tissues such as cardiovascular and respiratory system, testes, adrenal and spleen from rats, mice or human, CBS expression is rare or absent.

CSE is a protein of 405aa and is a tetramer formed by two homodimers, both contributing to the active site pocket [74]. CSE has been described as an exclusively beta-replacing lyase with a strict specificity for the primary substrate L-cysteine and for several sulfur-containing cosubstrates [40]. CSE can be regulated by several molecules. Among them, it has been shown that NO can

affect H_2S levels through two mechanisms: increasing CSE activity [42]; upregulating CSE expression [192]. CSE can be upregulated by bacterial endotoxin [46] and also upon stimulation with VEGF [193]. Testosterone is another endogenous CSE stimulator. It does not change the expression of CSE but stimulates CSE activity [194]. In rat thoracic aorta preparation, testosterone increased the production of H_2S from L-cysteine, which was significantly reduced by PAG and BCA. This would also explain testosterone-induced relaxation of rat aortic rings *in vitro*, which again was inhibited by PAG and BCA [194]. The mechanism for the transcriptional regulation of CSE is not clear, but there is evidence for the involvement of myeloid zinc finger 1 and specificity protein 1 (SP1) [46]. Conversely to CBS, CSE expression and activity is abundant in mammalian vasculature (aorta, mesenteric vessels, portal vein) and respiratory system [41, 42]. It also appears to be the main H_2S-forming enzyme in the liver, kidney, uterus, placenta, as well as pancreatic islets [42]. It has been showed that CSE is present in relatively large amounts in both vascular smooth muscle and endothelial cells [43] as

determined by Western blotting and immunohistochemical staining. CSE is absent in tissues of mammalian fetuses [44, 45], whilst mouse liver CSE activity increases significantly after birth and peaks at postnatal day 21 thereafter remaining constant in adulthood [46]. This developmental stage-dependent CSE activity seems to be related to the age-dependent posttranscriptional regulation of this enzyme [195]. Whether a similar age-dependent expression of CSE is present in cardiovascular tissues has not yet been determined. Animal experiments have identified the expression of CSE in portal vein, thoracic aorta [41], mesenteric artery, tail artery, and pulmonary arteries of rats [42]. The transcriptional expression level of CSE has been quantitated, using RNase protection assay, among different vascular tissues. The intensity rank of expression levels of CSE mRNA is pulmonary artery > aorta > tail artery > mesenteric artery [42]. D, L-propargylglycine is a specific covalent inhibitor of the cysteine desulfhydration, reaction catalyzed by cystathionase. This inhibitor considerably reduces hydrogen sulfide production in the rat liver and in both mouse and rat aorta [25]. Definitive evidence that CSE

is a physiologic source for H_2S comes from experiments employing CSE knockout mice [78]. H_2S levels in aorta and heart of homozygous CSE knockout mice are reduced by about 80% with a 50% reduction in heterozygous knockouts. Serum H_2S levels in homozygous and heterozygous CSE knockouts are reduced 50% and 20%, respectively. The residual H_2S in mutant serum may reflect non-enzymatic reduction of elemental sulfur to H_2S or H_2S generated from other tissues by CBS. The studies with CSE knockouts establish that H_2S is a product of normal mammalian physiology.

2.4 Mechanism of H_2S release from cells

H_2S is detectable within blood and many tissues. In some reports, concentrations of up to 160µM in the brain and 100µM in the blood are quoted. Although the liver has a high H_2S-generating capacity and may be responsible for maintaining the concentration of H_2S in the blood, 100µM H_2S would be readily detectable by the human nose while, clearly, blood does not normally smell of free H_2S. There is some uncertainty about the reliability of H_2S assay methods: total sulfate and sulfite may have been measured in

some studies, rather than free H_2S, which would account for this anomaly. Free (unoxidised) H_2S is likely present at lower concentrations than estimated initially. Indeed, Kimura and colleagues [48] have proposed recently that storage forms of H_2S such as bound sulfane sulfur may be critical in regulating the H_2S availability under different intracellular conditions. It means that H_2S, once produced in mammalian cells, can directly exert its biological effects via interaction with different signaling molecules, or it can be first stored and released upon a physiological signal.

Two forms of sulfur stores in cells have been identified [48, 49]: acid-labile sulfur and bound sulfane sulfur. Acidic conditions release H_2S from acid-labile sulfur, which occurs mainly in iron sulfur enzymes found in mitochondria and plays a critical role in a wide range of redox reactions in enzymes of the respiratory chain in mitochondria [50]. The critical pH below which H_2S is released from acid-labile sulfur is 5.4 [21]. As mitochondrial pH is between 7 and 8, acid-labile sulfur may not release H_2S under physiologic conditions. The sulfur of H_2S can be also incorporated into

proteins as bound sulfane sulfur [21, 51], which is divalent sulfur bound only to the other sulfur, such as outer sulfur atoms of the persulfides (R-S-SH) and inner chain atoms of polysulfides (RSnR, R is alkyl or aryl), or thiosulfate ($S_2O_3^{2-}$), thiosulfonate (R-S-SO_2-R), polythionates (SnO_6^{2-}), elemental sulfur (S_8) and disulfides which have an unsaturated carbon adjacent to the C-S bond. It seems that the main source of bound sulfane sulfur is MST enzyme. It has been showed that cells expressing MST and CAT increased the levels of bound sulfane sulfur, while cells expressing a defective mutant of MST, which does not produce H_2S, maintain the levels of bound sulfane sulfur at the control levels [52, 53]. Moreover, the bound sulfane sulfur is primarily localized in the cytoplasm and releases H_2S under the reducing conditions or at pH > 8.4 [50]. As it requires an alkaline microenvironment, the physiological implication of releasing H_2S from this store is questioned.

Interestingly, subcellular compartmentalization of sulfur store may facilitate differential H_2S release for specialized purposes and may have important physiological implications that are worthy of

further studies and in depth-research. For example, cross-talk between H_2S and NO could be highly regulated by their compartmentalization, accounting for the differential effects observed in different systems.

2.5 Catabolism of endogenous H_2S

Catabolism of H_2S is less recognized and most data were obtained by using exogenous H_2S; thus these studies have important toxicological but not necessarily physiological implications.

H_2S is broken down rapidly in the biological system and its half-life in mammalian plasma has been estimated at <30 min although the mechanism for this has not been identified [54]. Actually, several mechanisms have been suggested for H_2S degradation in the biological system (Fig. 2.3), such as:

1. expiration and excretion;
2. oxidation;
3. methylation;
4. scavenging.

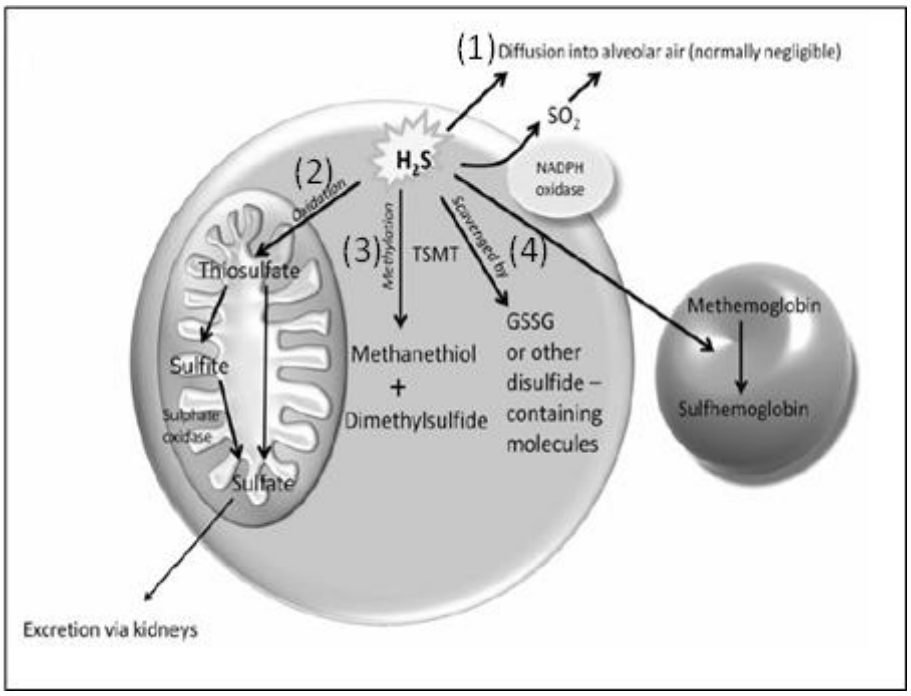


Fig. 2.3: *Catabolic routes for H_2S metabolism*

H_2S, like CO and NO, can be detected in human expired air [55]. In fact, a significant amount of exhaled H_2S has been measured after intravenous administration of sodium sulfide [56]. An increased amount of exhaled H_2S has also been reported after inhibition of endogenous NO synthesis. In healthy individuals, very little H_2S is lost via the lungs, because metabolic disposal keeps the free level of H_2S in the blood very low [57]. However, mammalian lungs may occasionally provide an escape route for H_2S, possibly during septic shock, hemorrhagic shock, or

pancreatitis when larger than normal amounts of H_2S may be generated [57]. Given the fact that the endogenous production of H_2S can be altered in several diseases, it may be useful to evaluate the possibility of using exhaled H_2S as a diagnostic measurement.

Endogenous H_2S, as a readily diffusible gas, can be rapidly metabolized in mitochondria by oxidation to thiosulphate, which is further converted to sulfite and sulfate by sulfate oxidase (Fig. 2.4) [58].

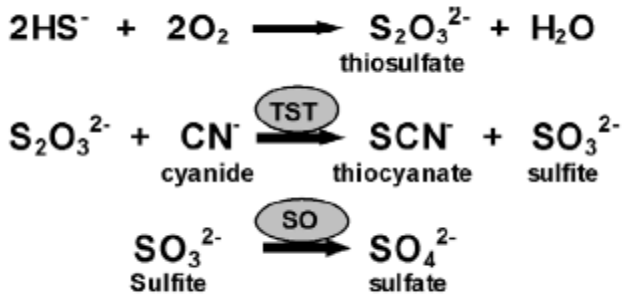


Fig. 2.4: *H_2S metabolim (II): oxidation.*

Oxidation of H_2S to thiosulfate is probably a non-enzymatic process associated with mitochondrial respiratory electron transport, although superoxide dismutase may also catalyze this reaction [59]. Conversion of thiosulfate to sulfite is catalyzed by

thiosulfate: cyanide sulfurtransferase (TST, rhodanase) which transfers sulfur from thiosulfate to cyanide or other acceptors [60]. The sulfite, which originates in this reaction, is rapidly oxidized to sulfate. Thus, under physiological conditions sulfate is a major end-product of H_2S catabolism. Although thiosulfate is also excreted in urine, its concentration is less than 1% of sulfate [61]. However, sulphate in body fluids cannot be used as a 'bio-marker' of endogenous H_2S production (in the same way that NOx is used as a marker of NO) since it is also derived from the direct oxidation of cysteine by cysteine dioxygenase (CDO). So, urinary thiosulfate seems to be the best biological indicator of whole-body H_2S production [62]. Mitochondria are very effective in oxidizing sulfides, however, if the supply of sulfide exceeds the ability of the mitochondrial enzymes to oxidize all of it, H_2S can poison enzymes [63].

Methylation is an additional, but perhaps quantitatively less important, metabolic pathway to degrade cellular H_2S. Conversely to oxidation, the methylation takes place in the cytosol. It consists of the methylation of sulfides by thiol S-methyltransferase (TSMT)

to methanethiol and dymethilsulfide, a non-toxic compound (Fig. 2.5) [58].

Fig. 2.5: *H_2S metabolism (III): methylation.*

TSMT is an ubiquitous enzyme with the highest activity in the colonic and cecal mucosa [64], but its activity has also been reported in the liver, lung and kidney.

Finally, H_2S can be scavenged by methemglobin to form sulfhemoglobin [65]. At this regard, Yang et al. reported that pretreating wild-type HEK-293 cells with methemoglobin for 1h prior to adding 100μM H_2S significantly abolished the antiproliferative effect of H_2S [66]. Since hemoglobin can scavenge NO and CO too, it may act as a common "sink" for all three gasotransmitters and these gas can compete with oxygen for binding, thus contributing to their toxicity on high exposure [67]. H_2S can also be scavenged by metallo- or disulfide-containing molecules such as horseradish peroxidase, catalase and oxidized

glutathione (GSH) [68]. Finally, H_2S is a reducing agent and could also be consumed by endogenous antioxidant species in the vasculature such as peroxynitrite [69], hypochlorite [70] superoxide [71] or hydrogen peroxide [72].

2.6 The biological function and signaling mechanism of H_2S in the vascular system

To date, much research into H_2S has centered upon its effects on individual body systems. Although many such systems have come under the spotlight, the effect of this gas on the cardiovascular system has attracted the most attention. Many other possible roles for endogenous H_2S have been postulated (Fig. 2.6), for example, in the peripheral and central nervous systems, pain appreciation and neurodegeneration, control of gastrointestinal and urogenital function, and endocrinology, respiratory system regulation and inflammatory response [28, 79-83].

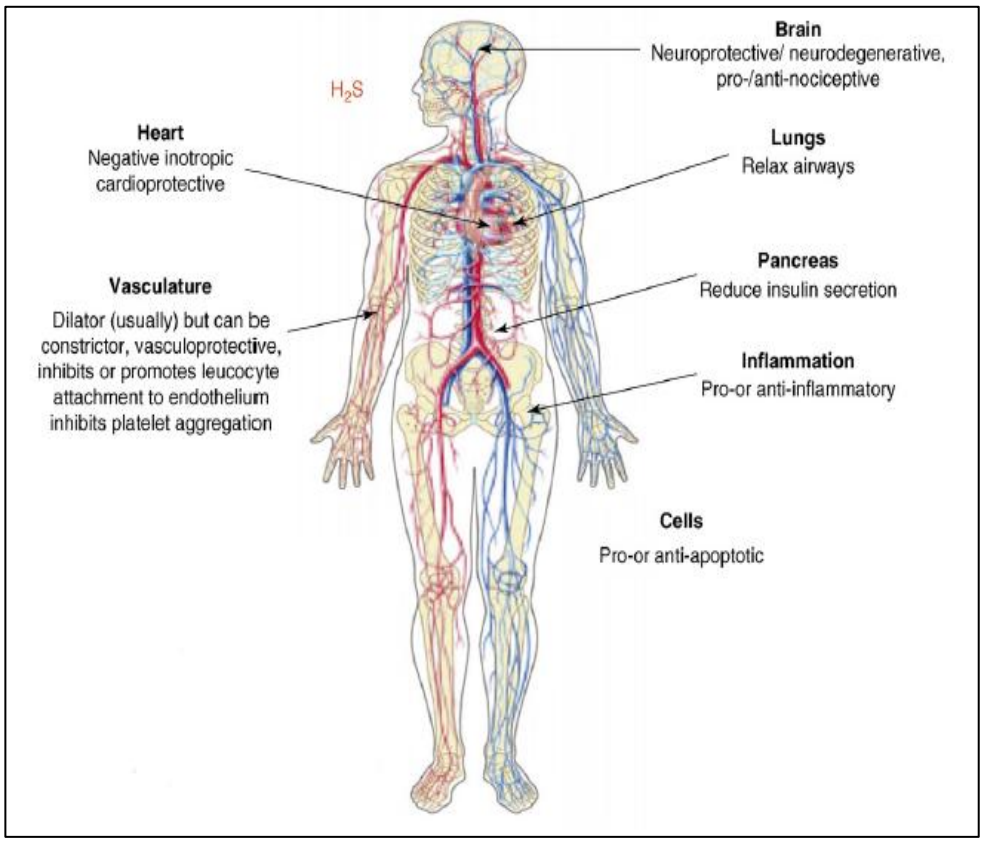


Fig 2.6: *Biological roles for H_2S in body systems.*

The biological effects of H_2S have been studied in a range of body systems including individual cells, intact and homogenized tissues, intact animals (both mammalian and no-mammalian) and also in man [28]. Its function has been investigated using mainly two approaches: 1) by inhibiting endogenous H_2S; 2) by administering exogenous H_2S mainly using NaHS as donor.

The modern era of H_2S research in the vasculature most likely started with the finding that H_2S dilates blood vessels. It was first demonstrated by Hosoki et al. in 1997 that arteries and veins express CSE and generate H_2S *in vitro* [41]. NaHS at concentrations above 100 μM may induce the relaxation of phenylephrine-precontracted isolated rat artery [41, 42, 84], but when it is used at concentration below 100μM may induce further contraction of precontracted isolated vessels [84-86]. The mechanisms underlying this vasoconstrictor effector are not fully understood, but the dilation is the most prominent effect in the vessels. Bucci et al. showed that the administration of L-cysteine elicited to vasodilation in isolated aorta rings of rat e, which was significantly blocked by both PAG and BCA, CSE-inhibitors [125]. Importantly, perfusion of the rat mesenteric arterial bed with the H_2S precursor, 1mM L-Cys, increased endogenous release of H_2S and relaxed the arterial bed, which are peripheral resistance vessels more significant for the regulation of vascular resistance and blood pressure than large conduit arteries [76]. Over the years H_2S has been reported to relax a long list of different blood

vessels, other than aorta and mesenteric vessels, such as gastric artery and portal vein [41, 84, 87] and dilate hepatic [88, 89] but not coronary [90] vascular beds. H_2S also relaxes the corpus cavernosum of rabbits [91] and men [92] and in this way may play a part, alongside NO, in control of erectile function [93, 92]. H_2S also relaxes the human internal mammary artery or other arteries in the organ bath [94]. The response of blood vessels to H_2S varies according to different several factors, such as: the preparation of H_2S; the precontraction agent used; the type of vessel; the condition of endothelium. The potency of vasorelaxant effect of H_2S is affected by the preparations of H_2S [101]. At the same concentration level, H_2S-gassed solution has much stronger vasorelaxant effects than NaHS solution does. The stimulus used to precontract vascular tissues also significantly affects the effect of H_2S. While H_2S relaxed phenylephrine-precontracted aortic tissues, high concentration of KCl (> 60mM)-induced vascular contraction was essentially not affected by H_2S. Different vascular tissues manifest different sensitivities to H_2S. Although rat aortic and mesenteric artery tissues produce similar levels of hydrogen

sulfide, H_2S is nearly six-fold more potent in relaxing rat mesenteric beds than relaxing rat aortic tissues. The higher sensitivity of mesenteric arteries, which are resistance vessels, to H_2S stands for the importance of this gas in regulating peripheral resistance as well as blood pressure [76]. The mechanisms for differential vasorelaxant effects of H_2S are not clear yet, but several possibilities exist. One explanation is the tissue-type specific distribution of the molecular targets of H_2S or it could be that the sensitivities of contractile proteins to H_2S may vary between conduit and resistance arteries [76]. Also different types of blood vessels face different shear stress levels, possess different cellular components (smooth muscle cells, endothelial cells, and connective tissues, etc.), and have different stiffness. In addition, oxygen-dependent sensitivity of blood vessels to H_2S should also be considered. It has been reported that H_2S induced vasorelaxation at physiological O_2 levels, and this vasorelaxation occurred much faster at below physiological O_2 levels. With higher than physiological O_2 levels, H_2S has the tendency to induce vasoconstriction [102]. This could result from the product of H_2S

oxidation, which may mediate vasoconstriction. Blood in small peripheral vessels has lower oxygen partial pressure, and these small vessels consume oxygen at higher rate due to high content of smooth muscle cells and low collagen. The situation is just opposite in large conduit arteries. The difference in tissue oxygen level may explain different vascular effects of H_2S. Finally, the condition of the endothelium can affect the H_2S-vasorelaxant action too. Initial studies demonstrated that neither endothelial denudation nor vascular denervation affected H_2S induced vasorelaxation, suggesting the direct effect on smooth muscle cells [42]. However, subsequent more detailed study revealed that NOS inhibitors or endothelial removal attenuated the relaxation of rat aortic tissues induced by H_2S and shifted H_2S concentration–response curve to the right with EC_{50} changed from 136 to 273 μM [101]. Although this observation did not address whether H_2S is endothelium derived or not, it shows that the presence of an intact endothelium does affect H_2S-induced vasorelaxation. The endothelium dependence of H_2S effect was again more pronounced in isolated and perfused rat mesenteric artery bed [76]. The

removal of the functional endothelium significantly reduced H_2S-induced relaxation of rat mesenteric artery bed by about sevenfold with EC_{50} of H_2S changed from 25 to 161 μM. As this tissue type-selective endothelium-dependent effect of H_2S is similar to that of endothelium-derived hyperpolarizing factor (EDHF), it has been considered that H_2S itself is a possible candidate for EDHF or that H_2S releases EDHF from endothelium. Both assumptions seem to be true. Indeed, Zhao and colleagues has shown that the co-application of apamin and charybdotoxin (Ca^{2+}-dependent K^+ channel, K_{Ca}) significantly weakened H_2S-induced relaxation of endothelium-intact rat aortic tissues [42]. Moreover, d'Emmanuele Di Villa Bianca et al. observed that H_2S could activate phospholipase A_2 (PLA_2), which in turn releases arachidonic acid leading, initially, to vasoconstriction followed by vasodilation mediated by cytochrome P450-derived metabolites. Because EDHF has been presumed to be a cytochrome P450 derivative of the arachidonic acid, their results suggest that H_2S acts through EDHF release [100].

In vivo studies confirmed such as vasorelxing effect of H_2S. In fact, in whole animals, intravenous injection (i.v.) of L-cysteine (endogenous source of H_2S [42]) and either 'fast' (e.g. NaHS [84, 42]) or 'slow' (e.g. GYY4137 [95]) H_2S-releasing donors all elicit dose-related falls in mean arterial blood pressure again indicative of a dilator effect on blood vessels. Interestingly some reports suggest a central component to the vasodilator effect of H_2S perhaps mediated by an action in hypothalamus [97, 98] or on baroreceptors [99]. In either case, the net effect of H_2S is to cause blood vessel dilation *in vivo*. The EC_{50} of H_2S in inducing vasorelaxation is quite close to the reported endogenous level of H_2S in plasma, which suggests that under physiological *in vivo* conditions the vascular tone of resistance arteries is likely regulated by endogenous H_2S.

The defining evidence for the role of endogenous H_2S in regulating vascular tone emerged after the CSE gene was knocked out from genetically engineered mice [43]. The endothelium-dependent relaxation of mesenteric arteries induced by methacholine, muscarinic receptor agonist, is virtually diminished in CSE

knockout mice. These results suggest that, similar to NO, acute production of H_2S from vascular endothelial cells is triggered by muscarinic activation. In agreement with this finding, Bucci et al. also showed that endothelium-dependent relaxations triggered by acetylcholine (Ach) were attenuated by CSE inhibition in rat aorta [125]. Methacholine increased H_2S levels in cultured endothelial cells by more than twofold. Atropine, a cholinergic antagonist, abolished methacholine effect. In the presence of the calcium ionophore A23187, endothelial production of H_2S is significantly increased. Chelating of intracellular-free calcium with BAPTA abolished the effect of A23187 on H_2S production as well as lowering the basal level of H_2S in endothelial cells. Furthermore, co-immunoprecipitation study demonstrated the binding of calmodulin to recombinant CSE *in vitro*. This binding is contingent on the presence of calcium, and abolished by calmodulin antagonist W7. These results demonstrated that a calcium–calmodulin system is the prerequisite for muscarinic activation of CSE in endothelial cells. Once CSE is knocked down from endothelial cells, the effects of methacholine and A23187 on

H_2S production were abolished, indicating that CSE, like eNOS, is activated by a calcium/calmodulin dependent mechanism [43]. Therefore, this could be a proof that, within the vascular wall, these 2 pathways coexist and serve a similar function.

Moreover, the fact that the lack of CSE from knocking down mice leads to age-dependent hypertension, similarly to NO synthase knockout mice, it underlies the important role of H_2S in the homeostasis of cardiovascular system, especially in the regulation of blood pressure; in addition, it indicates that H_2S is a vasoregulator of potency comparable to that of NO. Taken together, these findings strongly suggests that NO and H_2S either cooperate dynamically to maintain vessel homeostasis or serve as each other's backup system under pathological conditions.

2.7 A mechanism for H_2S regulation: H_2S-NO cross-talk

H_2S may provide a tonic influence on vascular tone under different conditions. It may also be acutely released upon the arrival of a specific stimulus. In either case, the production of H_2S needs to be

regulated should this gasotransmitter fulfill important physiological functions. We for the first time showed that NO is an endogenous regulator for the production of H_2S in vascular tissues [42]. The NO-stimulated H_2S production takes place following two schemes. Acutely, NO increases CSE activity in vascular tissues. Incubating the homogenized rat vascular tissues with different concentrations of SNP increased the accumulated H_2S production during a 90-min period. Putatively, NO increases the activity of cGMP-dependent protein kinases, which in turn stimulates CSE. This mechanism is supported by the finding that the blockade of PKG abolished the NO-induced increase in H_2S level in vascular tissues [120]. It is also possible that NO acts directly on CSE protein. Rat mesenteric artery CSE protein contains 12 cysteines that are the potential substrate of *S*-nitrosylation. Currently, the three-dimensional structure of CSE is unknown, and which cysteine contains a free –SH group cannot be assured yet. However, the nitrosylation of a certain free –SH group of CSE in the presence of NO does represent a possibility [121]. Chronically, NO up-regulates the expression of CSE [42]. NO has been shown

to regulate protein expression and synthesis, including growth factors [122], leukocyte adhesive proteins [123], and extracellular matrix proteins [124]. Wang et al. showed that, incubating the cultured vascular SMCs with SNAP, another NO donor, for 6 h significantly increased the transcriptional level of CSE. In rats, the NOS inhibitor L-NAME produced a time-dependent elevation in systolic blood pressure associated with inhibition of CSE gene expression and H_2S production whereas exogenous H_2S administration prevented the development of hypertension induced by L-NAME, highlighting the physiological significance of NO in the regulation of H_2S production in the cardiovascular system [13]. The mechanism, by which NO increased CSE transcription, is not clear yet. Published data have shown that pre-treating rat aortic tissues with 60 μM H_2S inhibited the vasorelaxant effect of SNP, an NO donor. Incubation of NaHS with a range of NO donors or NO gas *in vitro* was reported to form a nitrosothiol molecule. In this way, H_2S acts similar to an NO scavenger [116]. The formation of this nitrosothiol molecule inhibited the vasorelaxant effect of NO both *in vitro* and *in vivo*. NaHS also inhibited the

conversion of [^{3}H]-arginine into [^{3}H]-citrulline by recombinant eNOS [117]. In another study on isolated rat aortas and cultured human umbilical vein endothelial cells, NaHS incubation reduced eNOS activity, eNOS transcript abundance, and L-arginine transport in a concentration-dependent manner. H_2S treatment reduced protein expression of eNOS, but not of nNOS and iNOS. The downregulation of the vascular L-arginine/eNOS/NO pathway was observed after intraperitoneal injection of NaHS (14 μmol/kg) into rats [118]. In contrast, H_2S by itself had no effect on NO production from the rat vascular SMCs, but it augmented interleukin β-induced NO production and this effect was associated with increased expression of iNOS [119]. Whether H_2S and NO at low concentrations have a synergistic effect on vascular relaxation has been controversial. However, Hosoki et al. reported the additive effect of SNP and NaHS (30μM) in relaxing rat aortic tissues [41]. This discrepancy may be partially explained by the experimental conditions of these studies, including differences in tissue preparations and tension development before the application of H_2S. Although H_2S or NO alone relaxes vascular tissues, the

integrated vascular effects of the two gasotransmitters are more complicated than a simple algebraic summation of individual actions. In fact, it is clear that there is an interaction of physiological significance between H_2S and NO, which produce its effect in the cardiovascular system, however the precise relationships between H_2S, NO and NOSs are still far from clear.

2.8 Mechanisms of H_2S-induced vasorelaxation

The arising findings on the biological properties stimulated several groups toward the research on the mechanism of action of H_2S. Given that H_2S is a strong reducing agent and it may easily interact with other oxidative species, it has been suggested that some of its effects may be mediated by protection of protein thiol groups from oxidation. However, all studies performed to date indicate that effects of H_2S applied at physiological concentration are not reproduced or only partially mimicked by thiol-protecting agents, suggesting that this is not a major mechanism of H_2S action [17]. Furthermore, previous studies have shown that both superoxide dismutase (SOD) and catalase did not change H_2S-induced vasorelaxation of rat vascular tissues [42, 101]. These observations

suggest that the vascular contractility change induced by H_2S may not be linked to a changed redox status of vascular tissues.

Other than antioxidant effect, several signaling mechanism have been supposed to be triggered by H_2S with respect to vasorelaxing action, including opening of the ATP-sensitive K^+ channel (K_{ATP}) [42], inhibition of voltage-operated Ca^{2+} channels [114], effects on ATP generation (i.e. metabolic inhibition) [113], changes in vascular smooth muscle intracellular pH [115] and increasing in cGMP levels through inhibition of phosphodiesterase (PDE) [125]. Among them the activation of K_{ATP} channel and the inhibition of PDE seem to be the most relevant targets that mediated H_2S effect within the vasculature.

