UNIVERSITY OF BASILICATA

Department of Science

PhD in
Applied Biology and Environmental Safeguard
(International PhD Programme)

THESIS TITLE

“THE INTERFACE BETWEEN CARDIOVASCULAR DISEASE AND INFLAMMATION: HYDROGEN SULFIDE AND ANNEXIN A1 PATHWAYS AS NOVEL PHARMACOLOGICAL TARGETS”

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XXXV cycle

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ABSTRACT

Cardiovascular diseases are a heterogeneous group of disorders of the heart and the vasculature, in which different factors are responsible for their onset. In particular, among the various factors contributing the development of vascular disorders there are hyperlipidaemia, hyperglycaemia, cigarette smoke, obesity and hyperhomocysteinemia. Many of the pathologies that are part of vascular disorders, as hypertension, diabetes, atherosclerosis and heart failure, are characterized by two main conditions: oxidative stress and excessive prolonged inflammatory response. The interception of these dual aspects delineates the concept of inflammation-based vascular diseases (IBVD). In this context, it is of note to underline the decline of the nitric oxide (NO) and hydrogen sulfide (H_2S) levels, molecules that share vasorelaxant and anti-inflammatory properties. Deficiency of these two gaseous mediators, normally produced within the body, targets vascular endothelium resulting into one of the classical IBVD example.

In this project, we aimed to dissect the molecular mechanisms underlying the vascular impairment observed in hyperglycaemia and the cross talk between NO and H_2S in IBVD, with respect to vascular inflammatory response.

Indeed, we used *in vitro*, *ex vivo* and *in vivo* models to perform multiple approaches tackling different sides of disease conditions. We used bovine aortic endothelial cells (BAEC) and aorta rings harvested from mice to study the impact of hyperglycaemia and the effect of a novel mitochondrial-specific H_2S donor (AP123). In addition, we also performed specific experiments on human macrophages aiming to evaluate the effect of H_2S pathway in the physiological response by specialized pro-resolving molecules (SPMs) in inflammatory conditions.

First, we found that the decline NO levels associated to hyperglycaemia is paralleled by similar outcomes in H_2S levels. This impairment can be corrected in endothelial cells when AP123 was used as H_2S donor and the underlying mechanism is based on a transcriptional effect on eNOS, involving the activation of cAMP response binding protein (CREB) by PI3K. Furthermore, we found that this mechanism is also operative in the whole vascular tissue as demonstrated by ex vivo experiments.

In the context of experimental inflammation, we found that CSE pathway is downregulated in endothelial cells following administration of TNF-α. Interestingly, the

beneficial role of H_2S has been confirmed by administration of AP123. Therefore, the modulation of inflammatory response by H_2S has been also shown to be part of the mechanism of action of "unrelated" anti-inflammatory drug such as clodronate.

To evaluate whether H_2S activity could influence changes in the lipid mediators' activity, we evaluated the effect of slow-releasing H_2S donor, AP123, and a specific CSE inhibitor, PAG, on the concentrations of SPMs in M1 macrophages, stimulated with LPS, at two different time points. We found that the majority of LM are differentially regulated between two treatments, involving pro- and anti-inflammatory mediators. However, the administration of 1nM AP123, at 45 min, mainly triggered the involvement of anti-inflammatory lipid mediators, while H_2S donor (100nM and 1μM, 45min) seems to positively modulate the levels of a particular pro-resolving mediator, referred to as $RvD2_{n3\text{-}DPA}$.

Overall, our studies suggest that H_2S is a crucial vasculoprotective mediator in IBVD at vascular level and H_2S-releasing molecules may open new perspectives in the therapy of H_2S deficiency conditions with a pleiotropic mechanism not only related to a simple "replacement" effect.

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1. Introduction

1.1. Vascular diseases

The vascular system is an extremely complex network of arteries, capillaries and veins, lined by endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) that supplies tissues with oxygen (O_2) and nutrients. The function of the vascular system is to maintain cellular homeostasis (Pugsley & Tabrizchi, 2000). Indeed, the regulation of vascular function, in response to changing metabolic demands, is necessary for organ functions and the maintenance of normal tissue (Potente & Carmeliet, 2017; Chen *et* al., 2021).

Vascular diseases, caused by vascular injury and dysfunction, represent a heterogeneous group of disorders of the heart and the vascular system, in which there are several factors responsible of their onset. Therefore, these diseases are the largest cause of morbidity and premature death worldwide. Among the numerous factors contributing the development of vascular disorders there are: hyperlipidaemia, hyperglycaemia, cigarette smoke, obesity and hyperhomocysteinemia.

	Chronic patients	ACS	Acute stroke
Smoking (%)	29	36	42
Hypertension (%)	88	75	92
Diabetes mellitus (%)	39	29	29
Hypercholesterolemia (%)	65	61	42
Hypertriglyceridemia (%)	29	36	17
Obesity (%)	61	62	31

ACS Acute coronary syndromes

Table 1. Incidence of risk factors in patients with vascular disease (Horvath *et* al., 2004)

Consequently, all these elements could give a wide range of disorders in terms of the symptoms of initiation, the sites affected (i.e., heart, coronary arteries, etc), the severity and the disease progression (Cirino *et* al., 2017). Indeed, vascular diseases could influence several other diseases, such as metabolic disorders, nervous system diseases and heart diseases (Arokiasamy *et* al., 2017; Chen *et* al., 2021).

The abnormality of vascular endothelium is a major contributor to a plethora of several kinds of the pathologies, such as: atherosclerosis, heart failure, diabetes and hypertension.

1.1.1. Inflammation and oxidative stress as a major cause of inflammation-based vascular diseases (IBVD)

The majority of aforementioned pathologies are characterized by two main features: prolonged and exacerbated inflammation and oxidative stress (Ellulu *et* al., 2016). Therefore, the interception of these dual aspects delineates the concept of inflammation-based vascular diseases (IBVD) (Cirino *et* al., 2017).

1.1.1.1. Inflammation

Inflammation is a critical physiological response, arising in vascularized tissues, and plays a crucial role in the pathogenesis of cardiovascular diseases (Conte *et* al., 2018). In general, inflammatory process represents a complex network of events in the host response, to maintain homeostasis, against invading microorganisms, foreign substances or host self-disturbers, such as the molecules derived from damages cells (Brancaleone *et* al., 2014; Sugimoto *et* al., 2016b). Indeed, various pathogenic factors (i.e., infection, cardiac infarction, tissue injury) can induce inflammation by causing tissue damage. Specifically, the aetiologies of inflammation can be infectious or non-infectious (Chen *et* al., 2018).

Non-infectious factors	Infectious factors
Physical: burn, frostbite, physical injury, foreign bodies, trauma, lionizing radiation Chemical: glucose, fatty acids, toxins, alcohol, chemical irritants (including fluoride, nickel and other trace elements) Biological: damaged cells Psychological: excitement	Bacteria viruses other microorganisms

Table 2. Aetiology of Inflammation (Chen *et* al., 2018)

The five clinical signs of inflammation are redness (*rubor*), heat (*calor*), swelling (*tumor*), pain (*dolor*), and eventually loss of function of the injured tissue (Lo Faro *et* al., 2014; Recchiuti *et* al., 2019). In general, the acute inflammatory response is a complex coordinated sequence of events involving a large number of molecular, cellular and physiological changes, included vasodilation and increased blood flow,

elaboration of proinflammatory mediators, upregulation of adhesion molecule expression on cell surfaces, leukocyte/endothelial cell adhesive interactions, endothelial barrier disruption and edema formation, and tissue injury or dysfunction (Gilroy & De Maeyer, 2015; Zuidema & Korthuis, 2015). This mitigation process contributes to restoration of tissue homeostasis and resolution of the acute inflammation. Indeed, uncontrolled acute inflammation could become chronic, leading to a variety of chronic inflammatory diseases (Zhou et al., 2016; Chen et al., 2018). In particular, the inflammatory response involves several signaling pathways that regulate inflammatory mediator levels in resident tissue cells and inflammatory cells recruited from the blood (Lawrence, 2009). Although the anti-inflammatory response depends on the precise nature of the initial stimulus and its location in the body, all of them share a common mechanism:

- Cell surface pattern receptors recognize detrimental stimuli;
- Inflammatory pathways are activated;
- Inflammatory markers are released;
- Inflammatory cells are recruited (Chen *et* al., 2018).

Specifically, after the host has been incited, crucial microcirculatory events occur in response to local release of soluble proinflammatory mediators, such as cytokines, chemokines, leukotrienes, bradykinin, platelet-activating factors, prostaglandins, histamine and reactive oxygen species (ROS) by resident cells in the injured or infected tissue (i.e. tissue macrophages, dendritic cells, lymphocytes, endothelial cells, fibroblasts and mast cells), leading to higher permeability of microvessels and a consequent increased leukocyte recruitment (Medzhitov, 2010; Gilroy & De Maeyer, 2015; Sugimoto *et* al., 2016b). Usually, the role of these molecules is to destroy the pathogen or the cause of inflammation, protect the injured tissue and to prevent spreading of the injury (Lo Faro *et* al., 2014). Then, increased vascular permeability determines plasma fluid leakage and accumulation in tissues. These events lead to edema. Consequently, this is followed by polymorphonuclear neutrophil (PMN) recruitment, adhesion to vascular endothelial cells (ECs), diapedesis (or transmigration), and accumulation of swarming (Recchiuti *et* al., 2019). Their function is to eliminate bacteria or other damaging substances *via* phagocytosis. Indeed, phagocytes are able to engulf pathogens or foreign bodies, inside intracellular

vacuoles (phagosomes), thereby releasing ROS and proteases. (Lo Faro *et* al., 2014; Gordon, 2016; Recchiuti *et* al., 2019).

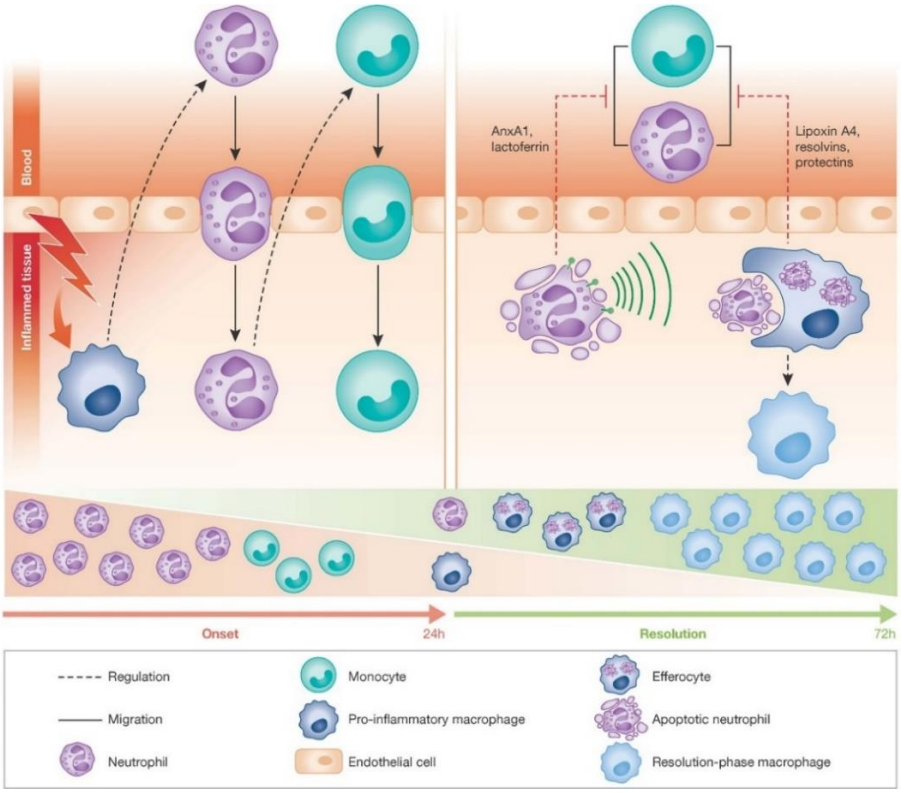


Fig.1 Cellular interplay during inflammatory process (Ortega-Gómez *et* al., 2013)

1.1.1.2. Oxidative stress

Therefore, another group of key players in the IBVD are the reactive oxygen species, commonly known as ROS (Cirino *et* al., 2017). ROS are normally produced within the body in limited quantity and they are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (Kumar *et*. al., 2015, Hussain *et* al., 2016). In particular, ROS such as peroxynitrite ($ONOO^-$), superoxide (O_2^-), hydroxyl (HO^-), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), are signal molecules that are involved in vascular homeostasis (Cirino *et* al., 2017). In general, these molecules are produced within the vessels by endothelium, smooth muscle cells and also in adventitia by different enzymic systems and regulate cell proliferation, apoptosis, oxygen sensing, defence from microbial injury and

inflammatory reactions (Zhang *et* al., 2007; Lakshmi *et* al., 2009; Santilli *et* al., 2015 Cirino *et* al., 2017). However, the problem occurs when ROS bioavailability overtake the antioxidant defenses (Sena *et* al., 2018). Indeed, the body undergoes what is called oxidative stress. This is a condition in which there is an excessive production of ROS in the cells and tissues and antioxidant system cannot able to neutralize them. Consequently, imbalance in this critical mechanism can lead to the damage of cellular molecules such as DNA, proteins and lipids, leading to cellular damage, tissue injury and inflammation (Hussain *et* al., 2016; Ďuračková, 2010). In particular, oxidative stress has been associated with the pathogenesis of several chronic disorders (i.e., diabetes, atherosclerosis, etc.) and an increased oxidative stress can lead to vasoconstriction, vascular remodelling, described as a major factor for the development of vascular diseases (Sena *et* al., 2018). Indeed, oxidative stress has a critical role in the initiation and progression of endothelial dysfunction and vascular diseases affecting cells in the vascular wall (Giacco & Brownlee, 2010; Sena *et* al., 2013; Rodriguez-Porcel *et* al., 2017). Furthermore, at vascular level, ROS reduce levels of vasorelaxant and anti-inflammatory molecules such as nitric oxide (NO) and hydrogen sulfide (H_2S). Indeed, the reduction of these two gaseous mediators, normally produced within the body, targets vascular endothelium leading into a classical IBVD condition. In particular, the impaired biosynthesis of NO by eNOS is the main consequence of oxidative stress in the endothelium, probably because in presence of high levels of ROS, the eNOS co-factor BH_4 is oxidised (Antoniades *et* al., 2007). Consequently, this event induces the uncoupling of eNOS, with a generation of O_2^-, instead of NO (Antoniades *et* al., 2007; Cirino *et* al., 2017).

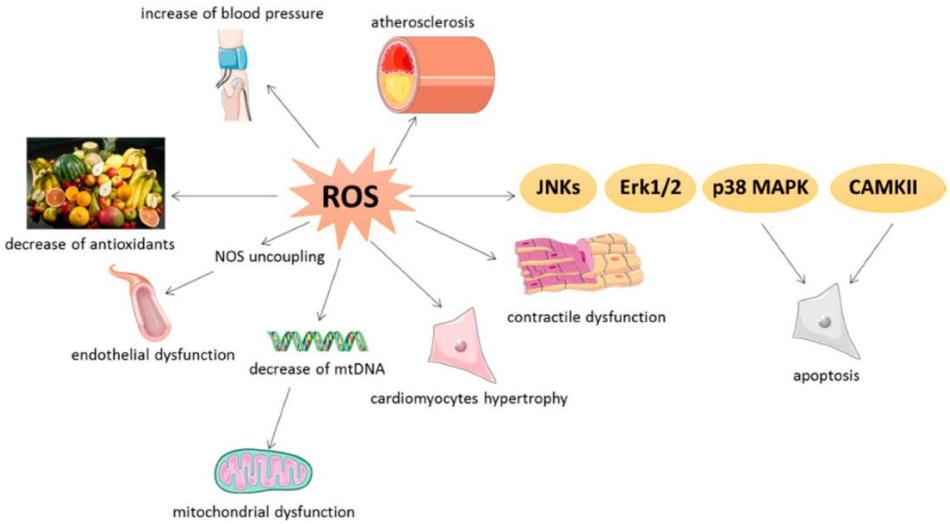


Fig.2 Pathological roles of oxidative stress at vascular level (Dubois-Deruy *et* al., 2020)

1.1.2. Hydrogen sulfide (H_2S) and nitric oxide (NO): two potential mediators in cardiovascular control

At vascular level, endothelial cell inflammation is directly linked to numerous cardiovascular diseases, such as hypertension, stroke, heart disease, aging, atherosclerosis, diabetes mellitus, obesity, venous thrombosis and intimal hyperplasia. Indeed, the ECs with inflammatory phenotype cause inflammation in the blood vessels, leading to endothelial dysfunction and consequently cardiovascular disorders (Sun et al., 2020). Within vasculature system, hydrogen sulfide (H_2S) and nitric oxide (NO) are known gasotransmitters that regulate many endothelial events, included integrity and vascular tone, but also inflammation. For many years, NO and H_2S have only been considered toxic gases. Nevertheless, they have both been discovered to be produced endogenously and to regulate key physiological functions (Lo Faro et al., 2014). However, the potential molecular mechanisms that trigger cardiovascular homeostasis mediated by H_2S and NO, in particular endothelium inflammation, are totally elucidated (Sun et al.,2020).

1.1.2.1. Nitric oxide (NO): an important mediator of the vascular integrity

Nitric oxide was first identified as an endothelium-derived endogenous mediator involved in the regulation of vascular tone (Furchgott and Zawadzki, 1980; Palmer et al., 1987). However, since then it has become clear that NO is the signalling molecule responsible for crucial physiological and pathophysiological processes, including immune defences, inflammation and neurotransmission (Shaw et al., 2005; Sharma et al., 2007). In general, nitric oxide, known as a short-lived molecule, is formed from guanidine nitrogen of L-arginine, through a series of redox reaction, and is synthesized by one of three different isoforms of nitric oxide synthase (NOS) that differ in enzymatic activity. In particular, these are known as NOS I, II and III, corresponding to inducible (iNOS), neuronal (nNOS), and endothelial (eNOS) isoforms respectively (Yuan et al., 2015; Nagpure & Bian, 2016). In this process, L-arginine is degraded to L-citrulline and NO in the presence of molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) (Chen et al., 2008).

