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"Hydrogen Sulphide, a novel gasotransmitter regulating pathophysiological processes and cross-talk with Nitric Oxide"

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Abstract

L'acido sulfidrico (H_2S) è stato considerato per lungo tempo come un gas tossico, ma recentemente i suoi effetti farmacologici sono stati rivalutati. E' stato dimostrato che tale mediatore è prodotto in vari tessuti grazie all'azione di due enzimi quali cistationina-γ-liasi (CSE) e cistationina-β-sintasi (CBS), ed è coinvolto in vari processi fisiopatlogici. In particolare da questi studi emerge per l'acido sulfidrico un ruolo di primo piano nella regolazione dell'omeostasi vascolare e nei processi infiammatori. E' noto, infatti, che H_2S induce vasodilatazione mediante interazione con i canali del potassio ATP-dipendenti (K^+_{ATP}) e che una riduzione dei livelli plasmatici di H_2S rappresenta un fattore che contribuisce allo sviluppo dell'ipertensione spontanea. Viceversa, in stati infiammatori ed in particolare in seguito a somministrazione di LPS nei topi, si riscontra un aumento dei livelli plasmatici di tale mediatore gassoso.

Queste evidenze hanno portato alla volontà di studiare il ruolo di H_2S nel compartimento vascolare ed in particolare in che modo esso possa interagire con l'endotelio e con i mediatori da esso prodotti, quali ad esempio il monossido d'azoto (NO) nella regolazione dell'omeostasi vascolare e quindi anche dei processi infiammatori. Pertanto, è stato utilizzato un primo approccio di tipo funzionale allo scopo di indagare sul coinvolgimento di tale molecola in un modello di disfunzione vascolare associato al diabete mellito insulino-dipendente (IDDM), utilizzando aorte espiantate da animali spontaneamente diabetici (NOD/Ltj).

E' da sottolineare il fatto che in questi animali la disfunzione endoteliale è principalmente legata ad una drastica riduzione dei livelli di NO. Il passo successivo è stato poi caratterizzato da uno studio molecolare mirato alla valutazione dell'espressione degli enzimi responsabili della sintesi di H_2S e alla quantificazione

della produzione di tale mediatore. In ultima analisi, è stato poi valutato il ruolo di H_2S nella regolazione della risposta infiammatoria, utilizzando diversi modelli animali e cellulari.

I primi risultati ottenuti hanno mostrato un chiaro coinvolgimento di NO nella vasodilatazione indotta da H_2S, in particolare NO sembra regolare positivamente la produzione enzimatica di H_2S a partire da L-cisteina, suo precursore endogeno, mediata da CBS e CSE. Inoltre, lo studio condotto sugli animali diabetici evidenzia una riduzione della capacità vasodilatante della L-cisteina, sintomo di un'alterata attività enzimatica probabilmente correlata ai ridotti livelli di NO.

Lo studio molecolare effettuato attraverso analisi di western blot e qRT-PCR ha mostrato un aumento nella espressione di CBS e CSE, sebbene i livelli di H_2S plasmatici e tissutali siano significativamente ridotti negli animali diabetici. Ciò potrebbe essere ascrivibile ad un fenomeno di tipo compensatorio in risposta ad una riduzione dei livelli di H_2S circolanti.

Infine, i risultati ottenuti attraverso l'utilizzo di diversi modelli animali di infiammazione hanno mostrato che H_2S è in grado di inibire la migrazione dei polimorfonucleati (PMN) nei tessuti in seguito a stimolazione con agenti flogogeni; in particolare, questa azione è mediata dai canali del potassio ATP-dipendenti. Inoltre, l'edema indotto da carragenina veniva significativamente ridotto dal trattamento con diversi donatori di H_2S.

In conclusione i risultati sperimentali ottenuti indicano in primo luogo che l'ossido nitrico è coinvolto nei meccanismi di vasodilatazione indotti da H_2S, suggerendo l'esistenza di un legame tra le vie metaboliche L-arginina/NO e L-cisteina/H_2S nella regolazione del tono vascolare. Inoltre, H_2S mostra un effetto anti-infiammatorio, che si esplica principalmente attraverso il blocco della migrazione

leucocitaria. Queste evidenze, nel loro insieme, suggeriscono per la prima volta che H_2S agisce in maniera diretta sulle cellule endoteliali vascolari influenzando molteplici fenomeni che avvengono all'interfaccia tra endotelio e sangue, in particolare intervenendo sul mantenimento dell'omeostasi vascolare e sulla regolazione della migrazione leucocitaria.

Summary

Sulphidric acid has been considered for a long time just a polluting gas and only recently its pharmacological effects have been investigated. H_2S is produced in many mammalian tissues by two enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) and it is involved in many physiological and pathological events. In particular this gas seems to have a critical role in vascular homeostasis and in inflammatory processes. It has been demonstrated that H_2S induces K^+_{ATP} channels-dependent vasorelaxation and that spontaneous hypertension is associated to its lowered plasmatic levels. On the other hand, during inflammatory process, such as LPS administration in mice, plasmatic H_2S levels result increased.

These evidences put us on way to investigate the role of H_2S in vascular tone regulation and the possible relationship with nitric oxide (NO). Furthermore the aim of this work was also to assess involvement of this gaseous molecule in inflammatory events. For these reasons, we first used a functional approach in order to study H_2S role in a model of endothelial dysfunction associated to diabetes, by using aortic tissue harvested from Non ObeseDiabetic mice (NOD/Ltj). These animals show endothelial disorder mainly associated to a reduction in NO levels.

The following step was characterized by a molecular approach in order to verify the expression of CBS and CSE and their enzymatic activity leading to H_2S production. The last part of this work dealt with evaluation of hydrogen sulphide in inflammatory responses. First data obtained showed a marked involvement of NO in H_2S-induced vasodilatation and in particular NO seemed to regulate production of H_2S from L-cysteine, its biological precursor. Furthermore, results of experiments carried out on diabetic mice highlighted a reduce vasodilatory effect by L-cysteine, likely linked to reduced NO availability. Molecular data showed that both CBS and CSE expression

are significantly increased, although plasmatic levels of H_2S were reduce in diabetic mice. This evidence could reflect a compensatory response in order to restore H_2S levels.

Finally, data obtained from experiments performed on different animal model of inflammation clearly demonstrated that H_2S inhibited polymorphonuclear (PMN) transmigration, following an inflammatory stimulus. In addition, this effect is mediated by K^+_{ATP} channels.

In conclusion, our experimental data indicate for the first time that nitric oxide is involved in vasodilatory effect induced by H_2S, suggesting a link between L-arginine/NO and L-cysteine/H_2S pathway in vascular tone regulation.

Furthermore, hydrogen sulphide showed an anti-inflammatory effect, mainly exerted through blockade of PMN transmigration. Altogether, these evidences suggest that H_2S is able to act directly on endothelial cells, where it is also produced, affecting all phenomenon occurring at blood-endothelium interface, particularly intervening on maintenance of vascular homeostasis and regulation of leukocytes migration.

CHAPTER 1

1. INTRODUCTION

In the second century A.D. Galen said: *"Throughout the body the animal arteries are mingled with veins and veins with arteries, and both veins and arteries are mingled with nerves and the nerves with these…And of course the usefulness of such a complete interweaving is very evident, if, that is to say, it is a useful thing for all parts of the animal to be nourished"*. Then, in despite of a very sophisticated and fine regulated human body, the most important and easy way for maintaining it alive and well working in all its functions is just represented by blood. Its flowing through a massive network of "tubes" makes all cells being fed with all nutrients needed and keep in constant communication all organs and tissues, even if they are far away from each other. In this kind of processes , these "tubes", called vessels, are simply fundamental (fig. 1.1)

Vessels are divided into two main categories, such as arteries and veins. Both of them have a particular structure formed by three layer of different cells types, even if there are some quantitative differences. The outer one is represented by *adventitia*, mainly formed by collagen and fibroblasts; in the middle, there is the *media*, constituted by smooth muscle cells; finally, the inner layer is the *endothelium*, formed by a monolayer of endothelial cells that have a proper direct contact with all molecules going around the body through the blood flow (fig. 1.2). The possibility of this interface contact between endothelium and blood makes the endothelium one of the most important actor in regulating physiology of vascular tone; and this also because it actually represents a "barrier" allowing or not molecules passing through it and going into tissues, and then exerting their own effects.

The activity of this "barrier" is regulated at various levels by a wide range of molecules but for sure the one playing a pivotal role is nitric oxide (NO). Through years, NO and its effects have been described in details and its scenario has been well drawn up (Palmer RM et al., 1988; Moncada S et al., 1991). Particularly, it has been appearing clear how just one simple molecule like NO could be responsible for vascular relaxation, pro-inflammatory actions and proliferation affecting stimuli (Cirino G et al., 2003; Garcia X et al., 2006; Moncada S et al., 1991). Furthermore, alteration of NO levels is consistent with many pathological conditions, overall endothelial dysfunction (Nagareddy PR et al., 2005; Bucci M et al., 2004; Pechanova O et al., 2007).

In this contest, another important gaseous mediator has been recently considered: the gaseous molecule in question is *hydrogen sulphide* (H_2S). H_2S has been known since early times and its chemistry has been studied since the 1600s; it is a colourless gas with a typical rotten eggs smell that represents the main reason it is well known for. This molecule was thought just like a toxic and polluting gas for a long time, even if pollution was later discovered to occur at concentration 400-fold higher than physiological one. Only in last decade its involvement in many physiological and pathological processes has been found out, and the aim of this work is just to assess its functions, contributing to the latest findings in the "pharmacology of hydrogen sulphide".

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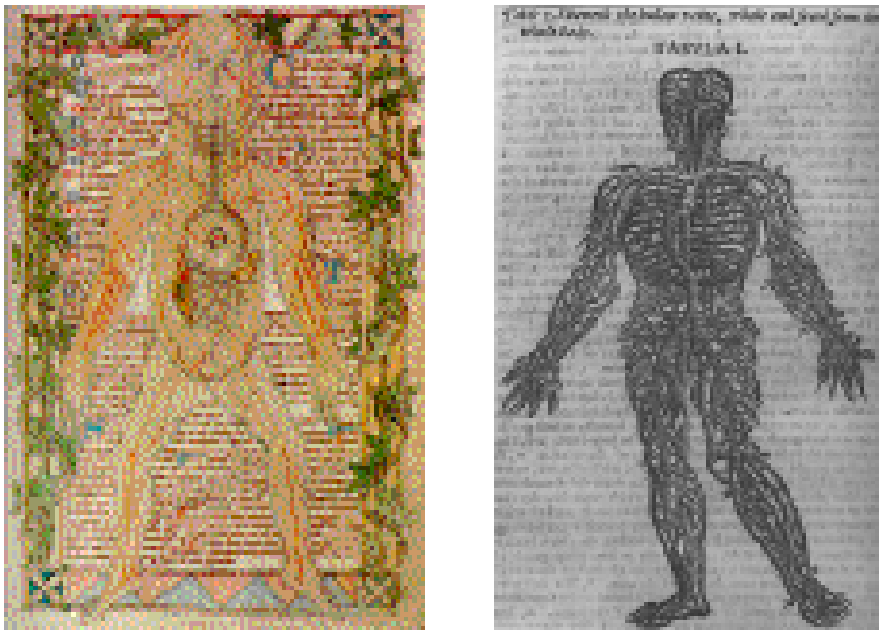


Fig. 1.1 Medieval images of circulatory tree

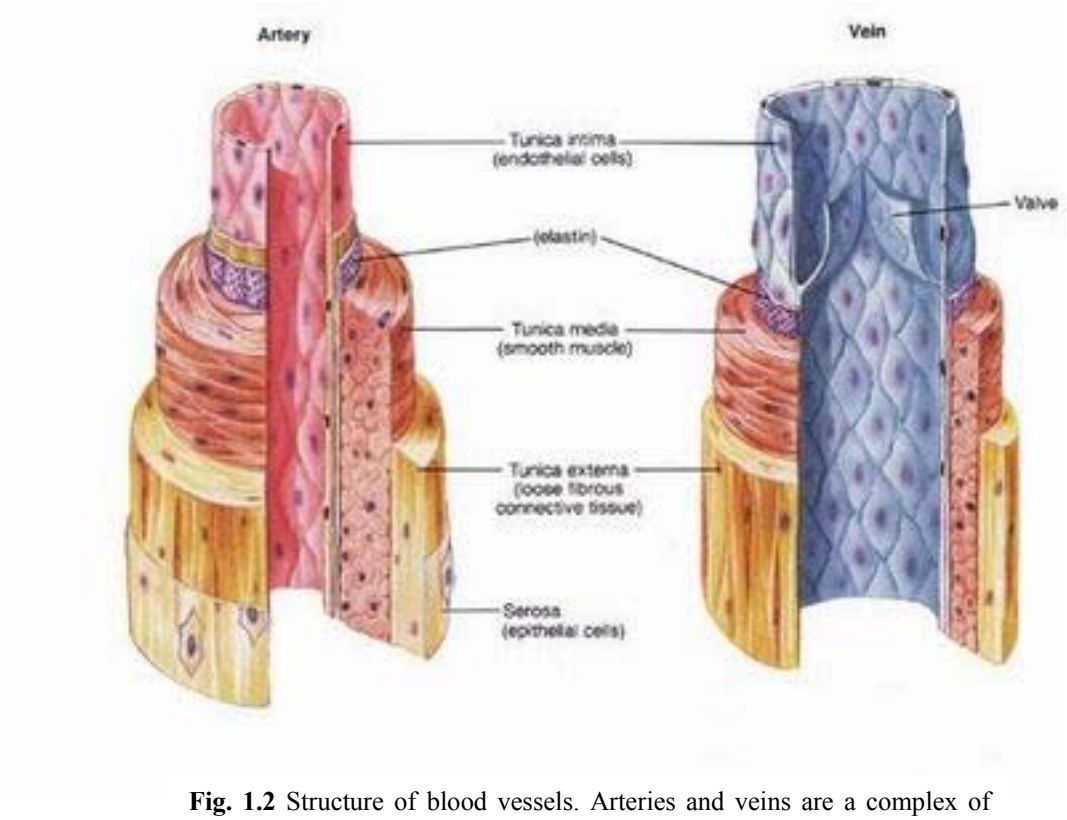


Fig. 1.2 Structure of blood vessels. Arteries and veins are a complex of multiple layers where endothelium represents the inner one and smooth muscle cells and connective tissue are stratified over it

1.1 THE ENDOTHELIUM AS A DETECTOR INDUCING VASCULAR CHANGES

1.1.1 Vascular endothelium and its multi-functional role

The *endothelium* is a specialized type of epithelial tissue consisting in a thin layer of cells that line the interior surface of blood vessels and forming an interface between circulating blood in the lumen and the rest of the vessel wall. In all types of blood vessels the endothelium of the tunica intima is highly specialised with endocrine, exocrine, cell adhesion, clotting and transport functions. Endothelial cells line the entire circulatory system, from the heart to the smallest capillaries and they reduce friction of blood flow allowing the fluid to be pumped further.

Endothelium is composed of flattened cells and in routine histological sections the cytosol of most cells is barely visible and only the small flattened nuclei are seen. Ultrastructurally, each cell can be seen to be anchored to an underlying basal lamina; individual cells are anchored together by adherent junctions, including prominent tight junctions which prevent diffusion between cells.

A major feature of endothelial cells is represented by pinocytotic vesicles which are involved in the process of transport of substances from one side of the cell to the other. In small blood vessels of the nervous system, endothelial cells express transport proteins which are responsible for active transport of all substances, for example glucose into the brain. Furthermore, they are able to sense changes in blood pressure, oxygen tension and blood flow by as yet unknown mechanisms. In response to changes in local conditions they respond by secreting substances which have powerful effects on the tone of vascular smooth muscle. Endothelium is also important in the control of

blood coagulation, since under normal circumstances its surface prevents blood clotting and allows a smooth blood flux (Santiago-Delpin EA, 2004; Ppova EN et al., 2004).

Another important feature of endothelium is characterized by its ability in adapting rapidly to changes in its environment. In fact, under certain circumstances, especially in response to adverse stimuli such as wounds, infections or irritation (e.g. insect sting), it becomes activated and changes its function (Pober JS et al., 2002). This activation is mainly operated by cytokines and develops specialisation for lymphoid cells migration. The endothelial cells become cuboidal in shape and express surface adhesion molecules which facilitate lymphocytes adhesion and migration. This type of endothelium is particularly present in the specialised venules in the lymph node paracortex (high endothelial venules). These changes normally occur after tissue damages and allows neutrophils to migrate into local tissues during acute inflammation. One of the first step in this pathway is represented by exposure of P-selectin, a cell adhesion molecule stored in special vesicles (Weibel-Palade bodies) inside the endothelium. Upon an appropriate stimulation, these vesicles dock with the endothelial cell membrane and P-selectin is then available on the cell surface for neutrophils adhesion (Arribas M et al., 2000; Burns AR et al., 1999) (fig. 1.3).

In physiological conditions, endothelium is locally impermeable to substances circulating in the blood but in presence of certain molecules, such as histamine, they lose attachment to each other and retract. This change allows fluid and proteins to diffuse out into local tissues causing its swelling, termed *oedema*. The reorganisation of cell-cell junctions is rapid and reversible and takes place in the space of a few minutes (Tab I).