2.8.1 H_2S as a K_{ATP} channels opener

The first and most recognized molecular target of H_2S in smooth muscle cells (SMCs) is the ATP-sensitive K^+ channel (K_{ATP} channel) [42]. H_2S directly stimulates K_{ATP} channels and leads to consequent membrane hyperpolarization. The closure of voltage-dependent calcium channels by membrane hyperpolarization

results in smooth muscle relaxation. The K_{ATP} channel is an inward rectifying channel composed of two types of subunits. The smaller pore-forming subunits are the inwardly rectifying potassium channel subunits (Kir6.1 or Kir6.2), while the larger regulatory subunits are the sulfonylurea receptors (SUR1, SUR2A, and SUR2B), so-called because of its sensitivity to high-affinity sulfonylurea inhibition and offering the site of action for MgADP and K^+ channel. The K_{ATP} channel isoforms come from differing combinations of subunits. For example, Kir6.1/SUR2B is found in vascular smooth muscle cells (VSMCs), whereas Kir6.2/SUR2B is found in nonvascular SMCs [103]. The K_{ATP} channels exist in the sarcolemmal, mitochondrial, and nuclear membranes of various tissues [104], and are characterized by their inhibition by micromolar concentrations of intracellular ATP [105]. Hence, an increase in intracellular ATP closes these channels, leading to cell depolarization. The K_{ATP} channels participate in the control of membrane potentials and the regulation of cellular activities such as energy metabolism, apoptosis, and gene expression. Zhao et al. first reported that glibenclamide, a K_{ATP} channel antagonist,

significantly attenuated the vasodilatatory effect of H_2S, whereas pinacidil, a K_{ATP} opener, mimicked the effect of H_2S in a concentration-dependent fashion [42]. In smooth muscle cells isolated from the rat mesenteric artery, H_2S increased the open-probability of K_{ATP} channels without altering their conductance [106]. Interestingly, CSE inhibitors reduced K_{ATP} channel current indicating that endogenous H_2S continuously stimulated the channel under baseline conditions [42]. Unlike direct effect on smooth muscle cells, the endothelium-dependent component of H_2S-induced vasorelaxation is independent of K_{ATP} channels [76]. In order to gain insight on the molecular mechanism for an interaction between H_2S and K_{ATP} channel proteins, Jiang et al. studied the effect of H_2S in HEK-293 cells expressing different K_{ATP} channel subunits, rvKir6.1 and rvSUR1, heterologously. They found that H_2S stimulated coexpressed rvKir6.1/rvSUR1 K_{ATP} channels, but had no effect on K_{ATP} currents generated by rvKir6.1 expression alone [107]. This strongly indicates that H_2S acts on SUR1 but not Kir6.1 subunit of K_{ATP} channel complex to cause the channel to open. Alternatively, the structural coupling

between Kir6.x and SUR.x subunits might have been altered by H_2S so that the channel complex changes its configuration more favorably for opening [107]. It was reported that expressions of SUR2B were higher in the aorta than in the pulmonary artery, suggesting that the greater K_{ATP} channel density in the aorta may underlie its greater extent of relaxation than that in pulmonary arteries [108]. It was further demonstrated that sulfhydryl group(s) located on the extracellular surface of the SUR subunit is the likely target of H_2S. Mutagenic studies confirmed that H_2S failed to affect K_{ATP} channel currents after one of the two cysteine residues (C6S and C26S) in the extracellular loop of rvSUR1 subunits was point-mutated. Hence, extracellular cysteine residues Cys6 and Cys26 of rvSUR1 are most likely the targets of H_2S actions [107]. In the most recent report published by the same group, the researchers found evidence that H_2S covalently sulfhydrylates cysteine-43 on Kir 6.1 to bring about the hyperpolarization which underlies H_2S-induced vasorelaxation [109]. S-sulfhydration is a covalent modification of a cysteine residue in which a sulfhydryl group is transferred to the cysteine residue of a protein, yielding to

hydropersulfide (-SSH) group, which result in increased reactivity of L-cysteine [110]. Importantly, the enhanced chemical reactivity of these modified cysteines may also be reflected in enhanced biological activity.

Furthermore, H_2S has been shown as enhancing the binding of phosphatidylinositol-4,5-trisphosphate to Kir 6.1 to elicit K_{ATP} activation [109] (Fig. 2.7).

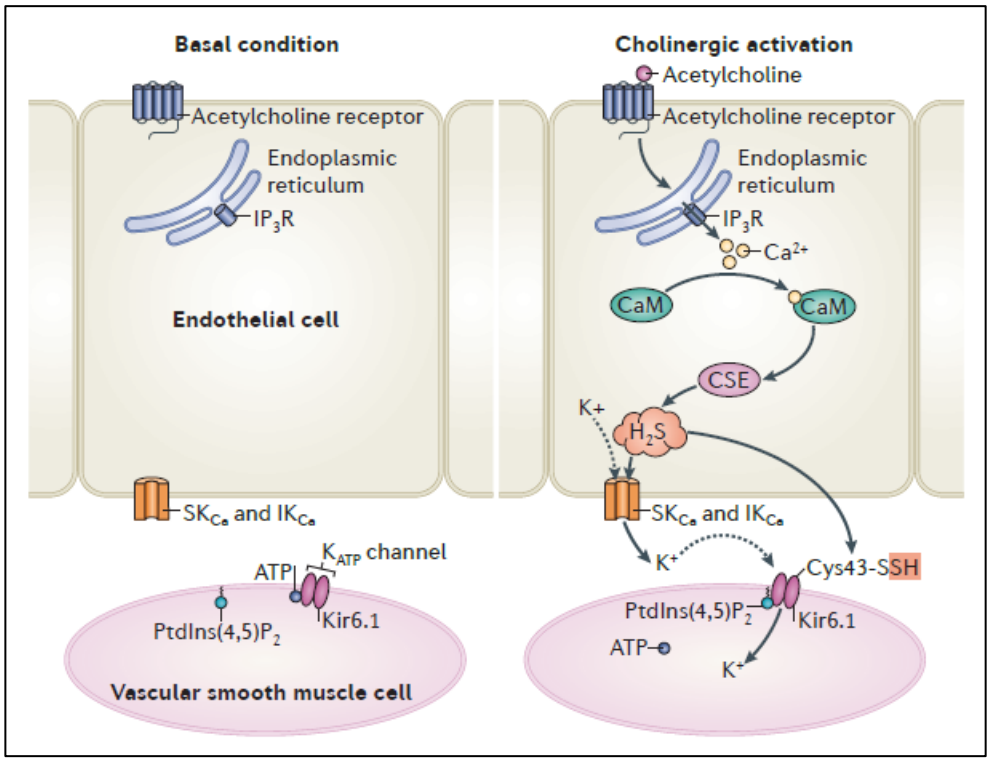


Fig. 2.7: *Mechanism of H_2S-induced relaxation (I): H_2S as a K_{ATP} opener.*

Indirect evidence also suggests that H_2S may act on small to medium conductance K_{Ca} channels in endothelial cells, as such fulfilling the role of an endothelium-derived hyperpolarizing factor (EDHF) [111]. The earliest speculation that H_2S may be an EDHF was derived from an observation that the co-application of charybdotoxin and apamin to endothelium-intact vascular tissues reduced the H_2S-induced vasorelaxation [42, 76, 100]. The endothelium dependent amplification of the vasorelaxant effect of H_2S is even more pronounced in peripheral resistance arteries than in large conduit arteries [76]. It is known that the contribution of EDHF to endothelium dependent vasorelaxation is much greater in smaller arteries, including mesenteric artery and coronary artery [111]. The involvement of voltage-dependent K^+ (K_V) channels in the vascular function of H_2S has been deduced as well. Cheang et al. recently investigated the effect of H_2S on membrane potentials of isolated rat coronary artery tissues. Using a membrane potential-sensitive fluorescent dye, the investigators found that addition of NaHS caused smooth muscle hyperpolarization. This effect was

inhibited by 4-aminopyridine (4-AP), but not by the K_{ATP} channel blocker glibenclamide [112].

Despite numerous reports and the widely accepted notion that the K_{ATP} channel is the main underlying mediator of most H_2S-induced vascular responses, H_2S-induced vascular relaxation is only often partially inhibited by glibenclamide and, in some cases, no inhibition can be achieved [94, 112, 113].

2.8.2 H_2S as an inhibitor of PDEs

It is well-known that cyclic guanosine monophosphate (cGMP)/PKG facilitates the dephosphorylation of MLC, preventing the association of myosin with actin and consequently leading to vessel relaxation. The cellular cGMP level is reached by a balance of cGMP production and degradation, which is regulated by the activation of guanylyl cyclases (sGC) and phosphodiesterase (PDE) isoenzymes respectively [126, 127]. Although H_2S, similarly to NO and CO, binds with high affinity to heme [128], it does not appear to activate soluble guanylate cyclase [129]. In fact, it has been shown that, conversely to NO

and CO, H_2S does not stimulate soluble guanilate ciclase (sGC), and its vasorelaxing effect is not inhibited by ODQ [1H-[1,2,4] oxadiazolo-[4,3-a] chinossalin-1-one], a sGC inhibitor [42]. Nevertheless, H_2S can affect cGMP levels within the vasculature. Indeed, Bucci et al. showed that the incubation of cultured rat aortic SMC with exogenous NaHS (10-200μM) increased cGMP levels in a concentration dependent-manner. More importantly, CSE overexpression (by CSE cDNA transfection) and CSE gene silencing (using small interfering RNA [siRNA] approach) greatly enhanced and attenuated cGMP levels, respectively, suggesting an endogenous role of H_2S in regulating cGMP levels [125]. The ability of H_2S to enhance cGMP levels was greatly reduced by the nonselective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), suggesting that H_2S and IBMX share a common target. By using a cell-free assay, they definitely showed that H_2S elevates cGMP levels by inhibiting PDE activity [125] (Fig. 2.8).

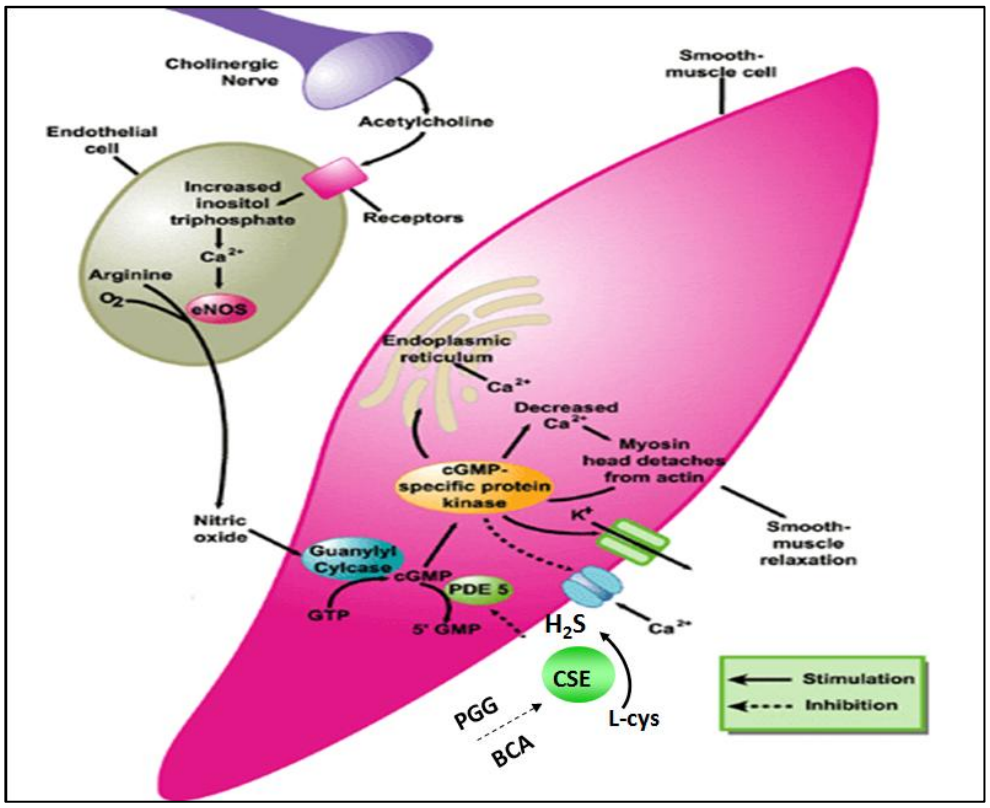


Fig. 2.8: *Mechanism of hydrogen sulfide-induced vasorelaxation (II)- H_2S as an endogenous inhibitor of PDEs.*

On the basis of the above, the mechanism through which H_2S promotes vasorelaxation would likely depend on the relative expression of K_{ATP} channels, the PDE isoform expressed, and the amount of H_2S present in the microenvironment. For example, tissues expressing K_{ATP} channels and no or low PDE levels would be expected to dilate in a glibenclamide-inhibitable manner. It could be possible that tissues expressing high PDE5 levels and abundant amounts of PKG, but no K_{ATP} channels, would dilate in a

cGMP-dependent manner. Another important observation that needs to be kept in mind is that PKG activates K^+ channels, leading to hyperpolarization and relaxation [130, 131]. This latter finding allows to the author to speculate that in cells and tissues in which H_2S responses are mediated by K channels, there might be both a cGMP-independent and a cGMP/PKG-dependent component. There is a need, therefore, to re-evaluate the conventional view that H_2S has no effect on cGMP levels [42, 101, 106], and to clarify the inconsistence of these observation.

2.9 Role of H_2S in cardiovascular disease: involvement in the pathogenesis of hypertension

Recently, an increasing number of diseases have been related to an imbalance of endogenous H_2S production [4]. The endogenous release of H_2S from mammalian tissues is likely to occur in a slow and constant rate and it appears to be involved in several processes such as neuromodulation [17], hypertension [132], inflammation [133, 134], hemorrhagic shock [135] and edema [136, 137].

The primary role of CSE in disparate cell and organ functions makes it likely that deficient generation of the product of its activity, H_2S, could be involved in human diseases. H_2S deficiency is a feature of rats with experimentally induced hypoxic pulmonary hypertension [138]. Reduced NO synthesis is the major factor underlying pulmonary hypertension in humans [139]. Given the functional parallelism and complementarity of H_2S and NO systems, a role of H_2S deficiency in this disease seems likely.

The vasorelaxant property of H_2S gives this gasotransmitter a role for regulating peripheral resistance and blood pressure. Reduced endogenous H_2S level, especially in blood vessel wall, would be in favor of hypertension development, logically. This was indicated in an early study in which it was found that the administration of PAG (37.5mg/Kg), an irreversible/suicide inhibitor of CSE, to rats for 5 weeks significantly elevated BP and increased the media cross-sectional aorta wall, indicating significant aortic structural remodeling [140]. A better alternative than using CSE inhibitors in animal experiments would be the use of genetically modified animals. CSE-KO mice were reported to exhibit pronounced BP

elevations compared with their wild-type counterparts [43], suggesting that the physiological production of H_2S may be critical for the regulation of basal BP. The elevated blood pressure in CSE-KO mice was not related to the contractile status of peripheral blood vessels since phenylephrine-induced constriction of isolated mesenteric arteries was not different between WT and CSE-KO mice [43]. On the other hand, endothelium-dependent vasorelaxation, triggered by the activation of muscarinic cholinergic receptors on the endothelium, was severely abolished in CSE-KO mice. Therefore, it was concluded that hypertension in CSE-KO mice is caused by the elimination of CSE expression in vascular tissues, especially in the endothelium, and the loss of H_2S as a critical EDRF in regulating peripheral resistance.

The role of H_2S in the pathogenesis of spontaneously hypertensive rats (SHR) was also examined. SHR develop hypertension spontaneously, coincident with decreased H_2S production and CSE mRNA expression in aortic tissues and lowered plasma level of H_2S compared to control Wystar-Kyoto (WKY) rats [140, 141]. These findings suggest that the hypertension in SHR involves a

reduction in the production and function of H_2S [140]. This is supported by the observation that chronic daily administration of NaHS (56μmol/kg/day/i.p.) for 5 weeks significantly reduced the SBP by 25 mmHg and inhibited aortic structural remodeling in the SHR but it had no effect in normotensive strain [140]. Consistently, Shi et al. reported significantly reduced SBP, diastolic blood pressure (DBP), and MAP of SHR to similar extents after NaHS (30 o 90μmol/kg/day, i.p.) for 3 months. A lower dose (10μmol/kg/day, i.p.) also reduced MAP, but the decrease in SBP was not statistically significant [142]. In addition, chronic treatment with GYY4137 also significantly reduced the SBP in SHR from day 2 after treatment [95]. Therefore, H_2S has a profound antihypertensive effect in SHR. Administration of CSE inhibitor, PAG, decreased plasma H_2S concentration and aortic H_2S production and elevated blood pressure in normotensive rats but not in SHR, indicating that vascular H_2S is involved in the regulation of vascular tone under baseline conditions, and that H_2S generating system is suppressed in the hypertensive strain [13]. Several mechanisms seem to underlie the antihypertensive effect

of H_2S. For example, H_2S inhibits renin-angiotensin system at different step of the enzymatic cascade. Lu et al. found that H_2S decreased plasma renin activity (PRA) in renin-dependent 2K1C rats, but had no significant effect on PRA in normotensive or renin-indipendent 1K1C rats [143]. H_2S suppressed renin degranulation and release was further shown to be mediated by the attenuation of AC activity and lowered cAMP production [144]. The same author also reported that neither acute nor chronic treatment with H_2S significantly affected the angiotensin converting enzymes (ACE) activity in normal (basal ACE activity) or hypertensive (elevated ACE activity) rat aortic tissues [143]. Conversely, Laggner et al. found that H_2S (60μM) inhibited ACE activity, but not its mRNA expression, on the surface of cultured HUVEC monolayers [145]. The inconsistent H_2S effect on ACE could be attributed to the different type of blood vessel used. Nevertheless, H_2S inhibition of renin or ACE would lead to significantly attenuated Ang II [143, 145].

On the other hand, CSE induction and excessive H_2S generation play a role in hypotension in septic shock [146] in the rat and,

again by analogy with NO, this gas may be a player in the corresponding human disease. A significant decrease in plasma H_2S has been reported in children with hyperlipidaemia and in patients with coronary heart disease [146].

In conclusion, it is clear that H_2S has a physiological importance in mammalian tissues and acts as an important mediator on the stage of the vascular homeostasis.

2.10 Physiologic regulation of blood pressure: role of renin-angiotensin system (RAS)

The mean arterial BP level is determined by the product of cardiac output (CO) and total peripheral resistance (TPR). These two principal determinants are importantly influenced by many physiologic factors. Cardiac output, for example, depends primarily on heart rate and stroke volume. Heart rate is governed by beta-1 and cholinergic receptors under the control of sympathetic and parasympathetic stimulation, respectively. The stroke volume is determined by the ventricular force of contraction (also under autonomic stimulation) and the filling pressure which

is in turn determined by the intravascular fluid volume status and the venous capacitance. Similarly, the systemic vascular resistance is influenced by multiple vasoactive mechanisms under the control of local, regional, and systemic neural, humoral, and renal factors. Importantly, all of these physiologic determinants of flow and resistance are interdependently governed by rapid-, intermediate-, and late-acting mechanisms that maintain the arterial BP within the normal range despite significant variations in the individual parameters [293, 294]. The complex systems and mechanisms that constitute the feedback and reflex control of BP have been extensively studied and characterized. Rapid responses and reflexes, for example, occurring within seconds after an acute rise in BP include the baroreceptor system, vagus nerve, and the central vasomotor center of the brainstem. In response to the rise in BP, a reflex decrease is noted in the sympathetic traffic to the arterioles and veins resulting in a decrease in peripheral resistance and cardiac filling pressure, respectively. In addition, slowing of the heart rate and reduced myocardial contractility occur as a result of simultaneous increases in parasympathetic outflow and decreased

sympathetic tone to the heart. Chemoreceptors acting at the vasomotor center also play a role in response to acute changes in BP level but, in addition, respond to changes in blood oxygen tension and carbon dioxide tension. Thus, the rapid-acting reflex and feedback mechanisms in response to acute changes in BP are almost entirely mediated by changes in activity of the autonomic nervous system [295]. Intermediate-acting responses that occur over minutes and hours involve the renin-angiotensin-aldosterone system, antidiuretic hormone, and the renal juxtaglomerular apparatus. Both local and circulating RAS play important roles in this feedback mechanism. In the systemic RAS, a drop in BP stimulates renin release via afferent arteriole baroreceptor mechanism and increased activity of the renal sympathetic nerves. A macula densa mechanism of renin release also exists [296-299]. In addition to these intermediate-acting responses, the RAS has a role in the long-term regulation of blood pressure. Late-acting mechanisms operate in days to weeks and are very important because of their long-term efficiency in BP regulation.

The Renin-Angiotensin-System (RAS) is the most carefully studied mechanism of blood pressure (BP) and volume regulation, and development of pharmacological antagonists to its various components has proved useful in the treatment of hypertension and related target organ disease (TOD) [148].

The traditional view of RAS is of a typical endocrine system. It consists of an enzymatic cascade that starts with the cleavage of angiotensinogen by renin to form the inactive decapeptide angiotensin I (Ang I). Thereafter, Ang I is converted by angiotensin-converting enzyme (ACE) to form angiotensin II (Ang II) (Fig. 2.9).

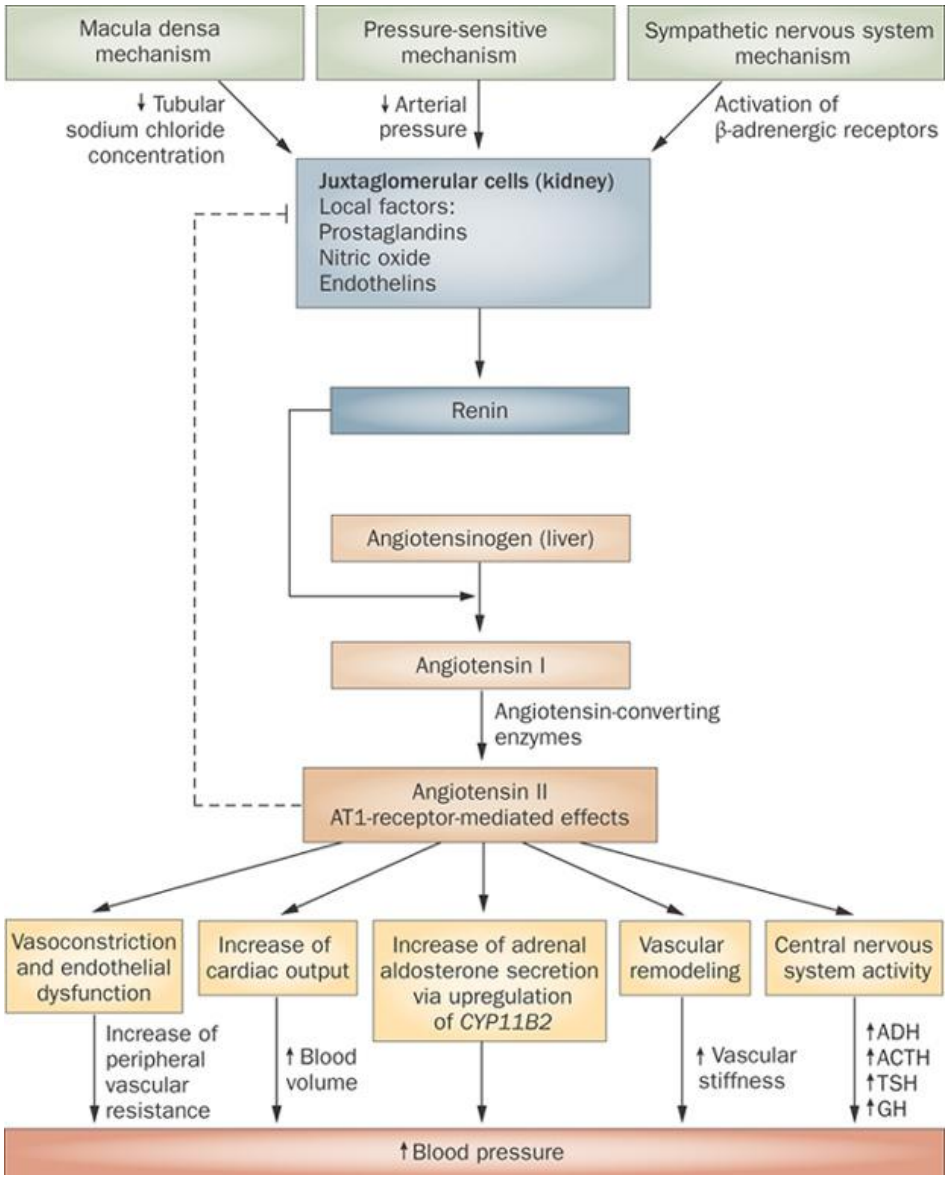


Fig. 2.9: *The renin-angiotensin system (RAS).*

Renin is an aspartyl protease that is synthesized as an inactive precursor, prorenin, primarly in the juxtaglomenrular cells surrounding the afferent arteriole of the glomerulus. Renin is activated by proteolytic cleveage of an N-terminal peptide while

still in the kidney. Both prorenin and activated renin are stored in granules in the juxtaglomerular apparatus and are released in a regulated fashion in response to a variety of stimuli, including decreases in BP or renal interstitial pressure via intrinsic juxtaglomerular baroreceptors, SNS activation of the renal nerves, and/or macula densa stimulation by decreased distal tubular sodium delivery. Importantly, the primary mechanism by which the RAS contributes to the acute changes in BP and volume homeostasis is by regulating renin release into the circulation. Renin acts on circulating angiotensinogen of hepatic origin to produce the biologically inactive decapeptide, Ang I, within the plasma. Ang I is cleaved to the octapeptide Ang II by ACE, present on the luminal surface of endothelial cells throughout the vasculature. In addition to this classical endocrine system, non-renin enzymes, including tonin and cathepsin, exist and can generate Ang II directly from angiotensinogen. ACE, or kininase, is a bivalent dipeptide carboxyl metallopeptidase present as a membrane-bound form in endothelial, epithelial, or neuroepithelial cells, including the heart, kidney, and brain, and as a soluble form

in blood and numerous body fluids [149]. ACE cleaves the C-terminal dipeptide from Ang I and bradykinin, by generating the vasoconstrictor agent Ang II and inactivating the vasorelaxant mediator bradykinin respectively. ACE strategically regulates the balance between the RAS and the kallikrein-kinin system [150]. Ang II acts on two major receptors known as AT_1 and AT_2 [151]. Both the AT_1 and the AT_2 receptors belong to the superfamily of G-protein-coupled receptors that contain 7 transmembrane regions [152]. Their amino acid sequence seems to be highly conserved across species and across tissues within a species. AT_1 and AT_2 receptors have distinct signal transduction pathways. AT_1 receptor is distributed ubiquitously and abundantly in adult tissues, including blood vessels, heart, kidney, adrenal gland, liver, brain and lung. Activation of the AT_1 receptor stimulates a variety of tyrosine kinases, which in turn phosphorylate the tyrosine residues in a number of proteins, leading to constriction of resistance vessels, cell growth and proliferation. Moreover, it induces aldosterone release, central sympathetic activation, renal salt and water retention, and other functions that tend to elevate BP and

cause hypertrophy or hyperplasia of target cells. NO can down-regulate AT_1 receptors in vascular tissues [153] and the adrenal gland [154] and mitigate the action of Ang II.

The AT_2 receptor was discovered more recently, and much less is known about its functions and signal transduction pathways than for the AT_1 receptor. In fetal tissues, the AT_2 receptor is highly and ubiquitously expressed, but expression in many organs is dramatically reduced after birth [155, 156]. However, expression levels are maintained in the adult adrenal gland, brain, uterus and ovary, and the receptor is also expressed by endothelial cells [157]. The physiologic role of the AT_2 receptor in adult humans is unclear, but it is thought to function under stress conditions (e.g. vascular injury, ischemia or reperfusion). Observation of increased AT_2 receptor expression in myocardial infarction [158] and skin wounds [159], as well as its much higher level of expression in fetal compared to normal adult tissues, have led to speculation as to a possible role for the AT_2 receptor in cell growth, development, and/or differentiation. Activation of the AT_2 receptor subtype stimulates a phosphatase that inactivates mitogen-activated protein

kinase (MAPK), a key enzyme involved in transducing signals from the AT_1 receptor. Thus, activation of the AT_2 receptor opposes the biologic effects of AT_1 receptor activation. In fact, conversely to AT_1 receptor, its activation leds to the synthesis and/or the release of both prostaglandins and NO and have been shown to exert antiproliferative effects [160].

The circulating RAS participates in short-term regulation of the cardiovascular system, which becomes activated in acute conditions including hypertension, hypovolemia and hemorrhage. In the case of chronic condition, including hypertension and chronic heart failure, activation of the RAS cause long-term regulation of cardiovascular homeostasis via sustained activation of local angiotensin and degradation of bradykinin, resulting in permanent structural changes [150].