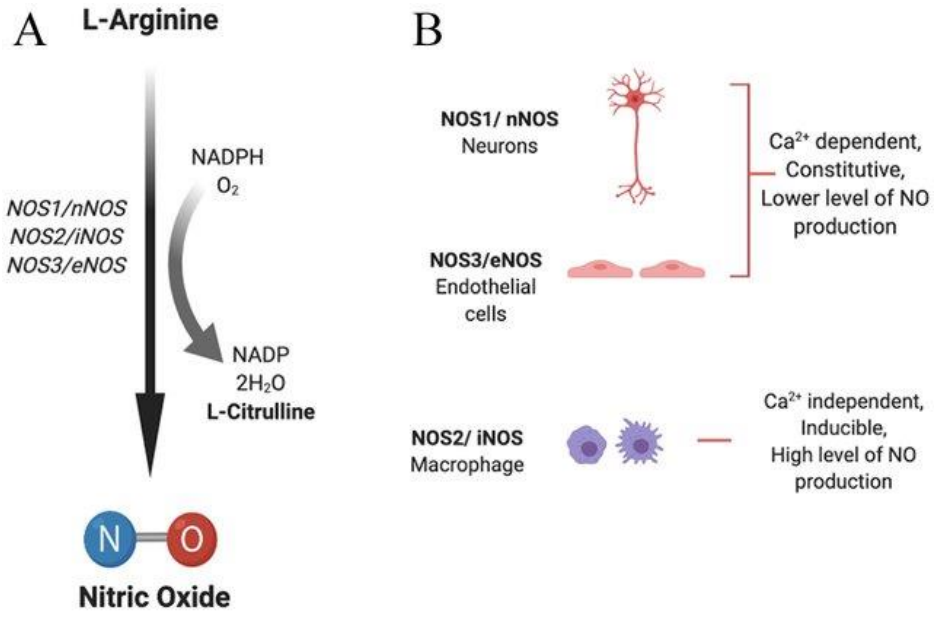


Fig.3 (A) Synthesis of nitric oxide. (B) Nitric oxide synthase (NOS) isoforms. (Mishra et al., 2020)

NOS1 and NOS3 are constitutive enzymes that are controlled by the availability of intracellular Ca^{2+}/calmodulin. Moreover, the regulation of NO production by eNOS is complex, however the pteridine cofactor (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4) has

a critical role on NO synthesis. Indeed, endothelial BH_4 availability is a key requirement for normal endothelial function (Alp & Channon, 2003). Instead, NOS2 is an inducible enzyme that is involved at the level of gene transcription and is expressed in the immune system, in response to inflammatory or proinflammatory mediators (Chen et al., 2008).

Once NO has been generated within ECs by eNOS, it diffuses to the vascular smooth muscle cells (VSMCs) and binds the haem group of its target enzyme, soluble guanylate cyclase (sGC), activating it and leading to the conversion of GTP to cGMP. Consequently, this second messenger, through a PKG dependent mechanism, lead a signalling which initiates a protein phosphorylation cascade and that induces a decrease on intracellular Ca^{2+} concentration (Cirino et al., 2017). The sGC-mediated production of cGMP is main responsible for the biological effects of eNOS, such as vascular relaxation and angiogenesis. PKG plays a key role in cell survival, endothelial permeability and vascular homeostasis. The importance of cGMP to NO signaling has led to find crucial catabolic enzymes that control this balance. Indeed, vascular cGMP levels are physiologically degraded by phosphodiesterase 5 (PDE5), leading its degradation to its consequent inactive metabolite, 5'-GMP (Kass et al., 2007; Szabo, 2016; Cirino et al., 2017). However, NO exerts another relevant role in modulating cellular signalling that leads a redox reaction between NO and the thiol group of L-cysteine. Consequently, this reaction determinates a modification of protein cysteine thiols with the formation of S-nitrosothiols (SNO). In general, cardiovascular (CVD) risk factors, such as hypertension, diabetes mellitus, cigarette smoking and hypercholesterolaemia, reduce bioactive NO. Previously, these several factors can lead to an enhanced production of reactive oxygen species (ROS) in the vessel wall. Main source of ROS is NAPDH oxidases, upregulated for CVD risk factors. Therefore, ROS interact with eNOS-derived NO, leading to the peroxynitrite ($ONOO^-$) formation. $ONOO^-$ leads oxidative damage, nitration and S-nitrosylation of biomolecules including proteins, lipids and DNA. The main NOS cofactor, BH_4, is highly sensitive to oxidation by $ONOO^-$. This induces the uncoupling of eNOS, with consequent generation of O_2^- instead NO, a mechanism linked to endothelial dysfunction (Förstermann & Li, 2010; Cirino et al., 2017).

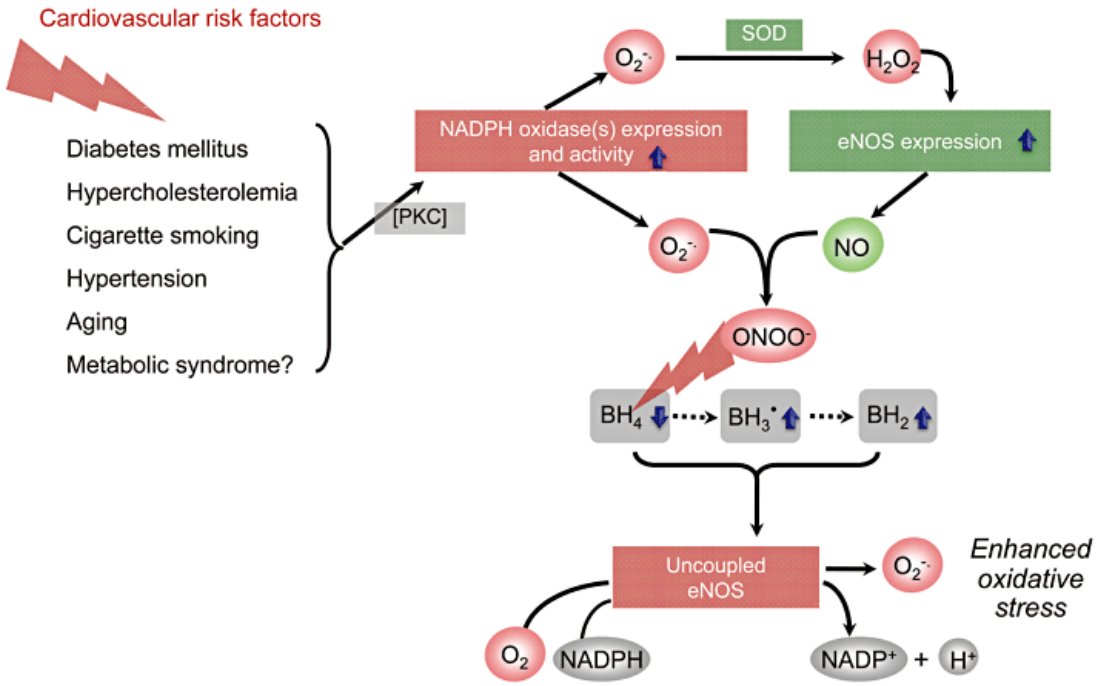


Fig.4 Endothelial nitric oxide synthase (eNOS) uncoupling and endothelial dysfunction (Förstermann & Li, 2010)

1.1.2.2. Hydrogen Sulfide (H_2S) and the vascular regulation

For much of past year, hydrogen sulfide (H_2S) has been long as a toxic environmental hazard and a pollutant. For this reason, it has often been associated to detrimental effects. However, recently, H_2S has emerged as an important gaseous signaling molecule with vast potential in pharmacotherapy, together with nitric oxide (NO) and carbon monoxide (CO). Indeed, all these small molecules possess significant physiological importance, like anti-inflammation and antiapoptosis (Wallace et al., 2015; Wang et al., 2015; Wen et al., 2018). In general, H_2S can be synthesized in mammalian tissues, can cross the cell membrane and can exert many biological effects in various systems. Indeed, several studies showed that H_2S can played a relevant role in neurophysiology, endocrine regulation, cardiovascular disease and other pathological and physiological systems (Zhang et al., 2018). In mammalian tissue, H_2S can be produced via both enzymatic and non-enzymatic pathways. About the enzymatic pathways, hydrogen sulfide is produced by three distinct enzymes known as cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS) and 3-mercaptopyruvate sulfotransferase (3-MST).

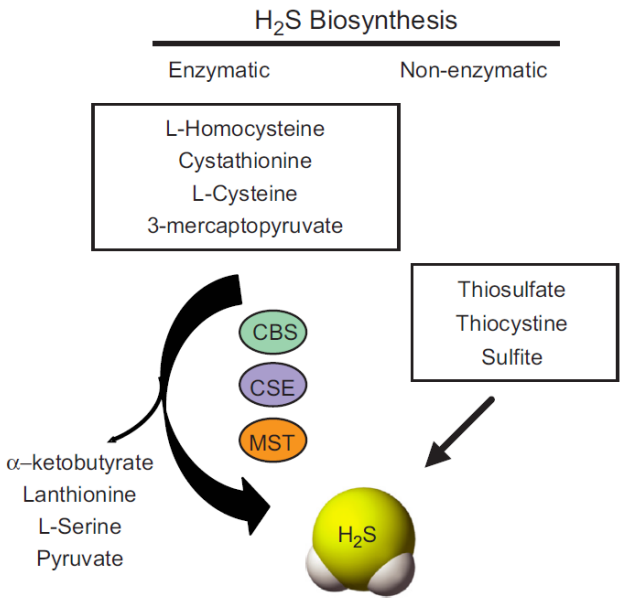


Fig. 5 H_2S biosynthesis (Kolluru et al., 2013)

The substrates of CBS and CSE are L-cysteine and homocysteine; the substrate of 3-MST is 3-mercaptopyruvate which is produced from L-cysteine (Szabo, C. 2017). In particular, CBS is a major contributor to H_2S production in the brain, while the hydrogen sulfide generation from CSE protects against injuries in the cardiovascular system (Shibuya et al., 2009a). Indeed, CSE is found in both endothelium and VSMCs (Cirino et al., 2017). However, presence of CBS has also been assessed in ECs (Wang et al., 1992).

	Cystathionine γ-lyase (CSE)	Cystathionine β-synthase (CBS)
Localization	Liver, heart, vessels, kidney, brain, adipose, small intestine, stomach, uterus, placenta, and pancreatic islets	Brain, liver, kidney and ileum, uterus, placenta, and pancreatic islets
Activators	Pyridoxal 5′-phosphate	Pyridoxal 5′-phosphate, S-adenosyl-L-methionine, and Ca^{2+}/calmodulin
Inhibitors	D,L-propargylglycine, β-cyano-L-alanine	Hydroxylamine, aminooxyacetate
Functional roles	H_2S production in the liver and smooth muscle	H_2S production in the brain and nervous system

Table 3. Characteristics of H_2S-producing enzymes (Wen et al., 2018)

About 3-MST, it appears to contribute to H_2S production in both the periphery and central nervous system (Shibuya et al., 2009b). However, in vascular tissues, 3-MST could be detected in both ECs and VSMCs (We et al., 2018). Finally, the non-

enzymatic route of yielding H_2S is the conversion of elemental sulfur and transformation of oxidation of glucose. It is presented in vivo, involving phosphogluconate (<10%), glycolysis (>90%) and glutathione (<5%) (Wang, 2002). H_2S exerts its cellular effects by directly transport across cell membranes, without a specific receptor, and it is involved in the modulation of several pathophysiological processes in cardiovascular system. Indeed, at physiological levels, it has an important role in cardiovascular homeostasis and the use of H_2S donors or specific inhibitors of the H_2S production showed significant effects in CVDs, such as atherosclerosis, heart failure, hypertension and ischemic myocardium (Pan et al., 2017). Recently developed H_2S donors show promising effect against several pathological processes in preclinical and early clinical studies, including cardiovascular diseases (Zaorska et al., 2020). The regulation of vascular tone by H_2S can be dependent on endothelium-independent and -dependent manners (Wang et al., 2015). Several studies demonstrated the vasodilation role of H_2S in aorta, gastric artery, internal mammary artery and mesenteric artery (Zhao et al., 2001; Cheng et al., 2004; Kubo et al., 2007; Webb et al., 2008). Zhao and co-workers demonstrated that the underlying mechanism by which H_2S relaxes blood vessels is related with activation of vascular smooth muscle ATP-sensitive K^+ (K_{ATP}) channels. The involvement of K_{ATP} channels in H_2S-induced relaxation was confirmed by using glibenclamide, a K_{ATP} channel blocker. The use of this inhibitor partially blocked the relaxation caused by higher concentrations of the H_2S donor sodium hydrosulfide (NaHS) in rat arterial smooth muscle. Conversely, the inhibition of CSE with l-propargylglycine (PAG), a specific CSE inhibitor, decreased the K_{ATP} channel current (Webb et al., 2008). Despite of these results, the exact mechanism of how K_{ATP} channels are directly activated by H_2S still remains unknown. Therefore, another study showed that the infusion of NaHS in the rat isolated and perfused mesentery caused a biphasic effect. Indeed, at low amount, NaHS caused vasoconstriction. Conversely, at higher concentrations, NaHS caused vasodilation. Both effects are dependent on arachidonic acid generated by phospholipase A2 (cPLA2) (d'Emmanuele di Villa Bianca et al., 2011). H_2S is also reported to stimulate endothelial proliferation, migration and angiogenesis (Wang et al., 2010). At the same way, the critical effects of H_2S donors in angiogenesis can affect in dual effect. Indeed, a low concentration, the H_2S donors may have cytoprotective effects, while at higher concentrations, cytotoxic effects. Moreover, it has been demonstrated that the H_2S-mediated angiogenic effects involved several

cellular signaling pathways, including the mitogen activated protein kinase (MAPK), the PI3K/Akt pathway and ATP-sensitive potassium channels (Szabo & Papapetropoulos, 2011). Furthermore, H_2S is also a powerful antioxidant and it can defend the endothelium against oxidative stressors. Xie and colleagues highlighted a novel role for Sirtuin3 (SIRT3), an important deacetylase under oxidative stress, in the protective effect of H_2S against oxidant damage in the endothelium both *in vitro* and *in vivo* (Xie et al., 2016). Instead, Wen and co-workers showed the pharmacological effects of H_2S on antioxidant effects and mitochondria protection against hydrogen peroxide (H_2O_2) in human umbilical vein endothelial cells (HUVECs) (Wen et al., 2013).

1.1.2.2.1. H_2S/NO crosstalk at vascular level

A number di reports suggest that NO-H_2S molecules can influence each other in their production and pathophysiological functions. Most of the research on NO-H_2S axis has been conducted in the cardiovascular system, where both gases exert extensive effects. Indeed, several studies showed that NO-H_2S crosstalk mediates their effects on vascular functions such as angiogenesis, vascular remodelling, vasodilation and inflammation (Kolluru et al., 2013). Both molecules can enhance their respective production through several mechanisms. For example, H_2S is able to prevent eNOS degradation and induce eNOS phosphorylation with subsequent NO production via PI3k/Akt activity and p38 MAPK pathways (Yong et al., 2008; Lei et al., 2010; Sojitra et al., 2012).

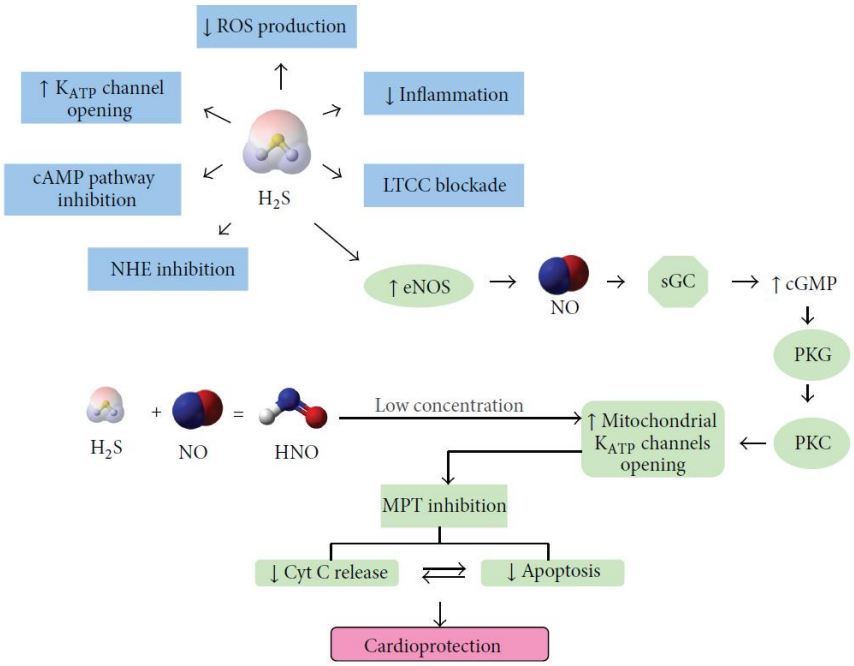


Fig.6 Cardioprotective effects of NO and H_2S (Nagpure & Bian, 2016)

Instead, NO can increase H_2S levels by elevating CSE and CBS expression in VSMCs (Zhao et al., 2001; Zhao et al., 2002). Furthermore, H_2S is able to cause vasorelaxation by acting as a nonselective endogenous PDE inhibitor that boosts cyclic nucleotide levels in tissues. Indeed, Bucci and colleagues evaluated the existence of cross-talk between NO and H_2S, although conflicting results have been obtained (Bucci et al., 2010). H_2S signaling has also been analysed in a genetic model of diabetes, the non-obese diabetic (NOD) mice. In these mice, endogenous H_2S production is impaired. Moreover, the ability of isolated aorta to respond to exogenous H_2S was enhanced and endothelium-derived NO appeared to be involved in the enzymatic conversion of L-cysteine into H_2S (Brancaleone et al., 2008). In the vascular system, hydrogen sulfide is now viewed as an enhancer of the NO/cGMP/sGC/PKG pathway, without which eNOS cannot function to its fullest physiological extent (Szabo, 2017). For example, exposure of ECs to H_2S increased intracellular cGMP in a NO-dependent manner and activated PKG signaling. Conversely, inhibition of eNOS abolished H_2S-mediated angiogenesis, and also attenuated H_2S- induced vasorelaxation (Coletta et al., 2012).

1.1.3. Chronic Inflammation

Continuous inflammatory stimuli can lead to aggressive and prolonged inflammatory stimuli can lead to aggressive and prolonged inflammatory responses, which may be detrimental to the host, leading to chronic inflammation (Nathan & Ding, 2010; Sugimoto *et* al., 2016b). Chronic inflammation is a kind of inflammation of prolonged duration and, although it could follow acute inflammation, it can also take place without initially showing any clinical symptoms (Whiteman & Winyard, 2011; Lo Faro *et* al., 2014). It is characterized from several events: the change in the cell type present at the site of inflammation, from granulocytes to mononuclear and plasma cells, tissue destruction mediated by the inflammatory cells and attempts to replace the damage tissue by the connective tissue (angiogenesis and fibrosis). In general, pro-inflammatory signalling pathways have the capacity to induce the parallel expression of anti-inflammatory mediators, such as IL-10 (Lawrence & Gilroy, 2007). For example, Hacker and colleagues demonstrated that the signalling pathway used by TLRs to activate expression of pro- and anti-inflammatory cytokines diverges at the level of the proteins TRAF3 and TRAF6. In general, TRAF3 is critical for induction of IL-10 expression. Consequently, its absence determinates a dramatically increased of the expression of the TRAF6-dependent pro-inflammatory cytokines IL-6 and IL-12 (Häcker *et* al., 2006). Although the balance between the TRAF3- and TRAF6-generated signals play a crucial role in controlling the inflammatory response, its alteration could interfere with the resolution of inflammation. Therefore, a perturbation in any one of these negative regulators could lead to "spontaneous" chronic inflammation. Indeed, genetic studies in mice showed that the absence of one negative regulator is sufficient to result in serious inflammatory disorders (Lawrence & Gilroy, 2007). Indeed, all these conditions can lead to different chronic inflammatory diseases, such as rheumatoid arthritis, asthma and inflammatory diseases, although there are many other disease states characterized from a chronic low-level inflammation, such as atherosclerosis, autoimmune diseases, diabetes, cancer, ischemia-reperfusion injury, Alzheimer's disease, etc (Lo Faro *et* al., 2014). For example, Mauro and Marelli-Berg reported T cell immunity in cardiovascular disorders. Indeed, they proposed that and altered metabolism in the cardiovascular system, initially induced by macrophages and innate immunity, can lead to the chronic inflammation and subsequent

migration of antigen-non-specific activated T cells to the affected site (Mauro & Marelli-Berg, 2012).