Tab 1 - Properties of endothelial cells of blood vessels

a)	Cells are bound together by junctional complexes and have many pinocytic vesicles
b)	Cells, although simple in appearance, have many complex roles
c)	Under normal circumstances secretes substances which prevent blood clotting
d)	Also under normal circumstances secretes substances which maintain the tone of vascular smooth muscle
e)	Can be activated by cytokines to express cell adhesion molecules which allow white blood cells to stick

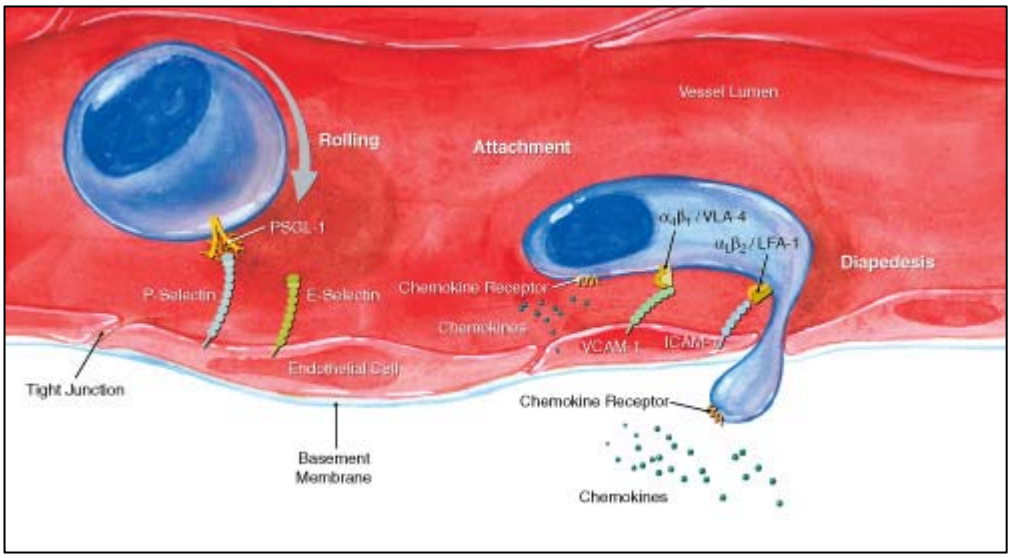


Fig 1.3 P-selectin and E-selectin are involved in rolling of circulating leukocytes and in transmigration process in inflamed tisues

1.1.2 Endothelial cells in the regulation of vascular tone

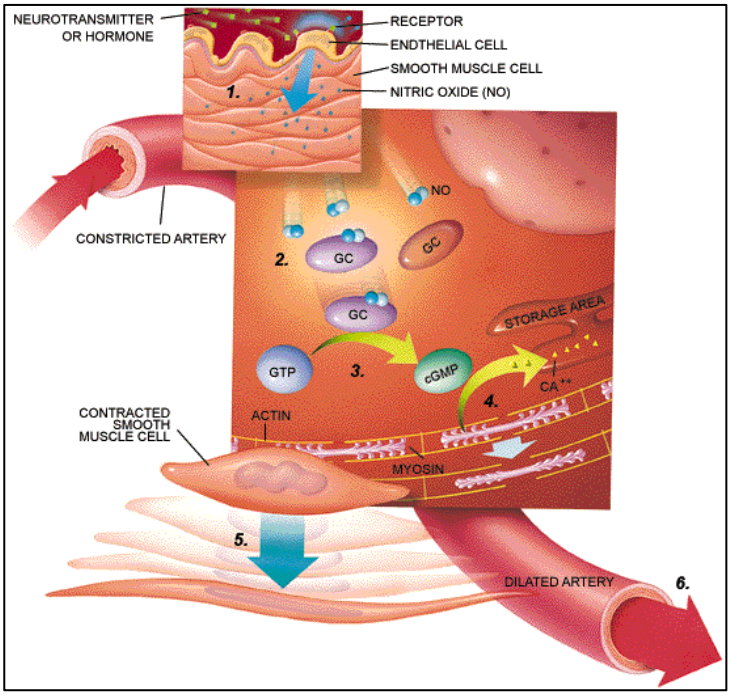
Arterial blood pressure is mainly regulated by a tight balance between different molecules having vasoconstrictor and vasorelaxant activities Pechanova O et al., 2007). Since endothelium represents a proper barrier between blood and tissues, it actually is the first element which comes in contact with all circulating molecules and it is also able to counteract any kind of misbalancing events. Endothelial cells produce and/or release a wide range of molecules responsible for regulation of vascular tone (Tab II). An alteration in production or release of these mediators could lead to an endothelial dysfunction, a hallmark for vascular diseases, typically associated to diabetes and hypertension (Bucci M et al., 2004; Li J et al., 2007; Punthakee Z et al., 2007).

One of the most important factor produced by endothelium is *NO*, released by enzymatic degradation of L-arginine operated by the endothelial isoform of nitric oxide synthase (eNOS). This radicalic molecule activates the soluble guanylate cyclase (sGC), increasing the amount of cGMP and leading to the final effect consisting in relaxation of smooth muscle cells. Furthermore, NO-dependent pathway activates cGMP-dependent protein kinase (PKG) that acts through opening potassium (K^+) channels and externalization of K^+ ions outside the cell. NO is extremely important in this environment since, its impaired release is associated to endothelial dysfunction (fig 1.4a).

Another important mediator involved in homeostasis of vascular tone is *prostacyclin* (PGI_2). As NO, also PGI_2 exerts vasorelaxant activity but through a different mechanism. Indeed, it activates adenylate cyclase and induces an increase in cAMP levels. Its increase activates PKA, a cAMP-dependent protein kinase, that in turn induces opening of K^+ channels PKG (fig 1.4b).

Belong vasoconstrictor mediators released by endothelium, there are *endothelins* (ETs). Endothelin-1 (ET-1), a peptide of 21 amino acid residues, is the most potent vasoconstrictor substance known. Originally isolated from porcine aortic endothelial cells,

a)



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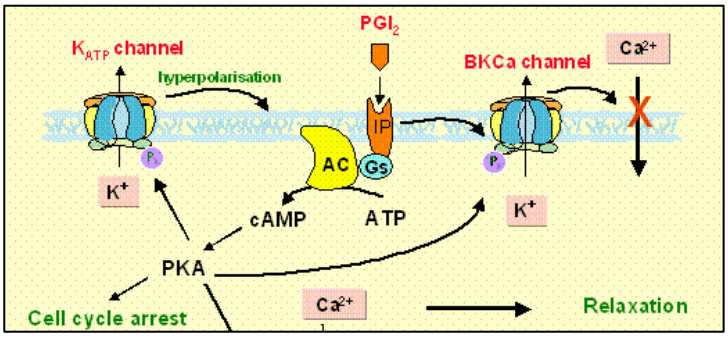


Fig 1.4 a) Nitric oxide signalling in vascular tissue. NO diffuses from endothelium to smooth muscle cells and there activate soluble guanylate cyclase, increasing production of cGMP that leads to vasorealxation. b) PGI_2 signalling in vascular tissues. PGI_2 activates adenylate cyclase increasing release of cAMP, responsible for relaxation through PKA activation.

ET-1 is now known to belong to a family of three mammalian vasoactive peptides that also includes endothelin-2 (ET-2) and endothelin-3 (ET-3). ETs have a double effect depending upon activation of two different receptors, ET_A and ET_B. Both receptors are expressed on smooth muscle cells and binding of ETs on these receptors is able to induce vasoconstriction. Conversely, only ET_B is expressed on endothelial cells and its activation leads to vasodilation via NO production (Rubanyi GM et al., 1994; Luscher T et al., 2000; Suzaki Y et al., 2003) (fig 1.5a).

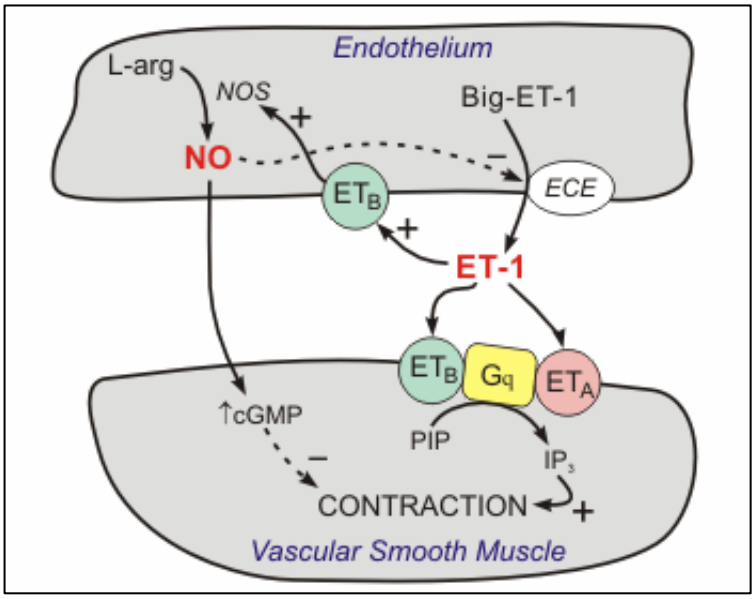
All mediators, so far mentioned, are just a part of the very complex network of molecules underlying the regulation of vascular functions and it has to be said about the existence of a neural system capable to interplay with NO, PGI_2 and ETs. In this network a prominent role is occupied by sympathetic system supplying hormones like noradrenalin and adrenalin, the major neuropeptides released upon nervous fibers activation.

In addition, it is noteworthy to underline the activity of the renin-angiotensin system and of vasopressin responsible, in association with ETs, of the constrictor effects in complex equilibrium that leads to the vascular homeostasis (fig 1.5b).

Tab II - Molecules produced by endothelial cells

Factor secreted by the endothelium	*Activities*
Prostacylin (PGI_2)	vasodilation, inhibits platelet aggregation
Nitric Oxide (NO)	vasodilation, inhibits platelet adhesion and aggregation
Tissue Plasminogen Activator (tPA)	regulates fibrinolysis
thrombomodulin	anticoagulant activity
thromboplastin	promotes blood coagulation
Platelet Activating Factor (PAF)	activation of platelets and neutrophils
von Willebrand Factor (vWF)	promotes platelet adhesion and activation of blood coagulation

a)



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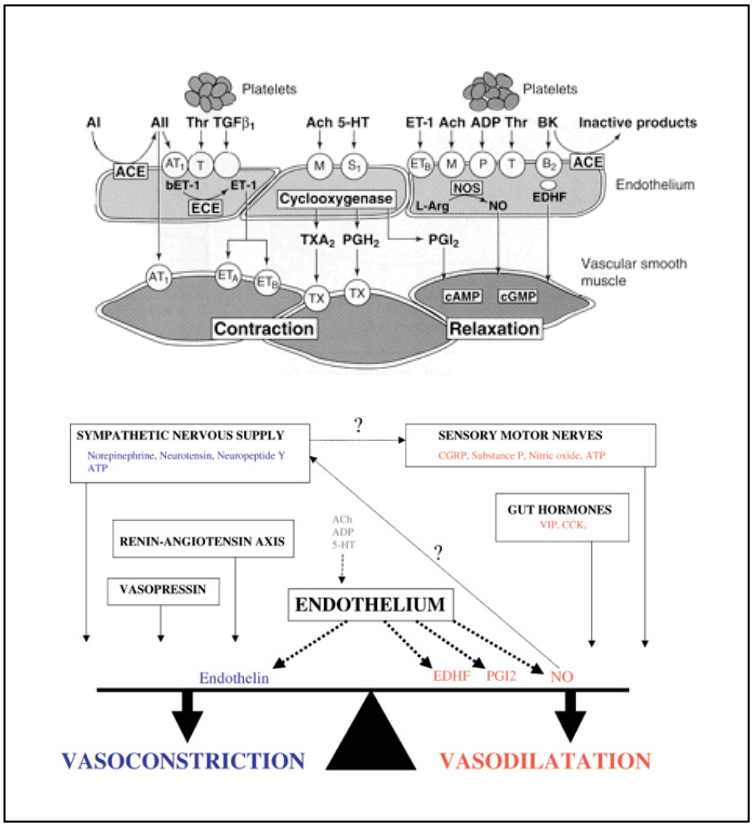


Fig 1.5 a) Endothelins signalling pathway. ETs have both constrictor and relaxant effects, depending upon activating ET_A or ET_B receptor. b) Balancing and interplay of vasoactive molecules in vascular tissue.

1.1.3 Inflammation and endothelial cells: interplay and communication

An inflammatory condition is characterized by a wide range of events one of which is the leukocyte transmigration. This event starts following an injury generating activation and release of cytokines by macrophages (IL-1, TNF-α), that in turn induce the expression of selectins (P- and E-selectin) on endothelial cell surface. Together *P-* and *E-selectin* slow the motion of leukocytes through the bloodstream by causing them to roll along the endothelial surface, allowing other molecules to interact with the slowed leukocytes in order to stop them and to promote their movement into the tissues (Leeuwenberg et. al. 1992). Leukocyte rolling occurs due to the brief, reversible binding of E and P-selectin with their complementary molecules, which are expressed on the surface of leukocytes.

At the same time, chemokines released by macrophages activate the rolling leukocytes and cause activation of integrins, another type of adhesion molecules. This activation consists in switching leukocytes from the default low-affinity state to a high-affinity state. This is assisted by juxtacrine activation of integrins by chemokines and soluble factors released by endothelial cells. In the activated state, integrins bind tightly to complementary receptors expressed on endothelial cells, with high affinity. The overall effect is the immobilization of the leukocytes, despite the shear forces of the ongoing blood flow (Aplin AE et al., 1998).

In order to migrate the cytoskeleton of the leukocytes is re-organized in such a way that the leukocytes are spread out over the endothelial cells. In this form, leukocytes extend *pseudopodia* and pass through gaps between endothelial cells. Transmigration of the leukocytes occurs as *PECAM* proteins, found on the leukocyte and endothelial cell surfaces, interact and effectively pull the cell through the

endothelium. The leukocytes secrete proteases that degrade the basement membrane, allowing them to escape the blood vessel – a process known as diapedesis. Once in the interstitial fluid, leukocytes migrate along a chemotactic gradient towards the site of injury or infection and become ready to counteract the inflammatory events (fig. 1.6).

Acute inflammation normally resolves by mechanisms that have remained somewhat elusive. Emerging evidence now suggests that an active, coordinated program of resolution initiates in the first few hours after an inflammatory response begins. After entering tissues, leukocytes promote the switch of arachidonic acid–derived prostaglandins and leukotriens of the starting phase of inflammation to lipoxins, which initiate the termination sequence. Belonging to this class of protein, we find Annexin 1 (AnxA1), well known as Lipocortin-1 too; this protein is playing a critical role in endothelial transmigration of neutrophils, being activated and exposed on cell surface once inflammation starts. In particular, it blocks adherence of leukocytes and diapedesis, promoting an anti-inflammatory effect (Kamal H. et al., 2005) (fig. 1.7).

Thus, when neutrophil recruitment ceases, programmed death by apoptosis is engaged and this event coincides with the biosynthesis of resolvins and protectins (Serhan CN et al., 2005). Then, apoptotic neutrophils undergo phagocytosis by macrophages, leading to neutrophil clearance and release of anti-inflammatory and reparative cytokines such as transforming growth factor-β_1 (TGF-β_1). The anti-inflammatory program ends with the departure of macrophages through the lymphatics.

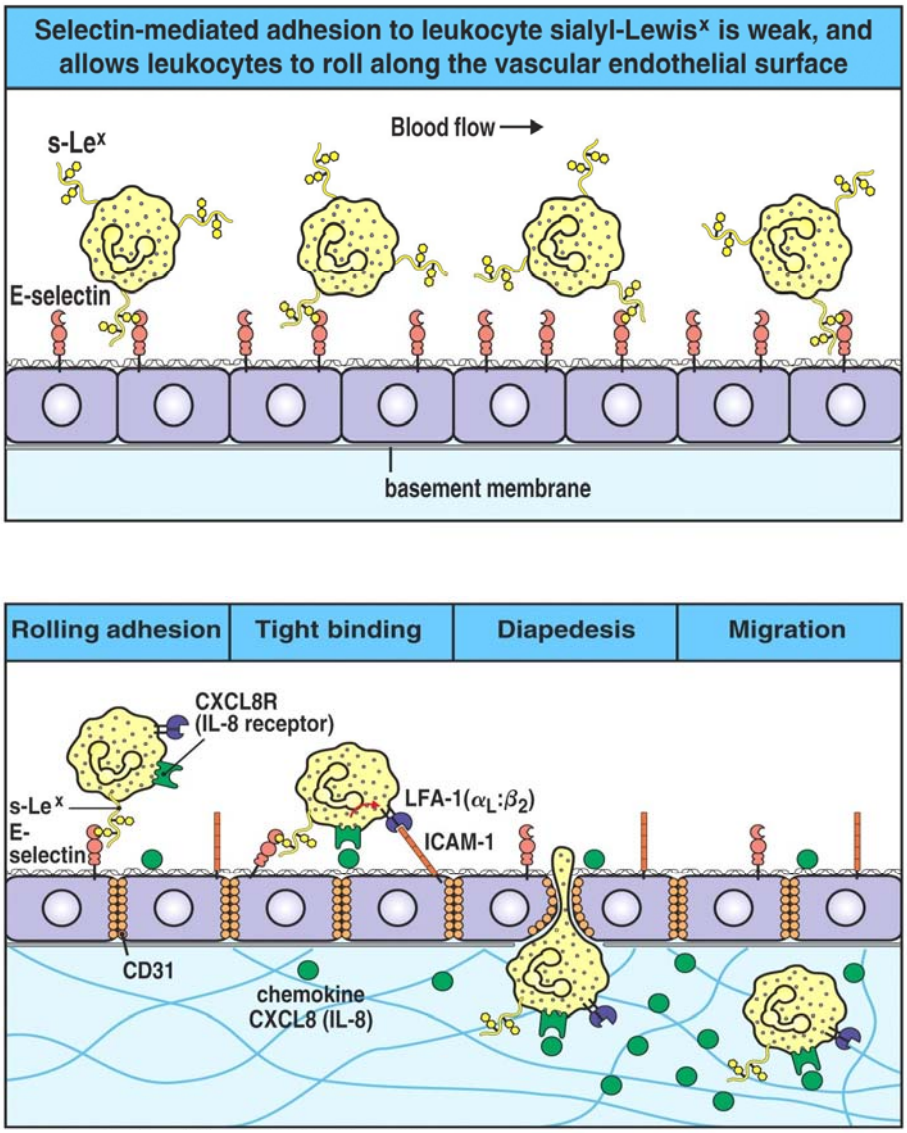


Fig 1.6 Rolling of leukocytes onto endothelial cells surface represents the first step of diapedesis. Then, leukocytes tightly adhere to adhesion molecules and changes in shape occur, allowing them to go through endothelium towards tissue.