The RAS induces vascular disease by multiple mechanisms including directly and indirectly (via aldosterone): stimulating vascular smooth muscle cell migration, proliferation, and extracellular matrix production; increasing the release of plasminogen activator inhibitor type 1 and enhancing the

expression of monocyte chemoattractant protein 1; augmenting the expression of adhesion proteins (e.g ICAM-1, integrins, and osteopontin) in vascular cells; and stimulating the production of inflammatory chemokines and cytokynes that enhance the migration of inflammatory cells [304].

2.11 Arterial hypertension: pathogenesis and pharmacological therapies

Arterial hypertension represents one of the most common conditions associated with increased cardiovascular risk, including stroke, renal failure, myocardial infarction and heart failure.

The term hypertension applies to any adult with a blood pressure persistently at or above 140mmHg systolic and at or above 90mmHg diastolic. It is distinguished: in primary (essential) or idiopathic hypertension and secondary hypertension.

Primary or idiopathic hypertension accounts for the majority (>90%) of cases of human hypertension and the causes are unkonwn [161]. Conversely, secondary hypertension has identifiable causes, such as tumors which produce epinephrine or

other vasoconstrictor hormones, primary aldosteronism, renovascular diseases which cause fluid retention, thyroid or parathyroid gland disorders, aortic coarctation and others. This list is much longer and more complicated when one tries to consider the possible causes in the remaining 90% of the hypertensive patients. The genesis of essential hypertension is a complex interplay of various pathophysiologic factors that include, but are not limited to, increased sympathetic nervous system (SNS) activity; heightened exposure or response to psychosocial stress; overproduction of sodium-retaining hormones and vasoconstrictors; long-term sodium intake; inadequate dietary intake of potassium and calcium; increased or inappropriate renin secretion with resultant increased production of angiotensin II (Ang II) and aldosterone; deficiencies of vasodilators, such as prostacyclin, nitric oxide (NO), the natriuretic peptides and a variety of other vasodilator peptides, including the angiotensin 1-7 peptide, calcitonin gene-related peptide (CGRP), substance P and adrenomedullin; alterations in expression of the kallikrein-kinin system that affect vascular tone and renal salt handling;

abnormalities of resistance vessels, including selective lesions in the renal microvasculature; diabete mellitus (DM); insulin resistance; obesity and the production of vasoactive adipocytokines; increased activity of vascular growth factors, alterations in adrenergic receptors that influence heart rate, inotropic properties of the heart, and vascular tone; and altered cellular ion transport. These factors interact with genetic, demographic and environmental influences, explaining the heterogeneity of the hypertensive population. In addition, increased vascular stiffness, endothelial dysfunction, inflammation and increased oxidative stress play a role in increasing BP and cardiovascular (CV) risk and offer potential therapeutic targets with benefits that may go beyond lowering BP. Analyses of BP patterns in families suggest that genetic factors account for 40% to 50% of BP variance, whereas shared environment accounts for 10% to 30% [148]. However, single gene mutations play a minor role, accounting for <1% of cases of hypertension. DNA variants in multiple so-called candidate genes (those having relevance to disease development or progression) with subtle effects on gene

expression or function, when taken cumulatively, may have major impact in raising BP. Improved techniques of genetic analysis, especially genome-wide linkage analysis, have enabled a search for complex sets of genes that may contribute to the development of primary hypertension in the population.

The unifying aspect of hypertension is that two pathological processes are manifest. Hypertension will cause pathological changes to: (1) the muscle of the heart and (2) the arteries. The muscle cells enlarge, with increased extracellular proteins. During the established phase of hypertension, total peripheral resistance occurs and may be due to a decrease density of diameter of smaller vessels, structural changes causing decreased compliance, or a change in activity or sensitive of the vessels to hormonal stimuli. In later stages of hypertension there is an increase in connective tissue with an associated decrease in vascular compliance. Hypertension is frequently associated with atherosclerotic plaques and it is likely that hypertension may increase some of the endothelial denuding, damaged endothelial cells causes or permits an injury repair response, the response causes infiltration of

macrophages, this infiltration continues to produce foam cells, and the atherosclerotic plaque cycle continues.

Pharmacological treatment of patients with hypertension decreases morbidity and mortality from cardiovascular disease. Effective antihypertensive therapy markedly reduces the risk of strokes, cardiac failure, and renal insufficiency due to hypertension. Currently therapies for hypertension can act on peripheral resistance, cardiac output or both. Drugs may decrease the cardiac output by inhibiting myocardial contractility or by decreasing ventricular filling pressure. On the other hand, drugs can decrease peripheral resistance by acting on smooth muscle to cause relaxation of resistance vessels or by interfering with the activity of systems that produce constriction of resistance vessels (e.g. the sympathetic nervous system, the RAS).

Antihypertensive drugs can be classified according to their sites or mechanisms of action as resumed in the following table (Tab.2.1):

Pharmacological Therapy of Hypertension

Diuretics

1. Thiazides and related agents (hydrochlorothiazide, chlorthalidone, chlorothiazide, indapamide, methylclothiazide, metolazone).
2. Loop diuretics (furosemide, bumetanide, torsemide, ethacrynic acid).
3. K^+-sparing diuretics (amiloride, triamterene, spironolactone).

Sympatholytic drugs

1. β receptor antagonists (metoprolol, atenolol, betaxolol, bisoprolol, carteolol, esmolol, nadolol, nebivolol, penbutolol, pindolol, propranolol, timolol).
2. α receptor antagonists (prazosin, terazosin, doxazosin, phenoxybenzamine, phentolamine).
3. Mixed α-β receptor antagonists (labetalol, carvedilol).
4. Centrally acting adrenergic agents (methyldopa, clonidine, guanabenz, guanfacine).
5. Adrenergic neuron blocking agents (guanadrel, reserpine).

Ca^{2+} channel blockers (verapamil, diltiazem, nisoldipine, felodipine, nicardipine, isradipine, amlodipine, clevidipine, nifedipine).

Angiotensin-converting enzyme inhibitors (captopril, enalapril, lisinopril, quinapril, ramipril, benazepril, fosinopril, moexipril, perindopril, trandolapril).

Ang II receptor antagonists (losartan, candesartan, irbesartan, valsartan, telmisartan, eprosartan, olmesartan).

Direct Renin Inhibitor (aliskiren).

Vasodilators

1. Arterial (hydralazine, minoxidil, diazoxide, fenoldopam).
2. Arterial and venous (nitroprusside).

Tab. 2.1: *Classification of antihypertensive drugs by their primary site or mechanism of action.*

2.12 ACE-inhibitors

ACE-inhibitors (ACEi) are widely used in controlling blood pressure in hypertensive patients. They act on the RAS by inhibiting the conversion of Ang I to the active Ang II. Inhibiton of Ang II production lowers blood pressure and enhances natriuresis. ACE is an enzyme with many substrates, and its inhibition may also induce effects unrelated to reducing the levels of Ang II. ACEi increase bradykinin levels and bradykinin stimulates prostaglandin biosynthesis; both may contribute to the pharmacological effects of ACE inhibitors. It has been demonstrated that ACE inhibitors enhance mediator release (eg, NO, prostaglandins or endothelium-derived hyperpolarizing factor) [312, 313] not only by protecting BK from degradation, but also by affecting bradykinin 1 and 2 receptor (BR1 and BR2) differently [311]. Several studies showed that ACE inhibitors are indirect allosteric enhancer of B2R signaling as interaction between B2R and ACE takes place on the cell surface. It seems that ACE inhibitors induce a conformational change in ACE that is transmitted to the B2R owing to their close contact, resulting in

increased mediator release [314–322]. On the other hand, ACE inhibitors are direct activators of B1Rs at an allosteric site that differs from the orthosteric site of peptide ligands. The consequence is a prolonged high-output NO production by iNOS in human endothelial cells [323-325]. Apart of BK-mediated beneficial properties, ACE-i may suppress oxidative stress by decreasing the activities of the NAD(P)H oxidase, PKC and MAPK [326] and by decreasing the expression of oxidative stresslectin-like oxidized LDL receptor-1 (LOX-1) [327]. Furthermore, ACE-i decrease tissue factor (TF) and MCP-1 after 1-month therapy in patients with MI and downregulated TF synthesis *in vitro* by inhibition of endotoxin-induced nuclear factor-kappaB translocation to the promoter region of the gene encoding for TF [328]. ACEi increase by 5-fold the circulating levels of the natural stem cell regulator N-acetyl-seryl-aspartyl-lysyl-proline, which may contribute to the cardioprotective effects of ACE-inhibitors [306]. In addition, ACEi increase renin release and the rate of formation of Ang I by interfering with both short- and long-loop negative feedbacks on renin release. Accumulating

Ang I is directed down alternative metabolic routes, resulting in the increased production of vasodilator peptides such as Ang (1-7).

ACEi can be classified into three broad groups based on chemical structure:

1) sufhydryl-containing ACE-inhibitors structurally related to captopril, such as alacepril and zofenopril;
2) dicarboxyl-containing ACE-inhibitors structurally related to enalapril (e.g. lisinopril, benazepril, quinapril, moexipril, ramipril, trandolapril, perindopril);
3) phosphorus-containing ACE-inhibitors structurally related to fosinopril, as ceronapril.

Many ACEi are ester-containing prodrugs that are 100-1000 times less potent but have a better oral bioavailability than the active molecules. They differ with regard to potency, whether ACE inhibition is primarily a direct effect of the drug itself or the effect of an active metabolite, and pharmacokinetics.

The main effect of ACE inhibition is the long-term fall of systemic blood pressure accompanied by a reduction in total peripheral

resistance. Cardiac function in patients with uncomplicated hypertension generally is little changed, although stroke volume and cardiac output may increase slightly with sustained treatment. Baroreceptor function and cardiovascular reflexes are not compromised, and responses to postural changes and exercise are little impaired. Other than antihypertensive action, several large, randomized, placebo-controlled clinical studies demonstrate that inhibition of ACE in patients with systolic dysfunction prevents or delay the progression of heart failure, decreases the incidence of sudden death and myocardial infarction, decreases hospitalization, and improves quality of life. Thus, ACE-inhibitors show additional protective effects in the cardiovascular system. Indeed, different studies have showed that ACEi induced many beneficial effects on endothelial function in both animal models of hypertension [307], diabete [308] and atherosclerosis [309, 310]. Interestingly, these beneficial effects seem to involve different mechanisms which are unrelated to Ang II inhibition. Moreover, recent findings reported the existence of significant differences between ACE inhibitors, as far as protection vs ischemic injury is concerned [329]. Early

studies, focused on the comparison between sulphydryl-containing ACEi captopril and dicarboxyl-containing ACEi enalapril, led to the hypothesis that the presence of a thiol group may be critical for cardioprotection [330-332]. With respect to the role of –SH group in the beneficial effect, more recently much attention has been paid to another sulphydrylated ACEi, e.g. zofenopril, which exerts protective action in the vasculature to a greater extent than captopril.

2.13 The sulphydrylated ACEi zofenopril

Zofenopril, [1(S), 4(S)]-1(3-mercapto-2methyl-1-oxopropyl) 4-phenyl-thio-L-proline-S-benzoylester, is the latest ACE inhibitor to reach European market in 1999. As the most part of ACEi, zofenopril is a prodrug, which is desterified *in vivo* to the active water-soluble inhibitor, the sulphydryl group containing compound, zofenoprilat (Fig. 2.10).

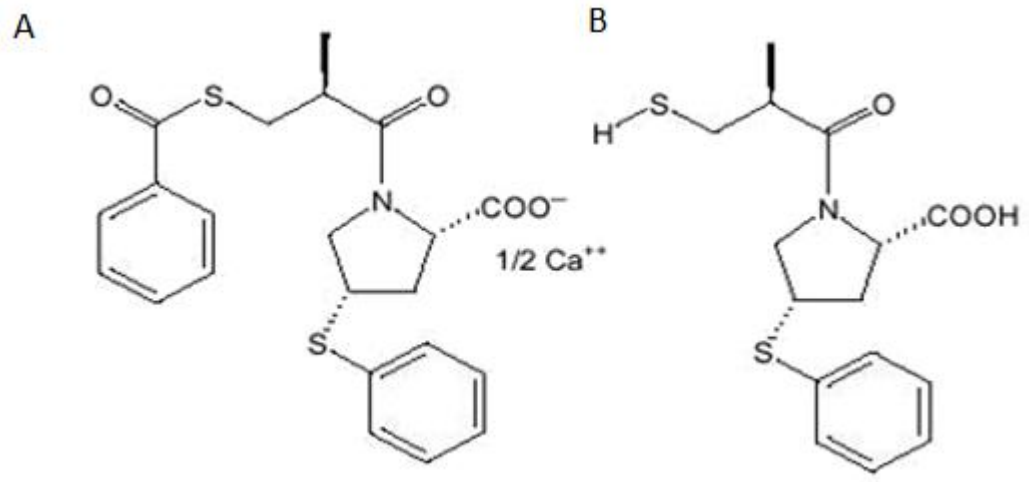


Fig. 2.10: *Molecular structure of (A) zofenopril and (B) its active metabolite zofenoprilat.*

Zofenopril is notably lipophilic and this feature allows it to undergo extensive and prolonged tissue penetration, which enhances ACE inhibition in tissues, more than other ACE inhibitors. Importantly, concentrations of zofenopril in the heart and aortic wall have been found to be greater than plasma concentrations, and penetration of cardiac tissue by zofenopril is particularly efficient [333]. Zofenopril, along with captopril, has sulphydryl moiety which was related to the cardiprotective effect of these drugs both *in vitro* and *in vivo*. *In vitro* studies showed that captopril (36-1080µM) and zofenopril (3.6-36µM) significantly increased coronary flow after 5 minutes of perfusion

in a concentration-dependent manner in isolated rat hearts, although the pharmacological efficacy as well as the relative potency of zofenopril was definitely higher. This effect was not accompanied by an increase in 6-keto $PGF_{1\alpha}$ overflow in the coronary effluent. On the other hand, ramiprilat induced coronary vasodilation after 20 minutes of perfusion and was associated with an increase in 6-keto $PGF_{1\alpha}$ outflow [334], suggesting a different mode of action for ACEi with and without –SH group. In another study, zofenoprilat and captopril, but not enalaprilat, potentiated the vasodilatator effect of bradykinin on coronary vessels in isolated rat hearts [334-336]. ACEi are likely to cause vasodilation by a bradykinin-mediated release of NO, which can be enhanced in presence of free –SH groups. Sulphydrylated ACEi may therefore potentiate nitrates and reverse tolerance to their therapeutic effects, as suggested by previous preclinical trials [337]. Several studies have investigated the cardioprotevtive effects of zofenopril in experimental model of global ischemia/reperfusion as well. The characteristic of reperfusion is the release of big amounts of lactate dehydrogenase (LDH, an indicator of cell viability), decreased

cardiac function, and increased end-diastolic pressure (EDP, an indicator of severe damage). It has been demonstrated that both zofenoprilat at 3 and 10µM and captopril 400µM improved contractile force and reduced EDP and LDH in a concentration-dependent fashion. Conversely, enalaprilat or ramiprilat, at concentrations up to 400µM and 100µM respectively did not show any cardioprotective effects [338]. In another model of cardiac ischaemia and reperfusion, it was compared the effect of zofenopril, captopril and fosinopril at the same dose of 50µM with respect to some typical parameter of ischaemia/reperfusion, such as reduction of left ventricular (LV) pressure and coronary flow, as well as increased release of creatine kinase (CK, a sensitive indicator of membrane injury). Treatment with zofenopril and captopril, but not fosinopril improved post-ischaemic LV function, increased coronary flow and reduced CK release. Moreover zofenopril, but not captopril, attenuated oxidative stress during reperfusion [339]. More recently, it has been found that zofenopril reduced infarct size in isolated rat hearts, which was partially reduced by BR2 antagonist, HOE-140 [340]. Moreover, the

cardioprotection afforded by zofenoprilat was also associated with better preservation of protein thiols at the end of the ischaemia. These findings reinforce the concept that zofenopril has a specific cardioprotective effect, which might be related either to interference with bradykinin metabolism and/or to preservation of protein SH groups. The ability of zofenopril to prevent ischemic myocardial damage was evaluated also *in vivo* in a chronic closed-chest pig model [342]. Zofenopril decreased the pressure-rate product and reduced the peak efflux of chatecolamines in coronary venous effluent during reperfusion in pigs pre-treated with zofenopril 2 days prior to ischaemia. The effects of zofenopril on the myocardial changes induced by sustained arterial hypertension or cardiac ischaemia have been studied in several trials. It has been showed that renal hypertension induced by aortic banding and constriction of the right renal artery was consistently reduced by oral administration of 6mg/Kg zofenopril daily for 8 weeks [343]. Studies performed with SHRs, zofenopril was given daily in food for 6 months and it was effective to reduce the mean arterial pressure from 106 to 84 mmHg. Its effect was similar to that of

hydralazine, but zofenopril also significantly reduced the LV systolic force–time integral (a measure of total ventricular load) and the LV weight to body weight ratio [344]. Because free radical damage of vascular etiology could be an important mechanism of myocardial ischemia-reperfusion injury [350], it has been suggested that the effect could be due to the free radical scavenging ability of the sulphydryl moiety of the agent. The protective effects of zofenorpil and other SH-containing ACEi from free radical-induced cell damage have been assessed in cultured endothelial cells exposed to a superoxide anion and hydroxyl radicals generating system [351]. Preincubation of the cells with captopril, epicaptopril (the stereoisomer of captopril which is devoid of ACE inhibitory properties), or zofenopril produced a concentration-dependent (10-200μM) inhibition of malonyldialdehyde formation. Both loss of cell viability and membrane blebbing were reduced by SH-ACEi at concentrations as low as 10μM, although zofenopril was 5 times more potent. In contrast, non-sulphydrylated ACEi were ineffective up to 200μM. Furthermore, it has been proposed that zofenopril can be

cardioprotective *via* modulation of K_{ATP} channels, because K_{ATP} openers were found to protect ischemic myocardium in various experimental models [352-355]. Sargent et al. showed that the effects of zofenopril were reversed by two structurally different blockers of the K_{ATP} channels: glyburide and 5-hydroxydecanoate. Isobolographic analysis demonstrated that treatment with a combination of zofenopril and cromakalim (a K_{ATP} opener) resulted in superadditive response in the ischemic myocardium, and K_B analysis demonstrated that glyburide is a noncompetitive antagonist in the presence of zofenopril and a competitive antagonist in the presence of cromakalim. The results suggest a link between the cardioprotective effects of zofenopril and the K_{ATP} channel. This activity appears to be a receptor-mediated event, involving a mechanism different from that of the classical K_{ATP} openers, such as cromakalim [356].

In other recent studies, zofenoprilat was shown to protect endothelial function in different experimental models [345]. It abolished the proapoptotic effects of doxorubicin, promoted mitosis of bovin coronary venular endothelial cells (CVEC) [346],

enhanced concentration-dependently cell survival, and improved vascular-endothelial-growth-factor–induced proliferation of CVECs [347]. Experimental evidences suggest also that zofenopril reduced stimulated and un-stimulated release of ET-1 secretion and concomitantly increased of nitric oxide production in HUVECs [348, 349]. Zofenoprilat improve the vascular reactivity and ameliorates endothelial dysfunction in non-obese diabetic (NOD) mice, animal model of type I diabete. It seems that it increases NO bioavaibility through by regulating the expression of Caveolin-1, which inhibits eNOS activity, conversely to the enalapril. Moreover zofenopril, but not enalapril ameliorates diabete oucome in diabete mice in terms of reduced polyuria and glycosuria [308].

Zofenopril inhibits LDL oxidation and attenuate atherosclerosis in different experimental model of atherosclerosis such as in apolipoprotein E deficient mice [309] or Watanabe Heritable Hyperlipidimic (WHHL) rabbits [310]. Cominacini et al. showed that zofenoprilat, but not enalaprilat, significantly and dose-dependently reduced the formation of intracellular ROS and

superoxide induced by oxidized low density lipoprotein (ox-LDL) and tumour necrosis factor-α (TNF- α) in human endothelial cells [357]. Zofenoprilat but not enalaprilat also decreased the consumption of intracellular glutathione induced by ox-LDL and TNF-α. While zofenoprilat significantly and dose-dependently reduced the expression of VCAM-1, ICAM-1 and E-selectin induced by ox-LDL and TNF-α in human umbilical vein endothelial cells (HUVECs), enalaprilat did not. In addition, pre-incubation of HUVECs with zofenoprilat dose-dependently reduced the increased activation of nuclear factor-κB (NF-κB) by ox-LDL and TNF-α [357]. It is known that the migration of circulating monocytes and ox-LDL into the subendothelial space is one of the key events in atherosclerosis [358] and is mediated by the occurrence and expression of adhesion molecules on endothelial cells. NF-κB, a redox-sensitive element, is a transduction factor in the molecular gene induction of adhesion molecules. Thus SH-containing ACE inhibitors, in addition to controlling blood pressure, may be useful in inhibiting atherosclerotic plaque progression, which slows the development

of atherosclerosis. Finally, the impact of zofenopril in nitric oxide production and cGMP concentrations was evaluated in bovine aortic endothelial cells (BAEC). Zofenopril enhance NO production which is associated to an increase in NO bioactivity, as demonstrated by the significant increase in endothelial cell cGMP accumulation. This effect was again notable only for zofenoprilat over the range of concentrations used (10-30-60µM); captopril also had an effect on cGMP, but only at the highest concentration tested. In contrast, enalaprilat was not significantly effective at the different tested doses. F2-isoprostanes were reduced, with the reduction correlating with the increases in nitrite/nitrate and cGMP produced by zofenoprilat, suggesting that the benefit of zofenoprilat is, in part, a consequence of reduced oxidant stress in the endothelial cell leading to improved bioacvtive nitric oxide production [359].

2.14 Objectives of the project

Hydrogen sulfide (H_2S) has been shown to exhibit a variety of biological effects in the cardiovascular system. It has been viewed especially as a vasorelaxing agent with antihypertensive properties. Indeed, both pharmacological inhibition and genetic disruption of CSE locus leads to an increase of blood pressure [78]. Thus, the interest in the biology of H_2S is growing up and a number of researches are devoting to the investigation of mechanism of action of H_2S and as well as to the development of drugs that can modulate H_2S release or biosyntheis in targeted tissues. Therefore, the thesis proposes two aims:

I) **Examine the molecular target of H_2S which triggers vasodilation.** Several mechanisms have been proposed to contribute to the effects of H_2S on vessel tone. Initially, H_2S was shown to enhance vasorelaxation by promoting K_{ATP} channel opening [42]. However, additional pathways contribute to vasorelaxation in response to H_2S, as K_{ATP} channel blockers fail to inhibit or do not completely abolish H_2S-induced relaxations in some tissues [360]. These

additional vasodilatory pathways might include other ion channels, as well as cGMP nucleotide regulated pathways. With respect to the latter, our group has recently observed that H_2S increases cGMP levels in smooth muscle cells [125]. Unlike nitric oxide that enhances cGMP synthesis by activating soluble guanylyl cyclase, elevations in cGMP in response to H_2S result from phosphodiesterase (PDE) inhibition [125]. By virtue of H_2S ability in inhibiting PDE, and thus to interact with L-arginine/NO/cGMP pathway, our purpose is to further analyze the role of cGMP in H_2S-induced vasorelaxation and to determine the contribution of cGMP-dependent protein kinase (PKG) in H_2S response.

II) **Investigate the role of sulphydrylated molecules, such as zofenopril, in the vascular function with respect to H_2S pathway.** Given that H_2S is involved in the pathogenesis of hypertension [140], it can be addressed as an additional therapeutic target for the management of this disease. Actually, ACE inhibitors are widely used in controlling blood pressure in hypertensive patients and they represent

first line treatment in different cardiovascular diseases, since they also show additional beneficial effects unrelated to ACE inhibition [309]. In particular, therapeutic use of zofenopril, a sulphydrylated ACE inhibitor, has raised hypothesis over a potential role of thiol group in such beneficial effects, other than antioxidant activity [357, 359]. Therefore, our aim is to investigate the link between the benefits of sulphydrylated ACEi zofenopril in therapies with respect to H_2S pathway activation.

Chapter 3

MATERIALS and METHODS I

3.1 Ethics statement

All animal procedures were in compliance with the European Community guidelines for the use of experimental animals and approved by the Committee Centro Servizi Veterinari of the University of Naples ‘‘Federico II’’; institutional regulations do not require the use of animal protocol numbers for approved protocols.

3.2 Animals

Male and female mice CD-1 6 to 8 weeks old and 129/Sv were purchased from Harlan Laboratories (Italy). Mice carrying a null mutation of the gene encoding PKG-I (PKG-$I^{-/-}$ mice, also termed $cGKI^{L-/L-}$ mice) and $CSE^{-/-}$ mice were generated as previously described [19,46]. PKG-$I^{-/-}$ mice were on a 129/Sv genetic background and analyzed at an age of 10 to 16 weeks. Animals were housed in our animal facility having free access to water and food.

3.3 Reagents

Cell culture media and serum were obtained from Life Technologies GIBCO-BRL (Paisley, UK). All cell culture plastic ware was purchased from Corning-Costar Inc. (Corning, NY). West Pico chemiluminescent substrate was purchased from Pierce Biotechnology (Rockford, Illinois); DC Protein assay kit, Tween 20 and other immunoblotting reagents were obtained from Bio-Rad Laboratories (Hercules, CA); penicillin and streptomycin were purchased from Applichem (Darmstadt, Germany). GYY4137, DT-2 and TAT were purchased from Cayman Chemical (Ann Arbor, Michigan), Biolog (Bremen, Germany) and Genscript (Piscataway, USA), respectively. The GAPDH, pVASP and secondary Abs were purchased from Cell Signaling Technologies (Beverly, MA), while the CSE antibody was obtained from Abnova Novus Biologicals (Littelton, CO). The PKG-I Ab used has been generated as previously described [47]. The anti-von Willebrand factor was obtained from Dako (Glostrup, Denmark), and the secondary anti-mouse and anti-rabbit antibodies used for the immunofluorescence studies were obtained from Life Technologies (Darmstadt, Germany). The cGMP

EIA kit was obtained from Assay Designs (Ann Arbor, MI). Sildenafil, sodium hydrosulfide (NaHS), L-cysteine (L-cys), S-nitroso-N-acetylpenicillamine (SNAP), phenylephrine, protease/phosphatase inhibitors and all other chemicals used in solutions and buffers were purchased from Sigma Chemical Co (Milan, Italy). All drugs were dissolved in distilled water.

3.4 *Ex vivo* studies

Animals were sacrificed with CO_2 and thoracic aortas were rapidly harvested, dissected, and cleaned of adherent connective and fat tissue. Rings of about 1 mm length were denuded of the endothelium, cut and placed in organ baths (2.5 ml) filled with oxygenated (95% O_2 -5% CO_2) Krebs solution maintained at 37°C. The rings were connected to an isometric transducer (type 7006, Ugo Basile, Comerio, Italy) and changes in tension were recorded continuously with a computerized system (Data Capsule 17400, Ugo Basile, Comerio, Italy). Exclusively in the set of experiments performed on aortic rings harvested from $PKG^{-/-}$ and their respective background 129/Sv the endothelium was preserved. The composition of the Krebs solution was as follow (mM): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4

1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10.1. The rings were stretched until a resting tension of 1.5 g was reached and allowed to equilibrate for at least 45 min, during which time tension was adjusted, as necessary, to 1.5 g and bathing solution was periodically changed. In each experiment, rings were first challenged with PE (1 mM) until the responses were reproducible. The rings were then washed and contracted with PE (1 mM) and, once a plateau was reached, a cumulative concentration-response curve of the following drugs was performed: SNAP (100 pM–3 mM); NaHS (1 mM–300 mM); L-cys (1 mM–300 mM); GYY4137 (1 mM–300 mM). Rings were treated with the PKG inhibitor DT-2 or its control peptide TAT (1–3 mM; 20 minutes), or with PDE5 inhibitor sildenafil (1 nM; 15 minutes). After incubation time, cumulative concentrationresponse curve to SNAP; NaHS; L-cys; GYY4137 were performed. A preliminary study on the optimal incubation time and concentration of the drug treatments was carried out (data not shown). In another set of experiments, a cumulative concentrationresponse of NaHS and L-cys were carried out on aortic rings from PKG-$I^{-/-}$ and 129/Sv strains.