1.1.4. Resolution of inflammation

The receptors and signalling pathways involve and that promote the inflammatory response have become well characterized; however, relatively a little is known about how acute inflammation resolvers to prevent chronic inflammatory diseases (Lawrence & Gilroy, 2007). The induction phase of inflammation is stimulated by the sensing of exogenous and endogenous danger signals from mechanically, chemically, or biologically induced tissue damage followed by the recruitment of effector cells, which induce an inflammatory response characterized by the release of crucial factors such as lipid, protein and gaseous mediators of inflammation (Schett & Neurath, 2018). Although for many years, the resolution of inflammation was considered a passive phenomenon, however several studies showed that it is an active process brought about by the biosynthesis of active mediators, which act on key events of inflammation to promote the return to homeostasis (Sugimoto *et* al., 2016a). Therefore, if we were to define the fundamental requirements for the successful resolution of inflammation it would become increasingly clear that the critical factor for the inflammatory response to switch off is the neutralization and the elimination of the injurious agents (Lawrence & Gilroy, 2007).

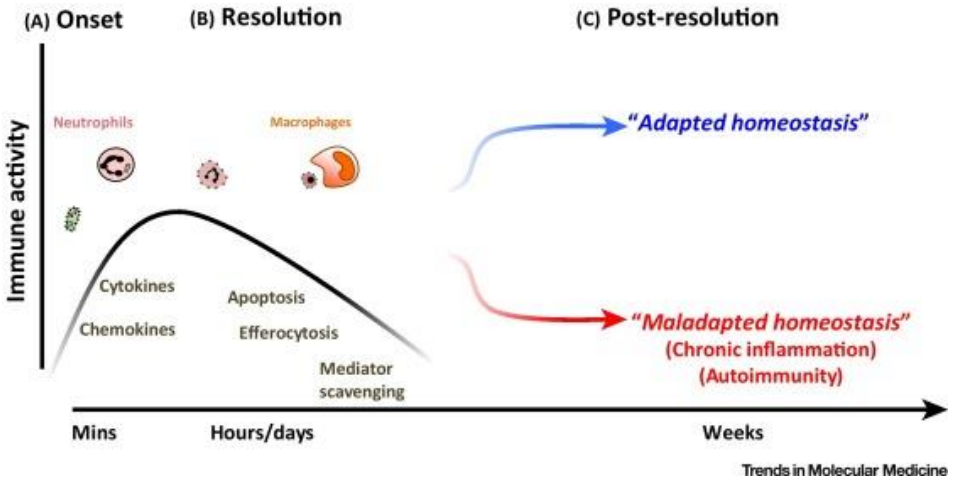


Fig.7 Activation, resolution and post-resolution (Feehan & Gilroy, 2019)

Inflammation resolution processes that rectify tissue homeostasis include reduction or cessation of tissue infiltration by neutrophils and apoptosis of spent neutrophils, counter-regulation of chemokines and cytokines, macrophage transformation from classically to alternatively activated cells, and initiation of healing (Chen *et* al., 2018). Successful resolution of inflammation is based on a number of keys events. First of all, at molecular level, pro-inflammatory factors need to be antagonized and catabolized. Then, at cellular level, immune cell numbers at inflammatory sited need to decline. Finally, at the macroscopic level, tissue integrity has to be restored by the activation of tissue-resident cells that induce repair (Schett & Neurath, 2018). The resolution process is characterized from three key mechanisms: cessation of neutrophil influx, neutrophil death and removal, and changes in macrophages function. In the first case, resolution of inflammation depends on stopping neutrophil recruitment, which is the most abundant leukocyte population at inflammatory sites (Schett & Neurath, 2018). The inhibition of continued leukocyte recruitment is essential to favour the return to homeostasis (Ortega-Gómez et al., 2013). This process is in part controlled by pro-resolving lipid mediators such as resolvins (Schett & Neurath, 2018). Several studies demonstrated that pro-resolving AnxA1 decreases neutrophil tissue accumulation by different mechanisms including downregulation of transendothelial migration (Perretti *et* al, 1996), promotion of neutrophil apoptosis (Perretti & Solito, 2004), and stimulation of the removal of dead neutrophils (Scannell *et* al., 2007). The combination of these mechanisms results in pro-resolving effects in *in vivo* models of inflammation (Dalli *et* al., 2008; Vago *et* al., 2012). Instead, in the second case, resolution processes require the death and the removal of tissue neutrophils (Schett & Neutorath, 2018). For effective resolution to occur, cessation of proinflammatory signaling is a prerequisite that pre-empts removal of infiltrating granulocytes. Indeed, during spontaneous resolution, neutrophils undergo apoptosis, a regulated cell death mechanism that prevents the release of histotoxic cellular contents (Savill *et* al., 1989). In particular, neutrophil apoptosis can be induced by expression of death ligands (i.e., TRAIL or FasL), produced by macrophages, or by transforming growth factor beta (TGFβ), produced by regulatory T cells (Brown & Savill, 1999; McGrath *et* al., 2011; Gagliani *et* al., 2015). Apoptotic neutrophils are rapidly engulfed by macrophages, a process known as efferocytosis, due to expression of "eat-me" signals on apoptotic neutrophils (i.e., photosphatidylserine). In particular, these signals are recognized by specific receptors on the surface of macrophages (Schett & Neurorath, 2018). Removal of apoptotic neutrophil is of a dual importance: it induces reprogramming

of macrophages and prevents spilling of potentially toxic contents from the neutrophil cytoplasm as they become necrotic (Ortega-Gómez *et* al., 2013). However, for effective resolution of inflamed tissues to occur cessation of the recruitment of granulocytes is required, followed by the recruitment of monocytes that differentiate into macrophages, which clear inflammatory cells and tissue debris, leading to the restoration of tissue structure and function (Henson, 2005; Barning & Levy, 2015). Indeed, the last key mechanism of resolution process is the change in macrophage function. In this phase, first of all, the monocytes-derived macrophages clear apoptotic cells (Schett & Neurorath, 2018). Clearance of apoptotic neutrophils prompts a switch from a pro-inflammatory (M1) to an anti-inflammatory (M2) macrophage phenotype, which is a prerequisite for macrophages egress via the lymphatic vessels favouring a return to tissue homeostasis (Wynn *et* al., 2013 Barning & Levy, 2015).

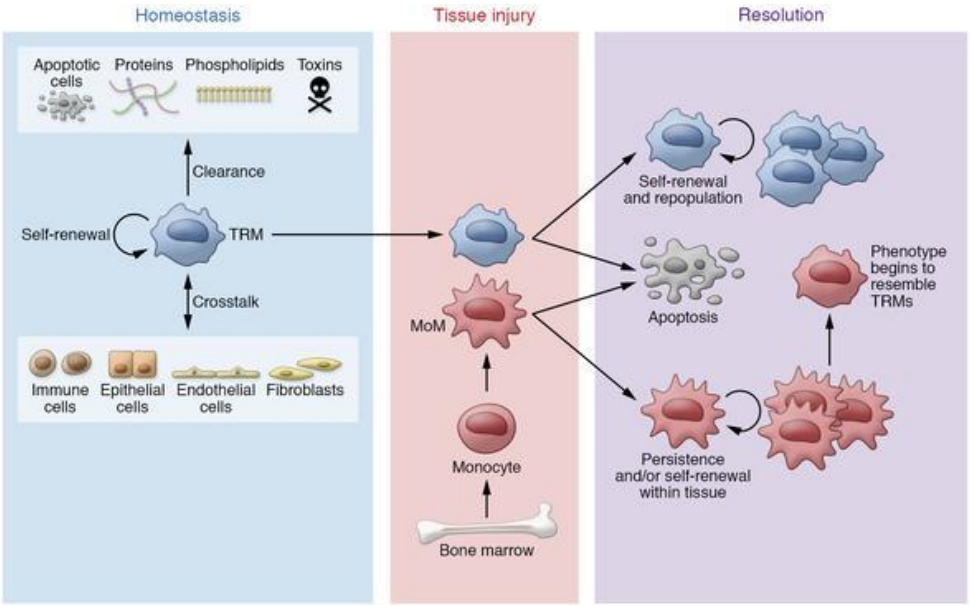


Fig.8 Tissue-resident macrophages and monocyte-derived macrophages play distinct roles in tissue injury and repair (Watanabe *et* al., 2019)

Then, the monocytes-derived macrophages start to release pro-resolving lipids, express anti-inflammatory receptors, such as TGF-R2 and FPR2, and synthesize increased concentrations of immune regulatory intracellular messengers, such as cAMP (Schett & Neurorath, 2018). Following efferocytosis, excessive macrophages and non-apoptotic macrophage leave the inflammatory site, leading a subsequent positive modulation of the

immune response. Indeed, at the end of inflammation resolution, resident immune cells regaining to form "adaptive immune", which creates a status of "post-resolution". Finally, all the events lead to induction of tissue repair, restoring the homeostasis without fibrosis or scar formation (Ortega-Gómez *et* al., 2013).

Clearance of apoptotic neutrophils leads to the production of crucial mediators that suppress the progression of inflammation and promote repair of damaged tissues (Barning & Levy, 2015). Indeed, anti-inflammatory and pro-resolving molecules such as specialized lipid mediators (lipoxin A4, resolvins, maresins, and protectins), peptides/proteins (melacortins, galectins and annexin A1), gaseous mediators (hydrogen sulfide and nitric oxide), and several other substances of different natures are released at the site of inflammation (Sugimoto *et* al., 2016b).

1.1.4.1. The role of gaseous transmitters in resolution

Therefore, several compounds are involved in the control of the resolution of inflammation, including gaseous mediators such as H_2S and NO. In general, these gaseous mediators act mainly as a signalling molecule. However, there are several studies showed their key actions with respect to their impact on inflammatory processes. For instance, eNOS-produced NO has both pro- and anti-inflammatory properties, depending on its location and concentration (Iwata et al., 2020). Indeed, excessive or inappropriate NO production can induce toxic reactions and tissue damage, directly (i.e., through damage to proteins, lipids and DNA) or indirectly (i.e., through the modulation of leukocyte activity), affecting as a proinflammatory agent. In contrast, low amount of NO derived from eNOS may act as an anti-inflammatory agent via its inhibitory action or apoptotic effects on cells (Tripathi et al., 2007). For example, $ONOO^-$ has a precise role in inflammatory cell apoptosis, although it still not clearly defined. Moreover, the generation of ONOO- is also involved, during inflammation, in the microbial killing (Pacher et al., 2007). However, the ability of NO to induce apoptosis is relevant during the resolution phase of inflammation. Indeed, various studies demonstrated that activated macrophages infiltrating murine tumors induce apoptosis through a NO-dependent pathway in both activated anti-tumor T cells and in the tumor cells themselves (Saio et al., 2001; Chattopadhyay et al., 2002). In another study, in a mouse model of kidney inflammation, macrophages

have been demonstrated to induce apoptosis in neighbouring mesangial cells priori to their ingestion by phagocytes (Duffield et al., 2000). At the vascular level, NO reduces oxidation in LDL, platelet reactivity and leukocyte stickiness, determining the vasculature protection (Cirino et al., 2007). Several studies have demonstrated that, in inflammatory-based vascular diseases, a crucial element could be caveolin-1 (CAV-1) expression, one of the major structural protein essential to the formation of the caveolae in ECs that keeps eNOS in a less active state (Fernández-Hernando et al., 2010). In normal condition, eNOS is bound to CAV-1 but the intracellular Ca^{2+} rise leads the Ca^{2+}- calmodulin complex binds eNOS, displaces it from CAV-1, leading to activation of the enzyme (Bucci et al., 2000; Gratton et al., 2000). CAV-1 overexpression has been found in several vascular disorders, such as hyperlipidaemia, diabetes, atherosclerosis and pulmonary hypertension (Cirino et al., 2007). Due to dual effect of NO, inhibition of NO production with specific NOS inhibitors (i.e., L-NAME and L-NMMA) has been explored as anti-inflammatory treatment (Laskin et al., 1994; Shan & Bourreau, 2000; Li et al., 2009). However, various NO donors have also been shown to exert anti-inflammatory effects (i.e., GEA3175 and NO-NSAIDs) (Keeble & Moore, 2002; Laursen et al., 2006). Of note, the role of NO in inflammation is complex and varied. For this reason, another level of complexity adds to this already complicate picture, if the interactions with the other gaseous mediators, in particular hydrogen sulfide (H_2S). Several studies have subsequently highlighted the importance of H_2S in inflammation. As was the case for nitric oxide, the literature on H_2S in inflammation was initially contradictory. Therefore, in the recent years, various findings demonstrated the anti-inflammatory effects of hydrogen sulfide, except at high concentrations (Wallace et al., 2012). Indeed, an important role of H_2S as an endogenous anti-inflammatory mediator has become increasingly clear. Among the different effects of H_2S as pro-resolutive mediators there are the inhibition of leukocyte adherence to the endothelium and the subsequent extravasation of leukocytes. These results have been shown in several models of inflammation, in which sulfide salts (i.e., NaHS) or other H_2S donors were able to inhibit the infiltration of neutrophils and lymphocytes (Zanardo et al., 2006; Wallace et al., 2012). There is also evidence that H_2S can trigger significant changes in macrophage function consistent with a shift to a pro-resolution phenotype (Dufton et al., 2011)

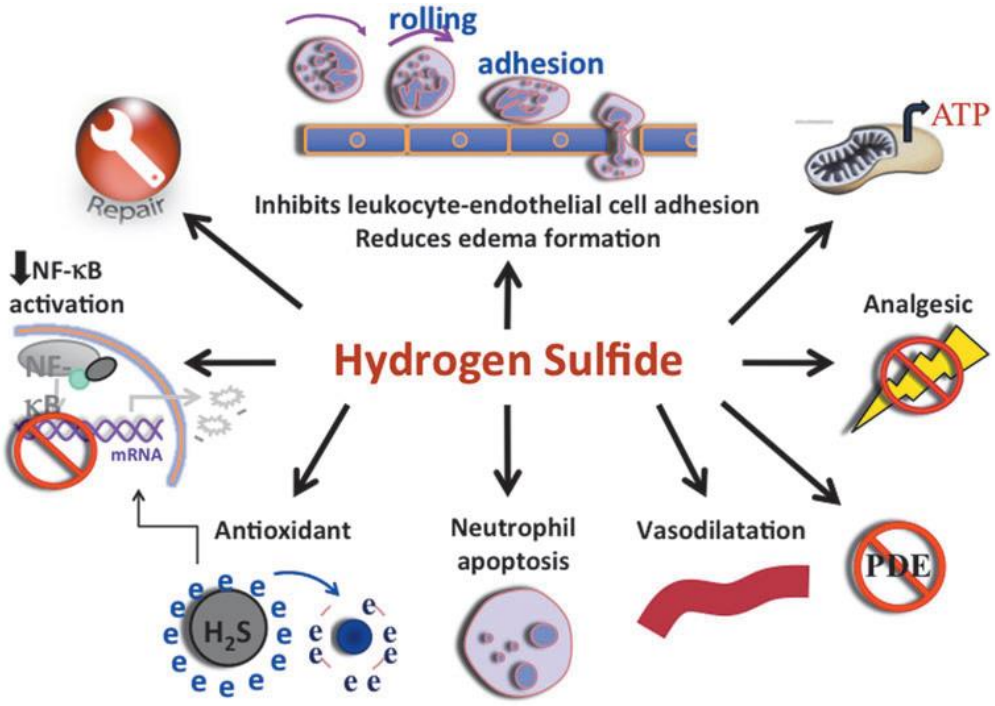


Fig.9 Anti-inflammatory effects of H_2S (Wallace et al., 2012)

Inhibition of nuclear transcription factor-kB (NF-kb) has been reported in numerous models, showing the potential ability of H_2S to reduce pro-inflammatory cytokine, chemokine, and enzyme (i.e., iNOS) expression (Wallace et al., 2012). For example, the inflammatory lungs saw decreased tissue IL-1β levels and increased IL-10 levels, and attenuated protein oxidation after Na_2S injection, a fast-releasing H_2S donor (Esechie et al., 2008). About the pro-resolutive action of H_2S, Brancaleone and co-workers demonstrated that its anti-inflammatory response involves activation of AnxA1 proresolutive pathway. Indeed, in inflammatory condition, AnxA1 cooperates with activity of CSE and H_2S pathway is affected by lack of function AnxA1 *in vivo* and *in vitro* model. It also markedly suppressed IL-1-induced leukocyte adhesion and emigration in mesenteric venules of wild type, but not AnxA1-deficient mice (Brancaleone et al., 2014).

Moreover, although many studies on NO-H_2S crosstalk in the vasculature have been observed, therefore investigations of this axis in inflammation have been less extensive. Indeed, also the interaction of NO with H_2S in inflammatory states could be significantly different from that which has been found for the vasculature (Lo Faro et al., 2014). Whiteman and colleagues studied the effect of a slow-releasing H_2S donor, GYY4137, on the LPS-induced production of inflammatory mediators on

RAW264.7 macrophages. GYY4137 dose-dependently inhibited the release of the pro-inflammatory mediators IL-1β, IL-6 and TNF-α and also inhibited NO production, via NF-kB inactivation. These authors demonstrated the dual effect of the sulfide salt NaHS, in that with low concentrations, the donor is able to inhibit the release of pro-inflammatory mediators. While, at higher concentrations, NaHS increased their production (Whiteman et al., 2010). In another study, the administration of NaHS reduced inflammation in a mouse model of viral myocarditis. The potential anti-inflammatory action is linked to the attenuation of inflammatory cell infiltration, reduction of cardiac edema and limitation of myocardial lesions. Therefore, the cardioprotective effects of H_2S are probably associated to NO over-production in inflammation and to increased expression of the protective enzyme HO-1. Indeed, NaHS reduced the levels of iNOS mRNA and increased HO-1 mRNA (Hua et al., 2013).