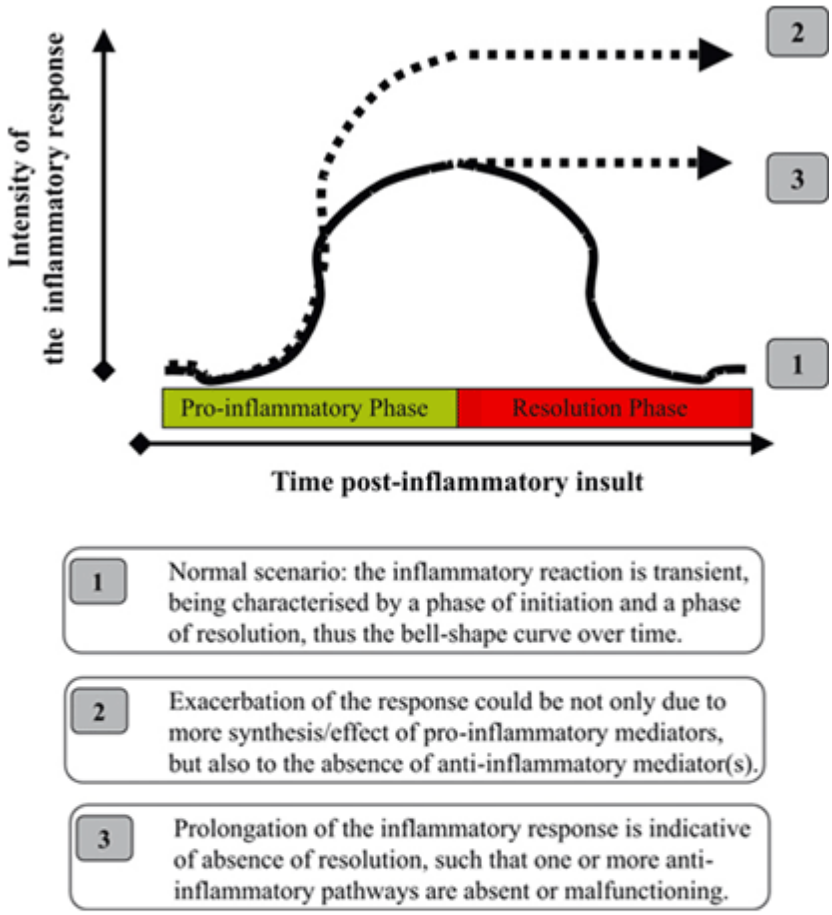


Fig 1.7 The concept of resolution and inflammatory pathology. This scheme summarises the concept behind the study of antiinflammation. It is proposed that the physiological scenario (1) is observed when a pro-inflammatory phase of inflammation is followed by an active phenomenon of resolution. Inflammatory pathology could be due to an overshooting of the pro-inflammatory phase (2) or by a lack of active resolution, with consequent prolongation of the inflammatory aetiology and symptoms (3). It is worth noting that scenario no. 2 could also due, at least in part, by lack of activity of anti-inflammatory mediators, tonically active to down-regulate the pro-inflammatory phase (classical example being the one of glucocorticoids, in view of the exacerbation of the response observed after adrenalectomy) (Flower RJ et al. 1986, Perretti M et al. 1989).

1.2 HYDROGEN SULPHIDE (H_2S): GASEOUS CHEMICAL WITH BIOLOGICAL ACTIVITIES

1.2.1 H_2S: an overview on chemical and biochemical properties

H_2S structure is very similar to that of water and this is where the similarity ends (fig 1.8). The sulphur atom is not nearly as electronegative as oxygen so that hydrogen sulphide is not nearly as polar as water. Because of this, comparatively weak intermolecular forces exist for H_2S and the melting and boiling points are much lower than they are in water. In fact, boiling temperature of hydrogen sulphide and water are -60.7 °C and +100.0 °C , respectively.

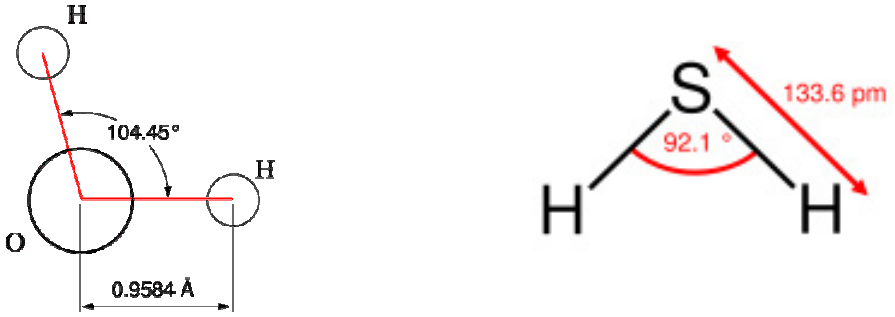


Fig 1.8 Similarity in molecular structure between water (H_2O) and hydrogen sulphide (H_2S)

Hydrogen sulphide is weakly acidic, dissociating in aqueous solution into hydrogen cations H^+ and the hydrosulphide anion HS^-, which subsequently may decompose to H^+ and sulphide ion S^{2-} (Ka_1 = 1.3x10^{-7} M, Ka_2 =1x10^{-19} M; Giggenbach W, 1971; Myers RJ, 1986).

$$H_2S \overset{Ka_1}{\rightleftharpoons} H^+ + HS^- \overset{Ka_2}{\rightleftharpoons} H^+ + S^{2-}$$

Under physiological conditions, i.e. at pH 7.4, one third of hydrogen sulphide is undissociated and this means that more than 30% of this gas may be revealed in biological fluids as H_2S. Conversely, the chemical form S^{2-} is not present in appreciable amounts, since the dissociation of HS^- occurs only at high pH values.

In a similar way to other biological relevant gases like carbon monoxide (CO) and nitric oxide (NO), H_2S has lipophilic features that confer on it the capability to permeate plasmamembrane. Nevertheless, due to partial dissociation, membrane are relatively less permeable to H_2S than to both other gases.

In mammalian, the "smelling gas" is endogenously produced by enzymatic reactions, even if some non-enzymatic pathways are involved in biochemistry of hydrogen sulphide. Enzymes regulating its release are cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) that work differently but both have a key role in H_2S metabolic pathway (Chen X et al., 2004). However, the only substrate for the generation of endogenous H_2S from these enzymes is L-cysteine, a sulphur-containing amino acid derived from alimentary sources, synthesized from L-methionine through a so called "trans-sulfuration pathway" with homocysteine (Hcy) as an intermediate, or liberated from endogenous protein (Meier M et al., 2001; Yap S et al., 2000) (fig 1.9).

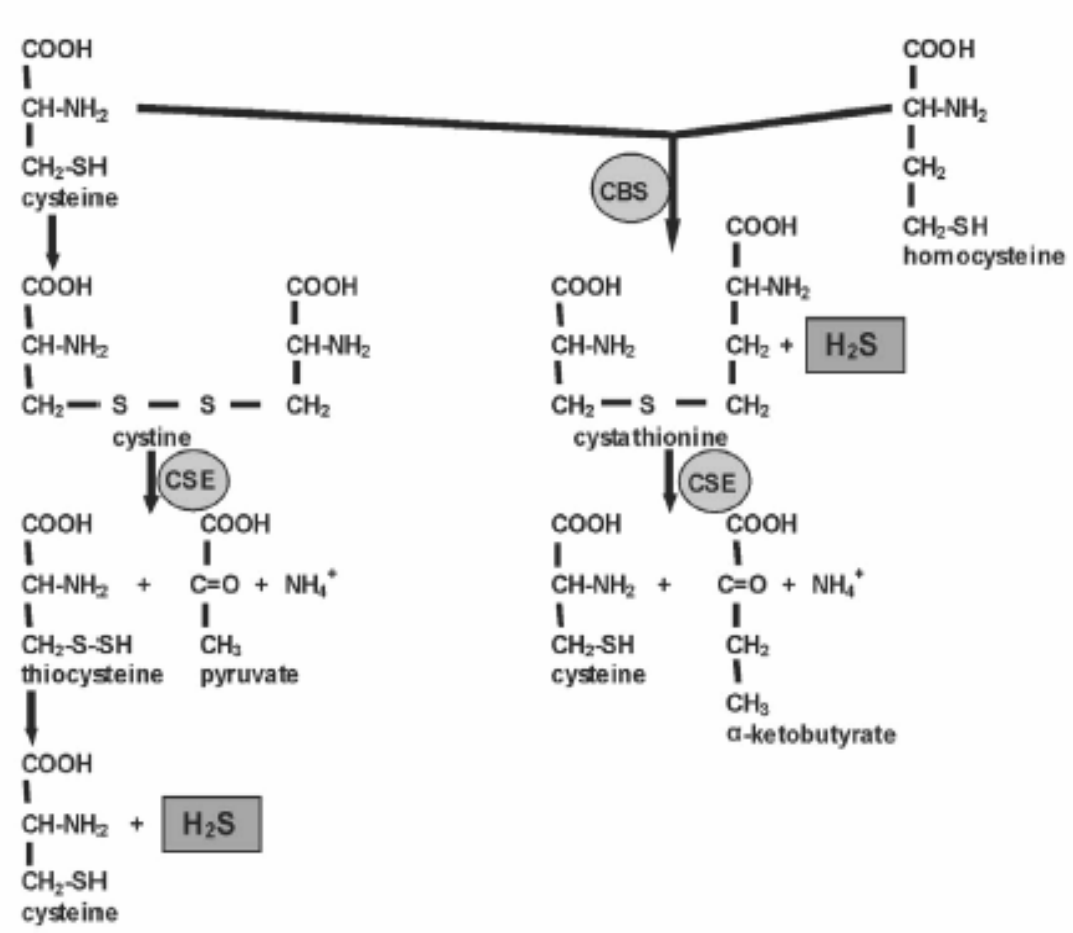


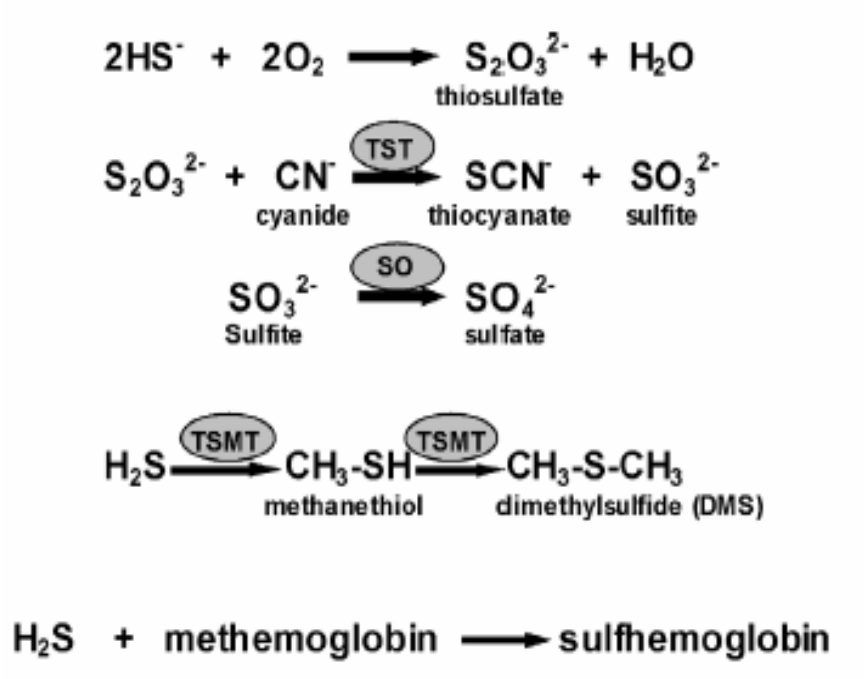
Fig 1.9 Endogenous hydrogen sulphide synthesizing pathways. Homocysteine is the physiological substrate for CBS, that in presence of cysteine releases both H_2S and cystathionine. Cystine and cystathionine are both substrate for CSE, that is able to produce both cysteine and thiocysteine; the latter releases H_2S in a non-enzymatic way

Another pathway that leads to the release of H_2S is represented by a "desulfhydration" reaction, consisting in the removal of cysteine sulphur atom without its oxidation and this process may be catalyzed by both trans-sulfuration pathway enzymes, CBS and CSE. On the other hand, H_2S catabolic pathway is not well defined yet, and most of the data we have been obtained by using exogenous H_2S; this implies that most of the studies carried out till now may not have biological and physiological relevance. Hydrogen sulphide is rapidly oxidized, mainly in mitochondria, initially to thiosulfate which is further converted to sulphite and sulphate. This oxidation is not enzymatically driven, whilst thiosulfate conversion in sulphate and/or sulphite is catalyzed by thiosulfate-cyanide sulfurtransferase (TST).

Also, sulfite originated by this reaction is quickly oxidized to sulphate, as sulphate is the major end-product of H_2S metabolism under physiological conditions; however urinary thiosulfate is considered to be an aspecific marker of whole-body H_2S production (Belardinelli MC et al., 2001).

Another catabolic pathway is represented by methylation by thiol-S-methyltransferase (TSMT) to methanethiol and dimethylsulfide. This reaction occurs mainly in cytosol and some studies question the significance of this pathway in the gastrointestinal tract (Levitt MD et al., 1999; Picton R et al., 2002). Finally, hydrogen sulphide is able to bind to methaemoglobin to form sulfhaemoglobin and since haemoglobin may also bind CO and NO, it is considered a "common sink" for all three endogenous gases (Kurzban GP et al., 1999) (fig 1.10)

a)



b)

c)

Fig 1.10 Catabolism of H_2S. a) mytocondrial oxidation; b) cytosolic methylation; c) bindind to haemoglobin. SO: sulfite oxidase; TMST: thiol-S-methyl transferase; TST: thisulfate-cyanide sulfurtransferase (rhodanese)

1.2.2 H_2S producing enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE)

H_2S formation is related to the activity of both cystathionine-β-synthase (CBS), and cystathionine-γ-lyase (CSE), two pyridoxal-5'-phosphate(PLP)-dependent enzymes involved in trans-sulfuration pathway that differ in their specific mechanism (fig 1.9).

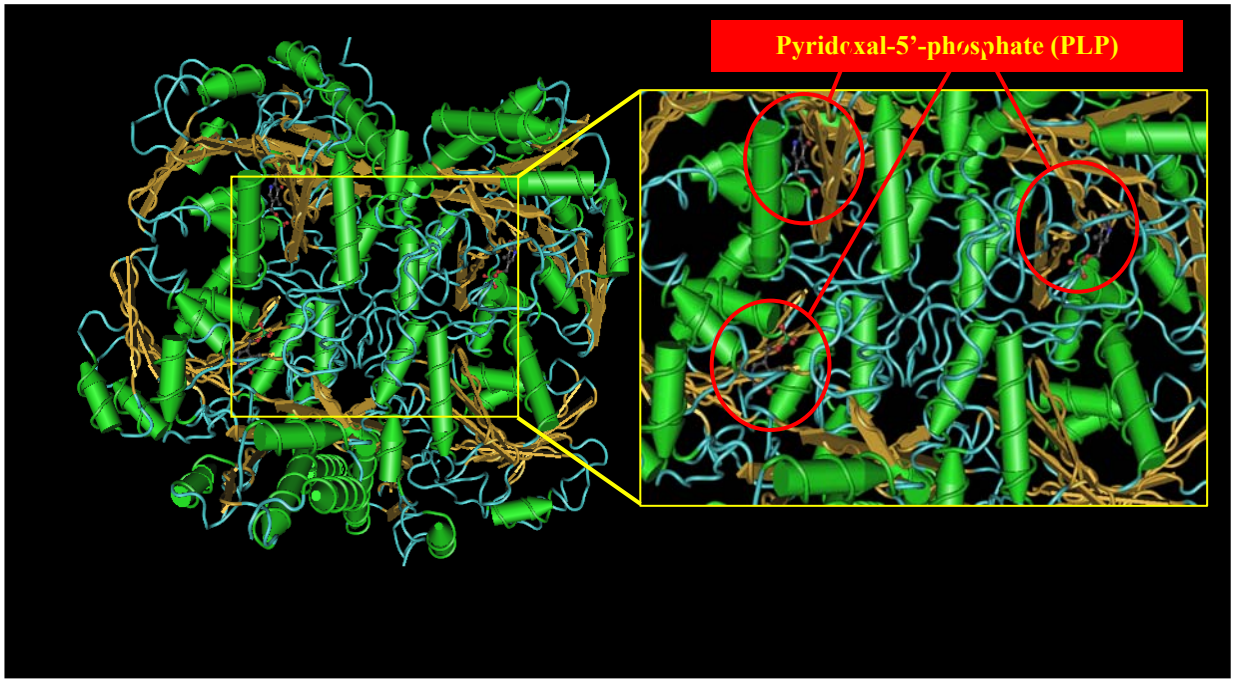
CSE, also known as cystathionase (CTH), is mainly able to convert cystathionine in cysteine; basically, it also catalyses elimination reactions of L-homoserine to form H_2O, NH_3 and 2-oxobutanoate, of L-cystine, producing thiocysteine, pyruvate and NH_3, and of L-cysteine producing pyruvate, NH_3 and H_2S (Braunstein AE et al., 1950; Braunstein AE et al., 1952; Flavin M et al., 1964). CSE is a protein of 405aa and it structurally is a tetramer formed by two homodimers both contributing to the active site pocket (fig 1.11a).

On the other hand, CBS is a cystathionine forming enzyme even if, in general, it catalyses β-replacement reactions between L-serine, L-cysteine, cysteine thioethers, or some other β-substituted α-L-amino acids, and a variety of mercaptans (Braunstein AE et al., 1971; Nakagawa H et al., 1968; Schlossmann K et al., 1962). In case that condensation reaction involves cysteine and homocysteine, the final product will be H_2S as well as cystathionine. The human CBS is a homotetramer consisting of 63 kDa subunits, which binds two cofactors, pyridoxal-5'-phosphate (PLP) and heme (Skovby et al., 1984; Kery et al., 1994). Each CBS subunit of 551 amino acid residues binds two substrates (homocysteine and serine) and is further regulated by S-adenosyl-L-methionine (AdoMet) (Kery et al., 1994). While the role of heme in CBS is unknown, catalysis by CBS can be explained solely by participation of PLP in the reaction mechanism (Kery et al., 1999) (fig 1.11b).

From a biological point of view, one thing to be considered is that CSE seems is described as the major H_2S-generating enzyme in cardiovascular system, while CBS is mainly involved in central nervous system (CNS) (Wang R, 2002). However, the presence of both enzymes could be required for producing H_2S in some tissues. Thus, it comes as no surprise that the expression of CBS and CSE is tissue specific. The expression of CBS (Hosoki R et al., 1997; Meier M et al., 2001) and CSE (Levonen AL et al., 2000; Lu Y et al., 1992; van der Molen EF et al., 1997) has been identified in many human and other mammalian cells; indeed, both were found in liver, kidney, brain, fibroblast and blood cells, and their activity is regulated by H_2S-dependent negative feedback mechanism since H_2S, the end product of cysteine metabolism, blocks CBS and CSE.