3.5 Conscious systemic blood pressure measurement

Systolic blood pressure (SBP) was measured in conscious mice using the pneumatic tail-cuff method (W+W Blood pressurereporter, model 8006, Ugo Basile). Before the measurement, animals were preheated in a room at 30°C for 30 min, then they were placed in a plastic chamber. A cuff with a pneumatic pulse sensor was attached to the tail. This procedure was performed every day for 1 week before starting the experiments in order to habituate the animals to this procedure. During the entire measurement period, the temperature was maintained at 30°C. Two consecutive measurements were always recorded. SBP was measured and, once basal SBP was assessed, intraperitoneal injection of (D)-DT2 (100 nmoles) was performed. A more stable form of DT-2 [(D)-DT-2] that is composed of D-aminoacids was chosen for the *in vivo* experiments. SBP was then evaluated twice every 5 minutes. Fifteen minutes after the (D)-DT2 injection, NaHS (1µmol/kg) was administered subcutaneously. SBP was then monitored every 5 minutes for three times. (D)-DT2 volume injected was 50µl i.p. NaHS volume injected was 30µl s.c. Both drugs were dissolved in saline.

3.6 Cell culture

Rat aortic smooth muscle cells (RASMCs) were isolated from 12- to 14-wk-old male Wistar rats, five rats per isolation, as previously described [412]. Animals were anesthetized with pentobarbital sodium (40 mg/kg ip). Once fully anesthetized as judged by the lack of reaction to a noxious stimulus, animals were exsanguinated; thoracic aortas were then removed. More than 95% of cells isolated stained positive for smooth muscle a-actin. Cells between passages 2 and 5 were used for all experiments. RASMCs were routinely cultured in DMEM containing 4.5 g/l glucose and supplemented with 10% FBS and antibiotics.

3.7 Western Blotting

Aortic tissues of CD-1 and 129/Sv were homogenized in modified RIPA buffer (Tris HCl 50 mM, pH 7.4, TritonX-100 1%, Sodium-deoxycholate 0.25%, NaCl 150mM, EDTA 1mM, phenylmethanesulphonylfluoride 1 mM, aprotinin 10 mg/ml, leupeptin 20 mM, NaF 50 mM) using a polytron homogenizer (two cycles of 10 s at maximum speed). In experiments performed to

determine the expression of CSE in wild-type and PKG-$I^{-/-}$ animals, aortas from three animals were pooled and then homogenized. After centrifugation of homogenates at 12000 r.p.m for 15 min, protein concentration was determined by Bradford assay using BSA as standard. 40 mg of the denatured proteins were separated on 10% SDS/PAGE and transferred to a PVDF membrane. Membranes were blocked in PBS-Tween 20 (0.1%, v/v) containing 3% non fat dry milk for 1 hour at room temperature, and then incubated with the primary antibody overnight at 4°C. The filters were washed with PBS-tween 20 (0.1%, v/v) extensively for 30 min, before incubation, for 2 hours at 4°C, with the secondary antibody (1:5000) conjugated with horseradish peroxidase anti-mouse IgG. The membranes were then washed and immunoreactive bands were visualized using a chemiluminescence substrate.

3.8 cGMP measurements

Rat aortic smooth muscle cells were incubated for 5 min with the indicated concentration of the H_2S donors. After the treatment, cells were washed with Hanks' balanced salt solution and cGMP was extracted using 0.1N HCl. cGMP content was measured in the

extracts using a commercially available enzyme immunoassay kit following the manufacturer's instructions. For cGMP measurements in tissue and plasma of CSE knockout mice ($CSE^{-/-}$), eight-week male $CSE^{-/-}$ and wild-type mice ($CSE^{+/+}$) were used in this experiments. Animals were anesthetized with pentobarbital sodium (40 mg/kg ip) and exsanguinated. Blood plasma was prepared by spinning a tube of fresh blood containing EDTA (1500xg for 15 min at 4°C). The aorta and mesenteric artery tissues were dissected and cleaned for immediate cGMP measurement. First, aortic rings were placed in Krebs solution at 37°C and incubated for 30 min. After that, rings were stimulated with sodium nitroprusside (10mM) for 2 min and then tissues were rapidly blotted weighted and quick frozen in liquid nitrogen. Tissues were snap frozen and homogenized in 1–3 volumes of buffer (containing 5% trichloroacetic acid) per gram of tissue and centrifuged at 1,500xg for 10 min. The supernatant was carefully removed and used in the next step. Residual TCA acid was removed by extraction into five volumes of watersaturated diethyl ether (repeated twice for a total of 3 extractions). Any residual ether was removed by warming the samples at 70°C for 5 min. The samples

were then processed according to the instructions provided with commercially available enzyme immunoassay kit following the manufacture's instruction. Blood plasma was prepared by spinning a tube of fresh blood containing EDTA (1500xg for 15 min at 4°C). The aorta and mesenteric artery tissues were dissected and cleaned for immediate cGMP measurement. First, aortic rings were placed in Krebs solution at 37°C and incubated for 30 min. After that, rings were stimulated with sodium nitroprusside (10 mM) for 2 min and then tissues were rapidly blotted weighted and quick frozen in liquid nitrogen. Tissues were snap frozen and homogenized in 1–3 volumes of buffer (containing 5% trichloroacetic acid) per gram of tissue and centrifuged at 1,500xg for 10 min. The supernatant was carefully removed and used in the next step. Residual TCA acid was removed by extraction into five volumes of watersaturated diethyl ether (repeated twice for a total of 3 extractions). Any residual ether was removed by warming the samples at 70°C for 5 min. The samples were then processed according to the instructions provided with commercially available enzyme immunoassay kit following the manufacture's instruction.

3.9 Statistical analysis

Data were expressed as mean ± s.e.m. Statistical analysis was determined by using one or two way ANOVA and Dunnett's or Bonferroni as a post-test or t-test analysis when appropriate. Differences were considered statistically significant when P-value was less than 0.05. GraphPad Prism software (version 4.02, GraphPad Software, San Diego, CA) was used for all the statistical analysis.

Chapter 4

RESULTS I

4.1 PDE regulates H_2S-induced relaxation

It has been demonstrated that exposure of smooth muscle cells to NaHS increases cGMP by inhibiting PDE [125]. To test whether our biochemical observations are functionally relevant, we pre-incubated rat aortic rings with a low concentration of the PDE5 inhibitor sildenafil (1 nM) and then contracted them with phenylephrine. Such pre-treatment did not have a significant effect on the ability of phenylephrine to cause tissue contraction, but differentially affected NO-induced vs H_2S-induced vasorelaxation. Incubation of rings with sildenafil led to a potentiation of NO-induced relaxation as evidenced by the leftward shift of the SNAP dose-response curve ($6x10^{-7}$ M vs. $1.4x10^{-7}$ M vehicle vs sildenafil, $p<0.001$; Fig. 4.1A). In contrast to the findings with the NO donor, pre-treatment with sildenafil attenuated the relaxing effect of NaHS in rat aorta (Fig. 4.1B). The observed rightward shift of the NaHS dose-response in the presence of sildenafil ($2.8x10^{-4}$ M vs. $8.5x10^{-5}$ M vehicle vs sildenafil $p<0.001$)

is consistent with the notion that NaHS-stimulated vasodilation is at least in part mediated by PDE5 inhibition.

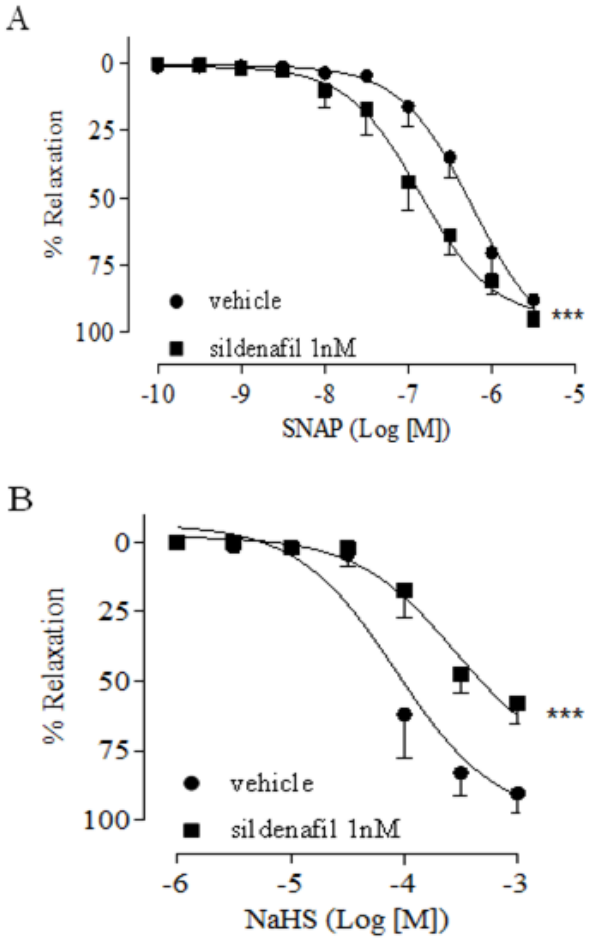


Fig. 4.1: The PDE5 inhibitor sildenafil differentially affects NO and H_2S-regulated vascular tone. *(A) Incubation of isolated aortic rings with sildenafil (1 nM) significantly inhibited NaHS-induced vasodilatation. (B) Incubation of isolated aortic rings with sildenafil (1 nM) significantly enhanced SNAP-induced vasodilatation. *** $p<0.001$ vs vehicle (dH_2O), n = 6 for each group.*

To provide proof that endogenously produced H_2S acts as a PDE inhibitor, we measured cGMP levels in the plasma and vascular tissues of $CSE^{-/-}$ mice. In these experiments we observed cGMP levels in the plasma (9.81±0.75 pmole/ml), aorta (9.55±0.80 pmole/mg protein), and mesenteric artery (0.42±0.04 pmole/mg protein) from $CSE^{-/-}$ mice were significantly lower than those in the plasma (20.74±1.97 pmole/ml), aorta (23.40±1.44 pmole/mg protein) and mesenteric artery (1.57±0.05 pmole/mg protein) from $CSE^{+/+}$ mice (Fig. 4.2). In addition, stimulation of vascular tissues with sodium nitroprusside increased cGMP levels in a statistically significant manner only in the vessels of wild-type, but not in the vessels of $CSE^{-/-}$ animals. The above observations taken together are consistent with the idea that H_2S is an inhibitor of PDE activity in vascular tissues.

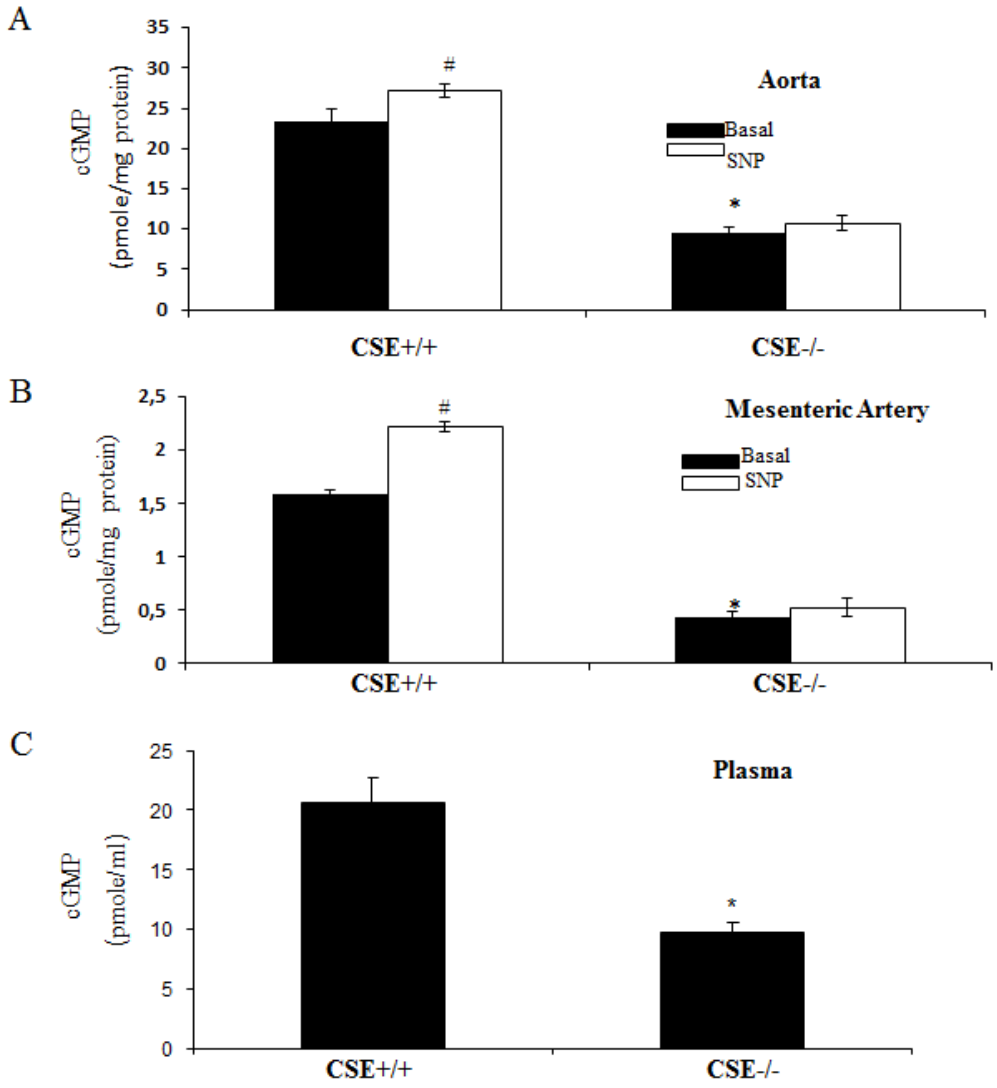
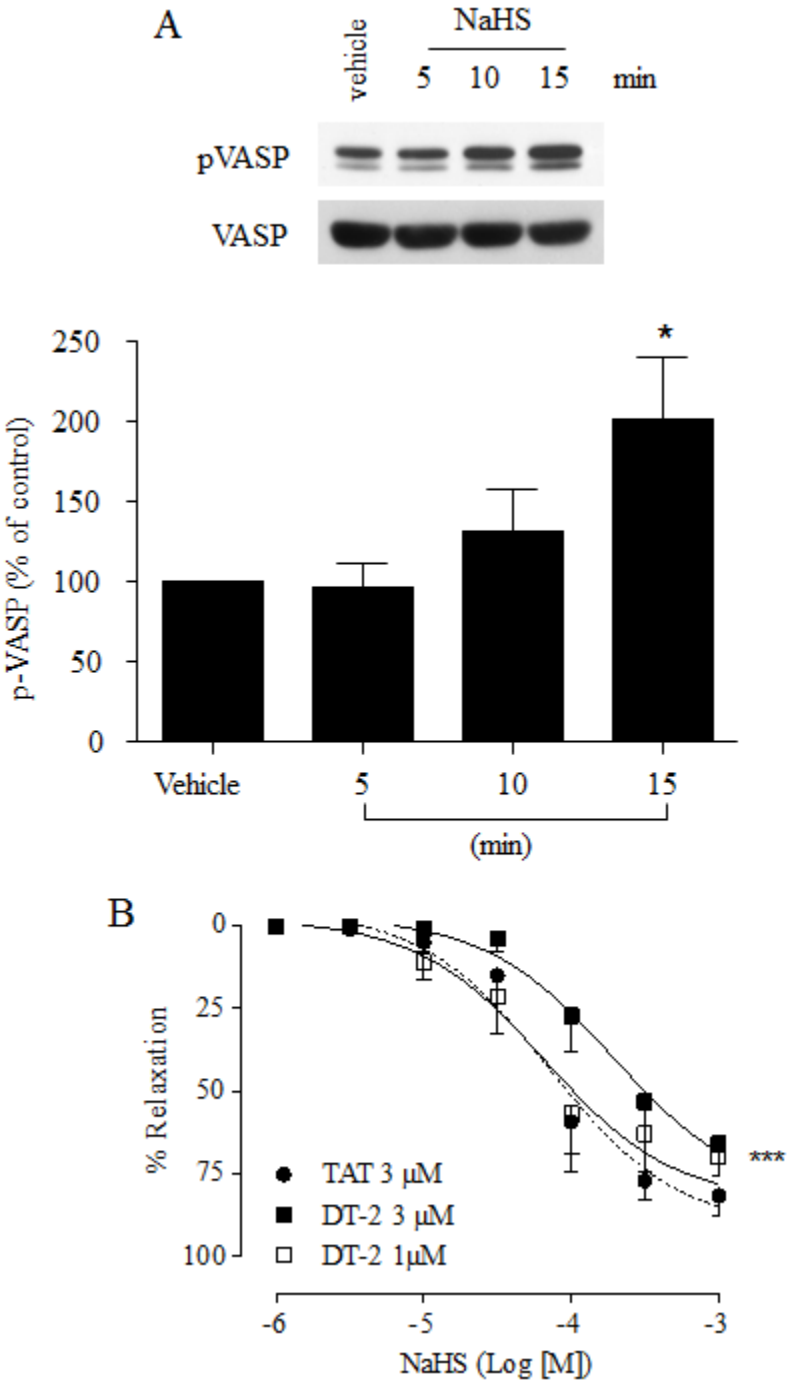


Fig 4.2: CSE deficiency reduces cGMP levels. *cGMP levels in the aorta (A), mesenteric artery (B) and plasma (C) of $CSE^{-/-}$ mice were significantly lower than those from $CSE^{+/+}$ mice. Sodium nitroprusside (SNP, 10 mM) significantly increased cGMP levels in aorta (B) and mesenteric artery (C) from $CSE^{+/+}$ mice but not $CSE^{-/-}$ mice; * p,0.05 vs $CSE^{+/+}$ mice, #p<0.05 basal, n = 5 for each group.*

4.2 H_2S activates PKG in vascular tissues

To elucidate the downstream signalling pathways activated in response to increased intracellular cGMP, we evaluated the ability of NaHS to stimulate cGMP-dependent protein kinase. To this end, we determined VASP phosphorylation on Ser239, as an index of PKG activation [24]. Indeed, exposure of aortic tissue to NaHS enhanced vasodilator stimulated phosphoprotein (VASP) phosphorylation in a time-dependent manner (Fig. 4.3A). Moreover, incubation of rings with DT-2, a PKG-I inhibitor, attenuated NaHS-induced vasorelaxation, while TAT (control peptide) had no effect (Fig. 4.3B); these findings provide evidence that PKG-I participates in H_2S-stimulated dilatation. To study the role of PKG-I in the hypotensive effect of H_2S in vivo, mice we pre-treated with DT-2 prior to being treated with the NaHS (Fig. 4.3C). DT-2 administration led to an increase in systolic blood pressure (SBP). Subcutaneous injection of NaHS resulted in a fall in SBP that reached a trough, 5 min after the injection, with a complete recovery within 15 minutes. In contrast, NaHS did not alter SBP in mice treated with the PKG-I inhibitor.


A
vehicle
NaHS
5 10 15 min
pVASP
VASP
p-VASP (% of control)
250
200
150
100
50
0
*
Vehicle
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15
(min)
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% Relaxation
0
25
50
75
100
TAT 3 μM
DT-2 3 μM
DT-2 1μM
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-6
-5
-4
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NaHS (Log [M])

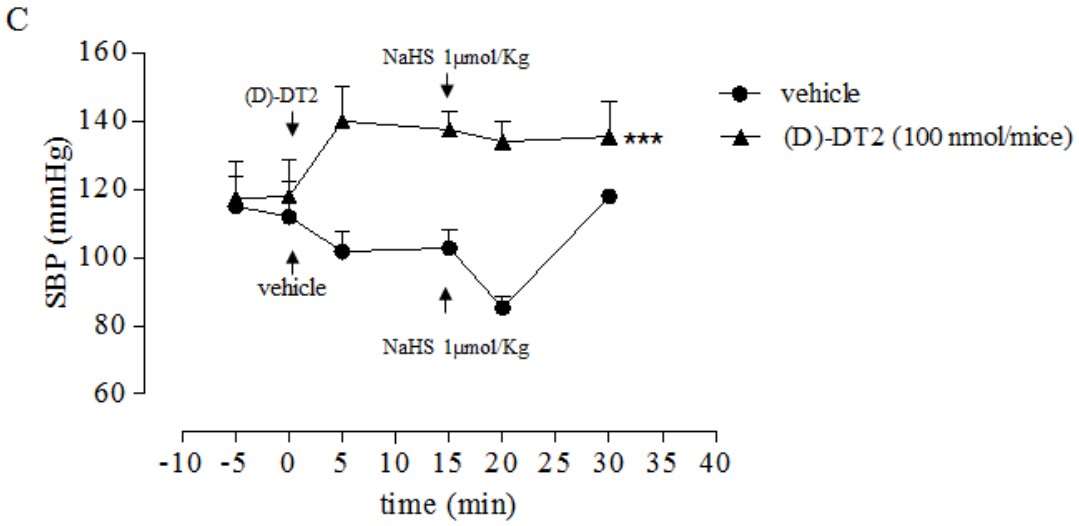


Fig. 4.3: H_2S activates PKG and triggers vasodilatation. *(A) Mouse aorta was incubated with NaHS (50 mM) for the indicated time and VASP phosphorylation on Ser239 was determined. Up: rapresentative blot; down: quantification of scanned autoradiograms, *p<0.05 vs vehicle, n = 3. (B) Incubation of aortic rings with the selective inhibitor of PKG, DT-2 (1, 3 mM) significantly inhibited NaHS-induced vasodilatation. TAT peptide (3 mM) was used as a control; *** p<0.001 vs. vehicle (dH_2O), n = 6 for each group. (C) Mice were injected with vehicle or DT-2 (100 nmoles, ip); after 15 min NaHS (1 mmol/kg) was administered subcutaneously. Systolic blood pressure (SBP) was monitored in conscious mice; *** p<0.001 vs vehicle, n = 4 for each group.*

In a different set of experiments we utilized GYY4137, a slow releasing H_2S donor. It should be noted that incubation of aortic smooth muscle cells with GYY4137, unlike NaHS, resulted in only minor increases in cGMP content (Fig. 4.4A&B). In agreement to what has been published, relaxation in response to GYY4137 took longer to manifest compared to the fast relaxations brought about by NaHS [95]. Moreover, GYY4137-stimulated relaxations were PKG-independent, as DT-2 failed to block the effects of this H_2S donor.

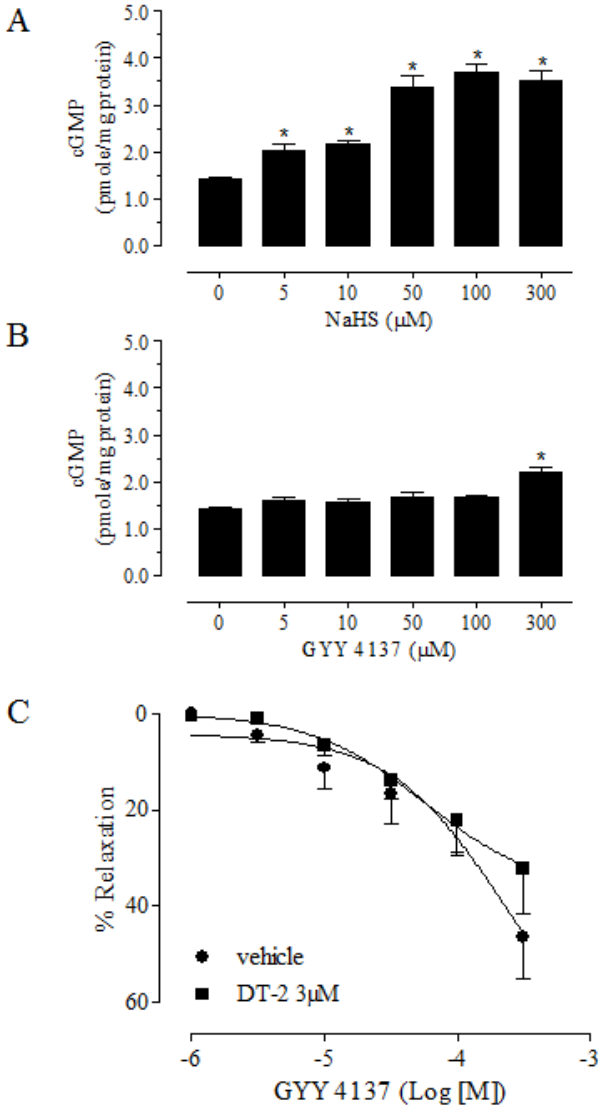
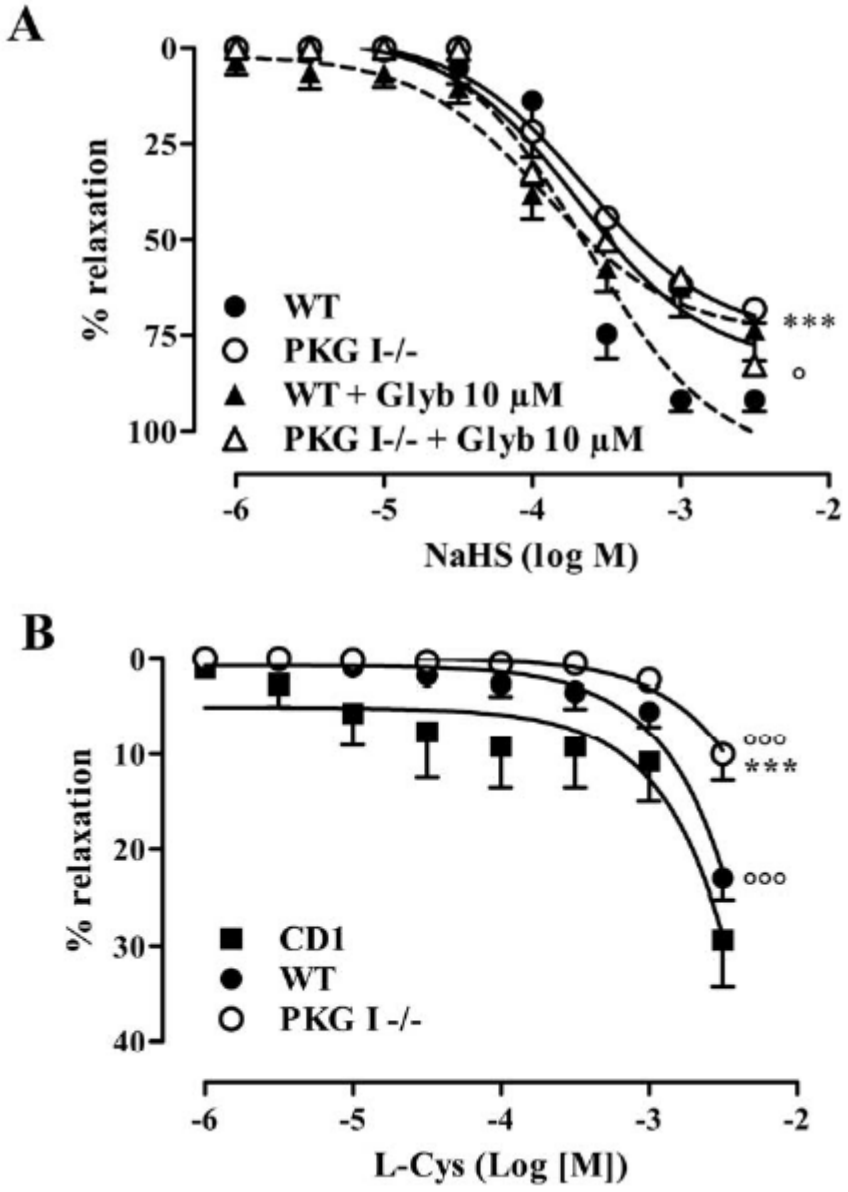


Fig. 4.4: GYY4137-induced relaxation is independent of PKG. *Aortic smooth muscle cells were exposed to the indicated concentration of NaHS (A) or GYY4137 (B) and cGMP levels were determined after 5 min. *p<0.05 control, n = 4 for each group. (C) Incubation of isolated aortic rings with PKG selective inhibitor DT-2 (3 mM) did not affect GYY4137-induced vasodilatation; n = 6 for each group.*

4.3 Genetic evidence for the role of PKG-I in H_2S-induced vasorelaxation

Although DT-2 is the most selective PKG-I inhibitor available, questions regarding its specificity have surfaced [413]. To confirm that H_2S uses PKG-I-regulated pathways to reduce vascular tone, we utilized blood vessels from PKG-$I^{-/-}$ mice. In these experiments we found that relaxations to NaHS were significantly hampered in PKG-$I^{-/-}$ vessels (Fig. 4.5A); however a significant residual response was observed, suggesting that complementary vasodilator pathways do exist. Importantly, glibanclamide, a K_{ATP} channel inhibitor, blocked NaHS-induced dilation in vessels from wild-type, but not PKG-$I^{-/-}$ mice, suggesting that both PKG-I and K_{ATP} work on the same effector pathway to trigger vasodilation. To study whether PKG-I is important for the dilatation in response to endogenously produced H_2S, rings were exposed to Lcysteine, the substrate for H_2S generation, and reduction in vessel tone was measured. L-cysteine promoted vasorelaxation in the vessels of wild-type mice; this response was greatly reduced in PKG-$I^{-/-}$ vessels (Fig. 4.5B). The diminished relaxation to L-cysteine observed in the PKG-$I^{-/-}$ mice was not due to

lower levels of the H_2S producing enzyme CSE in PKG-I $^{-/-}$ aortic tissue (Fig. 4.5D). It should be noted that relaxations in response to L-cysteine were of smaller magnitude in the 129/Sv mice (wt mice) compared to those observed in CD1 used in the first series of our experiments (max relaxation 30±4.89 vs. 23±2.14 in CD1 and 129/Sv respectively). This difference can be attributed to the lower levels of CSE expression in the aortas of 129/Sv mice (Fig. 4.5C).