1.1.4.2. The AnxA1/FPR2 axis as a key regulator in the resolution of inflammation

Therefore, the resolution of inflammation, a coordinated process, requires the activation of endogenous program, also leading to the production of protein and lipid mediators (Vago et al, 2012). The biological properties of G protein-coupled receptors are crucial in the resolution of inflammation. Within this context, a fundamental role is played by the duo annexin 1 (AnxA1) and formyl peptide receptor (FPR) type 2, its receptor (Bena et al., 2012).

1.1.4.2.1. Annexin A1 (AnxA1)

AnxA1 is a 37 kDa phospholipid-binding protein, previously known as lipocortin 1, expressed in several tissues, including endothelial cells, leukocytes, lymphocytes and epithelial cells. Structurally it consists of a highly conserved core region, characterized from four 70-amino acid annexin repeats containing calcium and

phospholipid-binding sites with a unique 42-amino acid N-terminus (Hutchinson et al., 2011).

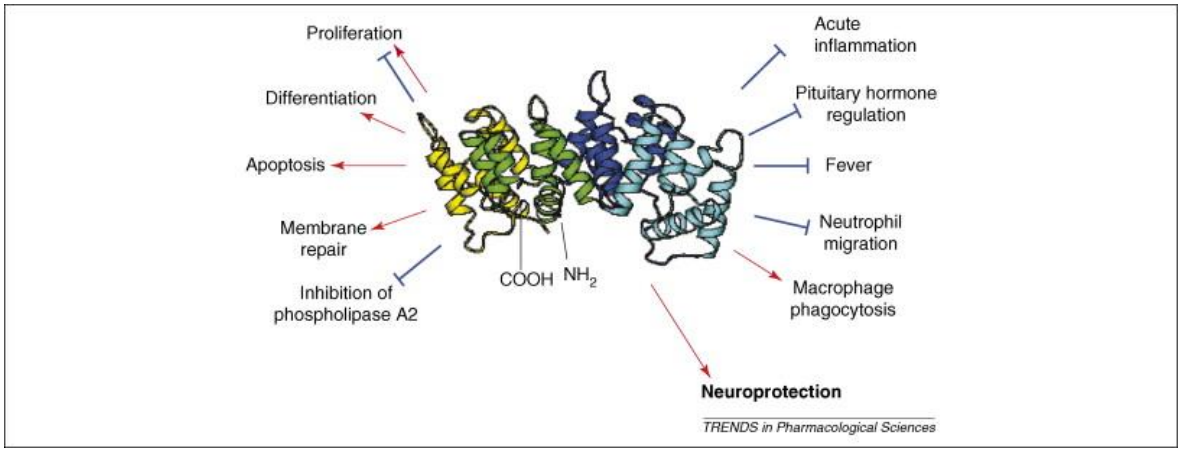


Fig.10 Structure and function of AnxA1 (Solito et al., 2008)

Furthermore, this type of protein can be present both intracellularly and at the membrane level, but can also secreted into the circulation (Purvis et al., 2019). Annexin A1 is a member of superfamily of 13 mammalian annexin proteins that bind acid phospholipids with high affinity in the presence of Ca^{2+} and a critical glucorticoid- (GC-) regulated protein, which contributes to the resolution through several ways (Perretti & D'Acquisto, 2009). In resting conditions, high levels of annexin A1 are contained in the cytoplasm of human and mouse peripheral blood cells, mainly neutrophils, monocytes, macrophages, eosinophils, mast cells and in minimal amounts in T-cells. Following cell activation (i.e., adhesion to endothelial-cell monolayers), intracellular AnxA1 is mobilized to the cell surface and secreted. Usually, the mechanisms, responsible of its secretion, are cell specific. Indeed, the protein can be externalized and/or secrets through several mechanisms. For example, in the neutrophil, annexin A1 is mainly stored in gelatinase granules and can be mobilized after an exposure of the cells to weak activating signals. Consequently, cell activation leads to the AnxA1 relocation to the outside leaflet of plasma membrane, to which it is bound in a calcium-dependent manner. Extracellular concentrations of $Ca^{2+} \geq 1$mM determine a conformation change in extracellular AnxA1 that leads to exposure of the N-terminal region, generating the active form of annexin A1 and binding to its receptor FPR2 (also known as FPR2), in an autocrine, paracrine and

juxtacrine manner. The last-mentioned could be the most reasonable mechanism of action in inflammatory conditions (Perretti & D'Acquisto, 2009).

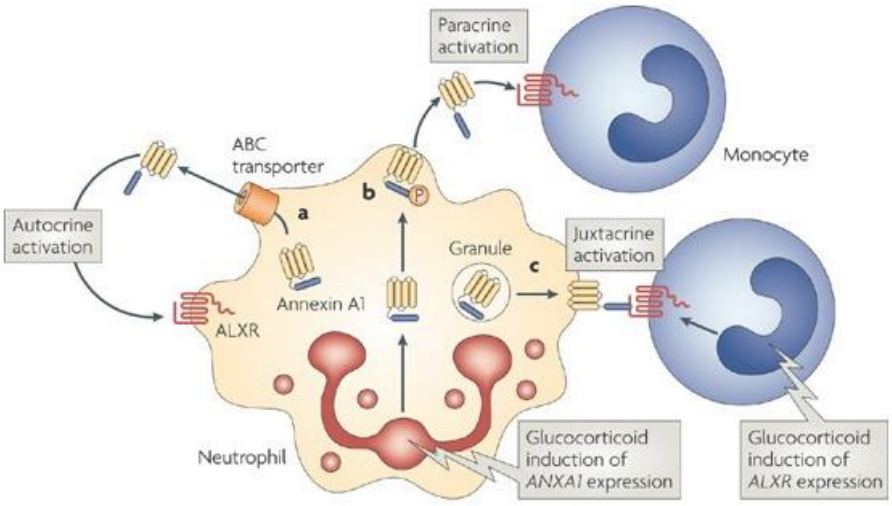


Fig.11 Mobilization of AnxA1 in activated cells (Perretti & D'Acquisto, 2009)

Perretti and D'Acquisto proposed that the AnxaA1 pathway is an important mediator of anti-inflammatory effects of glucocorticoids. Indeed, glucocorticoid administration to peripheral blood mononuclear (PBMN) cells increased annexin A1 expression in both a temporal and dose-dependent manner (Purvis et al., 2019). Several studies on the anti-inflammatory activity of this protein showed not only the different functions of AnxA1 are related to the N-terminal region, but also to the synthetic peptides from N-terminal domain, binding to FPR2 (Sugimoto et al., 2016). For example, Galvão and colleagues demonstrated that endogenous AnxA1 plays a fundamental role in promoting resolution of inflammation in a murine model of gout. Indeed, treatment with the AnxA1 peptide, $Ac_{2\text{-}26}$, decreased neutrophil recruitment and accumulation. Moreover, annexin A1 resolved acute gouty arthritis by inducing the apoptosis of neutrophils and leading the reduction of the IL1-β levels (Galvão et al., 2016). Rather, few studies reported the AnxA1 involvement in the context of intracellular parasite infection. Indeed, Oliveira and co-workers demonstrated that AnxA1 is expressed and can be required to control tissue inflammation in L. *braziliensis* infection BALB/c mouse model. Indeed, the levels of this protein increased the peak of tissue lesion and

parasitism in infected mice. Furthermore, it also increased after the infection of BALB/c bone marrow-derived macrophages (Oliveira et al., 2017). In the context of the discovery of new mechanisms of AnxA1, involved in the pro-resolution effects, Lima and colleagues demonstrated that rolipram (ROL), a specific phosphodiesterase-4 (PDE4) inhibitor that increases the cAMP levels, influences resolution of neutrophilic inflammation, associated with the rise of AnxA1 accumulation in inflammatory cells (Lima et al., 2017). Another study showed that high density lipoprotein (HDL)-induced AnxA1 inhibited cell surface VCAM-1, ICAM-1 and E-selectin, and secretion of MCP-1, IL-8, VCAM-1 and E-selectin in TNF-α-activated endothelial cells, thereby inhibiting monocyte adhesion (Pan et al., 2016). Furthermore, Brancaleone and coworkers investigated whether endogenous AnxA1 could modulate H_2S biosynthesis *in vitro* and *in vivo* model. Indeed, exogenous H_2S response involved activation of the AnxA1 pro-resolutive pathway (Brancaleone et al., 2014).

1.1.4.2.2. Formyl peptide receptor type 2 (FPR2)

In humans, the biological effect of Anxa1 and its mimetic peptides are mediated through a G-protein-coupled receptor, termed lipoxin A4 receptor/formyl peptide receptor type 2 (ALX/FPR2), so called because it also conveys the inhibitory signals induced by lipoxin A4 and resolvin D1 (Serhan et al., 2005; Senchenkova et al., 2019).

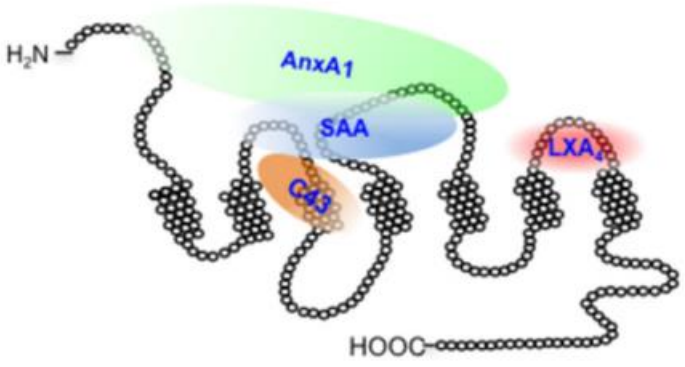


Fig.12 FPR2/ALX specific domains for ligand activation (Bena et al., 2012)

The FPR family, which includes FPR1, FPR2 and FPR3, is involved in immune response against pathogens or microbes. They are linked to inhibitory G proteins so that their activation leads to transient calcium fluxes, ERK phosphorylation, and in several cases cell locomotion (Dufton et al., 2010). In particular, FPR2 is a 7-transmembrane domain-, multifunctional G-protein-coupled receptor expressed on phagocytic leukocytes, vascular endothelial cells and fibroblasts (Lupisella et al., 2022). Furthermore, ALX/FPR2 can bind several peptides, proteins or lipids. Indeed, the ALX/FPR2 signaling pathway involves both pro-inflammatory and pro-resolution effects, depending on the ligand (Liu et al., 2020).

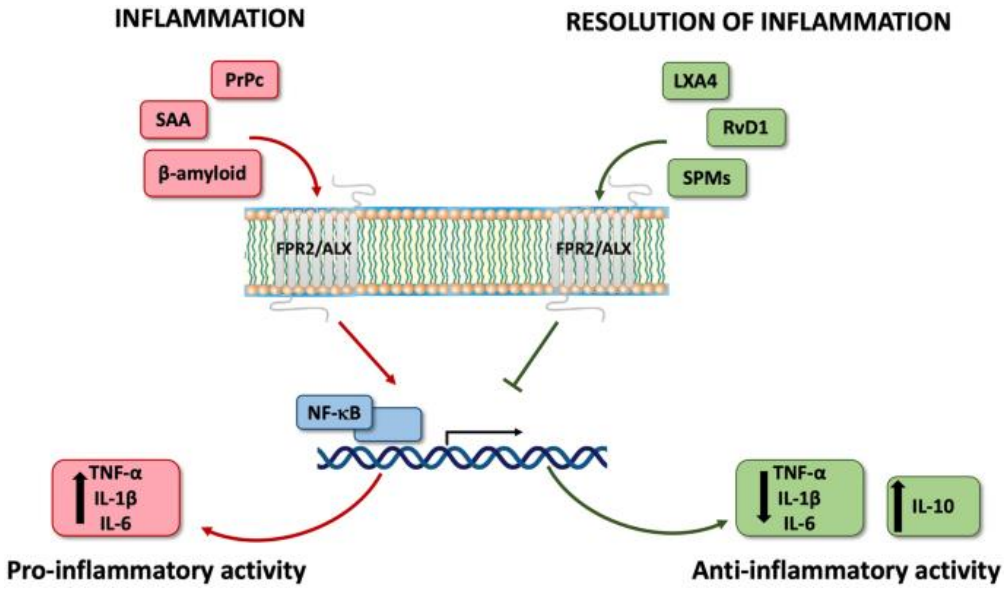


Fig.13 Pro- and anti-inflammatory activity of FPR2/ALX (Tylek et al., 2021)

The nature of FPR2 has remained unclear, despite data indicating its pro-inflammatory properties following receptor activation or other studies showing anti-inflammatory activities upon activation on target cells and tissues (Perretti & Godson, 2020). After binding of the ligand, FPR2 is activated and triggers several agonist-dependent signal transduction pathways, leading the activation of phospholipase A2 (PLA2), phospholipase C (PLC) isoforms, protein kinase B (Akt), phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), mitogen-activated protein kinase (MAPK) pathway (Tylek et al., 2021). Therefore, the nature of responses downstream of FPR2 activation is more complex, although the receptor could be defined as a master switch in promoting in the resolution of inflammation (Perretti & Godson, 2020). For

example, Hayhoe and co-workers demonstrated that blocking FPR2/ALX with a monoclonal antibody prevented AnxA1/Ac2-26 induced inhibition of human neutrophil transmigration and adhesion to the endothelial-cell monolayers under flow conditions (Hayhoe et al., 2006). Interestingly, Brancaleone and colleagues demonstrated the existence of an anti-inflammatory network centred on FPR2/ALX that operates to regulate PMN activation and trafficking in the microcirculation, though the activation and the mobilization of AnxA1 (Brancaleone et al., 2011). Moreover, Vital and co-workers showed that targeting the AnxA1/FPR2/ALX pathway can represent an interesting treatment strategy for resolving thrombo-inflammation, preventing the stroke in high-risk patient cohorts. This was possible using two distinct models of pathological thrombo-inflammation, lipopolysaccharide (LPS) and sickle transgenic mice (STM) (Vital et al., 2020). At the same way, Senchenkova and colleagues investigated that the lack of AnxA1 could lead to platelet recruitment and aggregation in the brain of the mice after ischemic stroke. Therefore, the exogenous administration of AnxA1, through its interaction with FPR2, can be a crucial pro-resolving mediator of thrombo-inflammation (Senchenkova et al., 2019). Moreover, Ni and co-workers evaluated the roles of AnxA1 and FPR2 in a mouse model of Streptococcus suis meningitis induced via intracisternal infection in FPR2 $^{-/-}$ and WT mice. Indeed, their data showed that AnxA1 determined several anti-inflammatory effects through its receptor (i.e, inflammatory mediator production, attenuation of leukocyte infiltration, and astrocyte or microglial activation in the brain. Of note, they demonstrated that AnxA1 is able to decrease neutrophil adhesion to the activated endothelium mainly through FPR2 (Ni et al., 2021). Furthermore, the crosstalk between AnxA1 and FPR2/ALX regulates ERK/MAPK signalling pathway which affects the activities of the transcription factors AP1, NF-kB and NFAT, leading to several T cells activities and exerting anti-inflammatory effects, in contrast to the regulative effects of glucocorticoids on T cell receptors (TCR) (Perretti & D'acquisto, 2009; Shao et al., 2019).

1.1.4.3. Specialized pro-resolving lipid mediators (SPMs)

Several studies have demonstrated that a series of endogenous lipid mediators produced during the resolution of inflammation can activate different mechanisms. Indeed, these mediators can bind to specific receptors, inhibit neutrophil infiltration, regulate the formation of cytokines and chemokines, and thereby promote extensive phagocytosis (Levy et al., 2001; Serhan et al., 2015; Yang et al., 2021). Novel endogenous lipid mediators, which can eliminate apoptotic cells and promote tissue repair, were identified as "specialized pro-resolving lipid mediators" (SPMs). They include resolving, protectin, maresins and lipoxins (LXs) (Yang *et* al., 2021). These lipid mediators are derived from polyunsaturated fatty acids (PUFAs) and include metabolites of arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) derivates (Homann et. al, 2014). Lipoxins are derived from endogenous fatty acids (arachidonic acid), while resolvins, protectins and maresins are derived from dietary fatty acids; in particular, the ω-3 fatty acids found in fish oil.

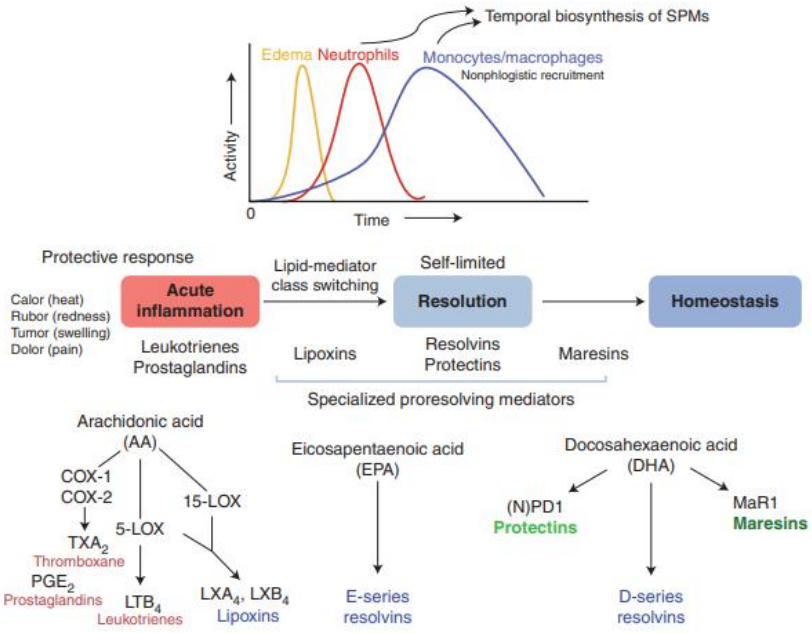


Fig.14 Lipid-mediator biosynthesis in exudate traffic in resolution of acute inflammation (Serhan *et* al., 2015).

In general, these mediators stimulate and accelerate resolution via multifactorial mechanisms at the tissue level (Freire & Van Dyke, 2013). Indeed, these mediators counteract the extent and regulate the pace of inflammatory response at several critical cellular events.