Furthermore, other regulatory mechanisms intervening in this pathway should be still clarified, although nitric oxide has already been shown upregulating CSE expression in smooth muscle cells in certain conditions (Sheng L et al., 2006; Zhang Y et al., 2005).

a)



b)

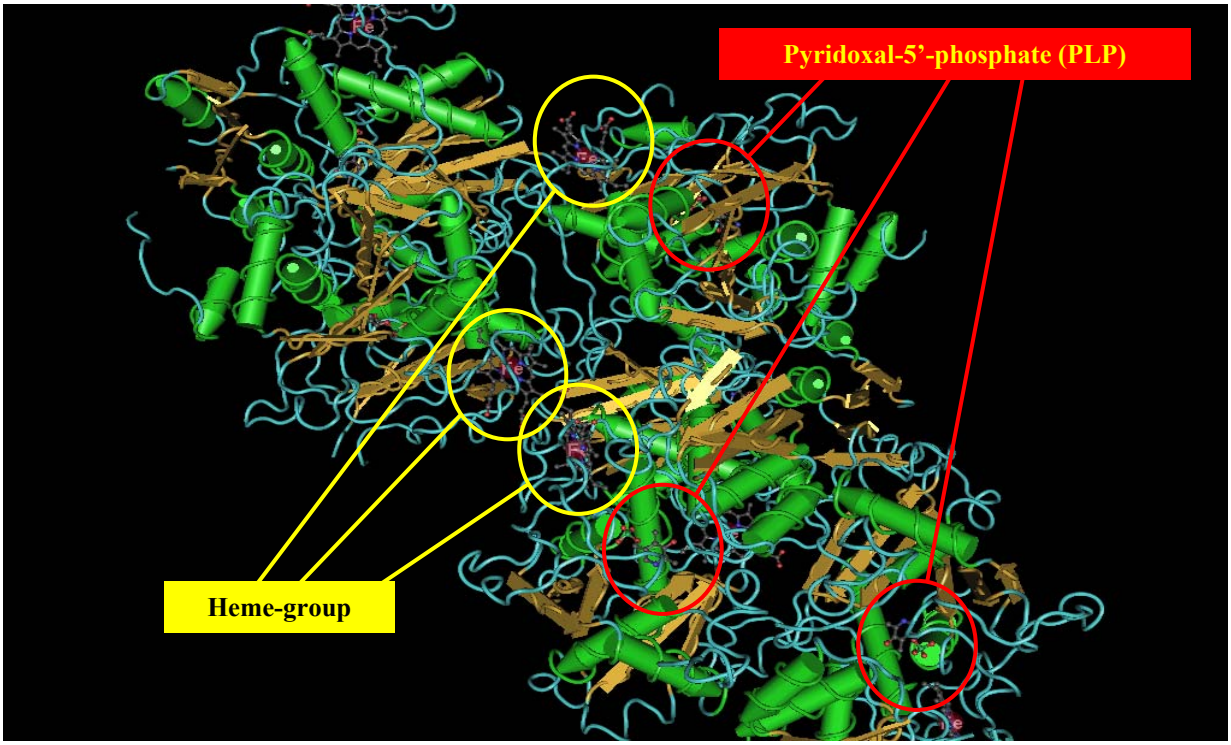


Fig 1.11 a) Cystathionine-γ-lyase (CSE) molecular structure. Red circles evidence pyridoxal-5'-phosphate (PLP) residues in quaternary folding of enzyme. b) Cystathionine-β-synthase (CBS) molecular structure. Red circles show pyridoxal-5'-phosphate (PLP) residues and yellow circles highlight the position of heme-groups.

1.2.3 Endogenous H_2S role in cardiovascular system

Hydrogen sulphide is a new gaseous mediator involved in both physiological and pathological processes in many mammalian tissues and the latest studies have shown a wide range of processes where it plays a regulatory role.

One of the first finding published in literature is the relaxant effect on vasculature exerted by H_2S, consisting in a decrease in tension *in vitro* and a transient fall in systolic blood pressure *in vivo* (Zhao W et al., 2001). This relaxant effect was reproducible in some vascular districts such as mesenteric artery, aorta and portal vein and importantly, the concentration of H_2S needed for obtaining the relaxing effect was quite lower in mesenteric artery rather than the latter two (EC_{50}: 25.2±3.6 μM for rat mesenteric artery; EC_{50}: 125.0±14 μM for rat aorta) (Cheng Y et al., 2004). Some earlier studies showed that neither endothelial denudation nor vascular denervation affected hydrogen sulphide-mediated vasorelaxation, suggesting a prominent role of smooth muscle cells in this process. However, subsequent and more detailed studies revealed that both NOS inhibitors and endothelial removal shifted the dose-response curve to the right side, i.e. higher EC_{50}, without affect the maximum response (Zhao W et al., 2002). In 1997 Hosoki R et al. showed a possible role of nitric oxide in H_2S induced vasodilation; in particular, they showed that relaxant effect of NO was increased in presence of hydrogen sulphide, although there was no apparent explication about mechanism through which H_2S exerted its effect. But in 2001, Zhao's research group described ATP-dependent potassium channel (K^+_{ATP}) as the target for molecular action of hydrogen sulphide. In fact, using glibenclamide, a specific K^+_{ATP} channels blocker, the vasorelaxant effect of H_2S was reverted, even if not completely abrogated. However, if this is the only molecular target for its action is to be further investigated.

As previously said, H_2S effects in vascular system are not only dealing with physiological environment, but there are some evidences suggesting a role for this endogenous mediator in many pathologies, other than in cardiovascular.

One of the pathological conditions involving H_2S levels impairment is represented by hypertension. This condition has a multifactorial aetiology since that the actors playing on the stage of vascular homeostasis regulation are several and one of the most important is for sure represented by NO. In fact, its reduced availability is mainly responsible of the increase in systolic blood pressure, even if sometimes this is only an effect and not properly the cause generating hypertensive conditions (Busse R et al., 1999).

In the same way, H_2S levels are reduced in plasma of two animal models of hypertension, such as treatment with L-$^{\omega}$Nitro-Arginine Methylester (L-NAME), a well known NOS inhibitor, and S*pontaneous Hypertensive Rats* (SHR). In particular, reduction in H_2S amount has been related to the downregulation of CSE mRNA expression and of its enzymatic activity (Zhong G et al., 2003; Yan H et al., 2004). Moreover, daily administration of CSE inhibitor D,L-propargylglycine (PAG) for a sustained time, i.e. 2-3 weeks, induces an elevation in systolic blood pressure in rats (Zhao W et al., 2003).

All these are good evidences supporting a clear involvement of hydrogen sulphide in regulation of vascular tone, even if the poor amount of clinical studies on the H_2S role in cardiovascular pathologies do not allow convincing conclusions.

1.2.4 Involvement of H_2S in inflammatory processes

Inflammation is a simple word hiding a very complex meaning. It is characterized by a massive amount of reactions and processes having the role of preserving the whole body functions. In general, inflammation occurs when a tissue damage is originated by several types of injuries, such as pathogen, irritant or mechanical stimuli. Moreover, it represents the starting point of healing process.

The first system triggering inflammatory reaction is the endothelium, which changes its features and starts to counteract the injury. The primary step is represented by release of preformed mediators, such as bradikinin (BK), histamine (H) and serotonin (5-HT) and leads to swelling, redness, pain, heat, and loss of function, all typical for inflammation. Blood vessels also permit the extravasation of leukocytes through the endothelium and basement membrane constituting the blood vessel. Once in the tissue, the cells migrate along a chemotactic gradient to reach the site of injury, where they can attempt to remove the stimulus and repair the tissue. The first cells to reach inflammation site are neutrophils, followed by macrophages. In addition, i-NOS-derived NO and COX-2-derived prostaglandins play a major role in maintaining of inflammatory state.

Recently, many studies were published on the involvement of hydrogen sulphide in inflammatory events, although there is not a clear evidence for pro- or anti-inflammatory role of H_2S. Hui et al., have demonstrated that H_2S levels were increased during septic shock in rats (Hui Y et al., 2003) and the same evidence has been shown in haemorrhagic shock conditions (Mok YY et al., 2004). Furthermore, H_2S inhibited chemotaxis and degranulation of polymorphonuclear leukocytes (PMN) and their adhesion to endothelium (Mariggiò MA et al., 1997; Zanardo RC et al., 2006). Several

subsequent studies confirmed that plasma H_2S concentration as well as CSE expression and activity were increased in LPS-treated mice (Li L et al., 2005). On the other hand, at least one study showed that inhibitor of H_2S synthesis propargylglycine (PAG) was able to reduce carragenaan-induced edema in mice paws (Bhatia M et al., 2005). This emerging discrepancy has not been clarified as yet and may be it is due to a different behaviour of H_2S in various tissues and at different concentrations.

1.2.5 How does H_2S behave in other tissues ?

As previously said, hydrogen sulphide is not only involved in vascular system and inflammation, but there are some scientific evidences showing important role for it in many other tissues.

The presence of H_2S and enzymes responsible for its synthesis have been largely detected in the nervous system, where the contribution of CBS seems to be predominant (Eto K et al., 2002). In particular, H_2S enhances NMDA-induced currents and increases the sensitivity of NMDA receptors to glutamate in cAMP-dependent manner (Kimura H, 2002). In addition, H_2S donors are able to decrease potassium-stimulated release of corticotropin release hormone (CRH) in rat hypothalamus (Dello Russo C et al., 2000).

Moreover, another source of endogenous H_2S is represented by the gastrointestinal system, where both CBS and CSE are markedly expressed. In this particular environment, H_2S seems to be a protective factor against gastric mucosa injury. In particular it has been shown that NaHS, a H_2S donor, prevents the reduction of mucosal blood low originated by NSAIDs (Fiorucci S et al., 2005 a). But H_2S effect

is also visible in isolated tissues, such as ileum, where it reduces both spontaneous and Ach-induced contractility (Teague B et al., 2002; Hosoki R et al., 1997).

Other effects for "the new gasotransmitter" have been identified in liver, where it contributes to relaxation of hepatic microvessels. It has been shown that in cirrhotic animals there is an increase in vascular resistance, likely related to a reduced expression in CSE and endogenous H_2S production (Fiorucci S et al., 2005 b).

These scientific evidences show that H_2S signalling is widely involved in many cellular pathways and more likely, many other implications are so far to be discovered.

1.3 SCIENTIFIC BASIS AND AIM OF THE STUDY

The role of hydrogen sulphide is still poorly understood although a large amount of studies has been already published. Furthermore the majority of experiments performed showed that endothelium seems do not have a key role in H_2S induced effect, since experiments performed in endothelium-denuded tissues do not highlight any significant difference in H_2S-induced vasorelaxation. It has to be underlined that mechanical endothelium removal is not necessarily reflecting the pathological condition known as endothelial dysfunction. Indeed, the alteration of endothelium functionality is a gradual and progressive process and cannot be directly related only to a endothelium disruption. For this reason, in the first part of this study we focussed on the role of endothelium in vasorelaxant effect generated by H_2S, by using *Non Obese Diabetic* (NOD) mice. This strain spontaneously and gradually develops type I diabetes associated to a progressive and gradual endothelial dysfunction (Bucci M et al, 2004).

Since the role of endothelium was demonstrated to be critical by our findings, in the second part of this study we focussed our attention on the ability of H_2S in modulating inflammatory events. In order to assess how this gasotransmitter could interfere with pathophysiological response to injuries, we used different approaches; first, we performed experiment of cell migration in animal model such as carragenaan-induced paw oedema and air-pouch model, in parallel with intravital microscopy (IVM) studies. Then, we used a cellular and molecular approach in order to identify a mediator for H_2S activity.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 H_2S IN VASCULAR TONE: FUNCTIONAL AND MOLECULAR STUDIES

2.1.1 Reagents

Acetylcholine (Ach), serotonin (5-HT), N^{ω}-nitro-arginine methyl ester (L-NAME), SQ-22,536 (SQ) and ODQ were purchased from Sigma Chemical Co. (Milano, Italy). Sodium hydrosulphide hydrate (NaHS) and L-Cysteine hydrochloride (L-Cys) were purchased from Aldrich (Milano, Italy). Kit for enzymatic measurement of glucose levels (Trinder reaction kit) was purchased from Bio-Gamma (Roma, Italy).

2.1.2 Non Obese Duiabetic(NOD) and Non Obese Resistant (NOR) mice

Non obese diabetic (NOD) mice is a strain with an elevated susceptibility in developing type 1 diabetes (IDDM) (Makino S et al., 1980). These mice show changes within evolution of pathology, in particular there is an early phase characterized by a localization of inflammatory cells, such as T-cells and activated macrophages, around pancreatic islet, inducing peri-insulite (4-10 weeks of age); consequently, these cells infiltrate islets and initiate a progressive destruction of pancreatic β-cells, resulting in a drastic reduction in insulin plasma level (12-30 weeks of age). The progression of diabetic pathology and its clinical evidences in these animals are similar to human ones and are associated to vascular disorders.

NOD mice were divided into three groups, as follows, by measurement of glycosuria and each group can be considered representative of different diabetic states.

Indeed, we can consider NOD-I group where diabetic state is not yet assessed; NOD-II group where we have the established pathology; NOD-III group where we find severe pathological conditions with all clinical manifestations of diabetes, followed by death for circulatory and renal complicances (Doi T et al., 1990). In aortic tissues of these groups, a gradual reduction of acetylcholine-induced relaxation was found and this correlated with a reduced availability of circulating nitric oxide (Bucci M et al., 2004).

NOR mice were used as control mice for NOD and both strains were purchased from Charles River Laboratories (Lecco, Italy) and then housed in an animal care facility.

2.1.3 Determination of glycosuria

Diabetic state was assessed by measurement of glycosuria. Animals were deprived of food for 2 hours to normalize glucose plasma levels and then put in metabolic cages for 4 hours, where urine samples were collected. Glycosuria levels were measured by glucose-6-oxidase colorimetric assay (Trinder reaction kit). According to glucose determination, mice were divided into three group, NOD-I (0 mg/dl<[glucose]<20 mg/dl), NOD-II (20 mg/dl<[glucose]<500 mg/dl), NOD-III ([glucose]>500 mg/dl), and sacrificed at different state of pathology, as described in following paragraph (fig. 2.1).

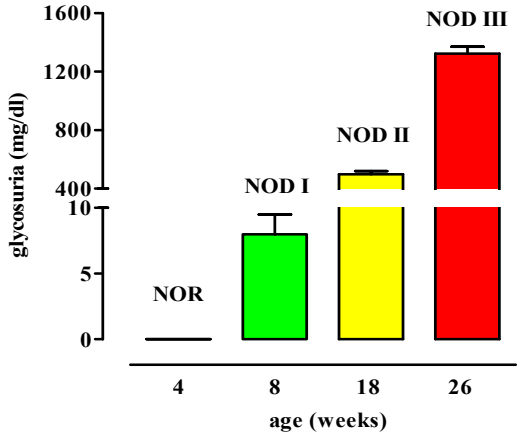


Fig 2.1 Determination of glycosuria in NOD mice. NOD mice show a progressive increase of urinary glucose levels that parallels the age.

2.1.4 Tissue preparation and experimental protocol

NOD mice diabetic state was monitored each week by measurement of glycosuria (gly). NOD animals were sacrificed at different state of pathology with age-matched NOR mice and aortas were explanted, cut in rings and used for functional study in isolated organ bath filled with oxygenated Krebs' Solution at 37°C, linked to isometric force transducers (Fort 10, World Precision Instrument, Ugo Basile, Italy). Krebs' composition was the following (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11. Rings were stretched until a resting tension of 1.5g was reached and allowed to equilibrate for at least 45 minutes during which tension was adjusted, when necessary, to 1.5g and bathing solution was periodically changed. In a preliminary study a resting tension of 1.5g was found to develop optimal tension to stimulation with contracting agents.

In each set of experiment rings were firstly challenged with serotonin (5-HT) (1μM) until the responses were reproducible. In order to verify endothelium integrity, acetylcholine cumulative concentration-response curve (10nM-30μM) was performed on 5-HT-precontracted rings. In this study we used both NaHS and L-Cys as source of H_2S, a direct H_2S donor and the physiological metabolic precursor respectively. In particular, H_2S release from L-Cys is correlated to enzymatic action of both CBS and CSE in examined tissues. Indeed, NaHS and L-Cys cumulative concentration-response curves were performed on 5-HT-precontracted rings both in presence and following removal of vascular endothelium.

A preliminary study on the optimal incubation time of the drug treatments and concentration was carried out (data not shown). L-NAME (100μM), SQ (100μM) and ODQ (5μM) were added in the organ baths on 5-HT-precontracted rings. After 15

minutes of incubation cumulative concentration-response curves to NaHS and L-Cys were performed.

2.1.5 Cell experiments with normal and high glucose environment

Bovine aortic endothelial cells (BAEC) were obtained by Istituto Nazionale per lo Studio e la Cura dei Tumori (Milano, Italy). The cells were cultured in 60mm Petri plastic dishes (FALCON, Microtech, Italy) and grown in medium (GIBCO, Invitrogen Corporation) supplemented with 2mmol/l glutamine (GIBCO), 10% heat inactivated fetal calf serum (GIBCO), 50U/ml penicillin, 50U/ml streptomycin. The Petri dishes were incubated at 37°C in a 5%CO2-95% air gas mixture. BAEC were subcultered on reaching confluence by use of 0.01% trypsin-EDTA. The cells were used between passage 5 and 6. Cells were grown until they reached 90% confluence and then were serum starved overnight. BAEC were pretreated for 3h with 11.5mM D-glucose solution (normal glucose) or 25mM D-glucose solution (high glucose) and H_2S production was examined.

2.1.6 Measurement of H_2S: plasma and tissues

Tissue H_2S production rate was measured according Stipanuk method with modifications (Stipanuk MH & Beck PW, 1982). Briefly, aortas were homogenised in a lysis buffer (potassium phosphate buffer 100mM pH=7.4, sodium orthovanadate 10mM and proteases inhibitors). Protein concentration was determined using Bradford assay

(Bio-Rad Laboratories, Milano, Italy). Homogenates were added to a reaction mixture (total volume 500µl) containing piridoxal-5'-phosphate (2mM, 20µl), L-Cysteine (10mM, 20µl) and saline (30µl). The reaction was performed in parafilmed eppendorf tubes and initiated by transferring tubes from ice to a water bath at 37°C. After incubation of 40 minutes, ZnAc (1%, 250µl) was added followed by TCA (10%, 250µl). Subsequently, DPD (20mM, 133µl) in 7.2M HCl and $FeCl_3$ (30mM, 133µl) in 1.2M HCl were added and absorbance of solution was measured after 20 minutes at a wavelength of 650nm. All samples were assayed in duplicate and H_2S concentration was calculated against a calibration curve of NaHS (3.12-250µM). To determine basal release of H_2S, L-Cys was not added to reaction mixture. Results were expressed as nmol per milligram of protein.