A
0
25
50
75
100
% relaxation
WT
PKG I-/-
WT + Glyb 10 µM
PKG I-/- + Glyb 10 µM
***
°
-6
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-3
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NaHS (log M)
B
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10
20
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L-Cys (Log [M])

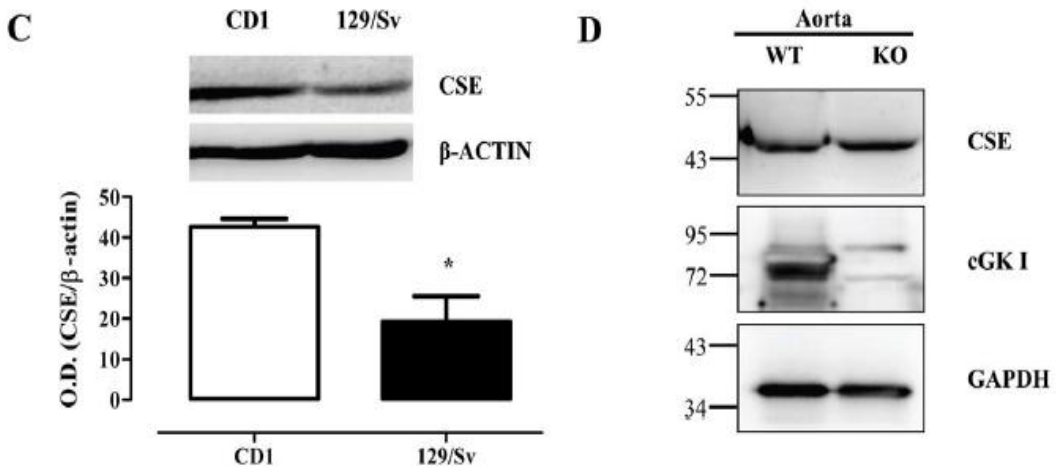


Fig. 4.5: PKG contributes to the relaxing effect of exogenous and endogenous H_2S. *(A) Mouse aortas from wild-type or PKG-I$^{-/-}$ animals were pre-treated with vehicle or glibenclamide (10 mM, 30 min) and then incubated with the indicated concentration of NaHS (n = 6 rings harvested from 3–4 animals); dashed lines are used for wild-type animals, while solid lines are used for knockouts. (B) L-cysteine-induced vasodilatation of aortic rings pre-contracted with phenylephrine from wild-type and PKG-I$^{-/-}$ mice. Note that cumulative concentration-response curves to L-cysteine were significantly different among the different strains of mice used CD1 vs 129/Sv (WT); °°° p<0.001 vs. CD1, *** p<0.001 vs WT, n = 8 rings harvested from 3–4 animals for each group (C) Representative blot and quantitation depicting aortic CSE expression in 129/Sv vs CD-1; n = 3 for each group, *p<0.05. (D) Representative blot showing expression of CSE in aortic homogenates in wild-type and PKG-I$^{-/-}$ mice. Experiments were performed twice with similar results.*

4.4 H_2S stimulates relaxations in both endothelium intact and denuded aortic rings

To test the relative contribution of each cell type (endothelium vs smooth muscle) to the relaxing effect of H_2S, endothelium intact or endothelium denuded mouse (CD 1) vessels were exposed to either a H_2S donor or a H_2S substrate (L-cysteine). While the vasodilatory response to NaHS was identical irrespective of whether endothelium was present or not (Fig. 4.6B), responses to L-cysteine were reduced in endothelium-denuded vessels (Fig. 4.6A). Staining of aortic tissue with an antibody against CSE revealed that although small amounts of the enzyme are present in the endothelium, the majority of CSE is expressed in the smooth muscle layer (Fig. 4.6C).

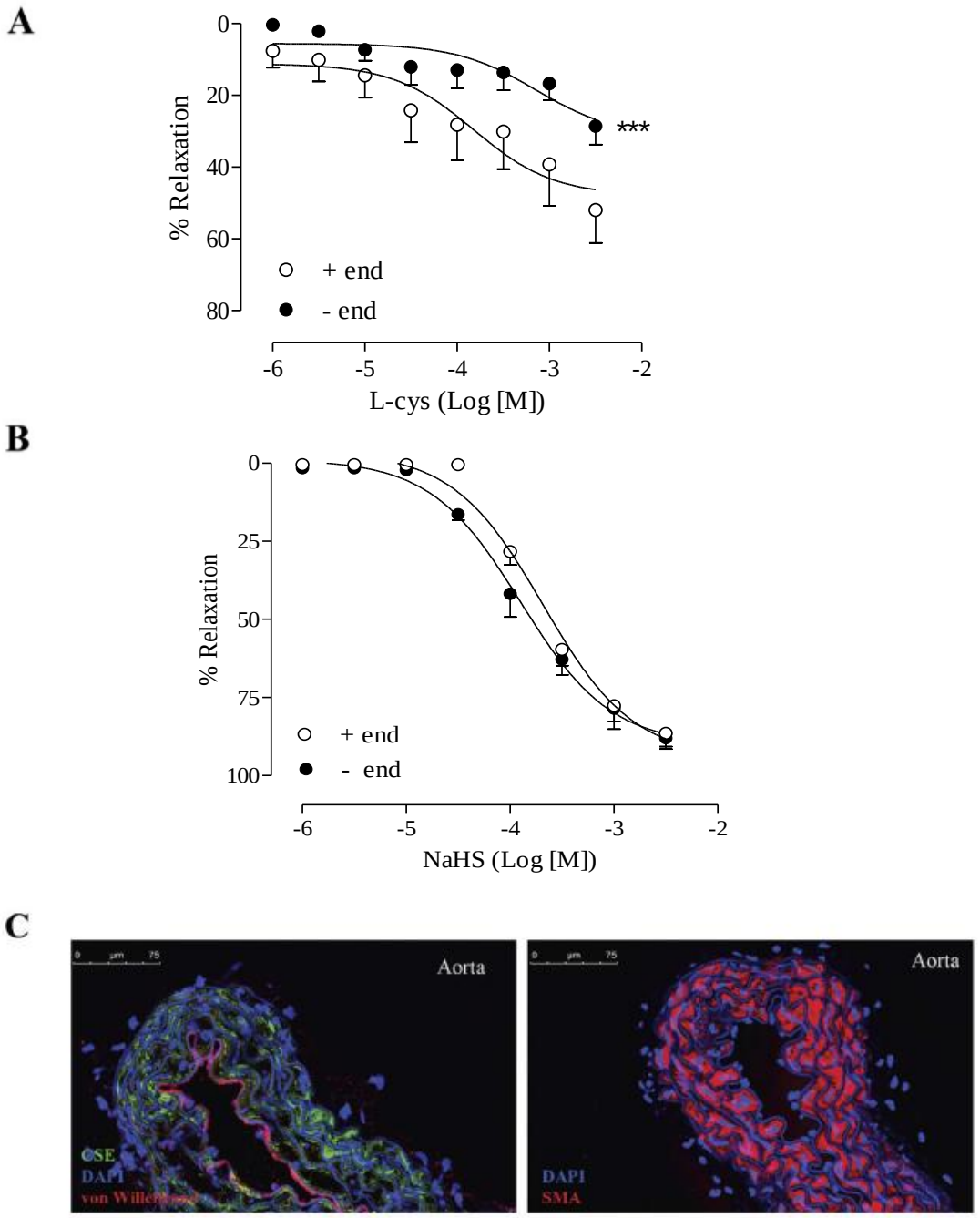


Fig. 4.6: Role of endothelium in H_2S induced-vasodilatation. *(A) L-cysteine-induced vasodilatation was significantly impaired in aortic rings without endothelium (–end). (B) NaHS-induced vasodilatation is not affected by endothelium removal; *** $p<0.001$ vs –end, $n = 6$ for each group. (C) Representative photomicrographs of aortas stained with a CSE antibody and counter-stained for von-Willebrant factor, smooth muscle α-actin (SMA) and DAPI, showing localization of CSE.*

Chapter 5

DISCUSSION I

Relaxation to H_2S is reported to occur through K_{ATP} channel activation [42], leading to the hypothesis that H_2S is an endothelium-derived hyperpolarizing factor [109, 111]. The effect of H_2S on these channels has been proposed to result from sulfhydration of Cys 43 of the pore-forming Kir6.1 subunit and/or interactions with Cys6 and Cys26 of the regulatory subunit SUR1 [107, 109]. Despite the large number of publications proving K_{ATP} channel involvement in the dilatory responses to H_2S, H_2S-induced vascular relaxation is only partially inhibited by glibenclamide [28, 42, 76, 414]. There are also instances where K_{ATP} channel inhibition does not attenuate H_2S-induced vasorelaxation [112, 117]. Based on the ability of H_2S to increase cGMP levels in vascular tissues, herein we investigated the role of this cyclic nucleotide in H_2S-induced vasorelaxation and the interaction between cGMP-regulated pathways and K_{ATP} channels in mediating the effects of H_2S. As we have previously shown that H_2S inhibits PDE activity [125], initially, we sought to determine whether H_2S-triggered relaxation is mediated by inhibition of PDE. Inhibition of PDE5 by sildenafil blocks cGMP

breakdown and leads to a reduction in vascular tone [415]. PDE5 blockade has been shown to potentiate the vasodilatory action of NO donors in the aorta [415]. In our experimental setup we confirmed that pre-treatment of mouse aortic rings with sildenafil potentiated the dilatory response to SNAP. In contrast, pre-incubation with sildenafil lead to a rightward shift and reduced the maximal response to NaHS, suggesting that H_2S, at least in part, relaxes vascular tissue by inhibiting PDE5. To obtain additional evidence that H_2S regulates cGMP levels we used tissues from $CSE^{-/-}$ mice. Under basal conditions cGMP levels in the aorta and mesenteric artery were lower in $CSE^{-/-}$ compared to wild-type controls. In addition, although a significant increase in cGMP levels was observed after exposure to a NO donor in vessels from wt animals, no such increase was seen in $CSE^{-/-}$ Taken together this data suggest that H_2S relaxes blood vessels by modulating cGMP levels. We next sought to determine the signalling pathways downstream of cGMP that become activated after H_2S exposure and lead to vasodilation. cGMP is known to activate cGMP-dependent protein kinases and to modulate the activity of cGMP-gated ion channels and phosphodiesterases [416]. To evaluate the ability of NaHS to activate PKG we used VASP phosphorylation on

Ser239, a site that is preferentially phosphorylated by PKG. Phosphorylation of this VASP residue in vascular extracts is widely used as an index of NO/cGMP pathway activity [417]. Exposure of aortic rings to NaHS resulted in a time-dependent phosphorylation [416]. In agreement with our findings, relaxation in response to H_2S in the mouse gastric fundus is partially blocked by a MLCP inhibitor [418]. So far, studies designed to evaluate the role of cGMP in H_2S-induced relaxation have reported mostly negative results. In many cases authors were unable to inhibit NaHS-stimulated relaxation by inhibiting soluble guanylyl cyclase activity (sGC) [42, 101, 112, 419]. cGMP levels might still rise in response to H_2S donors in ODQ-treated tissues, as PDE rather than sGC is the target for H_2S; cGMP in vascular tissues incubated with ODQ can be synthesized by the basal sGC activity (ODQ does not inhibit basal sGC activity [420]), as well as through natriuretic peptide receptors. In our next series of experiments we utilized GYY4137, a slow releasing H_2S donor and determined the contribution of cGMP/PKG pathway to vasorelaxation. In line with previous findings, GYY4137 relaxed pre-contracted aortic rings [95], but elicited a smaller dilatory response with slower kinetics; maximal relaxation to GYY4137 was 46% and took 90–

120 min to occur, while NaHS relaxed aortic rings >80% within 30 min. Another striking difference between the two donors was their differential sensitivity to PKG inhibition. Unlike what was observed with NaHS, GYY4137-induced relaxation was not inhibited by DT-2. Also, exposure of smooth muscle cells to GYY4137 at concentrations below 0.3 mM failed to enhance cGMP levels in smooth muscle cells. The difference in the rate of H_2S release and thus the concentrations of H_2S achieved after administration of a given dose of each H_2S donor could explain the difference in their ability to inhibit PDE and enhance cGMP levels in cells. Although it is frequently claimed and intuitively makes sense that slow H_2S release from donors is more physiologically relevant, endogenous H_2S production has never been compared to the rate of H_2S release from this slow H_2S donor, neither has the half-life of GYY4137 been determined in any biological system, *in vitro* or *in vivo*. The time required for GYY4137 to elicit vasorelaxation, compared to the fast responses triggered by L-cysteine, indicates that it might require bioactivation or that GYY4137 releases H_2S at a much slower rate than that produced endogenously. In line with the minimal amounts of H_2S liberated from this donor, when high GYY4137 concentrations are used,

PDE inhibition becomes apparent. Thus, one might hypothesize that lower GYY4137 concentrations that trigger sub-maximal vasodilation occur through cGMP-independent pathways (this would represent the 50% residual dilation seen in PKG-$I^{-/-}$ animals after NaHS), while at higher H_2S concentrations cGMP pathways become important. In addition to the herein presented finding that the fast H_2S donor NaHS increases cGMP levels in smooth muscle cells, we have demonstrated that slow releasing H_2S donors (thioglycine, thiovaline) are also capable of increasing cGMP in this cell type [421]. Taken together our findings suggest that researchers utilizing different H_2S donors with varying half-lives and modes of H_2S release, should not assume the participation of cGMP/PKG pathways in the observed responses, but should rather determine cGMP levels and PKG activation after application of the donor in their system. DT-2 is a peptide inhibitor that was originally described as being highly selective for the PKG isoform expressed in vessels, PKG-I [422]. It is 1000-foldmore selective for PKG vs. PKA and exhibits a 100-fold selectivity for PKG-I vs. PKG-II. However, questions regarding the behaviour and specificity of this inhibitor in intact cells have emerged [423]. Gambaryan et al., reported that DT-2 modulated the activity of

ERK, p38, PKB and PKC. To prove the involvement of PKG-I in H_2S-induced vasodilation we used a genetic model. Mice with targeted disruption of the PKG-I locus exhibited a reduced maximal relaxing response to NaHS. To test if endogenously produced H_2S also reduces vessel tone through PKG-I activation, vessels were exposed to L-cysteine and vascular tone determined. Similarly to what was observed with NaHS, relaxations to L-cysteine were reduced in the PKG-$I^{-/-}$ animals, providing genetic evidence that relaxation in response to both exogenously applied H_2S (NaHS) and endogenously produced H_2S are mediated in part through PKG-I. Moreover, inhibition of K_{ATP} channels with glibenclamide led to an inhibition of NaHS-induced vasorelaxation in rings from wildtype mice, that was of similar magnitude to that observed in $PKGI^{-/-}$ animals. It should be emphasized that glibenclamide did not exhibit an additional inhibitory effect on NaHS dilations in PKG-$I^{-/-}$ mice, suggesting that K_{ATP} and PKG-I work in tandem to promote vasorelaxation. Evidence that PKG activates K_{ATP} channels in the cardiovascular system has been previously reported [424-427]. It should however be noted that a substantial relaxation (approximately 50%) was still observed in the vessels of $PKG^{-/-}$ mice, providing proof that

additional pathways become activated by NaHS and allow H_2S to reduce vessel tone. The K_{ATP}-insensitive dilatory response to NaHS might occur through voltage-dependent K^+ channels [414], and intracellular acidification through activation of Cl^-/HCO_3^- [428]. The relative contribution of cGMP/PKG pathways vs alternative pathways in H_2S vasorelaxation are expected to vary with the vascular bed and species studied. In addition to the relaxing effect of NaHS on pre-contracted rings, we also observed that NaHS administration *in vivo* reduced systolic blood pressure in a DT-2 sensitive manner. However, since the requirement for PKG-I in the drop in blood pressure elicited by NaHS was only shown using a pharmacological inhibitor for which concerns have been raised [423], ultimate proof that PKG-I mediates the reduction in mean arterial blood pressure triggered by H_2S will have to await conformation by a genetic model. In the course of our experiments we noticed that L-cysteine exerted a somewhat smaller effect in the aortic rings of the control mice (WT) compared to the dilation we routinely get in response to this H_2S synthesis substrate. As the PKG-I-/- mice have been generated on a 129/Sv genetic background, we compared L-cysteine-induced relaxation in CD-1 and 129/Sv mice. Indeed, we

observed that L-cysteine-induced relaxations were attenuated in 129/Sv mice compared to CD-1 and this reduced response correlated with a lower expression of CSE in the vessels of 129/Sv animals. It should also be noted that relaxation of aortic rings from C57BL/6J mice are even smaller (approximately 15%, data not shown). These observations taken together confirm that strain differences in H_2S responses do exist, adding another level of complexity when comparing data from different studies. Zhao et al [42] have previously shown that CSE, but not CBS, is expressed in the endothelium-free rat pulmonary artery, mesenteric artery, tail artery and aorta; they also proposed that CSE localizes to the smooth muscle cell layer of blood vessels. It later became apparent that cultured endothelial cells, as well as the endothelium in native vessels express CSE [43, 193, 429]. To determine the relative functional importance of each layer in H_2S dilation, we tested the ability of endothelium-intact and endothelium-denuded aortic rings to relax to L-cysteine. Removal of the endothelium resulted in a significant decrease of L-cysteine stimulated relaxation without affecting that ability of NaHS to dilate the vessels. On the other hand, we observed that in the mouse aorta, CSE is primarily expressed in the smooth muscle cell layer;

however, lower CSE levels are present in the endothelium. The significant effect of endothelial denudation in L-cysteine dilation could be attributed to the fact that removal of the endothelial lining results in loss of NO production. Lack of NO is expected to inhibit H_2S responses as the action of the two gasotransmitters on vascular tone and angiogenesis has been shown to be interdependent [430]. In summary, we have provided pharmacological and genetic evidence for the existence of a cGMP/PKG pathway downstream of H_2S that regulates vascular tone. The two vasodilatory gasotransmitters, H_2S and NO, regulate contractility by acting on the degradation and synthesis of cGMP, respectively. Convergence of the two pathways on the same effector (PKG) in the vessel wall, would allow for the fine-tuning of vascular tone, but also provide the redundancy needed to maintain vascular homeostasis and prevent disease development.

Chapter 6

MATERIALS AND METHODS II

6.1 Animals and systolic blood pressure measurements

Male spontaneously hypertensive rats (SHR) and control Wistar Kyoto (WKY) rats 8 weeks old were purchased from Charles River and kept in 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 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 Declaration of Helsinki (European Union guidelines on use of animals in scientific experiments) ARRIVE guidelines and authorised by Centro Servizi Veterinari Università degli Studi di Napoli "Federico II".

6.2 Drugs and reagents

All reagents for Krebs buffer solution preparation were purchased from Carlo Erba Reagents (Milano, Italy). Phenylephrine (PE),

Acetylcholine (Ach) and L-cys were instead purchased from Sigma-Aldrich (Milano, Italy). Experiments were performed by using zofenopril or its water-soluble metabolite zofenoprilat. In particular we used S-zofenopril calcium salt, the diasteiroisomers S-zofenoprilat sodium and R-zofenoprilat sodium salt. Enalapril and its active metabolite enalaprilat were used as a non-sulphydrylated ACE inhibitor control. All ACE inhibitors were supplied by Menarini Research group (Firenze, Italy).

6.3 Experimental design: *in vivo* study

SHR and WKY rats of 8 weeks of age have been employed. Systolic blood pressure (SBP) was monitored for 4-6 weeks until hypertension was developed in SHR and a statistical difference ($p<0.05$) in pressure values was reached between SHR and WKY strains. Rats were divided in six different treatment groups (n=10 for each group) and organized as follows:

-SHR: vehicle, zofenopril and enalapril.

-WKY: vehicle, zofenopril and enalapril

Drug treatment was carried out on a daily basis for two weeks by using either 10mg/kg of zofenopril or 6mg/kg of enalapril, in 0.2% carboxymethylcellulose, per gavage. SBP was monitored during the treatment. After two weeks, animals were sacrificed. Carotid and aorta were harvested and *ex vivo* vascular reactivity assessed. Vascular tissues obtained were also used for western blot analysis and H_2S determination. Blood samples were also collected in order to measure H_2S levels. Zofenopril is a mixture of diasteroisomers and the active diasteiroisomer normally employed in therapy is the S isoform. Indeed, R-zofenoprilat has an ACE IC_{50} of 1700nM while the S isoform IC_{50} is 10.9 nM [437]. In another set of experiments, we evaluated blood pressure changes following treatment *in vivo* with S-zofenopril water soluble metabolites S-zofenoprilat or its inactive diastereoisomer R-zofenoprilat, at a dose that does not inhibit ACE [437]. Similarly to above, an *ex vivo* analysis of the aorta and carotid reactivity was performed.

6.4 Experimental design: *ex vivo* study

Rings from aortic and carotid arteries were mounted in an isolated organ bath system filled with Krebs' buffer solution (mmol/L: 115.3

NaCl, 4.9 KCl, 1.46 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 11.1 glucose) at 37°C and gassed with O_2/CO_2 mixture (95%/5%). Organ baths were equipped with force transducer (FORT10, Ugo Basile, Milano, Italy) connected to the acquisition data system PowerLab (ADInstruments, Colorado Springs, CO, US) with a computerized system where changes in tension were continuously measured. The rings were stretched until a resting tension of 1.5 g (for carotid) or 0.5 g (for aorta) was reached and allowed to equilibrate for at least 45 min, during which time 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 (0.1nmol/L-30µmol/L) or L-cys (0.1µmol/L-10mmol/L).

6.5 Western blot analysis

Aortic and carotid tissues were homogenized in modified RIPA buffer (50mmol/L Tris-HCl, pH 7.4, 1% v/v Triton, 0.25% w/v sodium deoxycholate, 150mmol/L sodium chloride, 1mmol/L EDTA,

1mmol/L phenylmethanesulphonylfluoride, 10mg/ml aprotinin, 20mmol/L leupeptin and 50mmol/L sodium fluoride). The denatured proteins (40 µg) were separated on 10% sodium dodecyl sulphate polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (PVDF). 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 minutes, followed by overnight incubation at 4°C with rabbit polyclonal CBS (1:1000) or mouse monoclonal CSE (1:500) antibody (Santa Cruz Biotechnology Inc, Hidelberg, Germany). Mouse anti-ACE antibody has also been tested (1:500, Santa Cruz Biotechnology Inc, Heidelberg, Germany). Membranes were extensively washed in PBST prior incubation with horseradish peroxidase conjugated secondary antibody for 2h. After incubation, membranes were washed and developed using Image Quant 400 (GE Healthcare, US). Mouse anti-β-actin antibody has been used as loading control (1:5000, Sigma-Aldrich, Milano, Italy).

6.6 H_2S quantification assays

6.6.1 Methylene blue assay-Tissue CSE/CBS activity assay

Tissue H_2S production rate was measured by using Stipanuk and Beck based assay with modifications [25, 194, 419]. Briefly, aorta and carotid vessels were homogenized in lysis buffer containing potassium phosphate buffer 100mmol/L pH 7.4, 10mM sodium orthovanadate and proteases inhibitors. Protein concentration was determined by using Bradford assay (Bio-Rad Laboratories, Milano, Italy). Homogenates were added to a reaction mixture (total volume 500µL) containing pyridoxal-5'-phosphate (2mmol/L, 20µL), L-cys (10mmol/L, 20µL) and saline (30µL). 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, 250µL) was added followed by trichloroacetic acid (10% w/v, 250µL). Subsequently, N,N-dimethylphenylendiamine sulphate (20mmol/L, 133µL) in 7.2mol/L HCl and $FeCl_3$ (30mmol/L, 133µL) in 1.2mol/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 H_2S concentration was calculated against a calibration curve of NaHS (3.12–250µmol/L).

6.6.2 Methylene blue assay-Plasmatic levels of H_2S measurement

Plasmatic levels of H_2S were determined in absence of L-cys in the reaction mixture. Results were expressed as µM concentration. H_2S determination in plasma samples was performed as follows: each sample (100µL) was added into eppendorf tubes containing trichloroacetic acid (10% w/v, 300µL), in order to allow protein precipitation. Supernatant was collected after centrifugation and zinc acetate (1% w/v 150µL) was then added. Subsequently, N,N-dimethylphenylendiamine sulphate (20mmol/L, 100µL) in 7.2mol/L HCl and $FeCl_3$ (30mmol/L, 133µL) in 1.2mol/L HCl was added to each tube and absorbance measured after 20 min at a wavelength of 650 nm. All samples were assayed in duplicate and H_2S concentration was calculated against a calibration curve of NaHS (3.12–250µmol/L). All chemicals used in this assay were purchased at Sigma-Aldrich (Milano, Italy).

6.6.3 Fluorimetric assay

The fluorimetric determination of H_2S released by zofenoprilat was performed according to a method previously described [439] with modifications. Briefly, vehicle or the indicated concentration of 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 μM) 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 H_2S (measured as thiobimane) was expressed as relative fluorescence unit (RFU). In experiments designed to quench H_2S, hemoglobin (10μM) was mixed with zofenoprilat before adding dibromobinane. All reactions were performed in triplicate.

6.6.4 Amperometric assay

The amperometric assay was performed by an Apollo-4000 Free Radical Analyzer (WPI) detector and 2mm H_2S-selective minielectrodes [95]. Following the manifacturer's instructions, a PBS buffer 10x was prepared ($NaH_2PO_4xH_2O$ 1.28 g, Na_2HPO_4 $x12H_2O$

5.97 g, NaCl 43.88 g in 500ml H_2O) and stocked at 4°C. Immediately before the experiment, the PBS buffer 10x was diluted in distilled water (1:10), to obtain the assay buffer; pH was adjusted to 7.4. The H_2S-selective minielectrode was equilibrated in 10ml of the assay buffer, until recovery of a stable baseline was achieved. Then 100µL of an aqueous solution of Zofenoprilat were added, at the final concentration of 1 mM. The H_2S generation was monitored for 20min. When required by the experimental protocol, L-cys (4 mM) was added, before the zofenoprilat. The correct relationship between the amperometric currents (recorded in nA) and the corresponding concentrations of H_2S were determined by opportune calibration curves, which were previously obtained by the use of increasing concentrations of NaHS (1µM, 3µM, 5µM, 10µM) at pH 4.0.

6.7 Statistical analysis

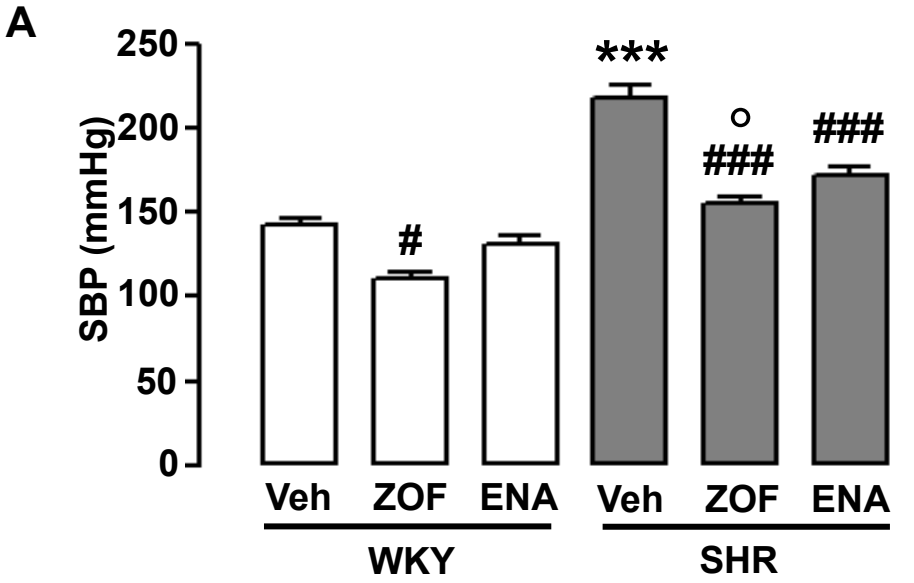
All data were expressed as mean±SEM. Statistical analysis was performed by using one-way ANOVA and Dunnet as post-test, or two-way ANOVA and Bonferroni as post-test where required (GraphPad software, San Diego, CA, US). Differences were considered statistically significant with a P value less than 0.05.