Mediator	Target cell	Action(s)	Refs
Lipoxin A_4	Neutrophil	Inhibits chemotaxis, *trans*-endothelial and *trans*-epithelial migration	140, 141
		Inhibits neutrophil–epithelial cell interactions	75, 77, 140
		Inhibits superoxide anion generation and degranulation	142,144
	Monocyte	Stimulates chemotaxis and adhesion	145
		Inhibits peroxynitrite generation	146
		Reduces IL-8 release by cells from individuals with asthma	147
	Macrophage	Increases engulfment of apoptotic neutrophils	62
	Eosinophil	Inhibits migration and chemotaxis	148
		Inhibits generation of eotaxin and IL-5	112
	NK cell	Inhibits NK cell cytotoxicity	149
		Increases granulocyte apoptosis	67
	ILC2	Inhibits IL-13 release	67
	Dendritic cell	Inhibits IL-12 production	110
	Epithelial cell	Increases proliferation after acid injury, blocks IL-6 and IL-8 release	77
	Endothelial cell	Stimulates PKC-dependent prostacyclin formation	150
		Blocks the generation of reactive oxygen species	151
		Inhibits VEGF-induced endothelial-cell migration	152
	Fibroblast	Inhibits IL-1β-induced IL-6, IL-8 and MMP3 production	153
		Inhibits CTGF-induced proliferation	154
	Smooth muscle	Inhibits LTC_4-initiated migration	155
Resolvin E1	Neutrophil	Inhibits *trans*-epithelial and *trans*-endothelial migration	156
		Inhibits superoxide generation	87
	Macrophage	Stimulates non-phlogistic phagocytosis of apoptotic neutrophils	30
	Dendritic cell	Inhibits IL-12 production	157
		Inhibits migration	41
	NK cell	Expresses CMKLR1 receptors	67
Resolvin E3	Neutrophil	Inhibits infiltration	158
Resolvin D1	Neutrophil	Inhibits transmigration	159
	Macrophage	Inhibits LPS-induced TNF release	160
		Increases phagocytosis of allergen and apoptotic cells	9,63
Protectin D1	Neutrophil	Inhibits TNF and IFNγ release	161
		Inhibits PMN transmigration	11
		Upregulates CCR5 expression	72
	Macrophage	Stimulates non-phlogistic phagocytosis of apoptotic PMNs	30
Maresin 1	ILC2	Inhibits IL-13 production and stimulates amphiregulin production	70
	Regulatory T cell	Induces regulatory T cell formation and stimulates amphiregulin production	70
	Bronchial epithelial cell	Inhibits organic dust-induced cytokine production	162

Table 4. SPMs display cell-type specific actions (Basil & Levy, 2016)

For example, the biosynthetic pathways required for lipoxin and resolvin formation are determinate via interactions of inflammatory leukocytes with other kind of cells such as epithelial cells, macrophages, endothelial cells and platelets, that constitute an inflamed tissue (Bannenberg & Serhan, 2010). Indeed, several studies showed that the vasculature is capable of endogenous production of SPMs to balance acute inflammation. In the cardiovascular system, endothelial cells (ECs) interact with a number of SPMs, such as LXA_4, PD1, MaR1, RvD1 and RvD2. The subsequent effects lead to the reduction of proinflammatory cytokines, adhesion molecule expression, and leukocyte-EC interaction, regulated by different SPMs receptors, such as ALX/FPR2, DRV1/GPR32, DRV2/GPR18, and ERV1/ChemR23 (Kim & Conte, 2020). Vascular smooth muscle cells (vSMCs) also play a critical role in cardiovascular disorders. Usually, vSMCs exhibit low rates of proliferation, migration, secretion of proinflammatory factors and apoptosis, in normal blood vells. However, the increased of these mechanisms contribute to cardiovascular diseases, typical of some disorders such as atherosclerosis and restenosis (Mill & George, 2012). Some studies on *in vitro* model showed that the treatment with RvD1, RvD2, and Mar1 reduce leukocyte-vSMC interactions, through the downregulation of cell adhesion molecules and other proinflammatory genes. At the same way, the treatment with other specific SPMs, such as RvD1, RvD2, RvE1 and LXA_4 attenuate vSMC migration and proliferation (Kim & Conte, 2020). Moreover, about the macrophages, pro-resolving lipid mediators also encourages transition to the M2 phenotype, usually associated to clearance of apoptotic cells, wound healing and tissue repair. Finally, neutrophil and platelet recruitment are essential events of any acute inflammatory response. Also in this case, the treatment with resolvins reduces neutrophil recruitment, while the treatment with different SPMs, such RvD1, RvE1, MaR1, determine different positive effects on platelet activity and function (Kim & Conte, 2020).

Cell Type	*SPMs studied*	*Effects*
Endothelial Cell (ECs)	RvD1, RvD2, Mar1, LXA_4, PD1	reduction of proinflammatory cytokines, adhesion molecule expression, and leukocyte-EC interaction
vSMCs	RvD1, RvD2, RvE1, MaR1, LXA_4	Reduced proliferation, migration, vasoconstriction
Platelets	RvD1, RvE1, MaR1	Enhanced hemostasis, reduced aggregation and inflammatory cytokine secretation
Macrophages	RvD1, RvD2, AT-RvD1, AT-RvD3, AT-PD1, AT-LXB_4, MaR1	Enhanced M2 polarization, efferocytosis
Neutrophils	Resolvins	Reduced NET release, recruitment

Table 5. Different effects of SPMs on various cardiovascular and inflammatory cell types (Kim & Conte, 2020)

These types of interaction permit gauging the number and activation of inflammatory leukocytes that participate in the inflammatory response (Bannenberg & Serhan, 2010). Therefore, SPMs have effects on a wide range of physiological and pathophysiological processes. In addition, some of them, such as resolvins, protectins and maresins are potent regulator of pain (Homann et al., 2014). Indeed, many preclinical and clinical evidence supports the role of these lipid mediators in neuroinflammation associated with chronic pain (CP) and long-term spinal potentiation (LTP), through the modulation of microglia, the regulation of nociceptors, or the regulation of the neuronal pathways involved in pain (Cháves-Castillo et al., 2021). While, resolvins, as well as protectins, were shown to have anti-infectious properties (Chiang et al., 2012). Indeed, many researchers speculated that SPMs are able to have therapeutic effects against the sequelae of SARS-CoV-2 infection. Indeed, the use of SPMs is likely to help regulate abnormal viral-mediated inflammation and prevent complications such as SARS-CoV-2 cytokine storm, typical of this infectious disease (Lee, 2021).

1.1.4.3.1. Lipoxins (LXs)

Lipoxins (LXs) were the first family of mediators identified in vivo with anti-inflammatory and pro-resolving actions (Serhan et al., 2008). Although lipoxins are present in low amount during the initiation of acute inflammation, their level increases during the resolution (Barning & Humbert, 2015). Derived from arachidonic acid, lipoxins have potent anti-inflammatory and resolution actions (Samuelsson et al., 1987; Serhan et al., 1995; Serhan et al., 2000; Freire & Van Dyke, 2013). LXs can be synthesized by three major routes from arachidonic acid through three major lipoxygenases: 15-LOX, 5-LOX and 12-LOX (Serhan & Sheppard, 1990; Serhan *et* al., 2015; Chandreasekharan & Sharma-Walia, 2015; Yang *et* al., 2021).

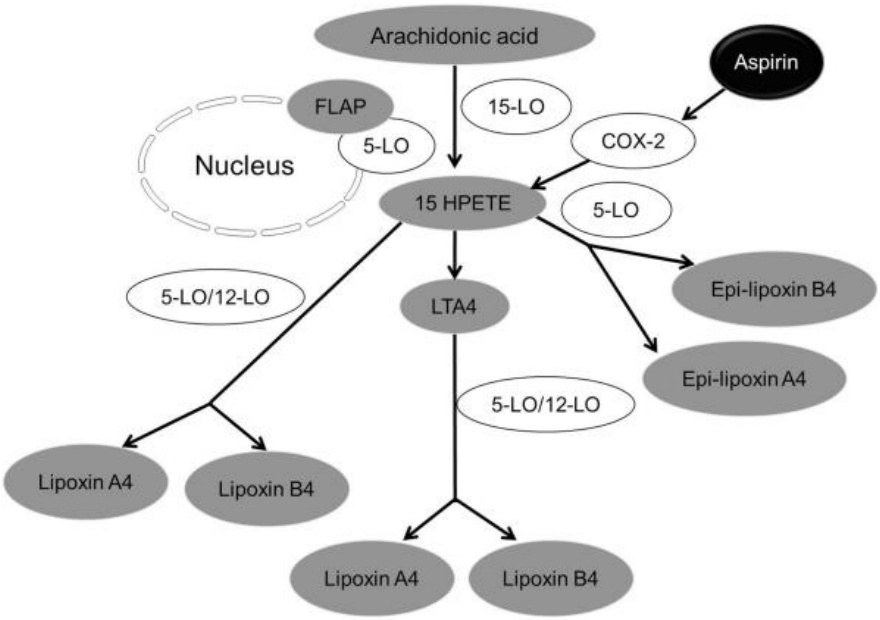


Fig.15 Synthesis of lipoxins (Chandrasekharan & Sharma-Walia, 2015)

Indeed, three main pathways of lipoxin synthesis have been identified. In the first pathway, sequential oxygenation of arachidonic acid by 15-lipoxygenase and 5-lipoxygenase, followed by enzymatic hydrolysis, leads to the production of lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4) (Levy et al., 2001; Freire & Van Dyke, 2013). In the second pathway, 5-lipoxygenase biosynthesizes lipoxin A_4 and 12-lipoxygenase in platelets produces lipoxin B_4. Finally, a third pathway is triggered by aspirin, that

promotes the acetylation of cyclooxygenase-2, leading to a change in cyclooxygenase action. There are several cells that express cyclooxygenase-2, such as macrophages, neutrophils, epithelial cells and endothelial cells (Freire & Van Dyke, 2013). *In vivo* biosynthesis of LXA_4 is triggered in an acute inflammatory process in which polymorphonuclear neutrophil (PMN)'s interaction with PGE_2 and PGD_2 activates 15-lipoxygenase, facilitating LXA_4 biosynthesis (Claria & Serhan, 1995). Moreover, the formation of LXs is preserved across a wide range of animal species, from fish to humans (Levy, 2005; Lee, 2021).

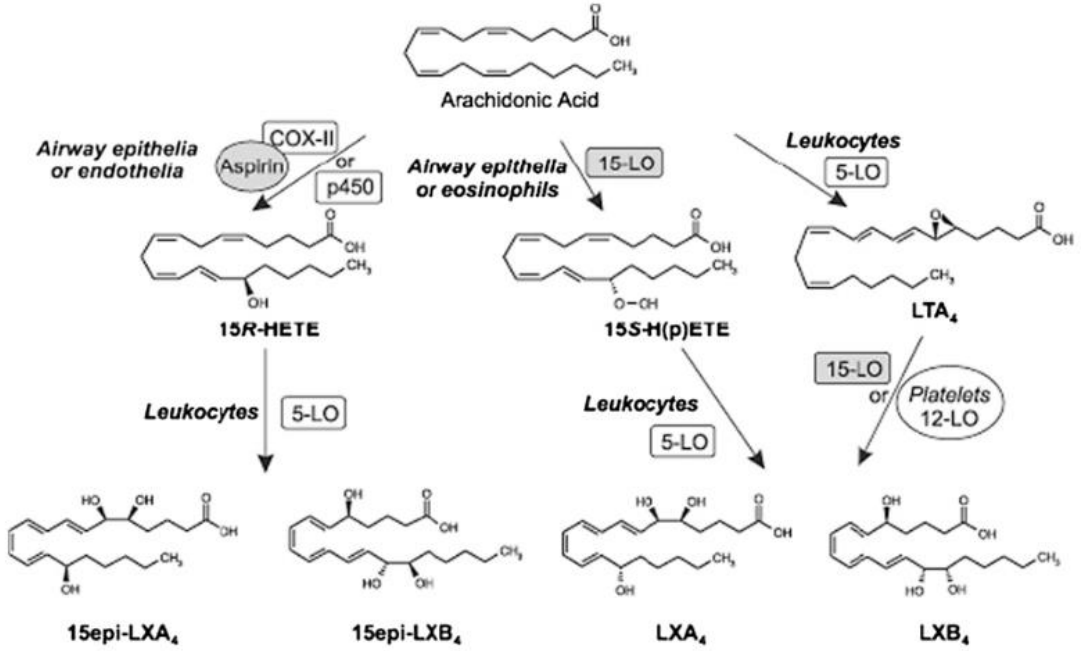


Fig.16 The metabolism of arachidonic acid to various lipoxins (Das, 2013)

Considering LXA_4 as an example, during the initial stages of inflammation, cells release the precursor protein AnxA1 of LXA_4, which is an agonist of ALX/FPR2 receptor. Usually, the latter is expressed from immune cells such as neutrophils to endothelial and epithelial cells (Barning & Levy, 2015; Jeong & Bae, 2020). ALX/FPR2 triggers the calcium-ion channels in cells to accelerate influx of calcium ions (Ca^{2+}). Consequently, the phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and phospholipase A2 pathways become activated, promoting cell-surface phosphorylation FPR2/ALX receptor and binding to LXA_4. In this way, FPR2/ALX receptor can induce cells to produce large amounts of

interleukin (IL)-10 and promote the regression of inflammation (Wu *et* al., 2019; Yang *et* al., 2021).

1.1.4.3.2. Resolvins (Rvs)

Resolvins (Rvs) were first discovered in the exudates of mice during inflammation resolution (Serhan *et* al., 2008). The term resolvins, resolution phase interaction products, was introduced to signify that the new unique structures, with temporal code in biosynthesis during resolution, are endogenous, local-acting mediators possessing potent anti-inflammatory and immunoregulatory properties (Serhan *et* al., 2002). Indeed, some studies showed that, in resolving inflammatory exudates, cell-cell interactions lead to the biosynthesis of active signals that limit neutrophil recruitment to the tissue and enhance the engulfment of apoptotic neutrophils by macrophages (Serhan et al., 2015). It is possible to identify two main resolvin groups, characterized from distinct chemical structures: E-series and D-series, produced from marine oils that enter humans via nutrition or supplementation (Calder, 2017). E-series and D-series are derived from omega-3 polyunsaturated fatty acids in form of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which combine with G protein-coupled receptors (GPCRs) and promote the elimination of bacteria and efferocytosis (Basil & Levy, 2015; Yang *et* al., 2021). Furthermore, based on the different unsaturated fatty acids derived from it, EPA is converted to resolvin E1 (RvE1) and resolvin E2 (RvE2) by 5-lipoxgenase (LOX) and resolving E3 by 15-LOX; while, DHA is converted to resolvin D1 (RvD1 by cyclooxygenase (COX)-2/aspirin/15-LOX (Ueno *et* al., 2019; Yang et al.,2021).

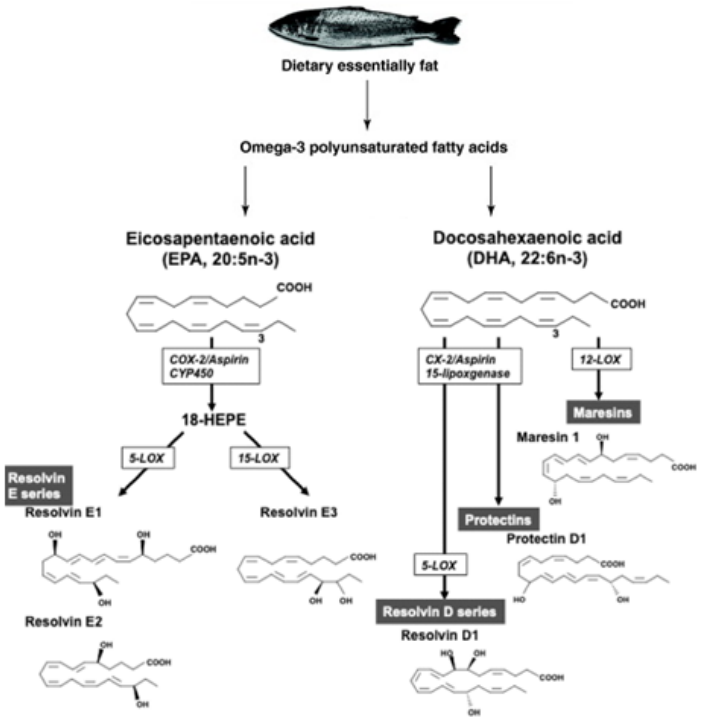


Fig.17 Omega-3 polyunsaturated fatty acids and specific pre-resolving lipid mediators (Ji et al., 2011; Ueno *et* al., 2019)

For example, in the vasculature, RvE1 transcellular synthesis in the presence of aspirin is notable for transformation of EPA to 18(R)-hydroxyEPA(18(R)-HEPE) by aspirin-acetylated COX-2 in endothelial cells and 18(R)-HEPE conversion to RvE1 by leukocyte 5-LOX (Basil & Levy, 2015). Several studies demonstrated that RvE1 mainly mediates the migration and activation of monocyte-macrophages system by binding with two types of receptors, ChemR23 and LTB4 receptor 1 (BLT1) (Arita *et* al., 2007; Ohira *et* al., 2010; Pirault & Bäck, 2018). Furthermore, other studies showed that RvD1 helps to promote inflammation by interacting with FPR2/ALX and GPR32 receptors on the cell surface (Basil & Levy, 2016).

1.1.4.3.3. Protectins and Maresins

Additional families of pro-resolution mediators derived from DHA have also been identified in resolving inflammatory exudates that display protective bioactivities,

namely protectins and maresins (Hong et al., 2003; Serhan et al., 2009). Protectin D1, neuroprotection D1 when generated by neural cells, is a member of the protectin family, generated from docosahexaenoic acid (Serhan et al., 2006). In particular, in this case, the lipoxygenase-mediated pathway converts DHA into a 17S-hydroxyperoxide-containing the intermediate that is taken up, by leukocytes, and converted into 10,17-Dihydroxydocosahexaenoic, known as protectin D1 or neuroprotectin (Freire & Van Dyke, 2013). Its production is regulated by human leukocytes, macrophages and eosinophils and appears reduced in exhaled breath condensates during acute exacerbations of asthma (Serhan & Levy, 2018). Furthermore, PD1 is also produced by human peripheral blood lymphocyte with a T-helper 2 phenotype. It reduces interferon gamma (INF-γ) and tumor necrosis factor alpha (TNF-α), promotes T-cell apoptosis and blocks T-cell migration (Anderson & Delgado, 2008). In addition, several studies also showed the protective actions of DHA-derived 10,17-docasatriene in animal models of stroke and Alzheimer's disease (Marcheseli et al., 2003; Lukiw et al., 2005). Another novel SPMs family, deriving from dietary fatty acids, in particular ω-3 fatty acid, are maresins (MaR), discovered more recently. These specialized mediators stimulate and accelerate resolution via multifactorial mechanisms at the tissue level. Furthermore, this family mainly regulates the inflammatory response by limiting neutrophil migration and stimulates macrophages phagocytosis (Yang et al., 2021). Biosynthesis of maresins starts in M2 macrophages and is initiated by an epoxygenation reaction. In general, Marenin 1 (MaR1) and maresin 2 (MaR2) are able to have a protective effect in inflammation, oxidative stress and immune diseases as protective mediators of macrophage function. Furthermore, maresins can protect the human body through several mechanisms such as limiting neutrophil infiltration, enhancing macrophage phagocytosis, reducing the production of pro-inflammatory factors, stimulating tissue regeneration and controlling pain (Ferreira et al., 2022). MaR1 exerts stereospecific leukocyte-directed actions, characteristic of the maresin family. In addition, this lipid mediator also exerts potent cancer-induced antinociceptive and tissue regenerative actions in wound healing in planaria (Serhan et al., 2015).