Plasma determination of H_2S was performed as following: 100µl of samples were added in eppendorf tubes containing ZnAc (1%, 150µl) and after TCA (10%, 300µl) was added. Subsequently, DPD (20mM, 100µl) in 7.2M HCl and $FeCl_3$ (30mM, 133µl) in 1.2M HCl were added to reaction mixture and determination follows the same protocol that is described above.

2.1.7 CSE and CBS expression: qRT-PCR

Quantization of the expression level of selected genes was performed by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) from mouse aortic rings. One µg RNA was incubated with DNaseI (Invitrogen, Carlsbad, CA) for 15 min at room temperature followed by 95°C for 5 min in the presence of 2.5 mM EDTA. The RNA

was reverse-transcribed with Superscript III (Invitrogen, Carlsbad, CA) with random-primers in volume of 20 µl. For real-time PCR, 10 ng template was used in a 25 µl reaction containing 0.3 µM each primer and 12.5 µl of 2X DyNAmo SYBR Green qPCR Kits (Finnzymes, Espoo, Finland).

All reactions were performed in triplicate using the following cycling conditions: 10 min at 95 °C, followed by 50 cycles of 95 °C for 10 sec, 60 °C for 20 sec and 72 °C for 20 sec, using an iCycler iQ instrument (Biorad, Hercules, CA). The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T: cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (GAPDH). Relative expression was calculated as the difference ($\Delta\Delta C_T$) between the ΔC_T values of the test and control samples for each target gene. The relative level of expression was measured as $2^{-\Delta\Delta C}{}_T$. All PCR primers were designed using the software PRIMER3-OUTPUT using published sequence data obtained from the NCBI database.

Mouse primers were as follows: CBS: (FWD) agaagtgccctggctgtaaa and (REV) caggactgtcgggatgaagt; CSE: (FWD) tgctgccaccattacgatta and (REV) gatgccaccctcctgaagta; GAPDH: (FWD) ctgagtatgtcgtggagtctac and (REV) gttggtggtgcaggatgcattg.

2.1.8 Western blot analysis

Aortic tissue or BAEC were homogenized in modified RIPA buffer (Tris-HCl 50mM, pH 7.4, Triton 1%, Na-deoxycolate 0.25%, NaCl 150mM, EDTA 1mM, PMSF 1mmol/L, aprotinin 10μg/ml, Leupeptin 20μmol/L, NaF 50mmol/L) using a Polytron homogenizer (2 cycles of 10 seconds at maximum speed). After centrifugation of homogenates at 10000 rpm for 10 minutes, 10, 30 μg of the denatured proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to a PVDF membrane. Membranes were blocked by incubation in PBS containing 0.1% v/v Tween 20 and 5% non-fat dry milk for 2h, followed by overnight incubation at 4°C with mouse polyclonal CBS (Abnova Novus Biologicals, Littleton, USA) antibody (1:500) or mouse monoclonal CSE (Abnova Novus Biologicals, Littleton, USA) antibody (1:500). The filters were washed extensively in PBS containing 0.1% v/v Tween 20, before incubation for 2h with anti-horseradish peroxidase-conjugate secondary antibody. Membranes were then washed and developed using Enhanced Chemiluminescence Substrate (ECL, Amersham Pharmacia Biotech, San Diego, CA, USA).

2.1.9 Statistical analysis

All data were expressed as mean ± s.e. mean. Statistical analysis was performed by using one-way ANOVA, two-way ANOVA and unpaired Student's t-test with Bonferroni's and Dunnet's post test where appropriate. Difference were considered statistically significant when *P* was less than 0.05.

2.2 H_2S AND INFLAMMATION: FUNCTIONAL STUDY

2.2.1 Reagents

Unless otherwise stated, all drugs were suspended in 1% carboxymethylcellulose. Aspirin, diclofenac sodium, N-formyl-Met-Leu-Phe (fMLP), glibenclamide, propragylglycine (PAG), β-cyanoalanine (BCA), λ-carrageenan, NaHS, Na_2S, pinacidil, N-acetylcysteine and Lawesson's reagent (2,4-bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane 2,4-disulfide) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2.2 Animals

Male Wistar rats weighing 175–200 g were obtained from Charles River Breeding Farms (Montreal, Canada, and Monza, Italy). For 18 h prior to an experiment, the rats were deprived of food, but not water. All experimental procedures described below were approved by the institutional animal care committees and were performed in accordance with the guidelines of the National Council on Animal Care.

2.2.3 Intravital microscopy

Examination of leukocyte-endothelial interactions *in vivo* was performed as described in detail (Wallace JL et al., 1993). Postcapillary mesenteric venules with a

length of at least 150 μm and diameters ranging from 25 to 40 μm were selected for the study. A video camera mounted on the microscope (Panasonic digital 5000) projected the image onto a monitor, and the images were

recorded for playback analysis using a videocassette recorder. Images of the mesenteric microcirculation were recorded over 5 min periods starting immediately before (baseline) and after aspirin administration or initiation of fMLP superfusion, and at 15 min intervals thereafter for 60 min. Aspirin was administered intragastrically at a dose of 50 mg/kg, whereas fMLP (10 μM) was dissolved in the buffer that superfused the mesenteric venules. In controls, vehicle (1% CMC) was given intragastrically instead of aspirin and vessels were superfused with buffer not containing fMLP.

Leukocyte adhesion was blindly quantified as the number of leukocytes that adhered to the vessel wall for at least 30 s per 100 μm venule length. Rolling leukocytes were defined as white blood cells moving at a velocity less than that of the erythrocytes in the same stream. The rolling leukocyte velocity was determined by the time required for a leukocyte to traverse a given distance along the length of a venule.

To assess the effects of H_2S on aspirin- and fMLP-induced leukocyte adherence, rats were pretreated intragastrically with Na_2S (1–100 μmol/kg), NaHS (100 μmol/kg), or Lawesson's reagent (0.1 to 3 μmol/kg) 30 min before aspirin or fMLP administration. Control rats received vehicle at the same time. In another group of experiments, glibenclamide was given 1 h prior to Na_2S or vehicle. In other experiments, rats were given a reversible inhibitor of CSE (β-cyanoalanine, 50 mg/kg i.p.) 1 h prior to aspirin. This dose of β -cyanoalanine (BCA) has been shown to significantly inhibit CSE activity in the rat (Mok YY et al., 2004).

2.2.4 Paw edema

Carrageenan (100 µl of a 1% w/v solution, prepared in sterile saline) was injected into a hind footpad of rats under halothane anesthesia. Paw volume was measured prior to any treatment, immediately before carrageenan administration, and at intervals of 1 h for 5 h thereafter using a Ugo Basile Model 7140 plethysmometer (Comerio, Italy). The person performing these measurements was unaware of the treatments the rats had received. Groups of at least 5 rats each were treated intraperitoneally 30 min before carrageenan administration with an H_2S donor (NaHS at 25–150 µmol/kg or Na_2S at 100 µmol/kg), an NSAID as positive control (diclofenac, 10 mg/kg), or a K_{ATP} channel agonist (pinacidil, 10 mg/kg). Other rats received BCA (50 mg/kg i.p.) 30 min before carrageenan administration. Additional experiments were performed in which groups of 5 rats each received glibenclamide (10 mg/kg) or vehicle (dimethyl sulfoxide) i.p. 30 min before administration of one of the H_2S donors.

2.2.5 Statistical analysis

All data were expressed as mean ± s.e. mean. Statistical analysis was performed by using one-way ANOVA, two-way ANOVA and unpaired Student's t-test with Bonferroni's and Dunnet's post test where appropriate. Difference were considered statistically significant when *P* was less than 0.05.

2.3 H_2S AND INFLAMMATION: MOLECULAR STUDY

2.3.1 Human neutrophils isolation and AnxA1 mobilization analysis by western blot

Human neutrophils were used in order to assess H_2S influence on cytosolic AnxA1 mobilization. Briefly, human blood was collected from healthy volunteers in presence of 3,6% of sodium citrate as anticoagulant and diluted (1:1) in RPMI-1640 (Sigma, Poole, UK). Then, it was centrifuged in Hystopaque gradient (Hystopaque 1119 Hystopaque 1077, Sigma, Poole, UK) at 1200 rpm for 30 minutes. Neutrophils fraction was then gently collected and erythrocytes removed by hypotonic lysis. Cells were washed and then resuspended in RPMI and plated for the experiment, performed through the treatment of neutrophils with NaHS (0.1 and 1 mM for 30 minutes, at 37°C). At the end of experiment, cells were spinned down and supernatants collected. Then, they were lysed in TRIS-HCl based lysis buffer added with proteases and phosphatases inhibitors, and then processed in order to separate cytosolic fraction from membrane fraction.

Western blot analysis was performed using 20 μg of the denatured proteins (cytosolic, membrane and supernatant fractions), separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to a PVDF membrane. Membranes were blocked by incubation in TBS containing 0.1% v/v Tween 20 (TBST) and 5% non-fat dry milk for 2h, followed by overnight incubation at 4°C with mouse monoclonal anti-AnxA1 antibody (1:1000). The filters were washed extensively in TBST, before incubation for 2h with horseradish peroxidase-conjugate anti-mouse antibody. Membranes were then washed and developed using Enhanced Chemiluminescence Substrate (ECL, Amersham Pharmacia Biotech, San Diego, CA, USA). AnxA1

antibody was raised in our lab through specific column purification of serum of mice treated with AnxA1.

2.3.2 Animals and BMD macrophages (BMDM)

Anx-A1$^{-/-}$ (KO) and wild-type littermate control (Anx-A1$^{+/+}$) (WT) mice were bred in-house (Hannon et al., 2003). All animals were fed on a standard chow pellet diet with free access to water and maintained on a 12-h light–dark cycle. Animal work was performed in accordance with U.K. Home Office regulations, Animals (Scientific Procedures) Act 1986. Mice 4–6 weeks old were killed, the femur was excised, and the epiphyses removed prior to flushing out the bone marrow. Cells were washed and re-suspended in RPMI 1640 supplemented with 25mM HEPES pH 7.4, 10% foetal calf serum (FCS) and 15% L929 cell-conditioned media and cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 7 days. On day 7, BMDM cultures were detached with PBS and plated in appropriate dishes for each assay. BMDM were then treated with LPS 10mg/ml, 6h and NaHS 100μM, 1h before LPS treatment.

2.3.3 qRT-PCR on AnxA1 KO mice

Real time PCR was performed on tissues harvested from Anx-A1$^{+/+}$ (WT) and Anx-A1$^{-/-}$ (KO). Quantization of the expression level of selected genes (CBS, CSE, COX-2, i-NOS) was performed by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) from cells and mouse tissues. Extracted RNA was incubated with DNA-free Kit

(Ambion Inc, Europe) for 30 min at 37°C. The RNA was reverse-transcribed with Superscript III (Invitrogen, Carlsbad, CA) with random-primers in volume of 20 µl. For real-time PCR, 40 ng of template were used in a 10 µl reaction containing 1µl of cDNA, 9µl of ABgene Mastermix and 1µl of specific QuantiTect Primer assay (Qiagen).

All reactions were performed in triplicate using the following cycling conditions: 10 min at 94 °C, followed by 40 cycles of 94 °C for 15 sec, 55 °C for 30 sec and 72 °C for 30 sec, using an ABI PRISM-7900HT (Applied Biosystem, CA, USA). The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T: cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (GAPDH). Relative expression was calculated as the difference ($\Delta\Delta C_T$) between the ΔC_T values of the test and control samples for each target gene. The relative level of expression was measured as $2^{-\Delta\Delta C_T}$.

2.3.3 Statistical analysis

All data were expressed as mean ± s.e. mean. Statistical analysis was performed by using one-way ANOVA, two-way ANOVA and unpaired Student's t-test with Bonferroni's and Dunnet's post test where appropriate. qRT-PCR were analysed by using REST© software analysis system (Pfaffl MW et al., 2002). Difference were considered statistically significant when *P* was less than 0.05.

CHAPTER 3

3. RESULTS

3.1 H_2S AND VASCULAR SYSTEM: SIGNALLING IN REGULATING VASCULAR TONE

3.1.1 Effect of NaHS and L-Cysteine on aortic rings harvested from NOR or NOD mice

NaHS (1μM-3mM) induced a concentration-dependent vasorelaxation of aortic rings harvested from NOR mice (fig 2A). NaHS-induced vasorelaxation was not affected by L-NAME pre-treatment (100μM) (fig. 2A). In NOD I and NOD II the relaxant response induced by NaHS on isolated aorta is not significantly different from NOR (fig. 2C), while in NOD III mice, where the pathology is well established and the endothelium is severely damaged, NaHS does induce a significant increase in vasorelaxation ($p<0.01$ vs NOR) (fig. 2C).

Addition of L-Cysteine (1μM-3mM) induced a relaxant effect on NOR mice aortic tissues (fig. 2B). L-NAME significantly inhibited L-Cysteine induced relaxation (fig. 2B). The disease progression leads to a significant decrease in L-cysteine-induced vasodilatation of aortic rings harvested from NOD II ($p<0.05$ vs NOR) or NOD III ($p<0.01$ vs NOR) (fig. 2D)..

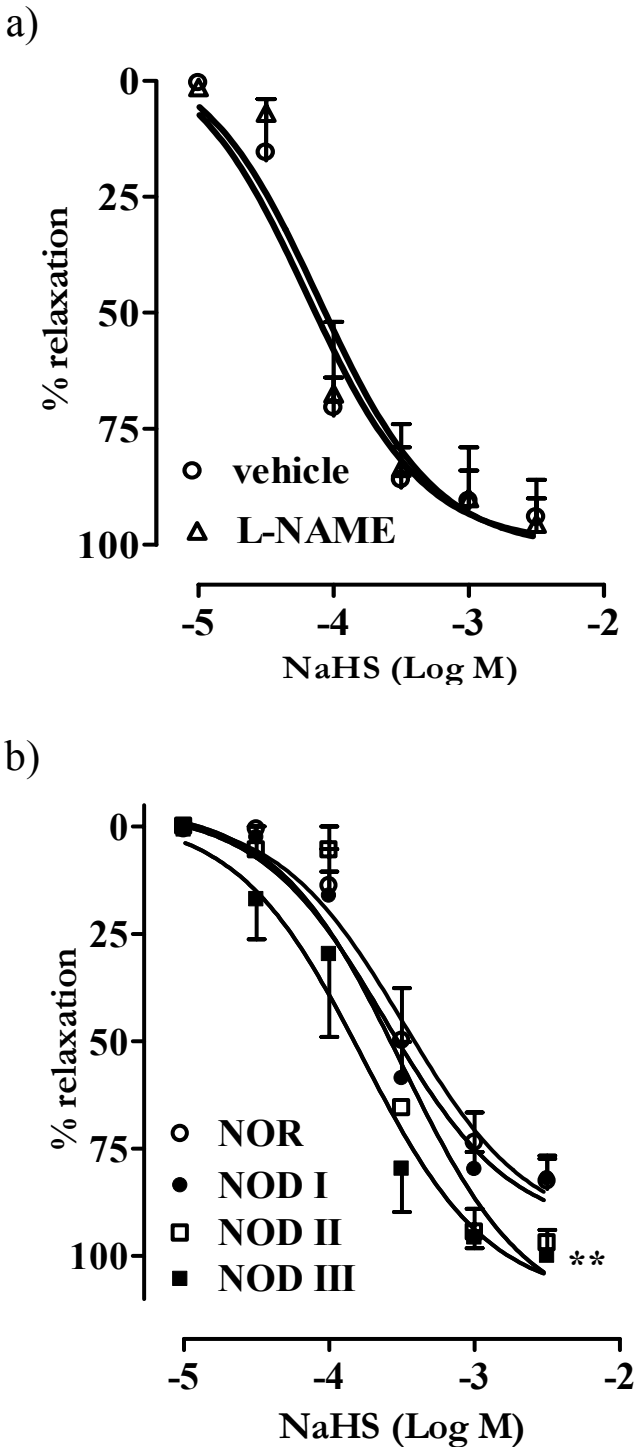


Fig 3.1 a) H_2S-induced vasorelaxation in aortic rings harvested from NOR or NOD mice. L-NAME pre-treatment does not affect NaHS-induced vasorelaxation in NOR mice. b) NaHS-induced vasorelaxation is enhanced in NOD-III mice ($p<0.01$ vs NOR)

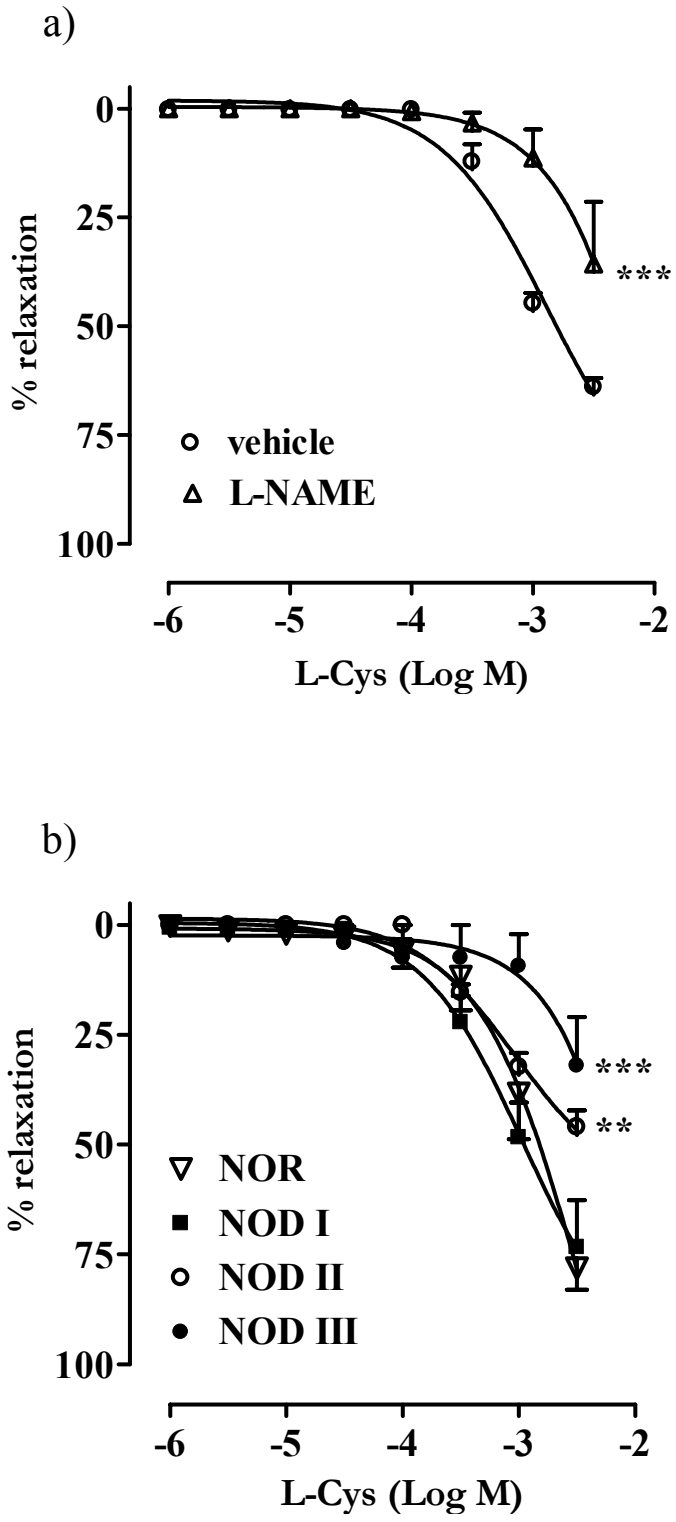


Fig 3.2 a) . L-NAME pre-treatment affected L-cysteine-induced vasorelaxation in NOR mice (*** $p<0.001$ vs vehicle). b) L-cysteine-induced vasodilatation was gradually reduced in NOD mice (D); (** $p<0.01$ vs NOR; *** $p<0.001$ vs NOR)

3.1.2 Contribution of cAMP and cGMP in H_2S-induced vasorelaxation

In order to evaluate a possible involvement of cAMP and cGMP we tested the effect of ODQ, a NO-sensitive guanylate cyclase inhibitor or SQ-22,536 an adenylate cyclase inhibitor, on H_2S-induced vasorelaxation. Incubation of NOR aortic rings with ODQ at the concentration of 5μM almost abrogated L-Cysteine-induced relaxation (fig. 3A). Conversely SQ (100μM) did not affect L-Cysteine-induced relaxation (fig. 3A).