Chapter 7

RESULTS II

7.1 Blood pressure changes following ACE inhibition

Animals received daily drug treatment by oral gavage administration and SBP was monitored as described. SHR rats treated with vehicle showed a significant increase in SBP compared to WKY (fig. 7.1A, $P<0.001$). Both S-zofenopril and enalapril significantly lowered SBP in SHR rats compared to vehicle group (fig. 7.1A, $P<0.001$). S-zofenopril showed a greater response when compared to enalapril (167.5±2.5 vs 178.8±4.6 mmHg, $P<0.05$, n=10). Both drugs reverted the increase in tissue ACE expression in aorta (fig. 7.1B), but only S-zofenopril was effective on ACE expression in carotid homogenates harvested from hypertensive animals (Fig. 7.1B and 7.1C).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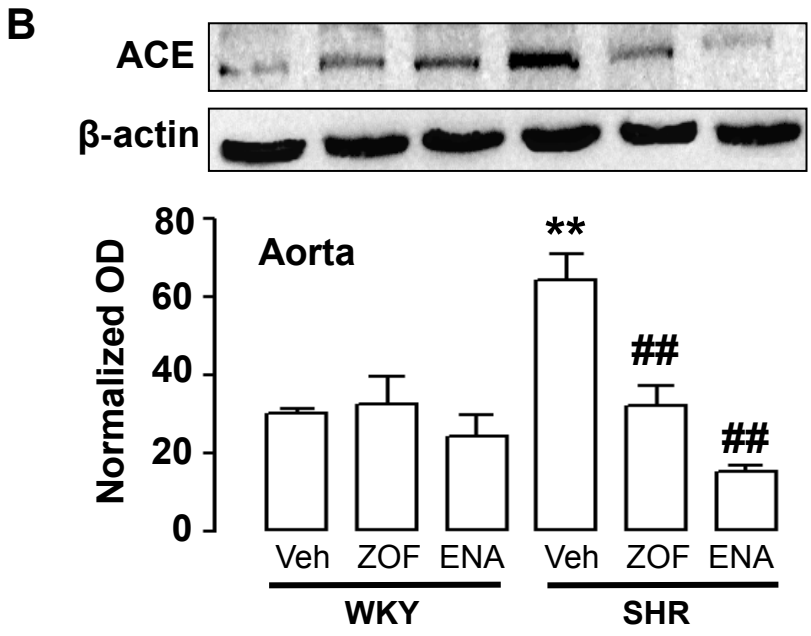
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ENA
Veh
ZOF
ENA
WKY
SHR

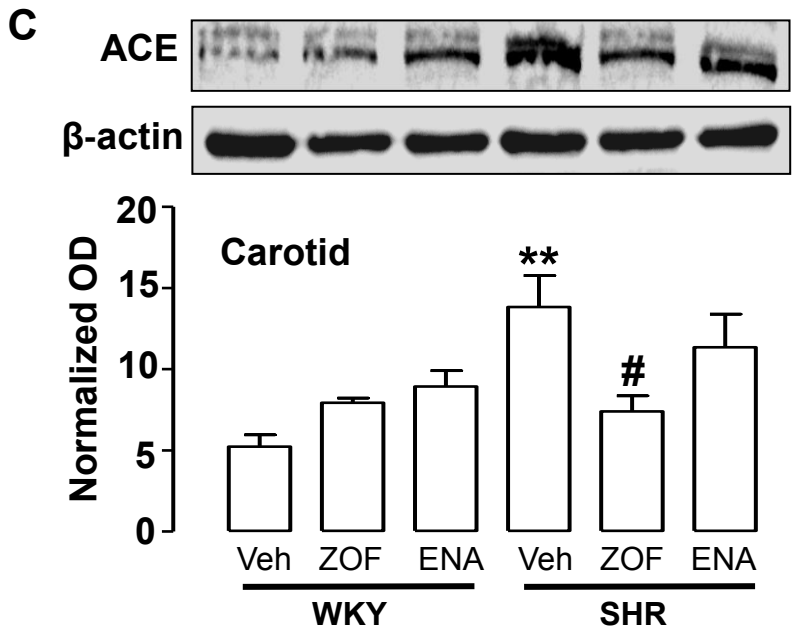


Fig 7.1: Blood pressure measurements *in vivo*. *A) Blood pressure values (SBP, mmHg) following two weeks treatment with S-zofenopril (ZOF) or enalapril (ENA) in SHR and WKY rats. Data are expressed as mean±SEM for n=10.* ***** *vs WKY vehicle, P<0.001; # vs SHR vehicle, P<0.001; ° vs SHR ENA, P<0.05. B) Expression of ACE in aortic tissue harvested from WKY or SHR rats, following ACE inhibitors treatment.* ***** *vs WKY vehicle, P<0.01; # vs SHR vehicle, P<0.01. C) Expression of ACE in carotid tissue harvested from WKY or SHR rats, following ACE inhibitors treatment.* ***** *vs WKY vehicle, P<0.01; # vs SHR vehicle, P<0.05. 40μg of total protein was loaded per each lane and β-actin has been used as loading control. Blots are representative of 3 different experiments. Optical density is expressed as arbitrary units (mean±SEM) and normalized to β-actin expression levels as housekeeping gene.*

7.2 Zofenopril treatment rescues endothelial dysfunction in hypertensive rats

Aorta and carotid arteries were harvested from rats treated with either enalapril or S-zofenopril and their response to Ach assessed *in vitro*. Ach-induced relaxation was significantly impaired in SHR compared to WKY rats in both aorta, (fig. 7.2A $P<0.001$) and carotid (fig. 7.2C, $P<0.001$). S-zofenopril treatment restored the Ach-induced vasodilatation in both aorta (fig. 7.2B, $P<0.001$) and carotid (fig. 7.2D, $P<0.001$). Enalapril did not significanly modify the vascular response to Ach in both vessel types (fig. 7.2B, $P<0.001$).

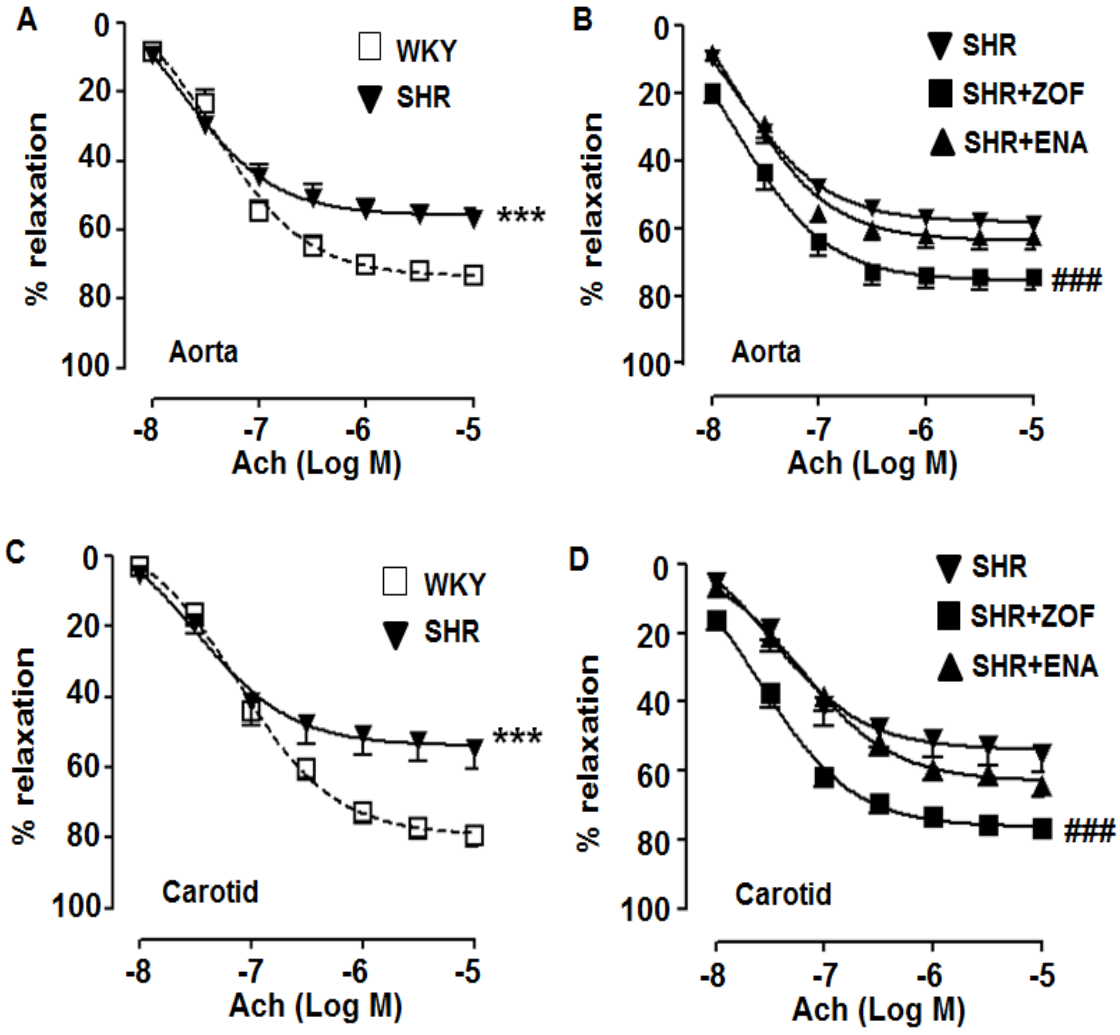


Fig. 7.2: S-zofenopril treatment ameliorates vascular reactivity in SHR. *Functional reactivity in vascular tissue harvested from WKY rats treated with vehicle (□, dashed line) and SHR rats treated with vehicle (▼), zofenopril (■, ZOF) or enalapril (▲, ENA). A) Ach-induced vasorelaxation in aortic rings is reduced in SHR rats compared to 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 SHR rats compared to WKY, 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±SEM for n=6).* ***** *vs WKY vehicle; # vs SHR vehicle.*

7.3 H_2S pathway is modulated following *in vivo* S-zofenopril treatment

Reduction in hydrogen sulphide levels has been associated with the development of hypertension in this rat strain, as reported by several studies [140, 431]. Therefore, we questioned whether or not prolonged ACE inhibition with enalapril or S-zofenopril could affect the H_2S 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 literature [140, 431], that expression of CSE in aorta samples was significantly lower in SHR compared to WKY rats, in naive animals (Fig. 7.3A, $P<0.05$). Treatment with S-zofenopril, but not enalapril, restored CSE expression in SHR rats to WKY level (Fig. 7.3A, $P<0.05$). Interestingly, CSE expression in carotid arteries of SHR was significantly higher than expression in WKY. Neither zofenopril nor enalapril did significantly modify CSE expression (Fig. 7.3B, $P<0.05$). CBS expression was not modified either in the carotid or in the aorta of SHR rats when compared to WKY (data not shown).

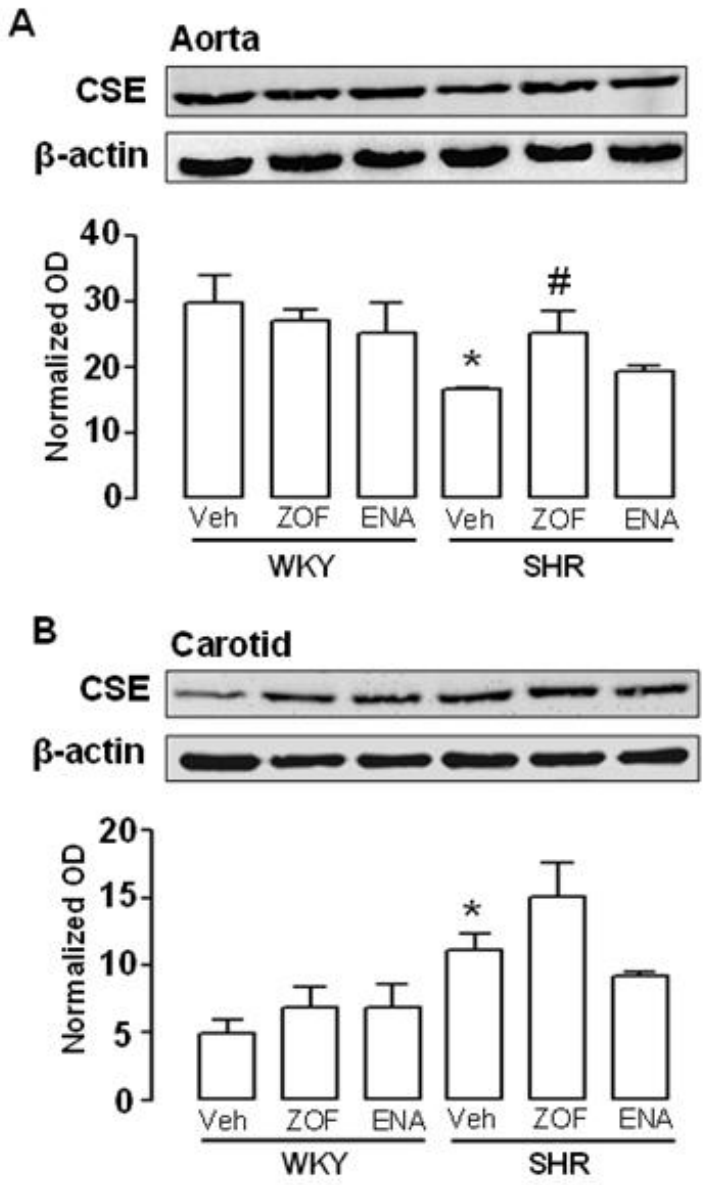


Fig. 7.3: S-zofenopril chronic treatment increases CSE expression in vascular tissues *A) Expression of CSE protein levels in aorta lysates. B) Expression of CSE protein levels in carotid lysates. 40µg of total protein was loaded per each lane and β-actin has been used as loading control. Blots are representative of 3 different experiments. Optical density as expressed in arbitrary units (mean±SEM) and normalized to β-actin expression levels as housekeeping gene. * vs WKY vehicle, P<0.05; # vs SHR vehicle, P<0.05.*

7.4 S-zofenopril treatment restores tissue and plasma H_2S levels and contributes to L-cysteine-induced vasodilatation

H_2S levels were measured both in aorta and carotid. As already shown, H_2S levels within the aorta (fig. 7.4A) and the carotid (fig. 7.4B) of SHR animals were significantly lower than WKY [140, 431]. S-zofenopril restored H_2S values to WKY levels in the aorta (fig. 7.4A, $P<0.01$), as opposite to enalapril (fig. 7.4A). In the carotid there was no significant reduction of H_2S levels between WKY and SHR. However, H_2S levels were significantly enhanced in carotid harvested from SHR rat treated with S-zofenopril (fig. 7.4B). Treatment with either S-zofenopril or enalapril did not affect H_2S production in WKY rats. Plasma H_2S values observed were significantly lower in SHR rats compared to WKY (fig. 7.4C, $P<0.05$). S-zofenopril treatment restored H_2S plasma concentration to WKY levels (fig. 7.4C, $P<0.05$), while enalapril did not modify H_2S plasma levels (fig. 7.4C).

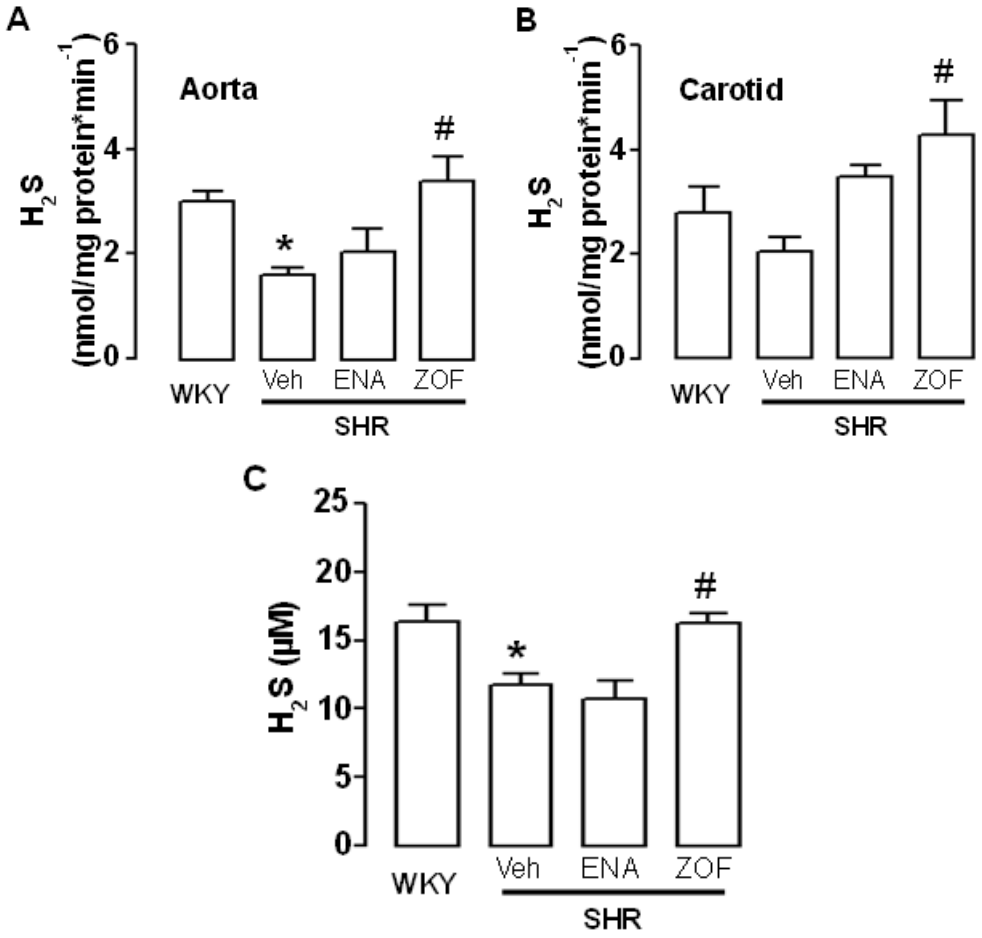


Fig. 7.4: Tissue and plasmatic levels of H_2S are restored by S-zofenopril chronic administration *in vivo*. *A) H_2S production rate in aorta harvested from WKY and SHR rats treated with vehicle, S-zofenopril or enalapril, after 40 minutes incubation in presence of L-cysteine as substrate. B) H_2S production rate in carotid harvested from WKY and SHR rats treated with vehicle, S-zofenopril or enalapril, after 40 minutes incubation in presence of L-cysteine as substrate. C) H_2S levels determination in plasma samples. Data are expressed as mean±SEM for n=6.* ***** *vs WKY vehicle, P<0.05; # vs SHR vehicle, P<0.05.*

In another set of experiments, concentration-response curves of L-cys were performed *in vitro*. The vasorelaxant effect induced by L-cys on either aorta (Fig. 7.5A), or carotid (Fig. 7.5C) harvested from SHR rats was not significantly different from the response obtained in WKY. However, S-zofenopril, but not enalapril treatment, enhanced L-cys- induced vasodilatation in aorta (Fig. 7.5B; $P<0.05$) and carotid rings (Fig. 7.5D; $P<0.01$) from SHR strain.

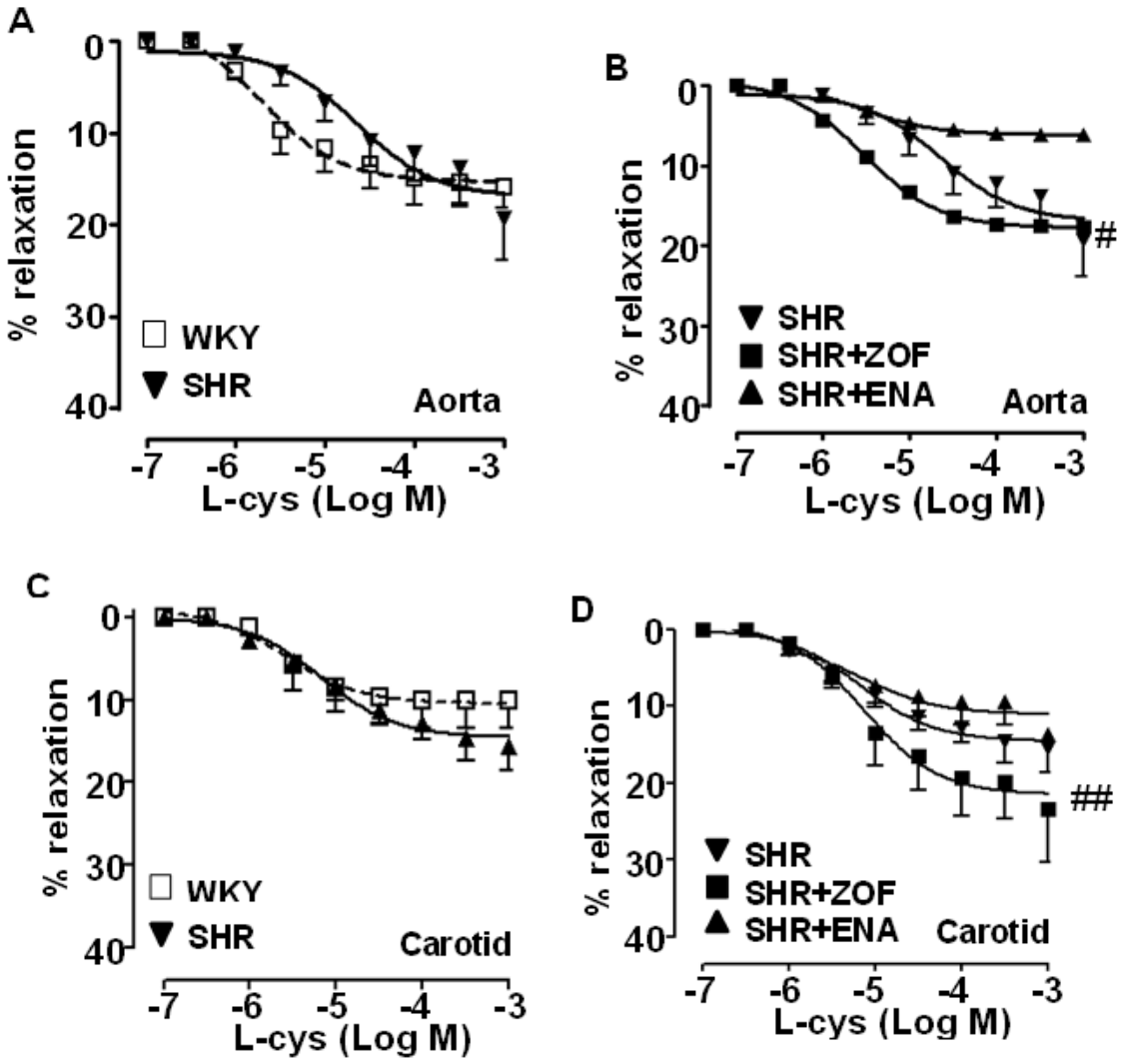


Fig. 7.5: L—cys-stimulated vasorelaxation is enhanced following zofenopril treatment. *L-cys-induced vasorelaxation in aortic rings from WKY rats treated with vehicle (□, dashed line) and SHR rats treated with vehicle (▼), zofenopril (■, ZOF) or enalapril (▲, ENA). A) L-cys-induced vasorelaxation in aortic rings is slightly, though not significantly, reduced in SHR rats compared to WKY. B) L-cys-induced vasorelaxation is significantly enhanced in aorta of S-zofenopril treated animals, P<0.05. C) L-cys vasodilation does not significantly change between carotid rings harvested from SHR compared to WKY rats. D) S-zofenopril treatment significantly increases vasorelaxation induced by L-cys administration in carotid rings, compared to vehicle treated animals, P<0.01. Data are expressed as mean±SEM for n=6. # vs SHR vehicle.*

7.5 S-zofenopril acts as an H_2S donor

In order to evaluate if S-zofenopril can act as an H_2S donor we measured the H_2S release in vitro by using S-zofenoprilat, the active metabolite bearing the free thiol. The evaluation was performed by using two different analytical approaches, a fluorimetric and an amperometric assay in order to overcome the bias due to the coloured S-zofenoprilat solution. The amperometric assay showed that S-zofenoprilat spontaneously released H_2S in solution and that H_2S release can be triggered by addition of a nucleophilic substance, such as L-cys (Fig. 7.6C). In order to further confirm these data we performed measurements in a fluorimetric assay. Also in this case zofenoprilat caused a time-dependent H_2S release. The fluorescent signal is significantly inhibited by haemoglobin (Hg), a H_2S scavenger (Fig. 7.6A). As expected, zofenopril, the prodrug, did not release H_2S. In parallel, we also assessed that H_2S is released by S-zofenoprilat in a concentration-dependent manner (Fig. 7.6B). These findings were further supported by amperometric assay, confirming that H_2S is released by S-zofenoprilat, especially in presence of L-cys, used in this case as a nucleophilic trigger (Fig. 7.6C).

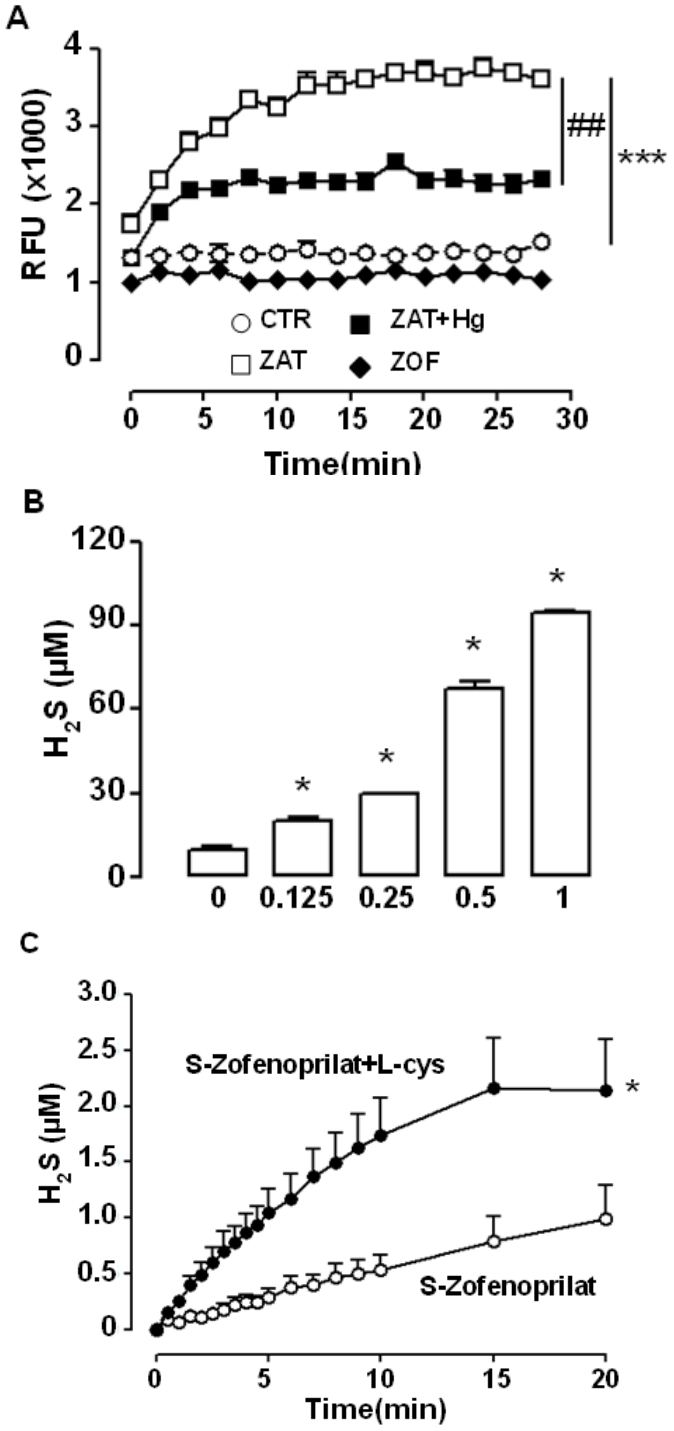
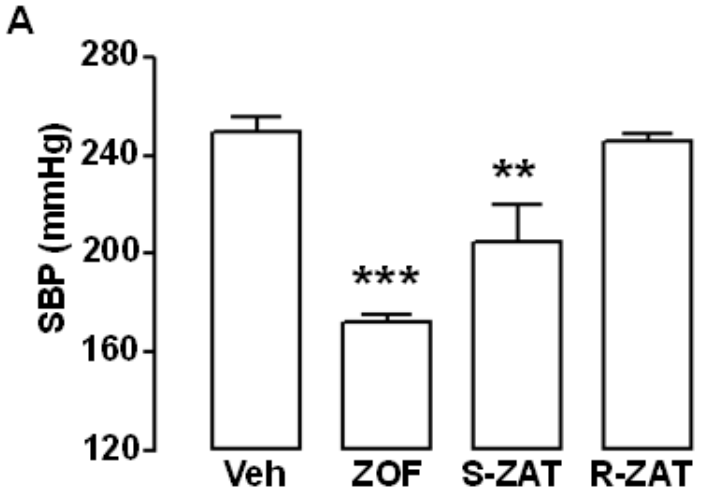


Fig. 7.6: S-zofenoprilat releases H_2S. *Free H_2S release determination. A) Time-dependent release of H_2S by S-zofenoprilat (□, 0.2mM) was detected using dibromobinane (0.25 mM) and fluorescence was measured every 2 min, P<0.001. S-zofenopril (♦, ZOF, 0.1mM) shows no H_2S release and haemoglobin (■, Hg,*

*10μM) reduces the amount of H_2S detected from S-zofenoprilat (ZAT), P<0.01. Data are presented as means±SEM for n=4. * vs vehicle (○, CTR); # vs ZAT. B) H_2S release from different concentration of S-zofenoprilat in DMEM, quantified again by using dibromobimane fluorimetric determination. C) Amperometric measurement vs time of H_2S release in aqueous buffer, P<0.05. *vs S-zofenoprilat. D) Vasorelaxant effect of S-zofenoprilat (□, ZAT) vs enalprilat (▲, ENAT) in rat isolated aortic rings. Data are expressed as mean±SEM for n=6. * vs ENA).*

7.6 ACE is involved in H_2S pathway activation

In order to further define the role of the thiol group we treated SHR rats *in vivo* with either the inactive form of zofenoprilat on ACE activity, namely R-zofenoprilat vs the active metabolite S-zofenoprilat. As expected, S-zofenoprilat significantly lowered blood pressure, while R-zofenoprilat did not (Fig. 7.7A). R-zofenoprilat, despite the lack of effect on blood pressure, was as active as S-zofenoprilat in restoring the vascular response to Ach in both aorta (Fig. 7.7B) and carotid (Fig. 7.7C). The treatment with either R-zofenoprilat or S-zofenoprilat, restored H_2S levels in aorta (Fig. 7.8A), carotid (Fig. 7.8B), as well as in plasma samples (Fig. 7.8C).