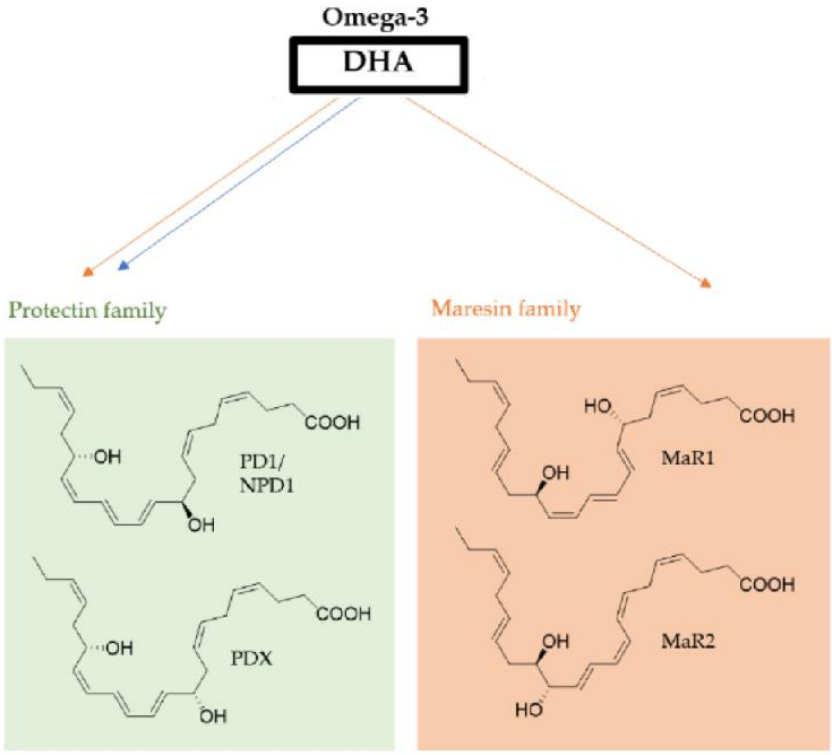


Fig.18 Structure of the SPMs protectins and maresins (Ferreira et al., 2022)

1.2. Aims

Inflammatory process represents a complex network of events regulated by engagement of a series of mediators, included protein, gaseous and lipid mediators.

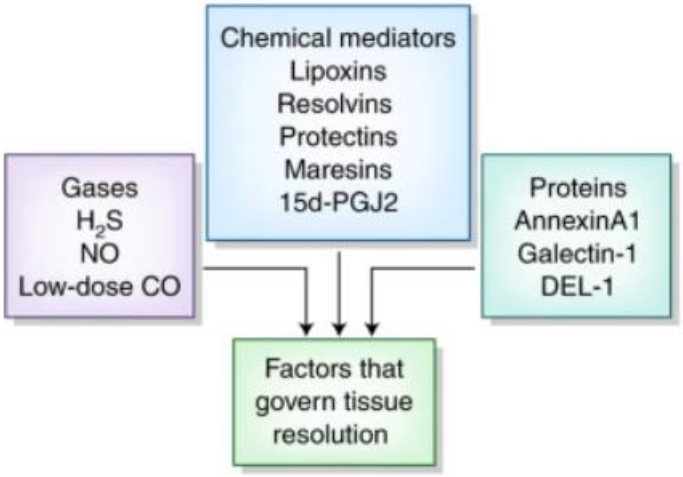


Fig.19. The main factors governing the resolution of inflammation (Fredman, 2019)

However, the interest in assessing mechanisms of the pro-resolution pathways have mainly been focused on immune system and leukocyte actions at the interface with endothelium. Conversely, the role of anti-inflammatory mediators involved in the resolution of inflammation, produced by endothelial cells, is not clear. Indeed, for instance, NO and H_2S pathways are able to interact, however such a cross-talk has not been sufficiently delineated in vascular inflammation. In addition, the role of AnXA1 has not been exploited in endothelial cells with respect to vascular function homeostasis. In particular, we expected to show the pathway involved within the ECs could modulate one or the other mediator, as it occurs in neutrophils. Therefore, these aspects lead us to evaluate multiple objectives. Especially, one of the aims of my PhD research project was to better define the cross-talk between NO and H_2S in terms of mechanisms and pathway involved in inflammatory conditions associated to vascular disease. In particular, we had the idea that NO and H_2S are somehow interchangeable, so, if one fails, the other may act as back up and take the control. The second objective was to determine the role of pro-resolution AnxA1/FPR2 axis in vascular function with respect to cardiovascular homeostasis, involved usually in the communication between blood cells and endothelial

surface. Change (increase or reduction) in their expression is very feasible and sometimes small changes could have big impact on tissue homeostasis, particularly on vascular reactivity. Thus, AnxA1 and FPR2 could possibly represent possible target that can be used (agonists or antagonists) to modulate vascular function together with inflammation. Finally, the last aim of this research project was to evaluate how the NO/H_2S interplay is active in inflammatory condition and whether it cooperates with AnxA1/FPR2 axis. Indeed, we expected to find some kind of interconnection between them all, including the specific pro-resolving lipid mediators (SPMs), to set a series of possible targets (direct or intermediate) to control alterations driven by inflammatory processes at vascular levels.

2. Materials and Methods

2.1. Bovine aortic endothelial cells (BAEC)

2.1.1. BAEC treatment

Bovine aortic endothelial cells (BAEC) were cultured in DMEM and used at passage 5 to 10, without any differences in cell response. Following overnight starvation, BAEC underwent normoglycemic (NG, 25mM) or hyperglycaemic (HG, 50mM) conditions for 3h and then challenged with calcium ionophore A23187 (3μM, 30 minutes, Sigma-Aldrich, Milan, Italy). Cells were grown until they reached 80% confluence and then plated for the experiments. AP123 or AP39 (Fig.20) (0.01nM-100nM) were administered at time 0 (t=0, same time as hyperglycaemia induction) or 1hr after hyperglycaemia induction (t=1). However, all experiments using AP123 were performed by using the 1h post-HG protocol. In a further series of experiments, BAEC cultured in HG conditions were also pre-incubated with PKA inhibitor KT5720 (1μM) or PI3K inhibitor wortmannin (100nM) 30 minutes before the addition of AP123 (10nM). Then, the supernatants and cell pellets were collected for NOx levels determination and western blot analysis, respectively.

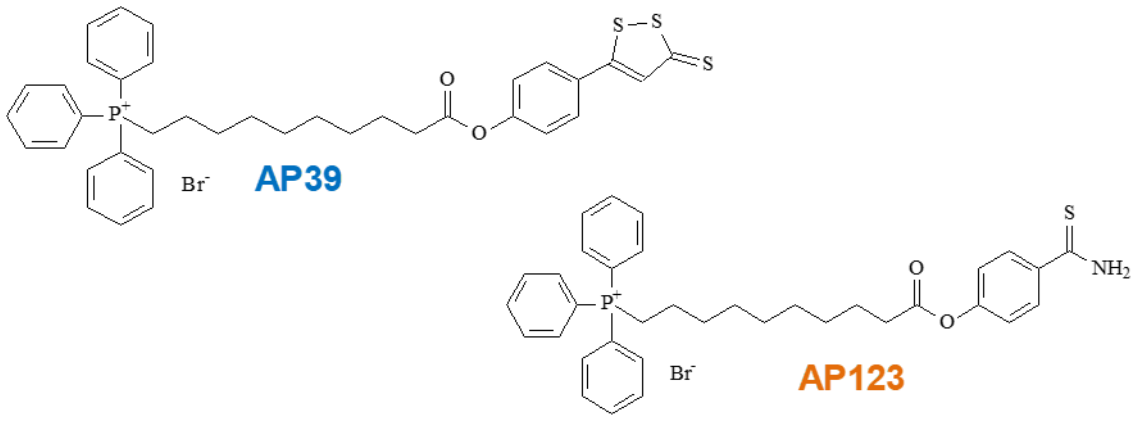


Fig.20 Chemical structures of AP39 and AP123

In inflammatory setting, obtained by administration of TNF-α (10ng/mL, 6h), endothelial cells were studied in terms of expression of CSE, eNOS, AnxA1 and FPR2 in presence

of FPR2 agonist Ac2-26 (Fig.21) (0.1 and 1µM) or H_2S donor AP123 (1 and 10nM). After 6h of treatment, both supernatants and cell pellets were collected to perform specific molecular analysis.

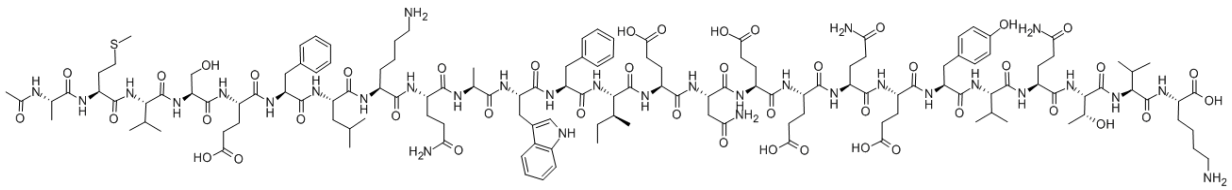


Fig.21 Ac2.26 structure

2.1.2. NOx determination

NOx (nitrite/nitrate) levels quantification was carried out in supernatant of BAEC undergone different treatments. The samples were added with 0.49M NH_4Cl and 0.06M $Na_2B_4O_7$ (3:1) in presence of elementary cadmium to reduce nitrate to nitrite for 2 hours at room temperature. After incubation, samples (160µl) were centrifuged and added with 10µl solution of 2,3-diaminonaphtalene (DAN, 0.05mg/mL) for 7 minutes in the dark and then 5µl of NaOH 2.8N were added in each sample to stop the reaction. Total nitrite levels were determined by fluorometric measurement of each sample (Ex. 365nm, Em. 450nm) against a calibration curve obtained with $NaNO_2$ (50nM-2µM).

2.1.3. Western blot analysis

BAEC pellets or aorta segments were homogenized in modified RIPA buffer (Tris-HCl 50mM, pH 7.4, Triton 1%, Na-deoxycholate 0.25%, NaCl 150mM, EDTA 1mM), added with protease inhibitor cocktail (Sigma-Aldrich). After centrifugation of homogenates at 6500g for 10min, 60µg of the denatured proteins were resolved on 10% SDS-PAGE gels and transferred to a polyvinylidene fluoride (PVDF) membrane. Unspecific binding on the membranes was minimized by using blocking buffer solution (phosphate-buffered saline, PBS, containing 0.1% v/v Tween-20 and 3% non-fat dry milk, 1h incubation), The incubation of membranes was carried out overnight at 4°C with following primary antibodies: mouse monoclonal anti-eNOS (1:1000, BD Biosciences, Milan, Italy), mouse

monoclonal anti-CSE (1:1000, Proteintech), mouse monoclonal anti-cAMP response element-binding protein (CREB, 1:1000, Cell Signaling, Leiden, NL), mouse monoclonal anti-p-CREB (1:1000, Cell Signaling, Leiden, NL), mouse monoclonal antibodies antihuman AnxA1 (1:1000; clone 1B), mouse monoclonal antibodies anti-FPR2 (1:2000; GeneTex) and mouse monoclonal β-actin (1:1000, Sigma-Aldrich). The filters were therefore washed in PBS containing 0.1% v/v Tween 20, before incubation for 2h with anti-mouse horseradish peroxidase-conjugated secondary antibody. Membranes were washed and developed using Enhanced Chemiluminescence Substrate (ECL; Amersham Pharmacia Biotech, San Diego, CA, USA). Images for western blot have been obtained by using Chemidoc System (Bio-Rad, Milan, Italy). Band intensity has been analysed by using ImageJ software and optical density (arbitrary units) have been reported.

2.2. Fibroblast-like synoviocyte cell line (K4IM)

2.2.1. K4IM treatment

With respect to inflammatory process, we also evaluated the role of H_2S in some anti-inflammatory properties associated to clodronate (Clo) (Fig.22), a bisphosphonate drug used in the treatment of osteoporosis. Immortalised non-aggressive fibroblast-like synoviocyte cell line (K4IM) were grown in T-25/T-75 flasks containing Iscove Modified Dulbecco's Medium (DMEM), supplemented with 10% heat-inactivated FCS, 1% penicillamine/streptomycin and 1% L-glutamine. This cell line was established from primary fibroblast-like synoviocyte cells by immortalization with SV40 T antigen47. Cells were cultured and splitted 1 to 5 every week and used starting from passage 4 to 6. Cells were treated with Clo at different concentration (0.1–10 μM, 6–30 h) following administration of TNF-α (10 ng/mL, 6 h), used as inflammatory stimulus. The effect of Clo on K4IM was addressed by comparing the effects to vehicle treated cells undergoing TNF-α stimulation only. Experiments were repeated at least three times in duplicates.

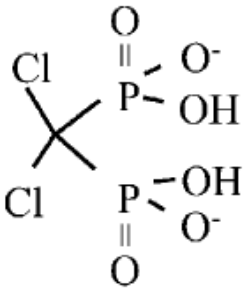


Fig.22 Clodronate structure

2.2.2. Western Blot analysis

Cell pellets obtained from each experiment were mechanically homogenised in lysis buffer (RIPA supplemented with protease inhibitors cocktail) and total amount of protein was determined by using Bradford assay. Denatured proteins (60 μg) were separated on 10% sodium-dodecylsulfate polyacrylamide gels and transferred to polyvinylidene fluoride membrane (PVDF). Membranes were blocked in phosphate-buffered saline containing 0.1% v/v Tween-20 (PBST) and 3% w/v non-fat dry milk for 30 min, followed by overnight incubation at 4 °C with primary antibodies: mouse monoclonal anti-CSE (1:1000, Sigma-Aldrich) or mouse monoclonal anti-tubulin (1:5000, Sigma-Aldrich). Membranes were extensively washed in PBST prior to incubation with horseradish-peroxidase conjugated secondary antibody for 2 h at room temperature. Following incubation, membranes were washed and chemiluminescence was detected by using ImageQuant-400 (GE-Healthcare). The target protein band intensity was normalised against housekeeping protein α-tubulin.

2.2.3. H_2S determination

H_2S levels have been determined by using a fluorometric assay based on SF7AM specific fluorochrome able to detect H_2S. Briefly, for what concerns cell supernatants, samples were diluted 1 to 4 in RIPA buffer and put in a 96-well black flat bottom plate. Then, SF7AM was added (10 μM) and plate was incubated a 37 °C under shaking conditions for 90 min to allow the fluorophore to quantitatively quench H_2S and give an established

fluorescence signal. In addition, H_2S biosynthesis has also been measured, by using a different approach. Briefly, cell lysates obtained in RIPA buffer (0.5 mg/mL) have been incubated in 96-well black flat bottom and SF7AM was added (10 μM). Plate was incubated a 37 °C under shaking conditions for 90 min to allow for H_2S biosynthesis and accumulation. In both cases, reading was performed (Ex 475 nm, Em 500–550) following incubation and H_2S concentration was calculated against a Na_2S standard curve (50 nM-200 μM).

2.3. Peripheral blood mononuclear cells (PBMCs)

2.3.1. PBMCs treatment

To obtain human monocyte-derived macrophages, cones were procured from the National Health Service Blood and Transplant Bank. Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque 1077 (Millipore Sigma) density centrifugation, and peripheral blood monocytes were incubated with macrophage colony-stimulating factor (M-CSF) (20 ng/mL; R&D Systems, UK) for 7 days in RPMI 1640 (10% human serum). After 7 days, the cells were stimulated for 24 hours with LPS L2630 (1 ng/mL; Sigma). Then, M1 macrophages ($1.0x10^6$ cells/1.5mL) were incubated with RPMI 1640, without phenol red, and treated with PAG (5mM) and AP123 (1nM, 10nM, 100nM and 1μM) at two different times (45 min and 24h). After incubation, cells were collected to perform LC-MS/MS analysis.

2.3.2. Lipid mediator profiling

Samples were thawed and 3 mL of ice-cold methanol containing deuterium-labelled internal standards (d_8-5S-HETE, d_4-LTB_4, d_5-LXA_4, d_4-PGE_2, d_5-RvD2, d_5- MaR1, d_5-MaR2, d_5-RvD3, d_4-RvE1, d_5-17R-RvD1, d_5- LTC_4, d_5-LTD_4 and d_5-LTE) representing each chromatographic region of identified LMs. Samples were then stored at -20˚C for a minimum of 45 min to allow for protein precipitation. Subsequently, supernatants were subjected to solid phase extraction, collecting methyl formate and methanol fractions.

Following solvent evaporation, samples were resuspended in methanol/water (1:1, vol/vol) phase for injection on a Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector, coupled with QTrap 6500+ (ABSciex) (Palmas et al., 2021). For the analysis of unconjugated lipid mediator eluted in methyl formate fraction, an Agilent Poroshell 120 EC-C18 column (100 mm by 4.6 mm by 2.7 μm) was kept at 50°C, and mediators were eluted using a mobile phase consisting of methanol/water/acetic acid of 20:80:0.01 (v/v/v) that was ramped to 50:50:0.01 (v/v/v) over 0.5 min and then to 80:20:0.01 (v/v/v) from 2 to 11 min, maintained until 14.5 min, and then rapidly ramped to 98:2:0.01 (v/v/v) for the next 0.1 min. This was subsequently maintained at 98:2:0.01 (v/v/v) for 5.4 min, and the flow rate was maintained at 0.5 ml/min. In the analysis of mediators eluted in the methanol fraction, an Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 mm) was kept at 50°C and mediators eluted using a mobile phase consisting of methanol/water/acetic acid 55:45:0.5 (vol/vol/vol) over 5 min, that was ramped to 80:20:0.5 (vol/vol/vol) for 2 min, maintained at 80:20:0.5 (vol/vol/vol) for the successive 3 min and ramped to 98:2:0.5 (vol/vol/vol) over 3 min. This condition was kept for 3 min (Pistorius et al., 2022). QTrap 6500+ was operated using a multiple reaction monitoring (MRM) method. Each lipid mediator was identified using established criteria; these included matching retention time of the primary transition with s/n≥5 and that of a secondary transition with s/n≥3 or a positive library match of the MS/MS spectrum with a “fit” score ≥70%. Quantitation was carried out in accordance with published methods that included calculating recoveries of deuterium labeled internal standards and linear calibration curves. Where curves for mediator of interest were not available calibration curves for surrogate molecules with similar physical characteristics were employed. Calibration curves were obtained for each mediator using lipid mediator mixtures at 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 pg that gave linear calibration curves with r^2 values of 0.98 and 0.99 (Fig. 23) (Koenis et al., 2021; De Matteis et al., 2022).