A similar pattern of inhibition was obtained with NODII and NOD III mice aortas. In particular L-cysteine-induced vasorelaxation, already impaired in NOD III mice, was abrogated by pre-treatment with ODQ, but unaffected by SQ-22,536 (fig. 3B). Neither ODQ or SQ affected NaHS-induced vasorelaxation in both NOR or NOD mice (data not shown).

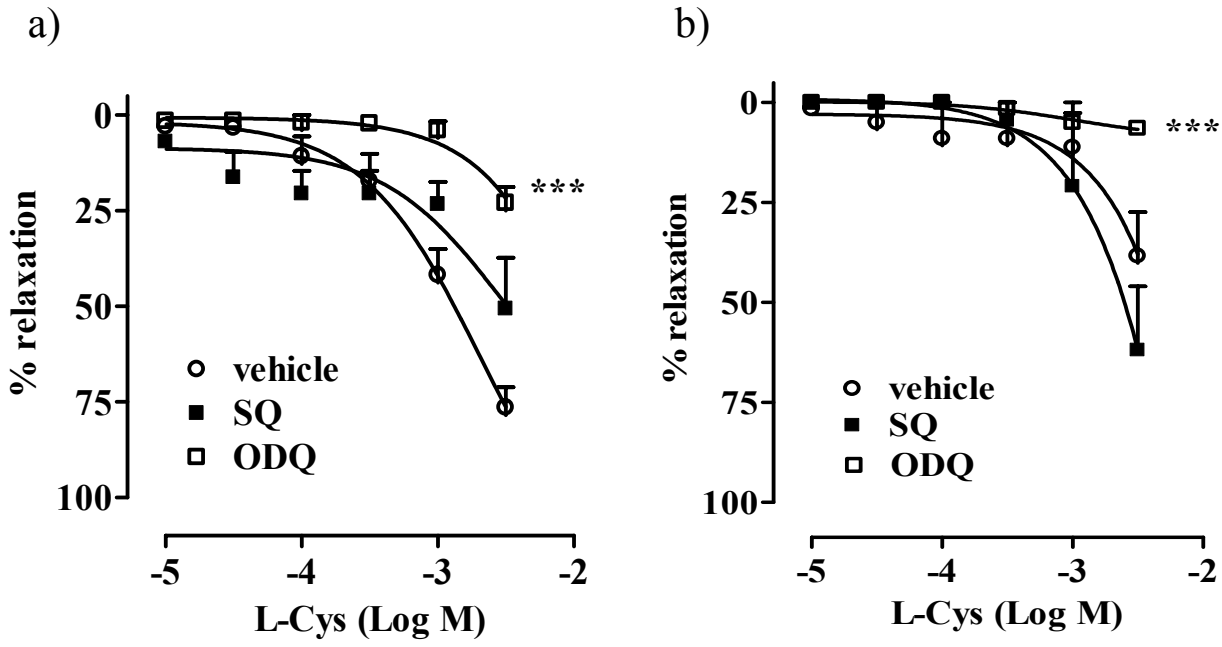


Fig 3.3 Effect of ODQ and SQ-22,536 on L-cysteine-induced vasorelaxation. ODQ (5μM, 15 minutes) significantly inhibited L-cysteine vasorelaxant response both in NOR (a) and NOD-III (b) mice (*** $p<0.001$ vs vehicle)

3.2 H_2S AND VASCULAR SYSTEM: RELEASE AND MOLECULAR CHANGES IN PRODUCTION

3.2.1 Impairment of CBS/CSE activity in NOD mice

Plasmatic concentration of H_2S was between 50-65μM in NOR mice. In NOD III mice there was a 50% reduction in H_2S levels (fig. 4A; ** $p<0.01$ vs NOR). To assess if the systemic H_2S impaired production was reflected by an analogue dysfunction at vascular level we evaluated the basal and the L-cysteine stimulated release of H_2S in aortic rings harvested by NOR and NOD mice. The basal production of H_2S in NOD mice aortas was not significantly reduced if compared to NOR mice aortas (figure 4B). Conversely, the conversion of exogenous L-cysteine to H_2S was significantly impaired in aorta harvested from NOD II and NOD III mice (* $p<0.05$; *** $p<0.001$; stimulated vs basal; # $p<0.05$; ## $p<0.01$ vs NOR). Although H_2S levels resulted progressively lowered during diabetes progression, both western blot (fig. 5A) and qRT-PCR (fig. 5B) showed a significant upregulation of both CBS and CSE in vessels harvested by NOD II and NOD III mice.

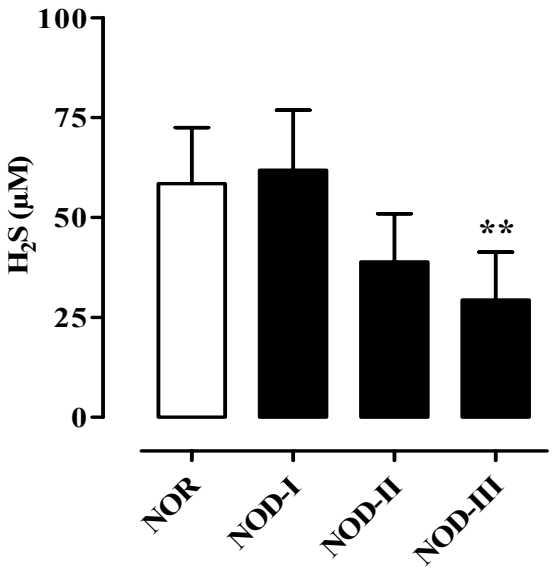


Fig 3.4 H_2S plasmatic levels were significantly reduced in NOD-III mice (** $p<0.01$ vs NOR; n=5)

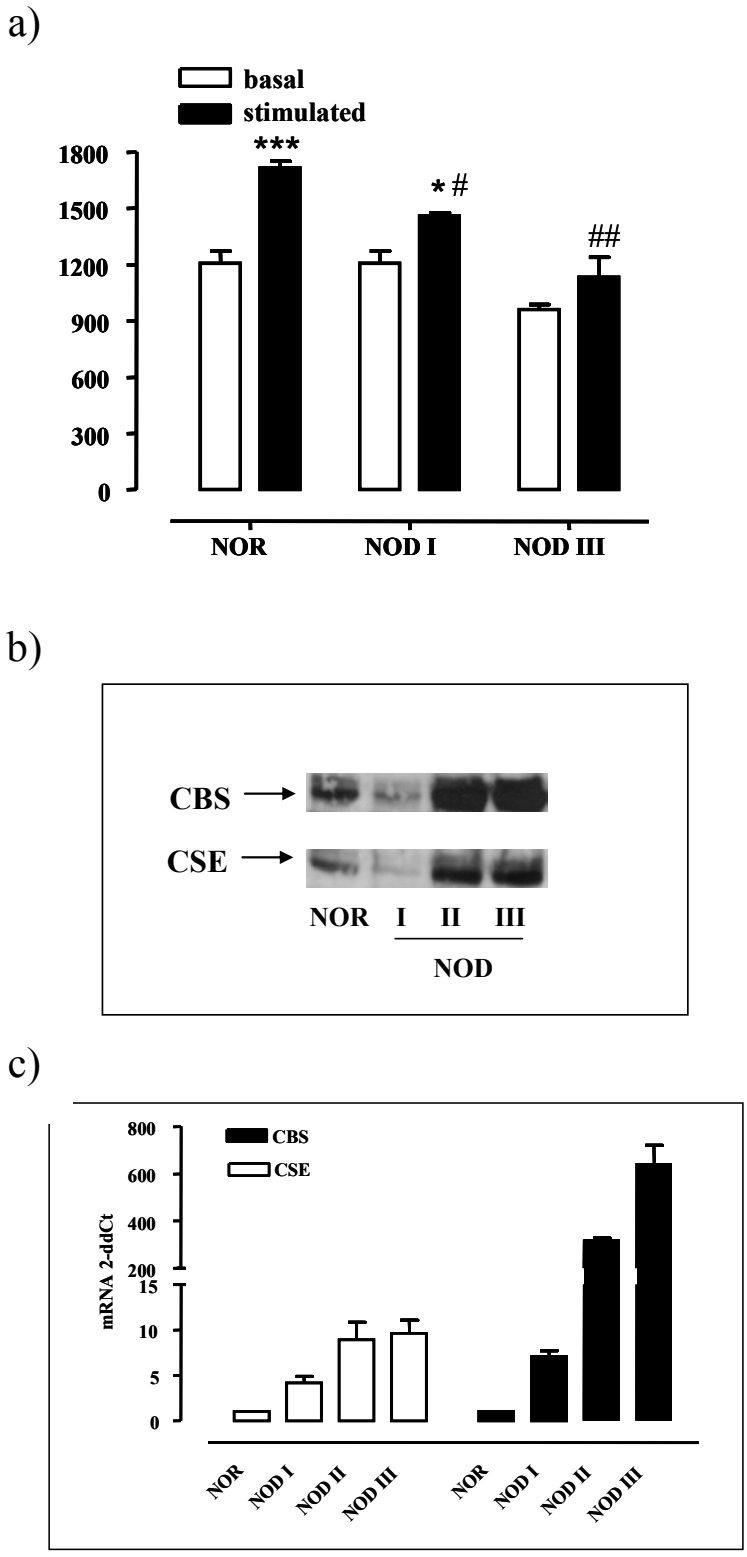


Fig 3.5 a) Tissue H_2S levels (basal) were not different in NOR and NOD mice, while H_2S production following L-cysteine challenge (stimulated) was significantly reduced in aorta harvested from NOD I or NOD III mice (# $p<0.05$ vs NOR; ## $p<0.01$ vs NOR; *** $p<0.001$ vs basal; * $p<0.05$ vs basal). Western blot (b) and qRT-PCR (c) analysis on aortic tissues harvested from NOR and NOR mice. Both western blot (A) and qRT-PCR (B) showed a progressive increased expression of CBS and CSE.

3.2.2 High glucose environment affects stimulated H_2S production in BAEC

Western blot analysis showed that BAEC express CBS but not CSE. To assess the specific role of endothelial cells we exposed BAEC to normal or high glucose environment using a well established protocol (26). In normal glucose medium we detected value of H_2S of ~70nmol/mg protein min^{-1}, whereas on stimulation with L-cysteine we measured values of H_2S of ~170nmol/mg protein min^{-1} with a 60% of increase in the response. When cells are cultured in high glucose medium, the unstimulated value was not affected, whereas L-cysteine-induced release was significantly reduced (fig. 6; * $p<0.05$ stimulated vs basal).

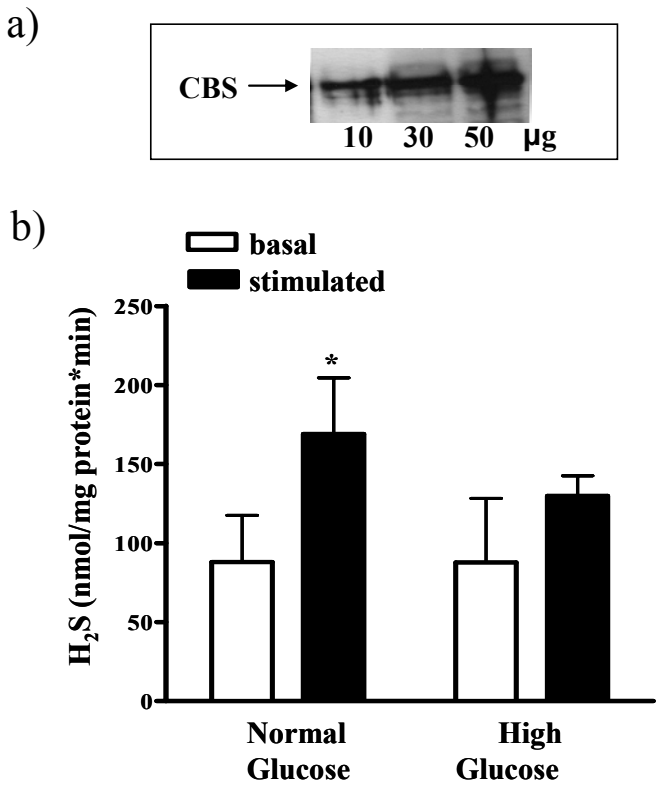


Fig 3.6 Evaluation of H_2S production in BAEC**.** a) Western blot analysis showed a specific expression of CBS in BAEC. b) When BAEC were cultured in high glucose medium, basal H_2S production value was not affected, whereas L-cysteine-stimulated release of H_2S was significantly reduced (* $p<0.05$ vs basal)

3.3 H_2S AND INFLAMMATION:

3.3.1 H_2S donors decrease ASA-induced leukocyte adhesion through the activation of K_{ATP} channels

Oral administration of aspirin (50 mg/kg) induced a significant time-dependent increase in leukocyte adherence compared with rats that received vehicle (fig. 3.7). Pretreatment of rats with Na_2S dose-dependently decreased aspirin-induced leukocyte adherence to the mesenteric microcirculation (ED_{50}: 5.0 μmol/kg). The reduction of leukocyte adherence by Na_2S likely occurred through activation of K^+_{ATP} channels, since pretreatment with an antagonist of those channels, glibenclamide, reversed the effects of the H_2S donor. Glibenclamide given to rats prior to vehicle or ASA did not alter basal leukocyte adherence or that induced by aspirin (data not shown). NaHS (100 μmol/kg) also inhibited aspirin-induced leukocyte adherence.

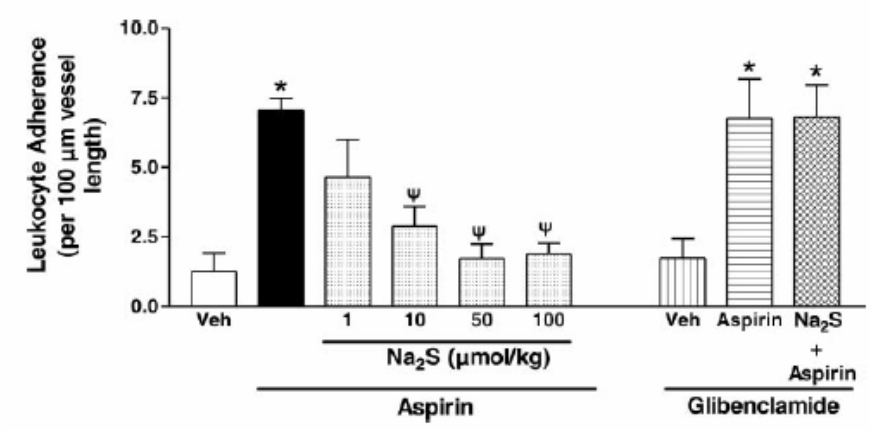


Fig 3.7 Hydrogen sulfide inhibits aspirin induced leukocyte adherence in mesenteric venules through activation of K^+_{ATP} channels. Na_2S dose-dependently suppressed leukocyte adherence induced by intragastric aspirin (50mg/kg). The inhibition of aspirin-induced adherence by Na_2S (100 μmol/kg) was abolished by pretreatment with glibenclamide (10 mg/kg), a K^+_{ATP} channel antagonist. **P*<0.05 vs. the corresponding vehicle-treated group. $^{\Psi}P<$ 0.05 vs. the corresponding group receiving aspirin alone. Data are expressed as mean ± SEM, n=5.

3.3.2.H_2S donors inhibit fMLP-induced leukocyte adherence

Pretreatment with Na_2S or NaHS (each at 100 μmol/kg) abolished fMLP-induced leukocyte adherence to the mesenteric microcirculation (fig. 3.8). Lawesson's reagent also inhibited leukocyte adhesion when administered at a dose of 1 μmol/kg. At a dose of 0.3 μmol/kg, Lawesson's reagent did not affect fMLP-induced leukocyte adherence (data not shown).