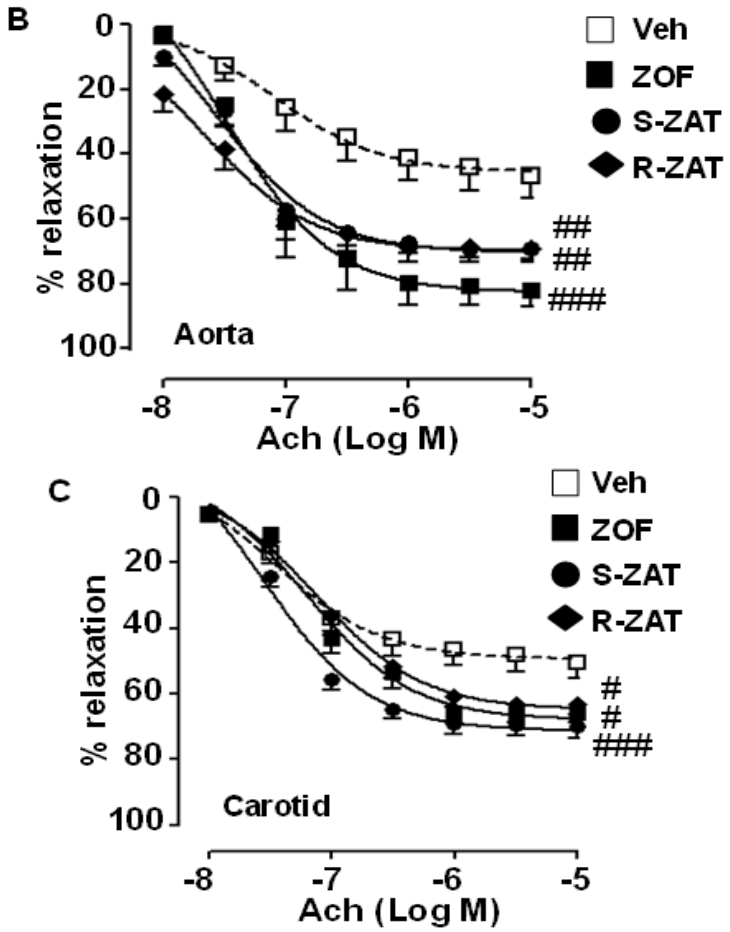


Fig. 7.7*:* R-zofenoprilat treatment does not affect blood pressure, but improves Ach-induced vasorelaxation *A) Blood pressure values (SBP, mmHg) following two weeks treatment with S-zofenopril (ZOF), S-zofenoprilat (S-ZAT) or R-zofenoprilat (R-ZAT) in SHR rats. Data are expressed as mean±SEM for n=10.* ***** *vs WKY vehicle, P<0.001; # vs SHR vehicle, P<0.001; ° vs SHR ENA, P<0.05. B) Functional reactivity in vascular tissues harvested from SHR rats treated with vehicle (□, dashed line), S-zofenopril (■, ZOF), S-zofenoprilat (●,S-ZAT) or R-zofenoprilat (♦, R-ZAT) . Ach-induced vasorelaxation in aortic rings is reduced in SHR rats compared to WKY, P<0.001, but it is restored following, S-zofenopril, S-zofenoprilat or R-zofenoprilat, but not enalapril, treatment, P<0.001. C) Ach-induced vasorelaxation in carotid rings is reduced in SHR rats compared to WKY, P<0.001, while it is restored after S-zofenopril, S-zofenoprilat or R-zofenoprilat, but not enalapril, treatment, P<0.001. Ach induced response is expressed as % of relaxation (mean±SEM for n=6). # vs SHR vehicle.*

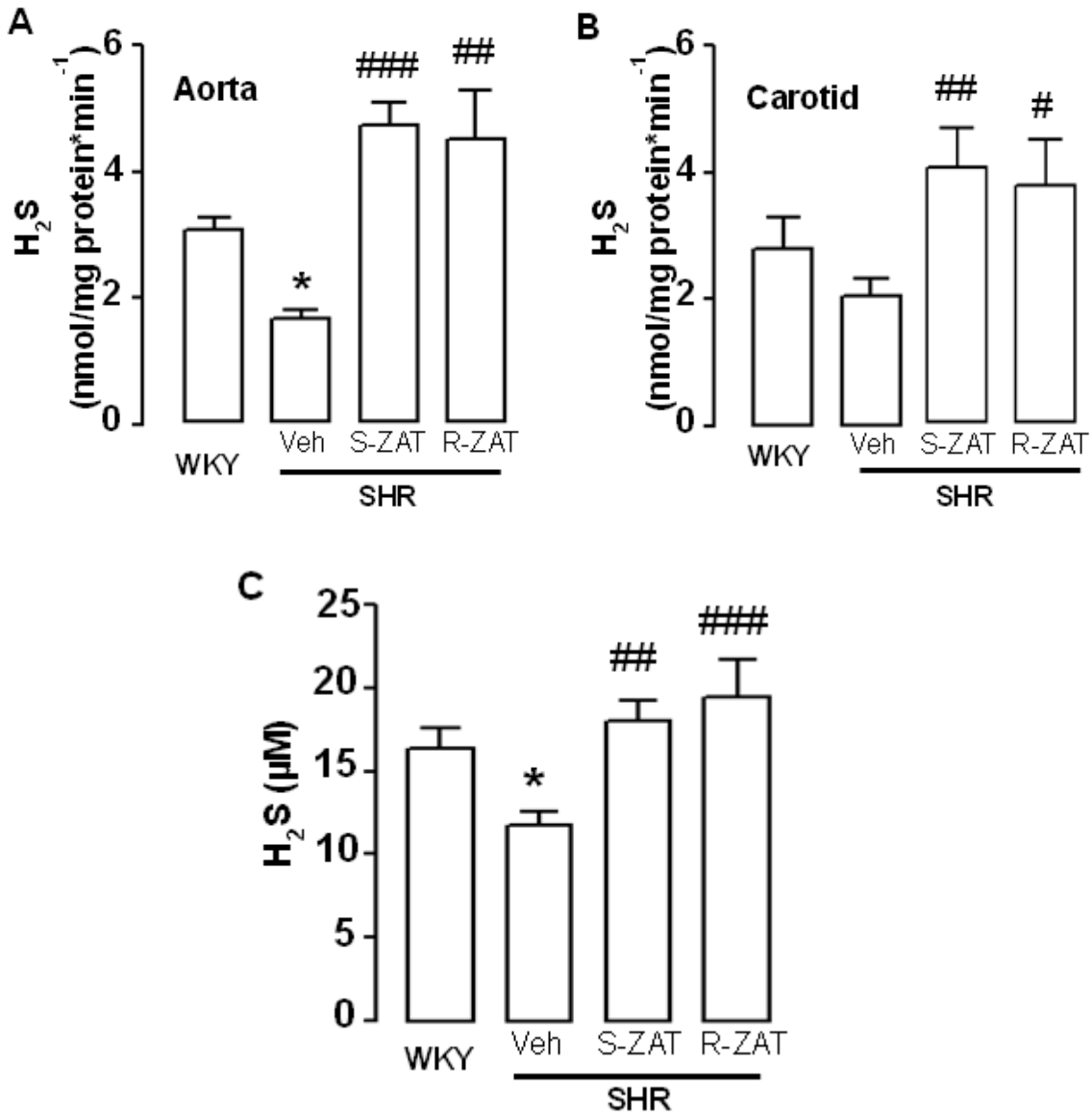


Figure 7.8: Both S-zofenoprilat and R-sofenoprilat restores plasmatic and tissue levels of H_2S. *A) H_2S production rate in aorta harvested from WKY and SHR rats treated with vehicle, S-zofenopril, S-zofenoprilat or R-zofenoprilat, after 40 minutes incubation in presence of L-cysteine as substrate. B) H_2S production rate in carotid harvested from WKY and SHR rats treated with vehicle, S-zofenopril, S-zofenoprilat or R-zofenoprilat, after 40 minutes incubation in presence of L-cysteine as substrate. C) H_2S levels determination in plasma samples. Data are expressed as mean±SEM for n=6.* ***** *vs WKY vehicle, P<0.05; # vs SHR vehicle, P<0.05.*

Chapter 8

DISCUSSION II

Clinical evidences have accumulated demonstrating that sulfhydrylated ACE inhibitors have some additional beneficial effects not related to ACE inhibition [340, 432-434]. Zofenopril is a highly lipophilic ACE inhibitor characterised by long-lasting tissue penetration that has cardioprotective properties and attenuates ventricular remodelling in animal models of myocardial injury. In particular the additional beneficial effects of zofenopril have been ascribed to the presence of the thiol group. Our working hypothesis was that the active metabolite of zofenopril e.g. zofenoprilat, bearing a free thiol, could act *in vivo* through modulation of the H_2S pathway accounting for the beneficial effect generically ascribed, in the current literature, to antioxidant activity. The study has been performed *in vivo* by comparing zofenopril to a non sulphydrylated ACE inhibitor belonging to the carboxylic class widely used in clinic, e.g. enalapril. Both enalapril and zofenopril are pro-drugs that are metabolized in vivo to their respective active form enalaprilat and zofenoprilat. In order to define which was the contribution of H_2S in

zofenopril action, we used SHR rats as model of hypertension. SHR rats and their matched control WKY were treated with S-zofenopril and enalapril at doses already known to cause a maximal reduction in blood pressure in this preclinical animal model [435]. As expected, both ACE inhibitors reduced blood pressure as well as ACE expression in both rat aorta and carotid in SHR rats. However, zofenopril was significantly more effective than enalapril in lowering blood pressure *in vivo.* The *in vitro* study demonstrated that Ach-induced vasorelaxation was significantly reduced in both isolated aorta and carotid harvested from SHR rats when compared to WKY as already described. S-zofenopril treatment rescued vascular responsiveness to Ach to WKY levels. Conversely, enalapril did not significantly modify such a response. This result strongly implied that the vascular effect exerted by zofenopril treatment was not to be ascribed solely to ACE inhibition but it involves additional mechanism(s). The most widely known additional mechanism through which ACE inhibitors works involves the inhibition of the degradation of bradykinin thereby promoting the generation of nitric oxide within the endothelium. The importance of this mechanism in driving the extra effect of ACE has been also clinically demonstrated in

patients by blocking the bradykinin receptor [436]. However, since in our condition both enalapril and zofenopril do inhibit ACE, it is clear that zofenopril possesses an additive effect over enalapril, the non-sulphydrylated ACE. Expression of CSE, as well as H_2S plasma levels, has been shown to be reduced in SHR rats [140, 431]. In line with these data S-zofenopril, but not enalapril, restored CSE expression in analysed vascular tissues as well as plasma and tissue H_2S levels. These data implies that following *in vivo* administration of S-zofenopril there is an amelioration or, in other words, a re-assessment of the L-cysteine/H_2S pathway within the vessels that restores Ach response. This hypothesis is sustained by the finding that L-cysteine-induced vasorelaxation, evaluated on both types of isolated vessels, was significantly enhanced exclusively following *in vivo* S-zofenopril treatment. In summary the *in vivo* treatment with S-zofenopril, as opposed to enalapril, in both vessels types analysed (resistance-carotid and capacitance-aorta) restores i) the Ach-induced relaxation; ii) the tissue and plasma H_2S levels iii) CSE expression coupled to an enhancement of the response to L-cysteine.

These findings strongly support our hypothesis that S-zofenopril, once converted into S-zofenoprilat, up-regulates H_2S pathways within the

vessel wall. We next asked whether S-zofenoprilat could itself release H_2S in addition to up regulating its endogenous production. In order to answer to this question, and to further demonstrate that zofenopril additional effects do involve H_2S, we run two different *in vitro* cell free assays to test whether S-zofenoprilat can release H_2S. The widely used methylene blue based colorimetric assay could not be employed since zofenoprilat colours the solution and interferes with the assay. Therefore we utilized two different approaches by using a fluorimetric and an amperometric based assays. Both assays clearly demonstrated that S-zofenoprilat can release H_2S. In the assay performed with the fluorescent probe zofenoprilat, but not zofenopril, released H_2S and the fluorescent signal could be significantly inhibited by haemoglobin. The amperometric assay confirmed that zofenoprilat can slowly release H_2S in solution and that this can be further increased by adding L-cysteine. The use of L-cysteine in the assay did not only stem from the idea that H_2S release from zofenoprilat is facilitated by nucleophilic substances, but was also based on the observation that the metabolism of zofenoprilat involves cysteine conjugation, as demonstrated by using radiolabelled zofenopril. The ability to release *in vitro* H_2S was also

confirmed by the finding that s-zofenoprilat, but not enalprilat, relaxes in a concentration dependent manner the isolated rat aorta. These findings imply that following *in vivo* treatment with zofenopril, S-zofenoprilat could supply a tonic pulse of H_2S by slowly releasing it within the cellular environment, where the ACE resides, leading to the beneficial vascular effects above described. In other words following the binding of zofenoprilat to endothelial ACE, H_2S is delivered locally. The vascular effect observed is also in line with the finding that S-zofenopril exerts a preferential inhibition on ACE expressed within the tissue, where it is metabolized to S-zofenoprilat, rather than on the plasma isoform. This effect is evident only when vascular homeostasis is disrupted since changes in H_2S do not play a role in physiological conditions as demonstrated by the parallel experiments performed on tissues harvested by WKY rats.

Zofenopril is a mixture of diasteroisomers and the active diasteiroisomer, as ACE inhibitor, is the S form. R-zofenoprilat has an IC_{50} of 1700nM while the S isoform IC_{50} is 10.9 nM [437]. In order to further strengthen our findings regarding the role of H_2S in the action of Zofenopril, we dosed SHR rats with either the R- or S- diastereoisomer of zofenoprilat .

R-zofenoprilat treatment did not reduce blood pressure in SHR rats in contrast to S-zofenoprilat. As expected, due to the first pass metabolism, the active metabolite S-zofenoprilat was less active in lowering blood pressure, compared to zofenopril. However, the Ach-induced vasorelaxation in both carotid and aorta from SHR rats were rescued by both isoforms of zofenoprilat. Furthermore, H_2S levels found in tissues and plasma of R-zofenoprilat treated animals were identical to the ones observed in S-zofenoprilat treated group. These data further confirm that the modulation of the H_2S pathway by zofenopril represents an additional mechanism that is unrelated to ACE inhibition.

In conclusion, we show here that sulfhydrylated ACE inhibitor zofenopril represents a unique molecule that up regulates the activity of the H_2S pathway *in vivo*. In line with this hypothesis it has been demonstrated that zofenopril, but not rampipril or atenolol, ameliorates flow-mediated dilation in hypertensive patients [438]. Overall these findings confirm that H_2S plays an important role in cardiovascular homeostasis and provide proof of concept for the development of H_2S-releasing therapeutic agents in cardiovascular disease therapy.

Chapter 9

INTRODUCTION

9.1 Reticulon 4B/Nogo-B: from ER structural protein to vascular function

Nogo-B belongs to the family of reticulon proteins (RTN), which are a large, ubiquitously expressed group of eukaryotic proteins ranging in size from 200 to 1200 amino acids. They are characterized by compartimentalization to the endoplasmic reticulum (ER) membrane. ER is an eukaryotic organelle with the most architecturally shape. It is composed of a nuclear envelope, sheet-like cisternae interrupted by nuclear pores and stabilized by connections to the underlying lamina, and a polygonal array of tubules connected by three-way junctions extended throughout the periphery of the cell [300-303] (Fig. 9.1).

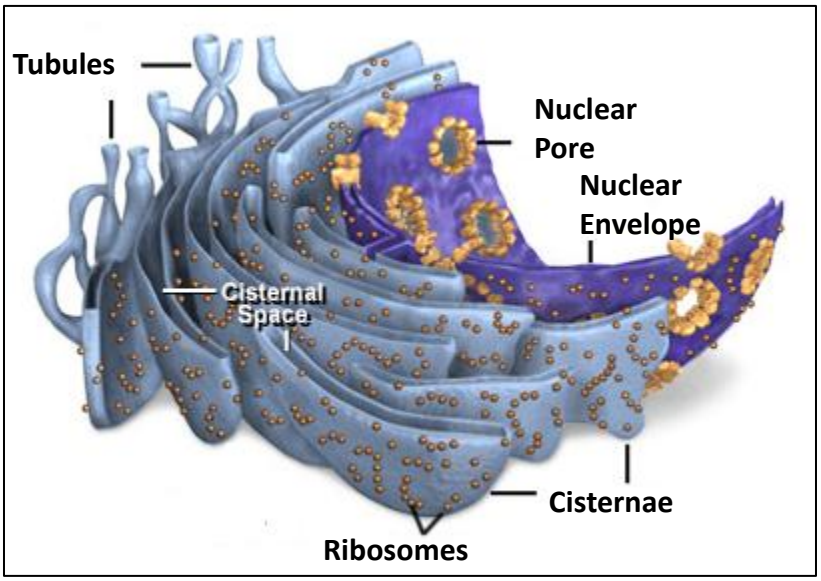


Fig. 9.1: ER structure. *It consists of two different shaped domains: the nuclear envelope (NE) and the peripheral ER. The NE consists of two continuous sheets of membranes, connected to one another at nuclear pores. The peripheral ER consists of a network of membrane tubules connected by three-way junctions.*

All these structural regions exist within the continuous membrane bilayer and therefore must be maintained by proteins that partition as they generate these ER domains [301]. ER sheets are flat, extended surfaces with little membrane curvature. ER tubules are long and cylindrical with high membrane curvature and intersect to form an elaborate network that pervades the cytoplasm [303]. The thickness of sheets and diameter of tubules varies depending on the cell type, but tipically is around 60-100nm suggesting that both domains are being

shaped actively [301]. These distinct morphological ER domains have drawn the attention of researchers not only because of their morphology, but also because of their critical functions. ER sheets are often characterized by the presence of membrane bound ribosomes (rough ER, RER) and perform all functions associated with the biosynthesis of membrane and secreted proteins, including the control of their proper folding and modification. Conversely, ER tubules are mainly formed of smooth ribosom-free ER (SER) and possibly involved in lipid biosynthesis, delivery as well as Ca^{2+} signaling. Defects in ER morphology, particularly the loss of tubular ER, can cause retarded growth in budding yeast and decreased embryonic survival in Caenorhabditis elegans [363, 364], as well as a neurodegenerative disease known as hereditary spastic paraplegia (HSP) in humans [365]. These facts strongly suggest that vital functions of the ER rely on its morphological integrity. The ER is an extremely dynamic organelle, which continuously undergoes significant rearrangement of its structure. The outgrowth and the retraction of tubules depend on the close association between the ER and the microtubules (MTs) cytoskeleton. There are two distinct

mechanisms whereby ER tubules move along MTs. One mechanism is defined as tip attachment complex (TAC) dynamics and involves the attachment of the tip of the ER tubule to the tip of a dynamic MT. During TAC dynamics, the ER tubule grows and retracts in concert with the dynamic of its MT partner [367]. The second mechanism is defined as sliding, and it is faster and much more common than TAC. During ER sliding events, tubules are pulled out of the ER membrane by the motor proteins kinesin-1 and cytoplasmic dynein along MTs that are marked by acetylation [368, 369]. However, although the cytoskeleton is required for the extension of the ER network, the alignment of membrane tubules with the cytoskeleton is not perfect, and the network does not rapidly collapse upon depolymerization of microtubules or actin filaments [370]. In addition, ER networks can be formed *in vitro* from small vesicles where an intact microtubule or actin network is not required [371]. Thus, the cytoskeleton is most likely unnecessary for determining or maintaining the shape of ER tubules. The most plausible models for shaping ER tubules are based on mechanisms generating or stabilizing high curvature in membranes. Although several different mechanisms can be

envisioned, recent results demonstrate that Rtn and DP1/Yop1 protein families are responsible for generating and maintaining ER tubules [364]. Deletion of these proteins in yeast or their depletion in mammalian cells reduce the tubulation of ER, indicating that they are required for tubule formation [363, 364]. The overexpression of reticulons or DP1/Yop1p lead to long tubules, which become resistant to the collapse that normally follows the depolymerization of microtubules, indicating that these proteins can stabilize ER tubules [373]. The RTNs and DP1/Yop1p might stabilize the high curvature of ER tubules by utilizing two cooperative mechanisms: hydrophobic insertion (wedging) and scaffolding [303]. The hydrophobic insertion mechanism is based on the two hydrophobic hairpins in these proteins, which are proposed to form a wedge-like structure that displaces the lipids preferentially in the outer leaflet of the lipid bilayer, thus causing local curvature. Recent studies have shown that both mammalian and plant reticulons suffer mislocalization and dysfunction when the hydrophobic segments are manipulated to disrupt potential hairpins [397-399]. The hairpin may be a common feature of integral membrane proteins that preferentially localize to

curved membranes. Examples of such proteins include atlastin (ATL), spastin, a microtubule-seveing ATPase on the ER, and caveolin-1, one of the major structural components of the caveoli [365, 401-404]. The scaffolding mechanism assumes that the reticulons and DP1/Yop1p form arc-like oligomers that mold the lipid bilayer into tubules. Arc-like scaffolds, rather than rings or spirals that wrap around tubules, are consistent with the observation that the reticulons also localize to sheet edge [372]. Because arcs are not entirely encircling a tubule, they would not block the long-distance diffusion of other membrane proteins. The reticulons and DP1/Yop1p conceivably combine the "wedge insertion" and "scaffolding" mechanisms to shape ER tubules (Fig. 9.2).

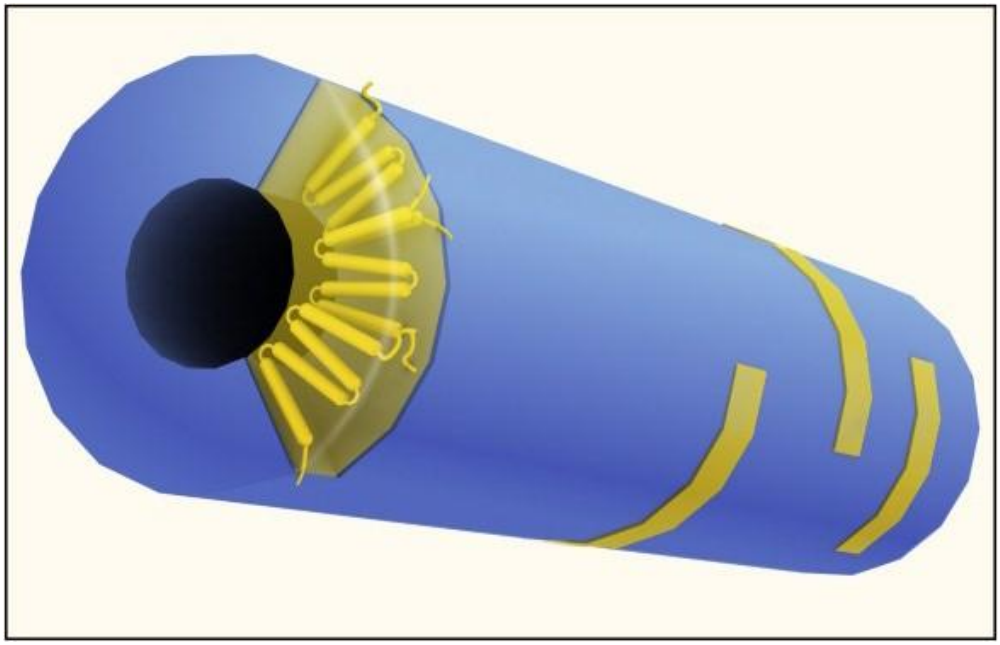


Fig. 9.2: Mechanisms for shaping ER tubules. *The reticulons and DP1/Yop1p induce and stabilize high curvature in ER tubules by inserting into the membrane as a "wedge" and forming an arc-like "scaffold".*

All RTN family members contain a conserved C-terminal domain (the reticulon homology domain, RHD), consisting of two hydrophobic regions, each 28-36 amino acids long, flanking a hydrophilic loop of 60-70 amino acids, followed by a C-terminal tail of approximately 50 amino acids [374, 375]. In contrast to the RHD domains, the N-termini of reticulons are completely divergent. The RHD hydrophobic regions are unusually long for transmembrane domains: each spans approximately 30-35 amino acids, whereas most transmembrane domains are about 20 amino acids in length. The topology of these hydrophobic regions within membranes is so far only partially

defined. The RHD loop region has been detected both on the surface of cells and intracellularly, and it has been suggested that the RHD hydrophobic regions might either span the ER membrane or plasma membrane completely or might double back on themselves to form a hairpin (Fig. 9.2).

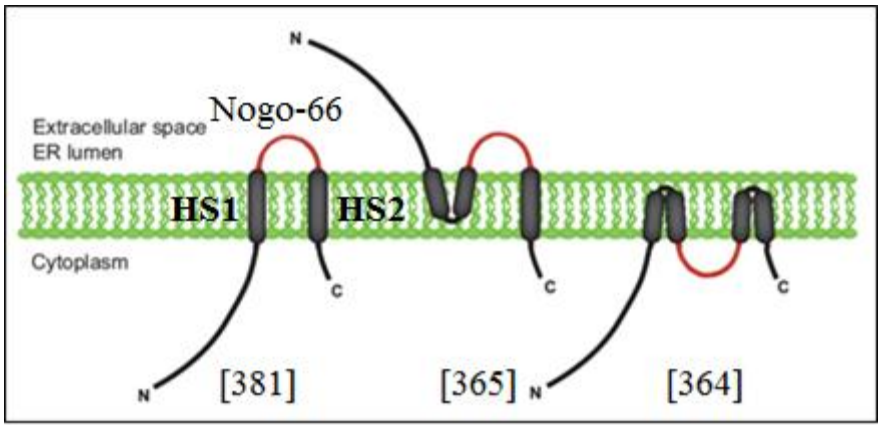


Fig. 9.3: Possible membrane topologies of Nogo. *(I) The topology proposed by GrandPre et al. [381] placed both the N- and C-terminus facing the cytoplasm and the Nogo-66 domain exposed extracellularly. (II) This topology shows that the N-terminus of Nogo is oriented extracellularly [365] and is also supported by studies on Nogo-B by Acevedo et al. [392]. (III) This topology has been recently proposed by Voeltz et al [364]. In this model, reticulon homology domain (RHD) does not span the membrane, but rather form hairpin loops.*

All reticulon genes contain multiple introns and exons, and most are alternatively spliced into multiple isoforms [374]. In mammals, there are four reticulon genes encoding reticulon proteins 1-4 (RTN_{1-4}). The expression patterns of different reticulons and their splice isoforms can be variable, even within the same organism [376-378]. Thus, there

is a complex array of RTN variants that are present in different combinations in a cell- or tissue-specific manner. An example of this variable pattern of expression is seen in Nogo/RTN4 expression. Nogo/RTN4 can give rise to 10 splice variants, of which the major isoforms are 3 (Fig. 9.4): Nogo-A, which is localized mainly in the central nervous system, whereas its splice variant RTN4B/Nogo-B is found in a variety of tissues, included non-neural ones [379]. The third splice variant RTN4C/Nogo-C was found in the CNS, but is particularly enriched in skeletal muscle.