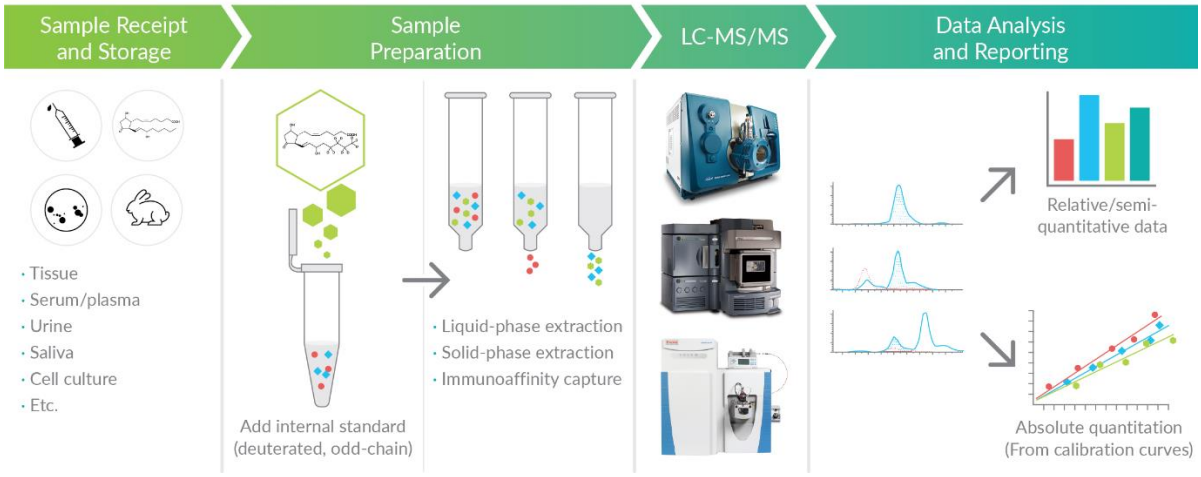


Fig.23 The different steps to identify and quantify SPMs

2.4. Animals

C57BL/6 and CD-1 mice were purchased from Charles River (Milano, Italy) and kept in animal care facility under controlled temperature, humidity and light/dark cycle and with food and water *ad libitum*. All animal procedures were performed according to the Declaration of Helsinki (European Union guidelines on use of animals in scientific experiments). All procedures were approved by the local animal care office (Centro Servizi Veterinari Università degli Studi di Napoli 'Federico II') and carried out following recommendations for experimental design and analysis in pharmacology as reported by Curtis *et al.*, 2015. C57BL/6 mice were divided into three groups. Indeed, with regard to CD-1 mice, we used 5 animals per each treatment.

2.4.1. Animals' treatment

2.4.1.1. C57BL/6 mice

The C57BL/6 mouse (Fig. 24) is the most widely used inbred strain in laboratory animal research and is used in a widespread of research fields, including cancer research,

diabetes/obesity research and behavioural/learning research (Hansen et al., 2022). All animals used were divided into three groups and each group can be considered representative of different treatments (5 animals per group). Indeed, the first group were untreated mice. While, the second group were mice treated with TNF (500ng/mouse, 6h). Finally, the last group were mice treated with TNF-α and AP123 (10μg/mouse, 6h).

Fig. 24 A common model of C57BL/6 mouse

2.4.1.2. CD-1 mice

CD1 mice (Fig.25) are an inexpensive, robust and readily available outbred population commonly used in preclinical studies (Aldinger et al., 2009). For what concern the first set of experiments, aorta was harvested exposed to hyperglycaemic (HG) conditions (50mM, 20h) in presence or absence of AP123 (10nM). Aorta exposed to 25mM was used as control vessel. In another set of experiments, pharmacological modulation with wortmannin as PI3K inhibitor was also performed (100nM, 20h).

Fig. 25 CD-1 mouse strain

2.4.2. Vascular tissue preparation

Thoracic aorta from C57BL/6 and CD-1 mice were used. Mice were anaesthetized with enflurane (5%) and then killed in CO_2 chamber (70%); the aorta was rapidly harvested, and adherent connective and fat tissue were removed. Rings of 1-1.5mm length were cut and placed in organ baths (Fig. 26) (3.0 mL) filled with oxygenated (95% O_2-5% CO_2) Krebs solution and kept at 37°C. The rings were connected to an isometric transducer (7006, Ugo Basile, Comerio, Italy) and changes in tension were continuously recorded with a computerized system (DataCapsule-17400, UgoBasile, Comerio, Italy). The composition of the Krebs solution was as follows (mM): 118 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 10.1 glucose. The rings were initially stretched until a resting tension of 1.5 g was reached and then were allowed to equilibrate for at least 30 min; during this period the tension was adjusted, when necessary, to 1.5 g and the bath solution was periodically changed.

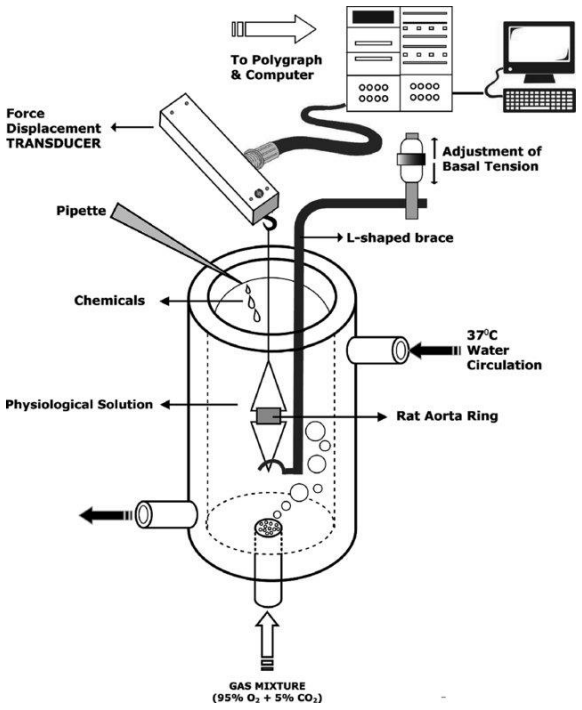


Fig.26 A schematic illustration of the aorta ring preparation in an organ bath (Yildiz et al., 2015)

2.4.3. Isolated organ bath study

In first set of experiments, rings were firstly challenged with phenylephrine (PE, 1nM-3μM) until the responses were reproducible. In order to verify the integrity of the endothelium, cumulative concentration-response curves to Ach (10 nM–30μM) were performed with PE pre-contracted rings. Rings not reaching a relaxation response of at least 75% were discarded. Moreover, for what concern the side of the inflammatory setting, tissues were then washed and contracted with PE (1nM-3μM) and, once the plateau was reached, cumulative concentration-response curves to L-cysteine (L-cys, 100 nM–1mM) were obtained (Brancaleone et at., 2016).

2.5. Statistical analysis

All data were reported as mean±SEM and the number of the replicated experiments is at least $n \geq 5$ for all data set. Statistical analysis has been performed by using one-way or two-way analysis of variance (ANOVA) where appropriated, followed by Bonferroni or Dunnett post-hoc test, where applicable. Data were analyzed by using Prism Graphpad 8.0. Data set were considered statistically significant when a value of $p<0.05$ was reached. While, Partial Least Squares-Discriminant Analysis (PLS-DA) was performed using MetaboAnalyst 5.0 and applying autoscaling on lipid mediator concentrations. PLS-DA builds a multivariate model that identifies the most relevant variables (lipid mediator concentrations) contributing to the separation of observations (samples). During classification, observations were projected onto their respective cluster. The score plot illustrates the clusters of observations where closer plots higher similarity in an observation profile (Palmas et al., 2021; De Matteis et al., 2022).

3. Results

3.1. BAEC undergoing hyperglycaemia: a model of vascular dysfunction

To better define the cross-talk between NO and H_2S in terms of mechanisms and pathway involved in inflammatory conditions associated to vascular disease, we evaluated a typical condition of vascular dysfunction, using endothelial cell culture to study the NO/H_2S cross-talk in diabetes-mimicking conditions.

3.1.1. NOx levels following APs administration

First of all, we evaluated NOx levels (nitrate/nitrite) of the samples following the treatment. In particular, these preliminary results showed the effects of the administration of H_2S donors, AP123 and AP39, used an established model of *in vitro* hyperglycaemia, were bovine aortic endothelial cells (BAEC) were grown in high glucose (HG, 50 mM) environment for 3h. AP123 and AP39 were added at same time as HG induction (t=0) and 1h later (t=1). These data showed that the incubation of BAEC in HG significantly reduced NOx levels. Indeed, administration of AP39 (Fig.27A) and AP123 (Fig.27B) (1µM) restored NOx levels in a similar fashion and independently from time of treatment.

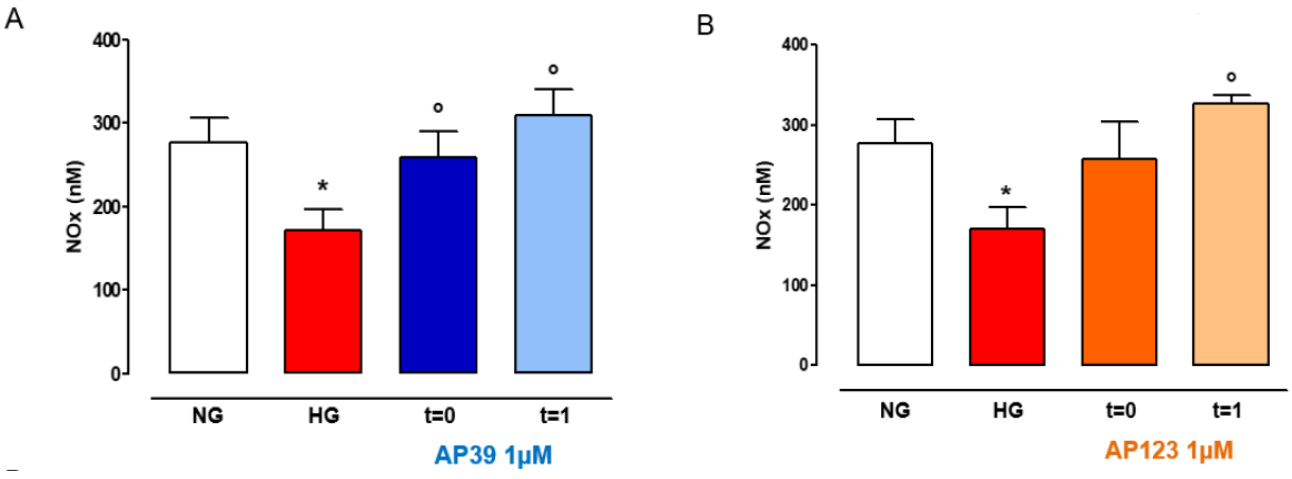


Fig.27 NOx levels following incubation of BAEC with H_2S donor molecules, AP39 and AP123, in HG environment at time 0 (t=0) or time +1h (t=1) ($p<0.05$; *vs NG; °vs HG).

Next, we wanted to evaluate the concentration-response effects for AP39 and AP123. In particular, the results obtained following concentration-response experiments at time 1, showed that AP39 and AP123 (0.01-100nM) affected NOx concentration in a different manner. Indeed, experiments performed showed that both AP39 and AP123 modulated NOx levels; however, AP39 induced an "on-off like" effect (Fig.28A), while AP123 showed an effect dependent from the concentration on NOx levels (Fig.28B).

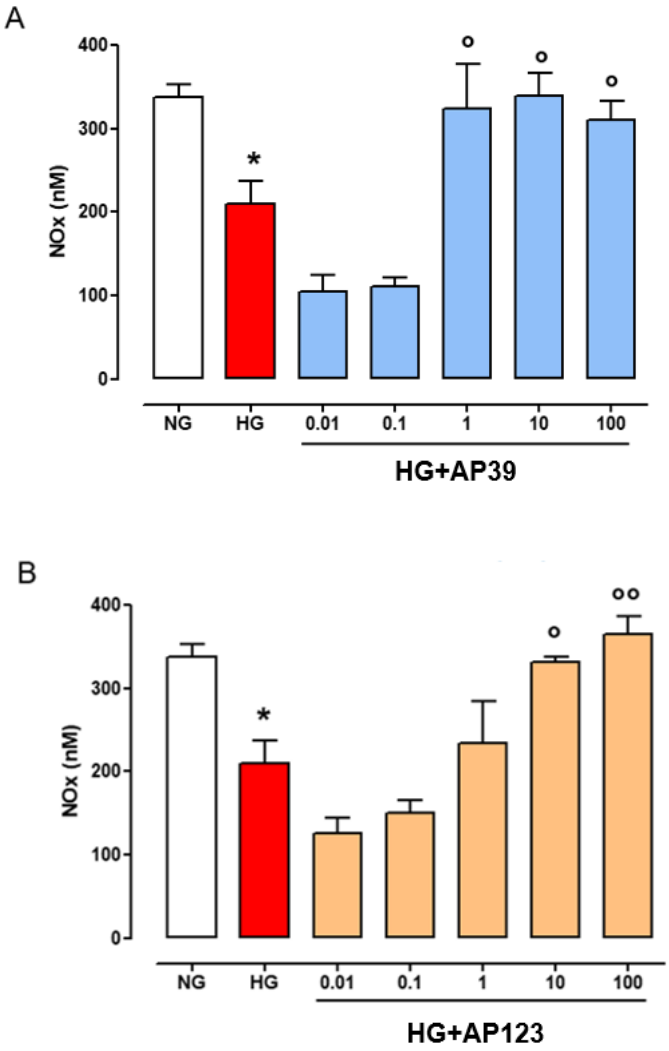


Fig.28 NOx levels as function of AP39 or AP123 concentration, expressed as µM ($p<0.05$; *vs NG; °vs HG).

3.1.2. The effect of AP39 and AP123 on the expression of eNOS and p-eNOS

Next, we evaluated the expression of eNOS through western blot analysis in hyperglycaemic environment. First of all, HG conditions reduced both expression and activation of eNOS (phospho-eNOS, p-eNOS). However, despite the restoration of NOx levels following the AP39 (1-10nM) administration, this slow-releasing hydrogen sulfide donor was unable to restore physiological levels of eNOS and p-eNOS (Fig.29A). Conversely, AP123 (1-10nM) was able to positively modulate p-eNOS and expression of eNOS (Fig.29B).

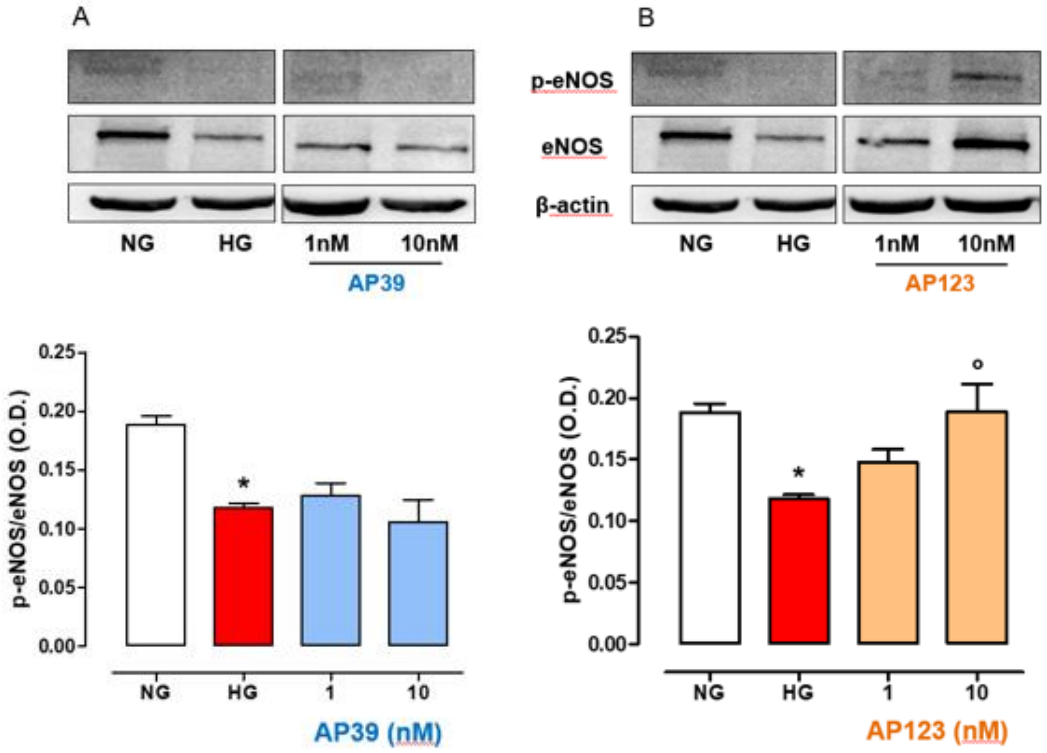


Fig.29 AP39 and AP123 effect on p-eNOS and eNOS expression ($p<0.05$; *vs NG; °vs HG).

3.1.3. CREB role in the action of AP123

The difference in mode of action between AP39 and AP123 led us to focus the attention on the signalling involved in eNOS expression, with particular attention to the transcription factor cAMP response element-binding protein (CREB), involved in eNOS modulation (Niwano et al, 2003). Thus, we evaluated the expression of CREB and its phosphorylated active form (p-CREB) in HG environment, in presence or absence of AP39 or AP123 (0.1-10nM). Western blot analysis showed that effect of AP39 does not involve CREB signalling. Conversely, AP123 seems to actively modulate this pathway (Fig.30).

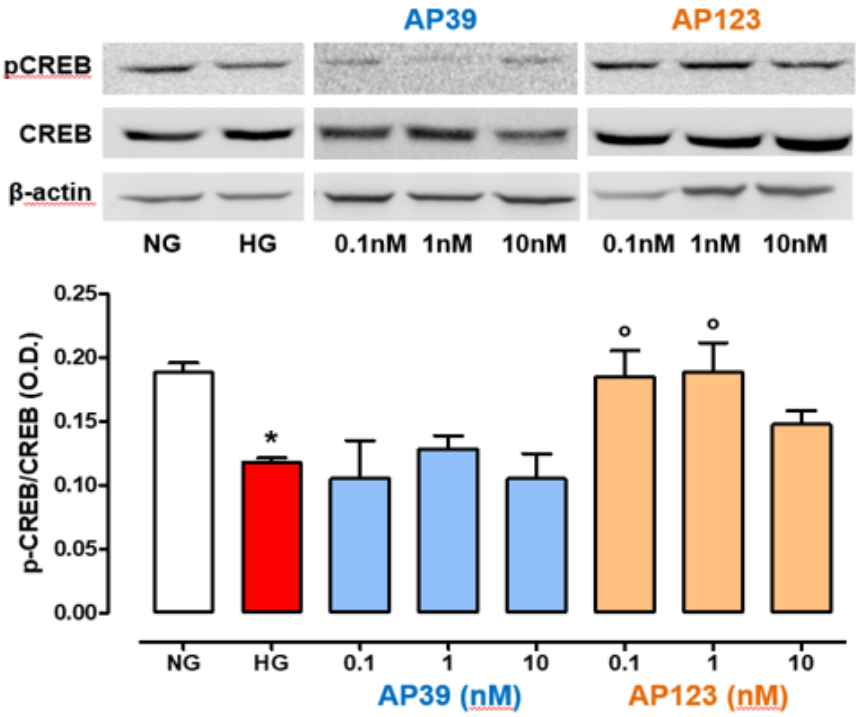


Fig. 30 Effects of AP39 and AP123 on pCREB/CREB expression ($p<0.05$; *vs NG; °vs HG).