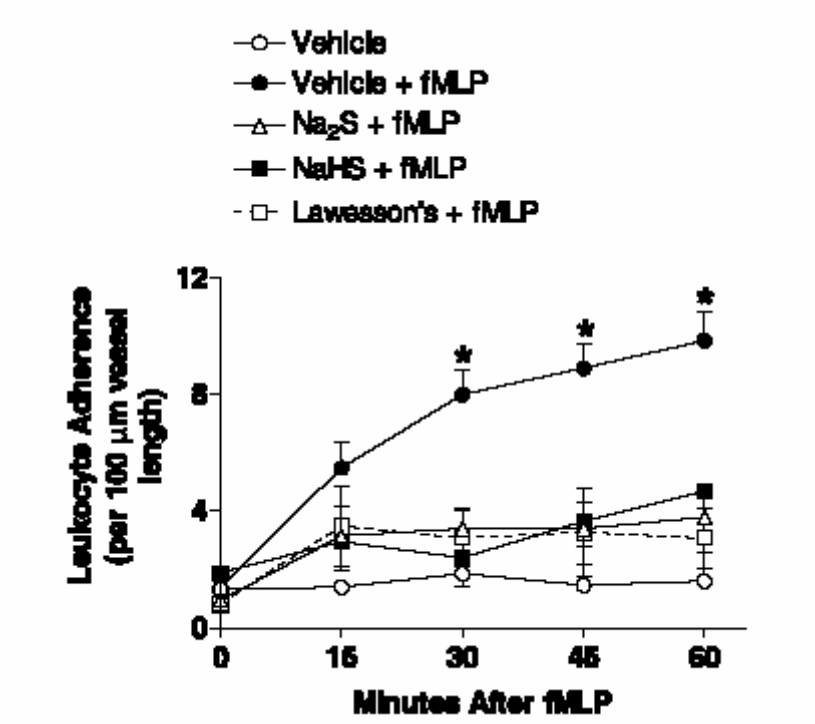


Fig 3.8 Hydrogen sulfide inhibits fMLP-induced leukocyte adherence in mesenteric venules. Superfusion of the vessels with N-formylated-Met-Leu-Phe (fMLP; 10μM) induced a time-dependent increase in leukocyte adherence. Hydrogen sulfide donors, given 30 min before fMLP, suppressed the increase in leukocyte adherence to levels not significantly different from control levels of adherence (*P<0.05 vs. the group receiving vehicle in place of fMLP). Na_2S and NaHS were given at 100 μmol/kg, and Lawesson's reagent was given at 1 μmol/kg. The results are plotted as mean ± SEM, n=5.

3.3.3 H_2S modulates oedema formation via effects on K_{ATP} channels

Injection of carrageenan into the hind footpads of rats resulted in a rapid and marked increase in paw volume as a consequence of edema formation (fig. 3.9a). The increase in paw volume could be significantly reduced by pretreatment with diclofenac (an NSAID). Pretreatment with NaHS or Na_2S similarly decreased carrageenan-induced paw edema (ED_{50}: 35 and 28 μmol/kg, respectively), as did pinacidil, a K^+_{ATP} channel agonist. In contrast, suppression of endogenous H_2S synthesis, through administration of BCA, resulted in a significantly greater paw swelling response to carrageenan. The reduction paw edema by either of the H_2S donors (NaHS or Na_2S) could be reversed by pretreatment with glibenclamide (fig. 3.9b).

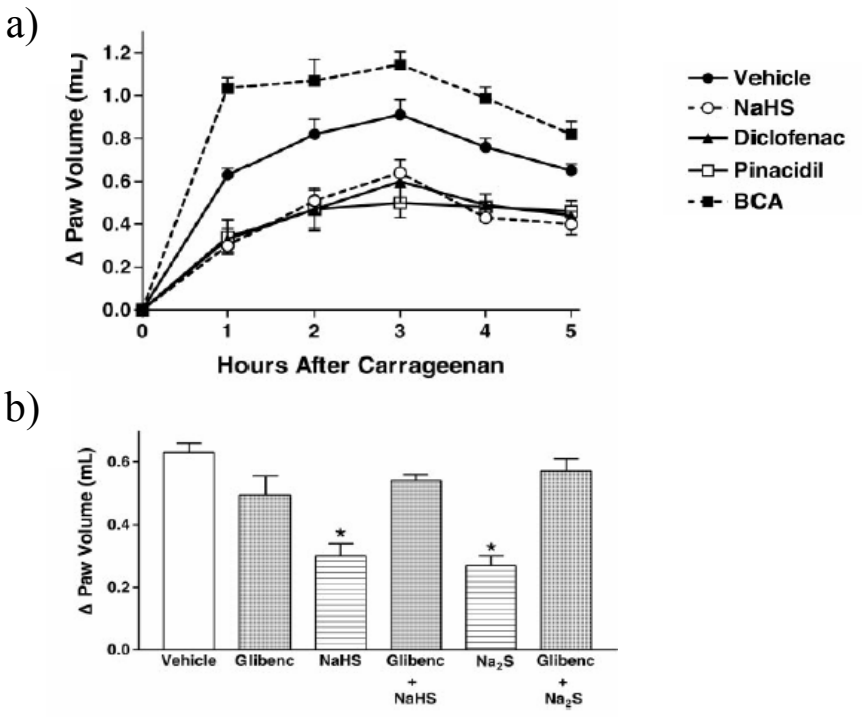


Fig 3.9 a) Injection of carrageenan into the rat hindpaw resulted in significant oedema formation as indicated by the increase in paw volume. Pre-treatment with NaHS significantly reduced paw oedema (P<0.05), as pre-treatment with diclofenac and pinacidil did. In contrast, administration of BCA significantly increased the oedema formation induced by carrageenan (P<0.05). b) The increase in paw oedema occurring during the first hour after carrageenan administration is shown. The reduction of paw oedema by both NaHS and Na_2S was abolished by pre-treatment with glibenclamide, whereas glibenclamide alone did not alter carrageenan-induced oedema formation. Data are shown as mean ± SEM, n=5

3.3.4 H_2S induced externalization of NaxA1 onto membrane surface

Neutrophils were isolated and treated with H_2S donor NaHS at 0.1 and 1mM for 30 minutes at 37°C. Western blot analysis clearly showed that AnxA1, normally resident in cytosolic compartment (C), mobilized towards cell membrane surface (M) after treatment with NaHS. In addition, NaHS also induced release of AnxA1 in extracellular environment (S) (fig. 3.10).

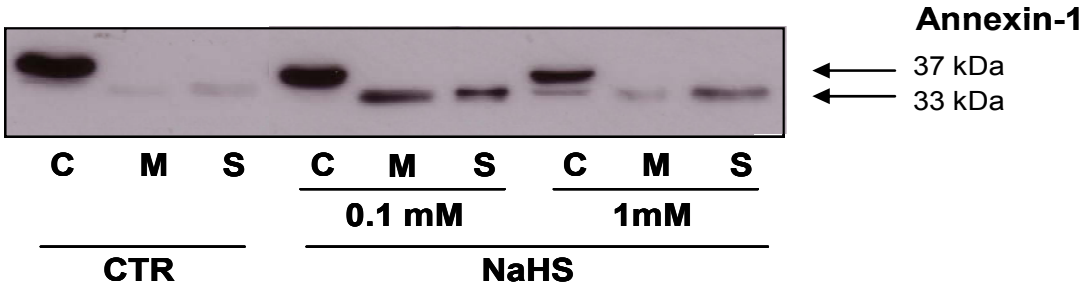


Fig 3.10 Western blot analysis on neutrophils isolated from peripheral human blood. Cells were treated and fractionated in cytosolic (C), membrane (M) and supernatant (S) fraction. AnxA1 is shown in two bands of 33 (cleaved) and 37 kDa (uncleaved) respectively. Blot is representative of three different experiments.

3.3.5 H_2S reverted CSE, COX-2 and i-NOS over-expression in LPS treated BMDM through AnxA1-dependent mechanism

qRT-PCR analysis on BMDM treated with LPS showed an increase in expression of CSE (1.3 fold increase, *p<0.05, n=3) and a very high expression of COX-2 and i-NOS (14 and 7 fold increase respectively, ***p<0,001, n=3). When BMDM were treated with NaHS, the observed effect was the reversion of LPS-induced increase in expression of CSE, COX-2 and i-NOS ($^{\#}$ p<0.05, n=3; $^{\#\#\#}$ p<0.001, n=3; $^{\#}$ p<0.05, n=3 respectively) (fig. 3.11). Conversely, the effect of NaHS was completely lost when the same experiment was carried out on BMDM from AnxA1$^{-/-}$ mice (fig. 3.12). These data may suggest that H_2S anti-inflammatory effect could be partially due to activation of AnxA1 signalling.

3.3.6 H_2S production in AnxA1$^{-/-}$ mice

We speculated on the role of CBS and CSE in AnxA1$^{-/-}$ mice, performing qRT-PCR analysis on several tissues. We found a marked increase in both CBS and CSE expression in the aorta of knock-out mice compared to wild-type (21 and 60 fold increase for CBS and CSE respectively). Similar results were observed in lymph nodes (LN), even if the increase in expression in AnxA1$^{-/-}$ mice was not comparable to the one found in the aorta (4 and 3 fold increase for CBS and CSE respectively). Liver, the most important producer of H_2S, did not show any significant change in the expression of both enzymes between wild-type and knock-out. Conversely, in spleen and kidney we found an upregulation only for CBS expression (10 fold increase) (fig. 3.13).

Furthermore, we dosed plasmatic levels of H_2S and we found that LPS induced an increase in H_2S amount in $AnxA^{+/+}$ mice compared to no-treated mice. In addition, is noteworthy that plasmatic levels of H_2S in no-treated $AnxA1^{-/-}$ mice were higher than ones detected in no-treated $AnxA1^{+/+}$ mice (fig. 3.13); these data fit with the higher expression of CBS and CSE in $AnxA1^{-/-}$ mice compared to $AnxA^{+/+}$ mice.

In addition to these data we have also performed H_2S production assay on tissues harvested from $AnxA1^{+/+}$ and $AnxA1^{-/-}$ mice. We found that basal production of H_2S in aorta was increased in LPS treated animals. On the other hand, basal H_2S produced in non-treated $AnxA1^{-/-}$ mice was lowerd compared to wild-type animals. There were not significant differences in production of H_2S in liver, fitting with data not showing any changes in CBS or CSE expression in liver. Conversely, in kidney we found a significant increase in basal production of H_2S in non-treated $AnxA1^{-/-}$ mice compared with $AnxA1^{+/+}$ mice (fig. 3.14).

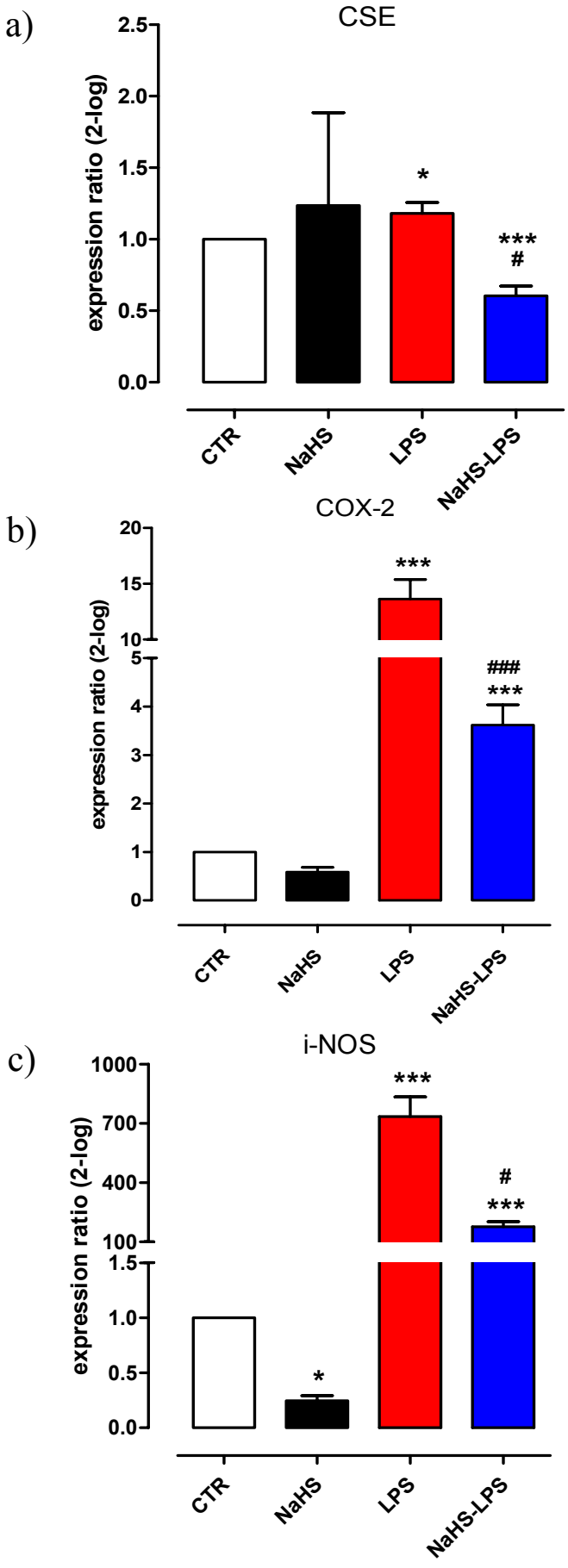


Fig 3.11 qRT-PCR on BMDM isolated from AnxA1$^{+/+}$ mice. NaHS pre-treatment reduces LPS-induced overexpression of CSE (a), COX-2 (b) and i-NOS (c). (* $p<0.05$ vs CTR; *** $p<0.001$ vs CTR; # $p<0.05$ vs LPS; ### $p<0.001$ vs LPS; n=3)

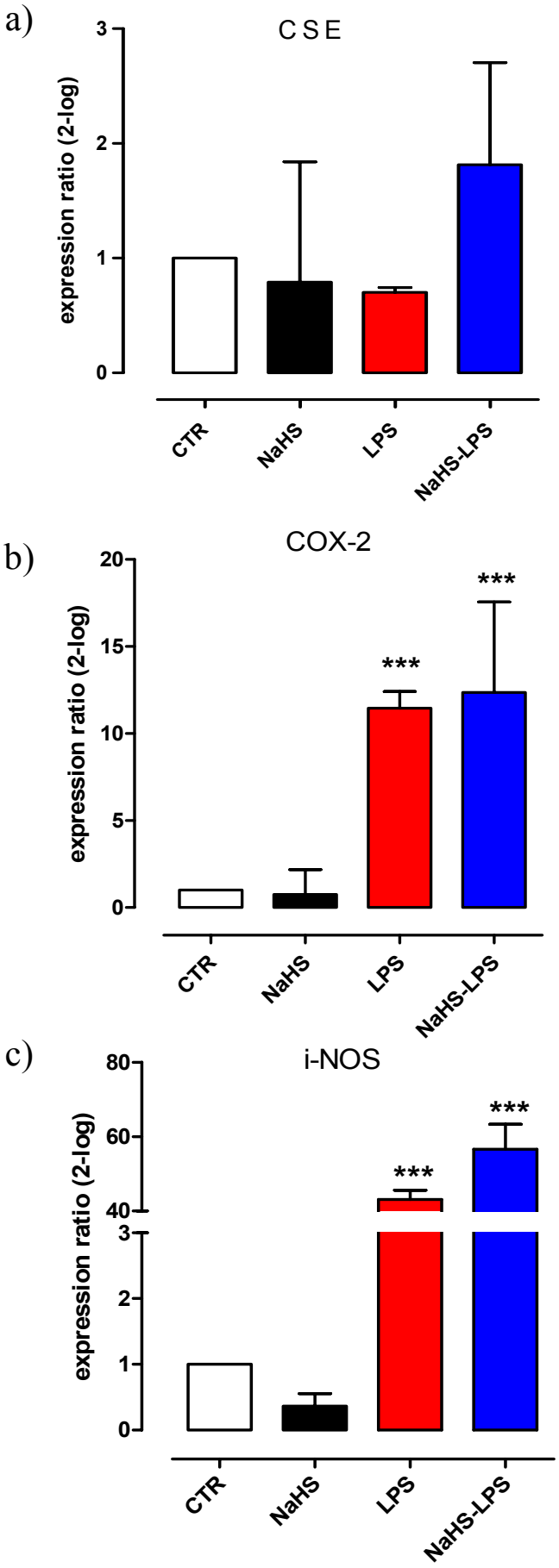


Fig 3.12 qRT-PCR on BMDM isolated from AnxA1$^{-/-}$ mice. After genetic deletion of AnxA1, the reducing effect of NaHS pre-treatment on expression of CSE (a), COX-2 (b) and i-NOS (c) after LPS treatment was lost (n=3).