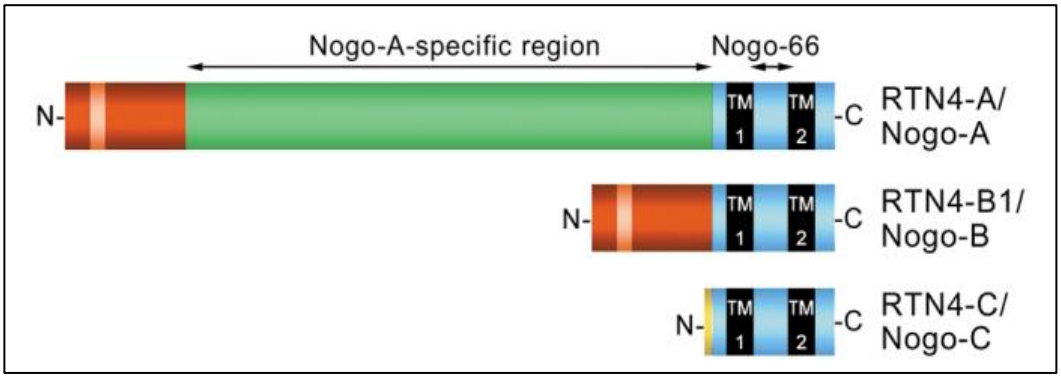


Fig. 9.4: The three main transcripts of mammalian RTN4/Nogo genes. *They are characterized by conserved C-terminal RHD, but they differ in length and tissue distribution.*

The regulatory processes underlying these variable patterns of expression remain undefined. Most of the information regarding the function of reticulons comes from studies of RTN4A/Nogo-A as a

myelin-derived inhibitor of neurite outgrowth [380-382]. Its potential to inhibit nerve growth is shown by the fact that peripheral nerves regenerate well after a lesion, whereas ectopic expression of Nogo-A in Schwann cells leads to delayed recovery of the crushed sciatic nerve [405]. Analysis of the regions of Nogo-A responsible for this inhibition identified the RHD-localized 66-amino acid hydrophilic loop (Nogo-66) as a potent inhibitor of neurite outgrowth. Following soon thereafter, a cell surface receptor for Nogo-66 was discovered and named Nogo Receptor (NgR) [383]. Another region, located within the N-terminus of Nogo-A, was also shown to have inhibitory activity and has been termed A20 [383, 384]. This region inhibits outgrowth in NgR-independent manner, yet a receptor associated with binding of this region remains unknown. Interestingly, the RHD from any Nogo/RTN isoform member is sufficient to delay nerve regeneration after nerve crush injury [385]. A number of *in vivo* studies have confirmed the role of Nogo-A as a mediator of inhibition of neural regeneration. These studies have relied upon genetic and pharmacological neutralization of Nogo-A, and conclude that its inhibition promotes axon growth and functional recovery following

spinal cord injury [386-390]. In contrast to these important advances made in understanding the physiological significance of reticulon protein function, little attention has been paid to the intracellular role of reticulons [391]. A number of studies of Nogo localization have shown that only a minor proportion of Nogo (2-5%) is localized to the cell surface [381, 392, 393], and topology analyses suggest that Nogo-66 is primarily arranged in a cytosolic orientation [364]. These data suggest that in addition to the neurite inhibitory function of Nogo-A, much remains to be studied regarding what is likely to be the major, intracellular function of Nogo-A.

Given the notable importance of the understanding of Nogo-mediated processes in the CNS, little attention has been paid to the role of Nogo isoforms in the non-neural tissues, especially in the vasculature. Using a proteomics-based approach to identify novel caveolae and lipid raft-enriched proteins in endothelial cells, Acevedo and colleagues demonstrated that the reticulon protein RTN4B/Nogo-B is higly localized in caveolin-1 enriched microdomains (CEM) and lipid rafts (LR), with its N-terminus oriented extracellularly [392]. They also indentified Nogo-B in both endothelial and vascular smooth muscle

cells of the vessel wall. For the first time they described that the N-terminus of Nogo-B (AmNgB) is a stimulant of adhesion of ECs and VSMCs and acts as a chemoattractant for ECs, conversely it inhibits PDGF-induced VSMC migration [392]. These results were shown to be physiologically relevant, as genetic loss of Nogo-B results in exaggerated neointimal proliferation, suggesting that endogenous Nogo-B regulates vascular remodeling especially in injured vessels [392]. Therapeutic delivery of Nogo-B in both murine and porcine models of acute vascular injury reduces the extent of vascular growth and neointima formation [406]. In humans, Nogo-B levels are reduced in atherosclerotic tissue and aortic aneurysms, and this reduction may contribute to plaque formation, destabilization, and vascular abnormalities [394, 407]. A negative role for Nogo-B was assessed also in lung inflammation, where its reduction contributes to the pathogenesis of asthma by enhancing the magnitude of Th2-driven allergic inflammation. However, in models of wound healing and tissue ischemia, endogenous Nogo-B participates in the extent of recovery, largely via regulation of macrophage motility and gene expression [408]. Furthermore, it plays a critical role in hepatic

fibrosis as hepatic Nogo-B levels are significantly elevated in cirrhosis, whereas the absence of Nogo-B ameliorates liver fibrosis and portal hypertension [409]. Finally, it has been shown that endothelial Nogo-B is a novel regulator of acute inflammatory response *in vivo*, controls ICAM-1–dependent leukocyte TEM *in vitro* [410]. Thus, it appears that Nogo isoforms exert diverse actions in multiple cell types, and the differences in function are likely attributable to the intracellular role of the RTN domain versus the biologically active fragments generated upon tissue damage.

9.2 Purpose of the study

Recent literature has supported the considerable importance of Nogo-B in the vasculature since it has been demonstrated to be abundantly expressed in both endothelial and vascular smooth muscle cells and to regulate vascular remodeling *in vivo* as its loss is associated with abnormal vascular function and stenotic atherosclerotic lesions in humans [392, 394]. However, all these studies were performed in injured vessels from both WT and Nogo-A/B$^{-/-}$ mice and they are still far from address to Nogo-B some mechanisms through which it regulates vascular homeostasis in physiological conditions. Therefore, the purpose of this focused project is to study the role of Nogo-B within the vasculature, in particular in resistance arteries such as the mesenteric artery from WT and Nogo-A/B$^{-/-}$ mice and to evaluate their vascular function by using the pressure myograph.

Chapter 10

MATERIALS AND METHODS

10.1 Animals

Nogo-A/B-deficient mice were generated as described by Kim et al. [400]. Age-matched male, 14 to 16 week-old Nogo-A/B$^{-/-}$ mice and their wild type (WT) littermates were used. All animal experiments were approved by the Weill Cornell Institutional Animal Care and Use Committee.

10.2 Reagents

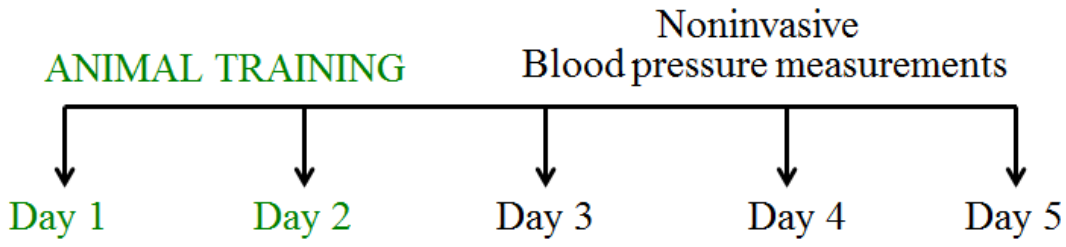
Phenylephrine (Phe), Acetilcholine (Ach) and all reagents for Krebs buffer solution preparation were purchased from Sigma (St. Louis).

10.3 Blood pressure measurements in conscious mice

Systolic, diastolic and mean blood pressure (SBP, DBP and MBP respectively) was measured in conscious mice using the pneumatic tail-cuff method (MRBP System, Life Science). The mice, left in a room at 30°C for 30 min in order to acclimate, were placed in a plastic

restrainer and a cuff with a pneumatic pulse sensor was attached to the tail. The restrainer was placed in the warm section of the system and the temperature was set at 34°C.This procedure was performed every day for 5 consecutive days according to the following protocol:

- Day 1 and 2: animal training. In order to acclimate the animals to this procedure, they were trained before to start the experiments.
- Day 3 to 5: blood pressure measurements. Three consecutive measurements were performed per each mouse every day and then the blood pressure values were obtained from averaged measurements.
- During the entire measurement period, the temperature was maintained at 34°C.



10.4 Preparation of vessel segments

Animals were sacrificed with CO_2. The intestine with mesentery attached was rapidly removed and placed in cold (4°C) Krebs solution, composed as follow (mM): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10.1. Second-generation mesenteric arteries were carefully cleaned from adhering tissue under a dissecting microscope (Zeiss, Discovery.V8).

10.5 Pressure myograph recordings

To measure acute changes in diameter in response to pharmacological agents and mechanical stimuli, in particular the stretch, the arteries with intact endothelium were mounted on glass micropipettes in a pressure myograph chamber (Danish MyoTechnology, Aarhus, Denmark), by keeping the orientation of the vessel in relation to the flow *in vivo* and were secured with silk sutures. The micropipettes were connected to a pressure interface which regulated intraluminal pressure and flow. The vessel diameter was monitored live using a microscope connected to a digital video camera (IC Capture) and computer software with edge detection capability. The edge chosen by

the software were examined critically in all experiments. The vessel was superfused with warmed (37°C) and oxygenated (95% O_2 and 5% CO_2) Krebs.

10.6 Experimental protocol

Before experimentation, intraluminal pressure was gradually increased to 95mmHg. At this pressure, the vessels were stretched manually in the longitudinal direction to 115% of its unstretched length. Preparations were subsewuently equilibrated at 37°C for 30 min at 45mmHg.

In each experiment, vessels were first challenged with PE (1 μM) until the responses were reproducible. The vessels were then washed and contracted with PE (1 mM) and, once a plateau was reached, a cumulative concentration-response curve of Ach (1nM-30μM) was performed to evaluate endothelium integrity. Vessels showing less than 70% of endothelium were discarded.

After the vessels were washed and stabilized for 15 min, cumulative dose-response curve of Phe (1nM-30μM) and AngII (0.1nM-3μM) were performed.

After washing, the pressure was brought to 20mmHg and left for 5min in way to stabilize the vessel at the new reached diameter value. Myogenic tone was provoked by raising intraluminal pressure from 20 to 120mmHg in 25-mmHg steps. Each pressure level was maintained for 5 min, and the vessel diameter was then measured. At the end of each experiment, the vessel was fully relaxed by using-Ca^{2+}-free Krebs supplemented with 2mM EGTA. Myogenic tone was expressed as $[(D_1-D_2)/D_1]x100$, where D_1 is the passive diameter in Ca^{2+}-free buffer and D_2 is the active diameter.

10.7 Vessel morphometry

Three secondary-branches of mesenteric arteries per mouse were harvested and freed from surrounding fat tissue. Following full relaxation with Ach 1µM, they were fixed with 4% paraformaldehyde (PFA) and left overnight at 4°C. Fixed specimen was washed with PBS and then paraffin-embedded. 5µm cross-sections were obtained and VVG (Verhoeff's Van Gieson) staining was performed to identify the elastic elamina. Wall thickness, area and radius were determined by using a computerized image-analysis system (Image-Pro). At least

three sections per vessel from each mouse were analysed. Wall thickness was obtained from three averaged measurements.

10.8 Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was determined by using Student's t-test analysis for unpaired data, or, in the case of multiple comparison, two way ANOVA and Bonferroni as a post-test. Differences were considered statistically significant when $p<0.05$. GraphPad Prism software (version 5.0, GraphPad Software, San Diego, CA) was used for all the statistical analysis.

Chapter XI

RESULTS

11.1 Vascular Nogo-B is involved in blood pressure homeostasis

To investigate the Nogo-B in vascular homeostasis, we measured the blood pressure in Nogo-A/B$^{-/-}$ and WT mice by using tail cuff system, which allow us to perform measurements in non-anesthetized mice. We found that systolic (fig. 11.1A), diastolic (fig. 11.1B) and mean (fig. 11.1C) blood pressure were significantly lower in Nogo-A/B-/- mice compared to matched WT (systolic 123.2±4.3mmHg and 90.7±3.3mmHg; diastolic 97±4.6mmHg and 62.9±2.3mmHg; mean 106±4.7mmHg and 72±2.6mmHg, WT and Nogo-A/B$^{-/-}$ respectively).

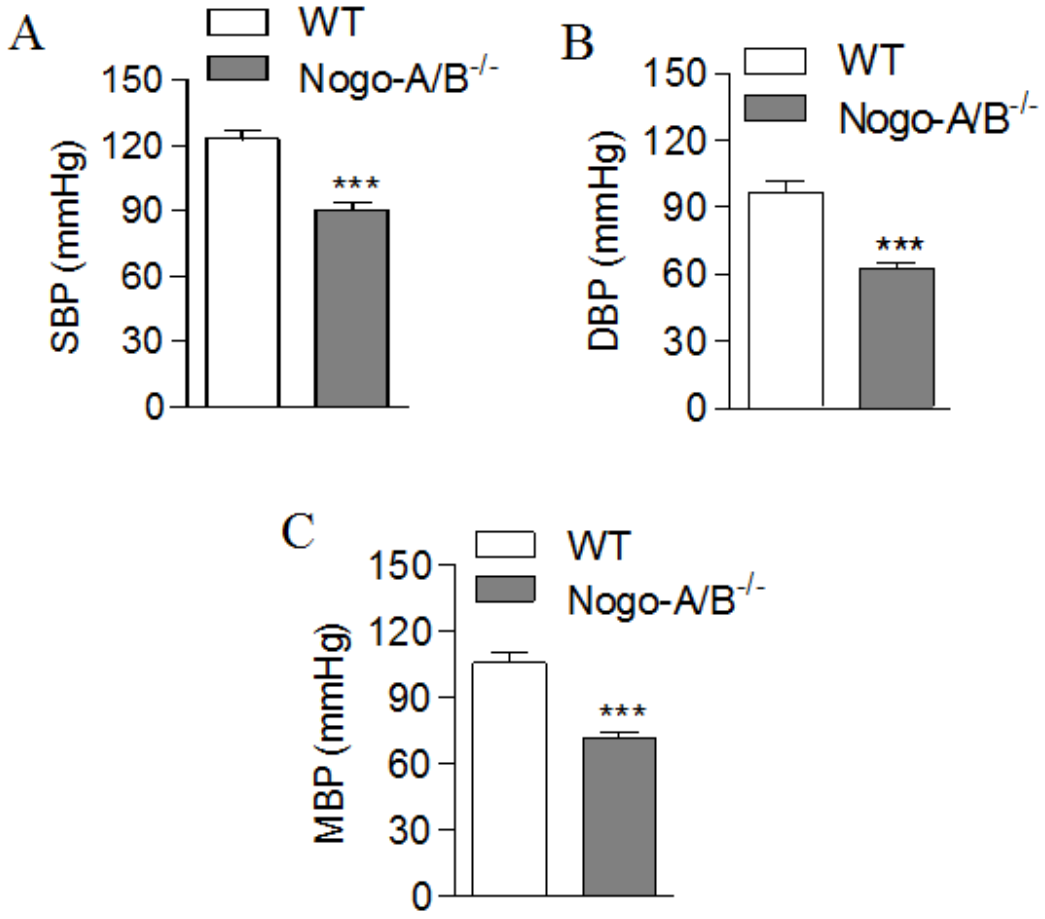


Fig. 11.1: Vascular Nogo B can regulate blood pressure *in vivo*. *Nogo-A/B$^{-/-}$ mice displayed a reduced (A) systolic, (B) diastolic and (C) mean blood pressure. (*** P<0.001 n=7 for each group).*

11.2 Loss of Nogo-B leads to arterial wall remodeling

It is well-known that increased or decreased pressure itself is responsible for the initiation of vascular remodeling. To assess vascular structure quantitatively, cross-sections of mesenteric artery were stained for elastic elamina (VVG staining) following morphometric analysis. The data indicated a reduction in both media wall thickness (4.4±0.26μm vs 3.7±0.24μm WT and Nogo-A/B$^{-/-}$ respectively) and media thickness-radius ratio (0.044±0.003μm and 0.034±0.001μm, WT and Nogo-A/B$^{-/-}$ respectively) (fig. 11.2).

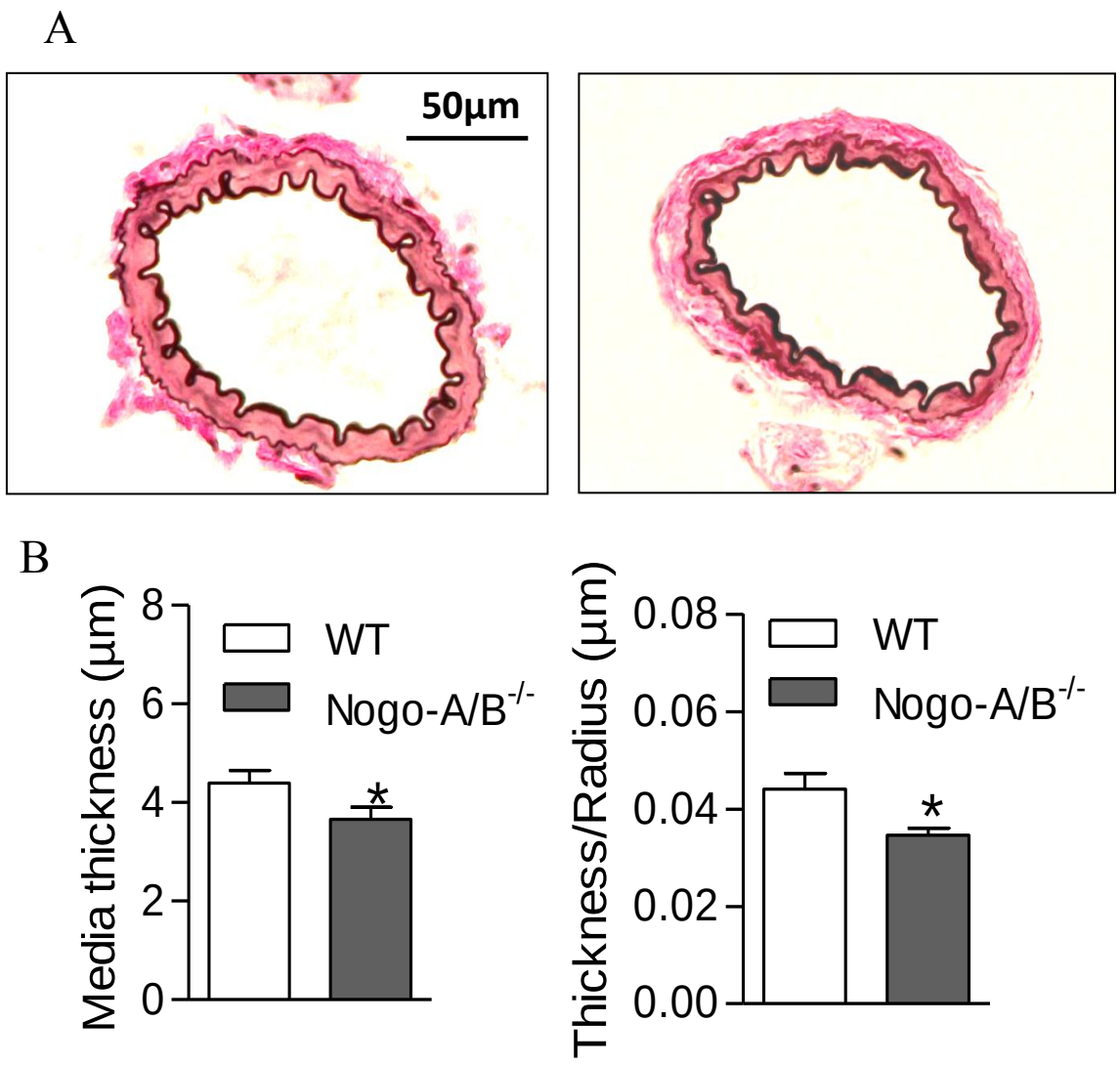


Fig. 11.2: Nogo-A/B$^{-/-}$ mice showed both media thickness and media thickness:radius ratio reduction compared to WT mice. *(A) VVG staining of mesenteric vessel sections from WT and Nogo-A/B$^{-/-}$ mice, respectively. (B) summarized analysis of morphometry (* P<0.05. n=10 per group).*

11.3 The lack of Nogo-B impairs the myogenic tone, but not the pharmacological response to Ach and Phe in mesenteric arteries

Since the lack of Nogo-B causes hypotension in mice, we evaluated the vascular reactivity of mesenteric artery from both WT and Nogo-A/B$^{-/-}$ mice. In particular we evaluated the pharmacological response and the myogenic tone by using the pressure myograph.

First we performed cumulative Ach dose-response curve and we found that vasodilation triggered by activation of cholinergic receptor is not affected in Nogo-A/B$^{-/-}$ mice compared to WT mice (E_{max} 87.5±4.2% vs 86.6±2.7% WT and Nogo-A/B$^{-/-}$ respectively) (fig. 11.3A). Then, we evaluated whether adrenergic system was affected by the loss of NogoB. As shown in fig 11.3B, Phe-induced vasoconstriction was not modified in Nogo-A/B$^{-/-}$ mice (Emax WT 52.3±1.8% vs KO 54.4±2.6%). This suggested that both cholinergic and adrenergic tone were not influenced by the loss of vascular Nogo-B.

In another set of experiments, pressure was increased in steps from 20 to 120mmHg to induce myogenic response. Active lumen diameter was significantly increased in Nogo-A/B$^{-/-}$ compared to WT mice (Emax 265.2±13.8µm and 301.2±13.7µm, WT vs Nogo-A/B$^{-/-}$(fig. 11.3C).

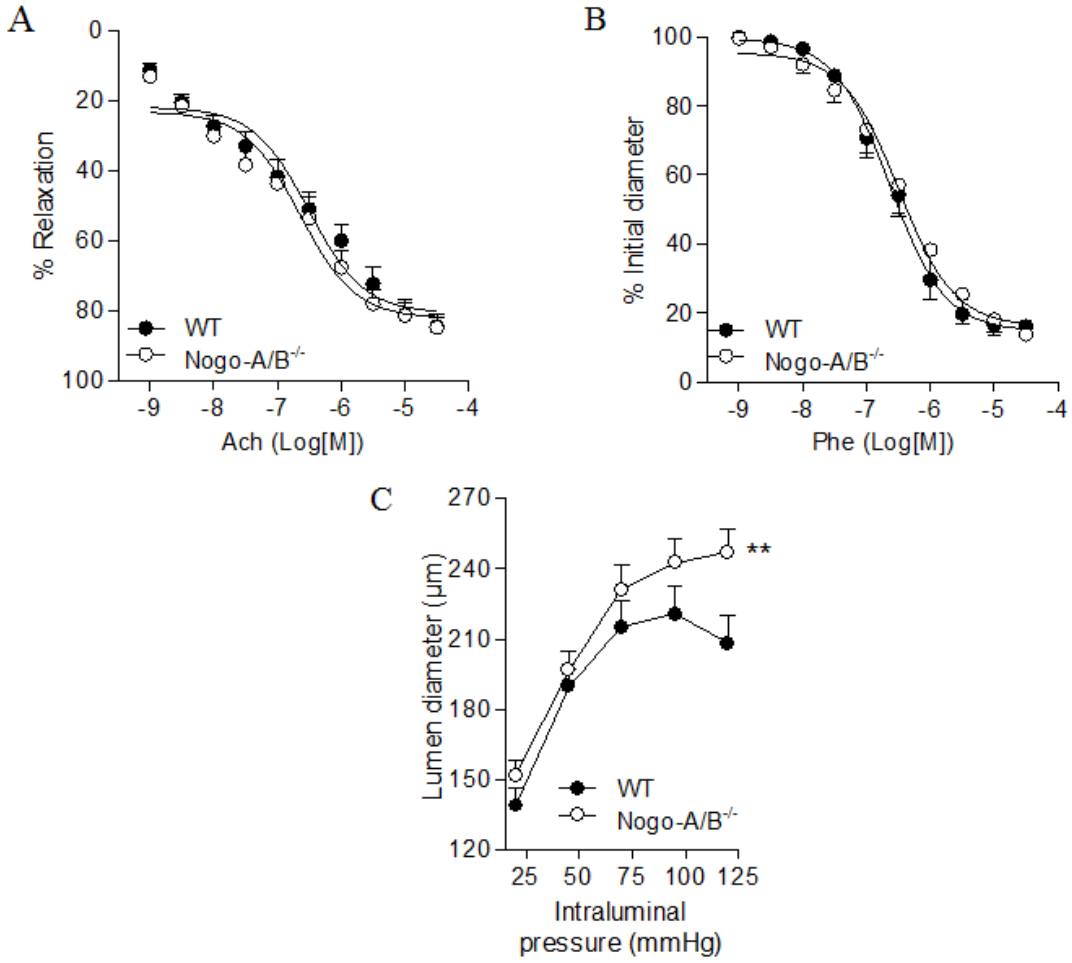


Fig. 11.3: Myogenic tone, but not cholinergic and adrenergic response is impaired in Nogo-A/B$^{-/-}$ mice *(A) Ach-induced vasorelaxation and (B) Phe-induced vasoconstriction are not influenced by the lack of Nogo-B. Conversely (C) myogenic tone is impaired in Nogo-A-B-/- mesenteric artery (** P<0.01, n= 10).*

Chapter 12

DISCUSSION

Nogo is a novel member of the RTN family of proteins named after the main subcellular localization of RTN-1 in the endoplasmic reticulum [302]. These proteins share a conserved 188 amino acid residues long C-terminal reticulon homology domain with two large hydrophobic regions of unknown function [381, 393]. The N-terminal sequences share some physicochemical properties but differ among the RTN family members. Of the three majorisoforms of Nogo, Nogo-A and Nogo-C are highly expressed in the central nervous system, with Nogo-C being additionally found in skeletal muscle, while Nogo-B is found in a ubiquitous pattern, such as the central and peripheral nervous systems, vessel wall, heart, spleen, skeletal muscle and testis [439, 440]. Previous works have focused on the biology of Nogo-A, Nogo-A receptors and Nogo-A co-receptors as negative regulators of axonal regeneration in the central nervous system [376, 385, 390]. However, it is still unknown the biological function of Nogo-B in the peripheral system. Acevedo et al [392] investigated the

expression and the role of Nogo-B in peripheral blood vessels and reported that Nogo-B was highly expressed in endothelial and smooth muscle cells of vessel wall, and that it was a regulator of cell migration *in vitro* and vascular remodeling *in vivo*. Although they suggested that Nogo-B participates in vessel injury and vascular remodeling, the physiological importance of Nogo-B in vascular homeostasis, remains still undefined. Given the abundance of Nogo-B in both endothelial and vascular smooth muscle cells, we investigated the function of Nogo-B in the contractile machinery of vascular system and thus in the regulation of blood pressure.

We found that Nogo-A/B$^{-/-}$ mice were markedly hypotensive compared to WT mice. It is well established that blood vessels undergo remodeling when chronically exposed to pressure changes. Indeed, blood vessels adapt to the hemodynamic stress in order to maintain constant the circumferential wall stress, which is the blood pressure value per media wall thickness. Thus, the lumen diameter of resistance arteries is governed not only by the magnitude of vasoconstriction, but also by vascular remodeling, providing a chronic change in wall geometry [228], Therefore, we looked at mesenteric

arteries morphometry and we found that Nogo-A/B$^{-/-}$ mesenteric arteries displayed a decreased media thickness as well as media thickness:radius ratio compared to WT mice.

It is well known that vessel tone is regulated by both intrinsic and extrinsic mechanisms. Circulating vasoactive mediators such as cathecholamines, as well as locally released mediators from autonomic nervous system, confer extrinsic regulation of smooth muscle contraction. Intrinsic regulation of vessel diameter is mainly mediated by two mechanosensitive mechanisms. One is the sensing of shear stress on the endothelium caused by an increase in blood flow. This leads to release of vasodilators from the endothelium including NO or EDHF, which relaxes the smooth muscle cells themselves, resulting in narrowing of the vessel lumen. The other mechanism involves stretch sensing by the smooth muscle cells themselves, resulting in narrowing of the vessel lumen. The latter mechanism is referred to as myogenic tone, the intrinsic ability of vessels to constrict in response to increased intraluminal pressuret.

We found that both Ach-induced vasorelaxation and Phe-induced vasoconstriction were not affected in mesenteric arteries from Nogo-

A/B$^{-/-}$ mice, suggesting that both cholinergic and adrenergic systems are not involved in vascular effects of Nogo-B. On the other hand, we found that myogenic tone in response to the increase of intraluminal pressure was notably lower in KO than in WT mesenteric vessels. This suggests that Nogo-B can be involved in the regulation of mechanotransduction rather than participates in the signaling triggered by extrinsic factors such as catecholamines or NO..

In conclusion, the present study reports a previously unidentified role of Nogo-B plays in the regulation of blood pressure in physiological conditions. Further studies are needed to understand the mechanism defining the physiological and biological function of Nogo-B.

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