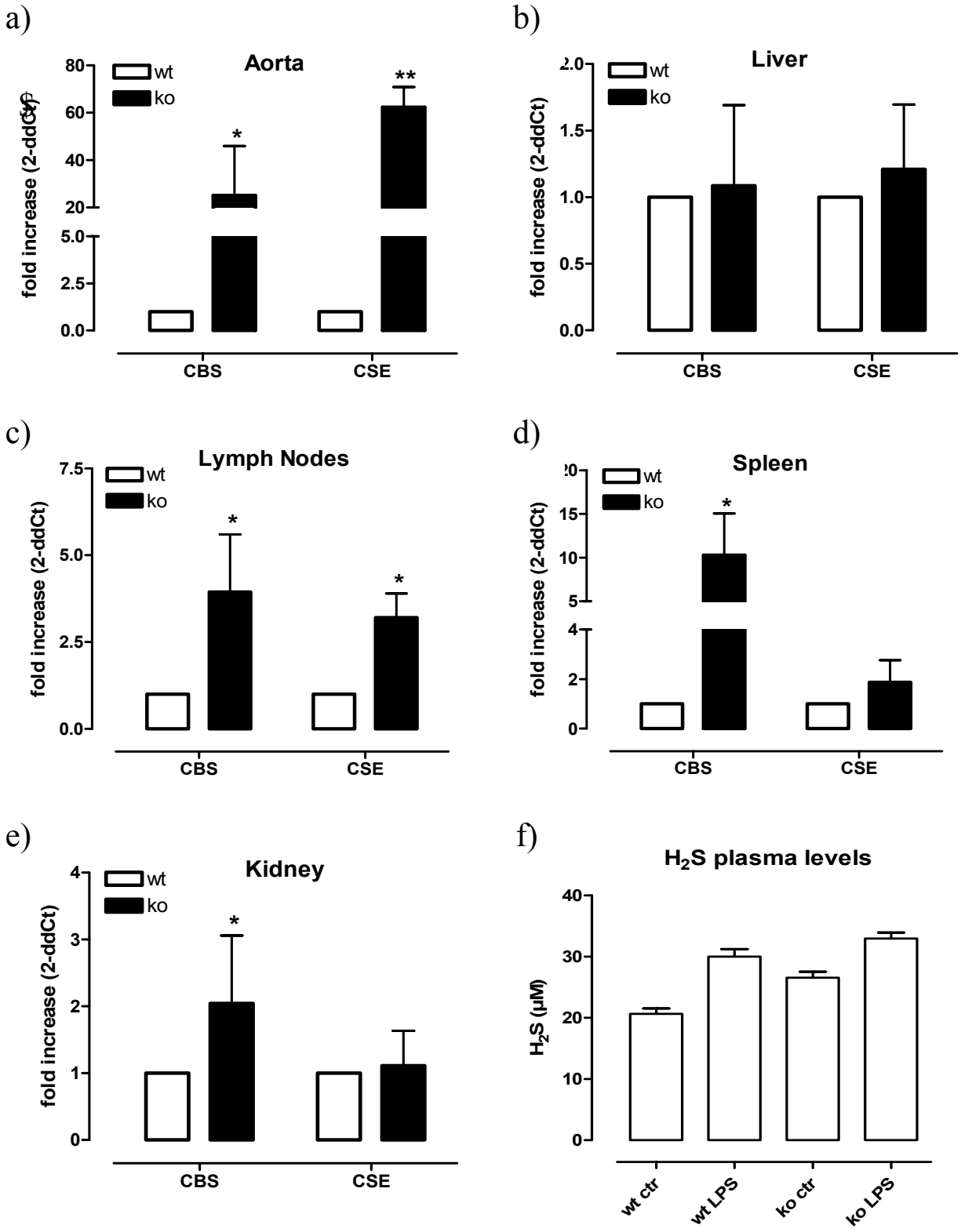


Fig 3.13 qRT-PCR analysis for CBS or CSE expression performed on aorta (a), liver (b), lymph nodes (c), spleen (d) and kidney (e) harvested from AnxA1$^{-/-}$ mice. Data are expressed as mean of fold increase ± SEM. e) Plasmatic H_2S levels in AnxA1$^{+/+}$ or AnxA1$^{-/-}$ mice under normal condition and after stimulation with LPS

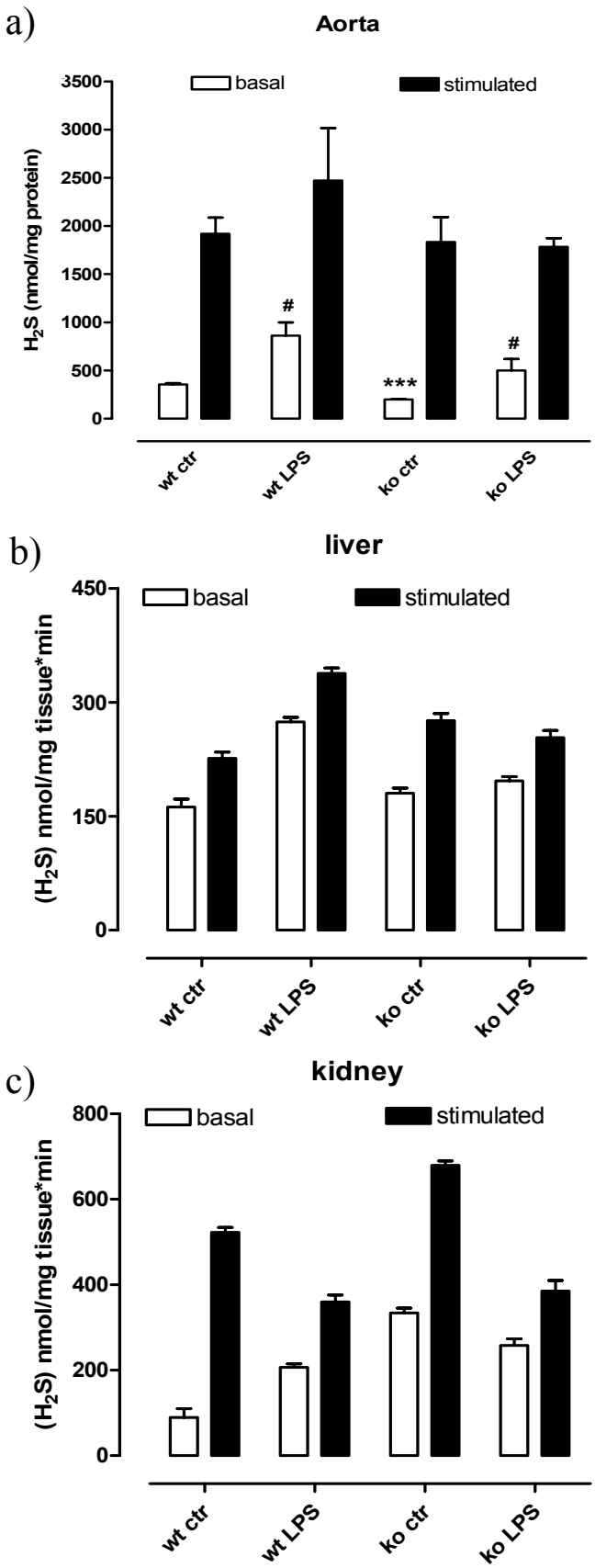


Fig 3.14 H_2S production in aorta (a), liver (b) and kidney (c) harvested from AnxA1$^{+/+}$ and AnxA1$^{-/-}$ mice under basal condition and after exogenous L-cysteine feeding (stimulated). Animals were treated with LPS (10mg/Kg) and with saline (n=3).

CHAPTER 4

4. DISCUSSION

The biological relevance of hydrogen sulphide has been recently addressed by different research group and in many papers it has been shown that H_2S participates to various pathophysiological conditions.

Vascular studies have shown that H_2S-induced vasorelaxation is not affected by endothelium removal, suggesting that endothelium is not involved in H_2S pathway at vascular level (Zhao W et al., 2001; Wang R, 2002; Hosoki R et al., 1997). Mechanical endothelium removal only partially mimics the pathological condition known as endothelial dysfunction. Indeed, the alteration of endothelium functionality, in the majority of the cases, is a gradual and progressive process and cannot be simply and directly related to a mechanical non specific endothelium disruption. In the first part of this work, we used NOD mice, a strain that spontaneously and gradually develops diabetes associated to a progressive endothelial dysfunction, in order to assess the involvement of H_2S pathway in an endothelial progressive disorder and a possible interplay with nitric oxide.

In physiological conditions, we have found by using NOR mice tissues that administration of NaHS or L-cysteine, on pre-contracted aortic rings, induced a concentration-dependent vasorelaxation. When we blocked the NO contribute by using L-NAME, we found a marked inhibition of L-cysteine-induced relaxation, but not of NaHS. A similar pattern was observed in NOD-I mice that have normal glycosuria/glycaemia. Conversely, as expected, when an H_2S donor is used, as exogenous source of the gas, there are no changes in the vasodilatory pattern. Thus, it appears that L-Arginine/NO pathway is involved in L-cysteine-induced vasorelaxation in physiological condition.

In pathological conditions L-cysteine-induced vasodilatation was impaired. Indeed, aortic vessels harvested from NOD II and NOD III mice showed a reduced vasodilation that paralleled the disease progression and endothelial dysfunction. As we have already demonstrated, NOD mice vessels display an impaired Ach-induced vasorelaxation strictly related to a specific and progressive disorder in eNOS post-translational activation (Bucci M et al., 2004). Thus, in pathological conditions, where there is a reduced production of NO we mimicked the effect of L-NAME on normal rings (physiological) further supporting the previous hypothesis that basal production of NO is involved in the machinery necessary for endogenous production of H_2S. This hypothesis is further corroborated by the finding that NaHS-induced vasorelaxation was significantly enhanced in NOD III mice but unaffected in NOD I or NOD II mice. The enhanced response of NaHS in NOD III mice, where a strong impairment of Ach-induced relaxation is present (Bucci M et al., 2004), could be explained by a reduced direct interaction (chemical quenching) between basal NO and exogenous H_2S .

Soluble guanylate cyclase is the primary target for NO, thus if the previous hypothesis is valid, modulation of the enzyme should in turn modulate L-cysteine induced response without affecting NaHS–induced vasodilatation. In order to assess this theory we compared the vasodilatory response elicited by either NaHS or L-cysteine following pharmacological modulation with either ODQ an inhibitor of soluble guanylate cyclase or with SQ-22,536 an inhibitor of adenylate cyclase in NOR versus NOD III aortic rings. As expected incubation with ODQ or SQ-22,536 did not affect NaHS-induced vasorelaxation in both NOR and NOD III mice. Conversely, ODQ but not SQ-22,536 caused a marked inhibition of L-cysteine-induced vasorelaxation in rings harvested from both NOR or NOD III mice. This finding together with the previous data

strongly implies that endogenous H_2S production through L-cysteine may be modulated by nitric oxide through guanylate cyclase.

Recently, Jiang HL et al. have demonstrated a similar correlation between NO and H_2S in patients with coronary heart disease. Indeed, in these patients H_2S levels decrease parallels the disease severity and is further impaired if patients were smokers and/or hypertensive patients as NO does (Jiang H et al., 2005). Interestingly similarly to NO, H_2S levels are inversely correlated to glycaemia as in our experimental model. When we measured plasma levels we found that H_2S concentration declined following the disease severity as well as the glycosuria. In particular H_2S levels were reduced of about 50% in NOD III mice. Evaluation of H_2S levels in aorta harvested from NOR, NOD I, NOD II or NOD III mice was similar. However, when aorta tissues were fed with exogenous L-cysteine, in order to assess the ability to convert the substrate, we found a progressive reduction of H_2S production that paralleled the disease progression. Overall these data suggest a role for the endothelium and in particular of endothelial nitric oxide in modulating endogenous production of H_2S.

To further confirm this hypothesis we tested the direct effect of L-cysteine on endothelial cells by using BAEC. Indeed, we have recently demonstrated that BAEC incubated in high glucose environment display an impaired NO production as well as an increased expression of caveolin-1, an endogenous inhibitory protein for eNOS activation. The same increased caveolin-1 expression coupled to impaired NO production was present in vessels harvested from NOD III mice (Bucci M et al., 2004). Administration of L-cysteine to BAEC in normal glucose environment induced an increase in H_2S production, confirming the ability of endothelial cells to convert L-cysteine to H_2S. When L-cysteine was fed to BAEC grown in high glucose environment

there was a significant decrease in H_2S production. This pattern paralleled the pattern found in aortic tissue harvested from NOD III mice.

In conclusion these results show that endothelial dysfunction significantly impairs endogenous H_2S signalling without affecting the ability of vascular tissue to react to exogenous H_2S. Furthermore, these data suggest that endothelium has an active role in modulating H_2S endogenous production and that the reduction in L-cysteine-induced relaxation may be originated by the lost ability by vascular tissue in converting L-cysteine to H_2S following eNOS impairment activity.

This part of this work has clearly demonstrated that endothelium is one of the machinery involved in H_2S signalling in vascular responses. Thus, we focussed our attention on the role of endothelium in H_2S-induced response in inflammatory processes, since that endothelial cells have a critical role in both triggering and resolving phases of inflammation.

Studies over the past 5 years have provided convincing evidence that H_2S is an important modulator of vascular tone and acts as a neuromodulator as well (Wang R, 2002; Abe K et al., 1996). The results coming from the second part of the present study suggest that H_2S also plays important roles in the context of inflammation. H_2S is generated at sites of inflammation and can influence the ability of neutrophils to cause tissue injury (Whiteman et al., 2004); it was recently shown to reduce visceral pain perception (Distrutti E et al., 2005). In the present study, we have demonstrated that several H_2S donors can suppress leukocyte adherence to the vascular endothelium and can reduce leukocyte infiltration and oedema formation. These effects of H_2S were seen irrespective of the inflammatory stimulus used (carrageenan, aspirin, fMLP). Suppression of endogenous H_2S synthesis, through blockade of CSE, resulted in enhanced leukocyte adhesion, leukocyte infiltration, and oedema formation. These

actions appeared to be mediated via K^+_{ATP} channels, as they were reversed by pre-treatment with glibenclamide and mimicked by pinacidil, a K^+_{ATP} channels blocker and a K^+_{ATP} channels opener respectively.

Our findings therefore suggest an important role for endogenous H_2S as a modulator of some of the key components of acute inflammatory responses, particularly those occurring at the leukocyte-endothelial interface. As for other gaseous mediators (carbon monoxide, NO), H_2S was recognized for its toxicity long before its importance in physiological processes was described. H_2S is synthesized, primarily from L-cysteine, through actions of the enzymes CSE and CBS. In rats, blood and plasma levels of H_2S are in the 10–100 μM range (Richardson CJ et al., 2000). In the present study, we used three different H_2S donors at doses that would approximate concentrations of H_2S that fall within this physiological range. Differences in the potency of Lawesson's reagent vs. Na_2S and NaHS in suppressing leukocyte adherence/infiltration are consistent with observed differences in their ability to elicit H_2S-mediated vascular smooth muscle relaxation. Moreover, the observation that suppression of endogenous H_2S synthesis with β-cyanoalanine led to increased leukocyte adherence and infiltration is consistent with a role for this mediator as a tonic inhibitor of leukocyte adherence/extravasation. Our observation that leukocyte rolling velocity decreased sharply after administration of the CSE inhibitor is consistent with previous observations that P-selectin expression can be regulated by H_2S (Fiorucci S et al., 2005). Since leukocyte expression/affinity of LFA-1 has also been shown to be suppressed by H_2S (Fiorucci S et al., 2005), it is possible to hypothesize that H_2S by modulating expression of proteins on both cell types regulates leukocyte migration.

It has been recently reported that NSAIDs suppress H_2S synthesis by reducing expression of CSE (Fiorucci S et al., 2005). The subsequent reduction of H_2S synthesis

may contribute to the increase in leukocyte adherence that is seen after NSAID administration (Asako H et al., 1992; Wallace JL et al., 1993), which has been shown to contribute significantly to the gastric injury induced by this class of drugs (Wallace JL et al., 1990; Wallace JLet al., 1991; Wallace JL et al., 1997). Indeed, co-administration of an H_2S donor with an NSAID resulted in inhibition of NSAID-induced leukocyte adherence and reduction of the severity of gastric damage (Fiorucci S et al., 2005). Pertinent to the present study, administration of an H_2S donor prevented many of the other "proinflammatory" effects of NSAIDs, including the elevation of ICAM-1 and LFA-1 expression and the increase in mucosal TNFα expression (Fiorucci S et al., 2005).

Among the four H_2S donors used in the second part of this study, only N-acetylcysteine requires metabolism in order for H_2S to be released. N-acetylcysteine is a precursor of Lcysteine (Fujii K et al., 2005), which is the substrate for H_2S generation via CSE and/or CBS. The observation that the anti-inflammatory actions of N-acetylcysteine were reversed by an inhibitor of CSE (β-cyanoalanine) is consistent with the effects being mediated by H_2S.

Irreversible inhibitors of CSE and CBS have been reported to interfere with other enzymes (Moore PK et al., 2003; Smith SB et al., 1977). As with any pharmacological agent, we cannot exclude the possibility that the reversible inhibitor of CSE, β-cyanoalanine, could exert non-specific effects. For these reasons we have developed and studied four different H_2S donors in order to adderss this issue.

Genetic deletion of CSE and CBS are lethal, ruling out the use of these "knockouts". CBS heterozygotes are viable, expressing half as much CBS as wild-type (WT). When fed a diet high in homocysteine, $CBS^{-/-}$ mice have been shown to exhibit increased leukocyte adherence, increased P-selectin expression, and increased vascular

permeability (in the brain) (Kamath AF et al., 2006), all consistent with a role for H_2S in mediating acute inflammation. However, use of these mice for direct studies of inflammation is of questionable value, as they have drastically altered vascular responsiveness to cholinergic agents and bradykinin (Weiss N et al., 2002).

A consistent finding in the various models used in the present study was that the anti-inflammatory effects of H_2S appeared to be mediated via activation of K^+_{ATP} channels. Similarly, the analgesic effect have been ascribed to modulation of K^+_{ATP} channels (Distrutti E et al., 2005). It is possible, therefore, that K^+_{ATP} channels represent a novel target for anti-inflammatory and analgesic agents.

In addition to these data, hydrogen sulphide also plays a primary role in resolving phase of inflammation. In fact, our results suggest that H_2S is able to induce an anti-inflammatory effect, likely relying on annexin-1-dependent responses, as we have demonstrated in our experiments performed on human neutrophils, bone marrow-derived macrophages and $AnxA1^{-/-}$ mice.

In summary, the results showed in the second part of the present study demonstrates a role for endogenous H_2S as a modulator of key inflammatory events occurring at the interface of leukocytes and the vascular endothelium. H_2S may act as a tonic regulator of leukocyte adherence to the endothelium and of endothelial permeability. The anti-inflammatory effects of H_2S appear to be mediated via activation of both K^+_{ATP} channels and annexin-1. These results, and recent reports that H_2S donors can down-regulate adhesion molecule and pro-inflammatory cytokine expression, therefore identify H_2S, the key enzymes responsible for H_2S synthesis, and K^+_{ATP} channels as potential targets for novel anti-inflammatory therapies.

Finally, we can conclude that H_2S is a very complex modulator in despite of a very simple chemical structure.

Indeed, it is involved in vascular homeostasis directly and also through interaction with L-arginine/NO pathway; most likely through K^+_{ATP} channels and annexin-1.

Furthermore, the ability of endothelial cells to generate H_2S is very consistent with the function that H_2S exerts at the endothelium-blood interface. These findings open a new horizon in the pharmacology of the gaseous mediators. Thus, the scenario we have in front of us is far to be completely understood, since now there are three mediators, NO, H_2S and CO, all contributing to the complex phenomenon of inflammation and still there is much to be understood on each single mediator as well as on the possible cross talk among them.

Unravelling these issues will allow researchers in the future to develop new therapeutic targets and design new and effective drugs.

CHAPTER 5

5. REFERENCES

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