3.1.4. The role of AP123 in the presence of the inhibitors KT5720 and wortmannin

Then, we tested the involvement of PKA and PI3K/Akt pathways in the effect triggered by AP123 on the eNOS pathway. In particular, PKA and PI3K are some protein kinases responsible for the activation of CREB through its phosphorylation (Zhang et al., 2020; Narasimhamurthy et al., 2022). Indeed, we evaluated NOx levels in supernatants collected from BAEC grown in high glucose (HG) conditions treated with AP123 (10nM) and specific kinases inhibitors, KT5720 (KT, 1μM) and wortmannin (WM, 100nM), PKA and PI3K inhibitors respectively. In HG environment, nitrite/nitrate (NOx) levels appaired reduced, in presence of KT or WM, nevertheless the administration of AP123. Indeed, KT5720 and wortmannin (Fig.31) abrogated the beneficial effect induced by H_2S donor molecule.

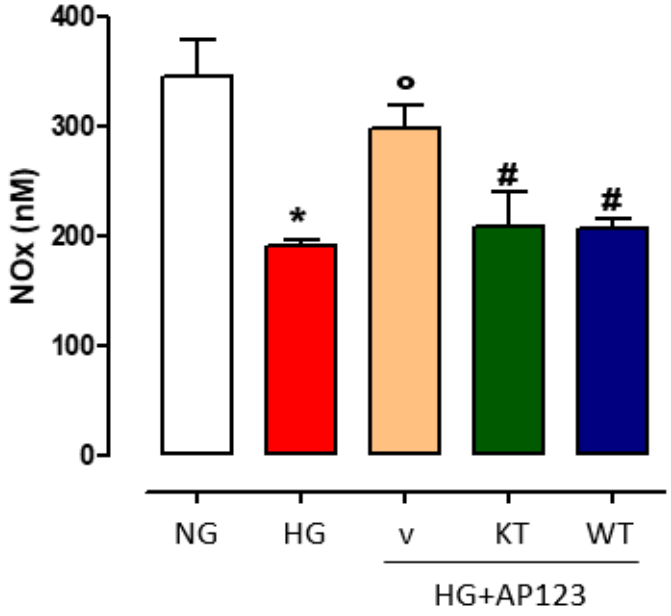


Fig.31. NOx levels following incubation of BAEC with H_2S donor molecule, AP123, and specific kinases inhibitors, KT5720 and wortmannin, in HG environment (*p<0.05 vs NG; °p<0.05 vs HG; #p<0.05 vs AP123).

Next, we evaluated the expression of eNOS through western blot analysis, following of the administration of KT5720 (1μM) and wortmannin (100nM), in HG environment. Our results demonstrated that KT (Fig.32) did not affect AP123 (10nM) associated increase in eNOS expression. Conversely, WM (Fig.32) abrogated the beneficial effect induced by AP123 in HG conditions.

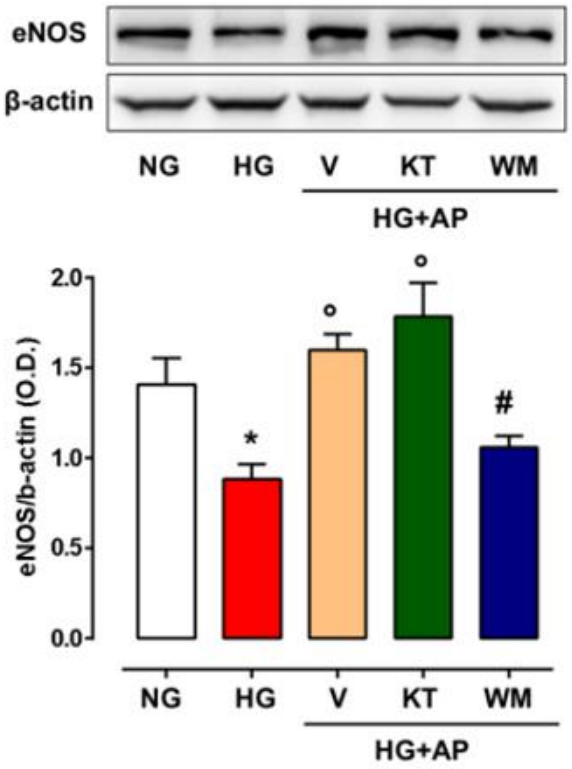


Fig.32 The effect of KT5720 and wortmannin on eNOS expression (*p<0.05 vs NG; °p<0.05 vs HG; #p<0.05 vs AP123).

Similarly, when we also evaluated the effects of KT and WM on CREB signalling. In particular, western blot analysis demonstrated that KT5720 (1μM) (Fig.33) did not influence CREB expression and its phosphorylated active form (p-CREB) in presence of AP123 (10nM) in HG environment. Conversely, we observed the loss of the effect by AP123 on p-CREB expression when HG exposed BAEC were pre-treated with WM (100nM) (Fig.33).

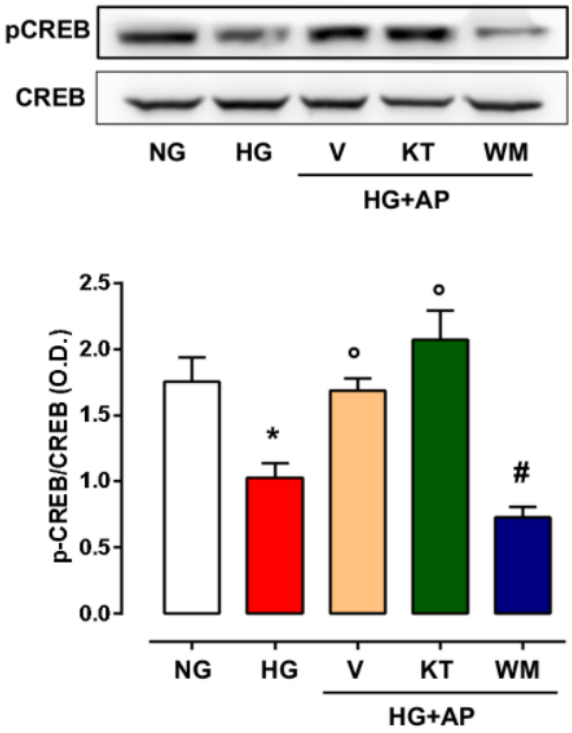


Fig.33 The effect of KT and WM on CREB expression and p-CREB (*p<0.05 vs NG; °p<0.05 vs HG; #p<0.05 vs AP123).

3.1.5. The effects of HG environment on *ex vivo* model and positive modulation of AP123

To corroborate our data *in vitro* and to evaluate the role of H_2S in an *ex vivo* model of hyperglycaemia, we used an *ex vivo* model by testing vascular reactivity in isolated aorta harvested from CD1 mice and exposed to normo- or hyperglycaemic environment in presence or absence of AP123. Vascular reactivity in response to acetylcholine (Ach, 10nM-30μM) was reduced in HG-exposed vessels compared to a normal glucose environment. However, exogenous H_2S source rescued the vascular function in HG conditions mice, thus restoring the normal response to Ach (Fig. 34).

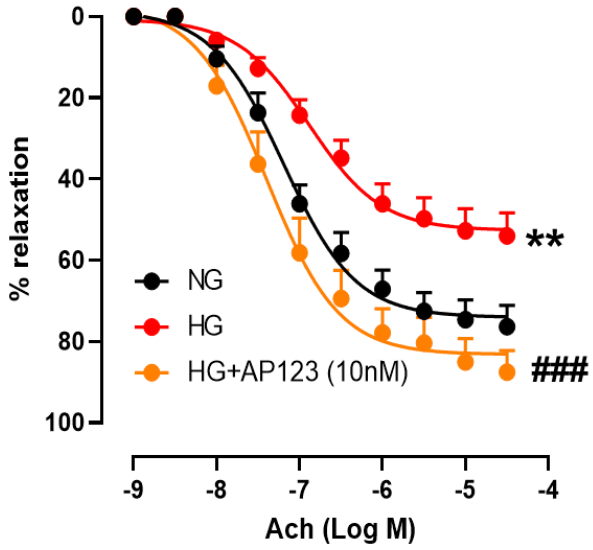


Fig.34 The response of the HG-exposed mice aorta rings, to Ach, alone or treated with AP123 (**p<0.001 vs NG; ###p<0.001 vs HG)

Next, we evaluated whether the specific PI3K inhibitor, wortmannin (100nM/mouse, 20h), influenced the restoration of vascular reactivity observed in AP123 treated aorta during HG. In particular, we observed that, similarly to what happens in vitro, the administration of WM was able to abrogate the positive action of the H_2S donor (Fig.35).

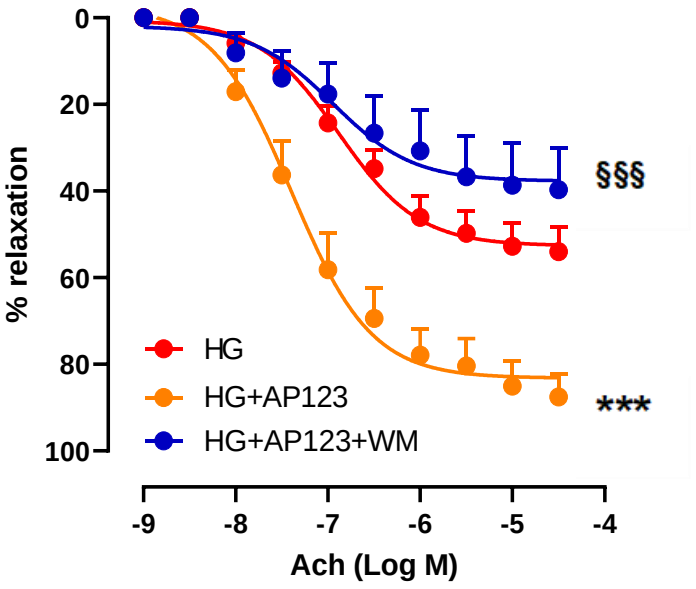


Fig.35. The response of the HG-exposed mice aorta rings, to Ach, and treated with AP123, alone or in combination with WM (***p<0.001 vs HG; §§§ p<0.001 vs HG+AP123).

3.2. Hydrogen sulfide role in inflammatory response

To better determine the role of H_2S in inflammatory response and pro-resolution AnxA1/FPR2 axis in vascular function with respect to cardiovascular homeostasis, we used *in vitro* models of vascular function impairment.

3.2.1. The interplay between AnxA1 and H_2S/NO pathway

Indeed, for what concern the side of the inflammatory setting, we stimulated BAEC with TNF-α (10ng/ml, 6h) and observed the expression of AnxA1 and FPR2, to evaluate the possible NO/H_2S influence in inflammatory condition and their interplay with AnxA1/FPR2 axis. Firstly, western blot analysis demonstrated that, in a treatment of 6h, the exposure of BAEC to TNF-α induced a downregulation of AnxA1 expression. Conversely, the administration of AP123 (1-10nM) in inflammatory condition positively influence AnxA1 expression (Fig.36).

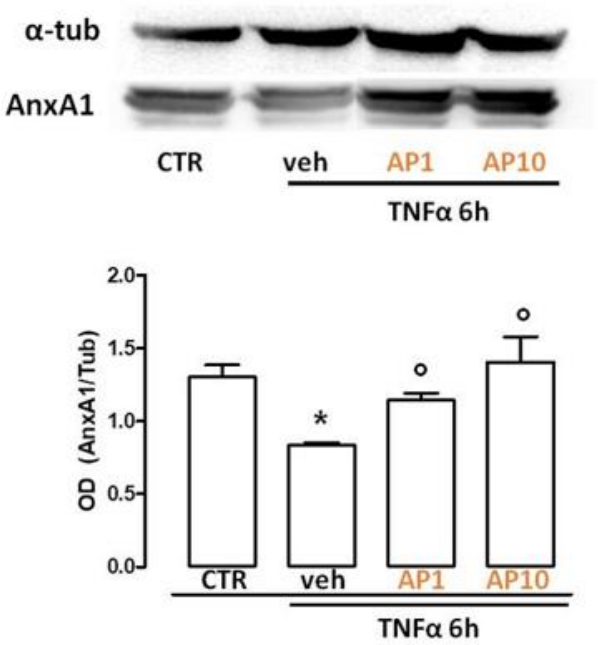


Fig.36 AP123 effect on AnxA1 expression in inflammatory condition ($p<0,05$; *vs CTR; °vs TNF)

After evaluating the role of slow-releasing H_2S donor on the expression of AnxA1, we demonstrated the effect of AP123, in inflammatory conditions, on formyl peptide receptor 2 (FPR2), to confirm the existence of a crosstalk between H_2S and AnxA1/FPR2 axis at vascular level. Notably, our data showed that BAEC exposed to TNF-α (10ng/mL) showed a significant reduction in FPR2 expression. However, H_2S donor molecule (1-10nM) seems to actively modulate this pathway in inflammatory condition (Fig.37).

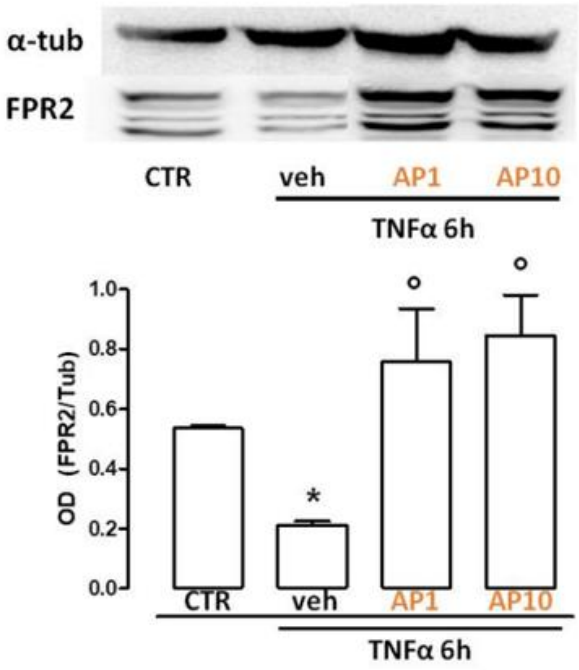


Fig.37 AP123 effect on FPR2 expression in inflammatory condition ($p<0,05$; *vs CTR; °vs TNF)

The next passage was to verify whether Anxa1 was able to influence H_2S/NO crosstalk at endothelial level, in a typical inflammatory condition. For this reason, we evaluated eNOS and CSE expression by treating our cells with AnxA1-derived peptide Ac2.26 (0.1µM - 1µM), in combination with TNF-α (10ng/mL). In particular, our data showed that the treatment of ECs with TNF-α (6 h) can lead to a decrease of the expression of CSE and eNOS. Conversely, endothelial cells treated with TNF-α, in presence of FPR pan agonist Ac2.26 (Fig.38), showed that both eNOS and CSE expression is rescued through the inflammatory condition.

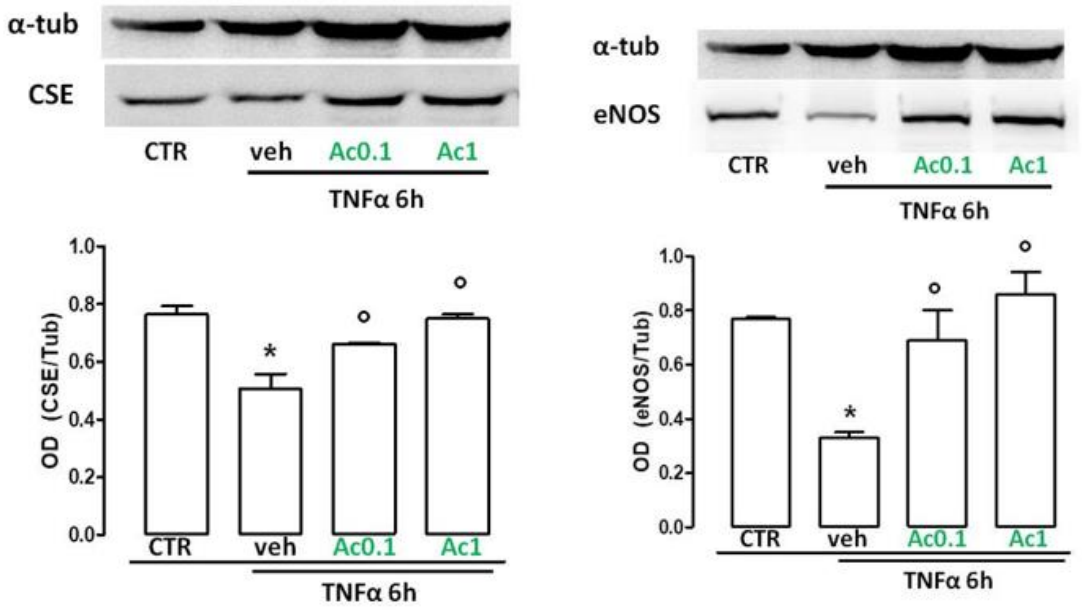


Fig.38 Ac2.26 effect on CSE and eNOS expression in inflammatory condition ($p<0.05$; *vs CTR; °vs TNF)

3.2.2. The role of H_2S in clodronate action

Clodronate (Clo) is one of the most known bisphosphonates (BPs), commonly used as anti-osteoporotic drug in the treatment of several osteometabolic disorders featured by excessive bone resorption (i.e., osteroporosis, Paget's disease etc.) (Rossini et al., 2015). In addition, Clo also displays additional anti-inflammatory and analgesic properties, although the benefits described in the literature are unclear. With respect the evaluation of H_2S role played in the clodronate therapeutic effect, we found that clodronate displayed antinflammatory properties through of H_2S pathway, thus assuring the control of the inflammatory state. In some set of experiments, in which we used fibroblast-like synoviocyte cell line (K4IM), we measured levels of H_2S and expression of CSE. Western blot analysis demonstrated that CSE expression was significantly reduced by TNF-α (10 ng/ml, 6h) (Fig.39). However, the administration of clodronate (10μM) partly restored CSE towards levels similar to those observed in unstimulated cells (Fig.39).

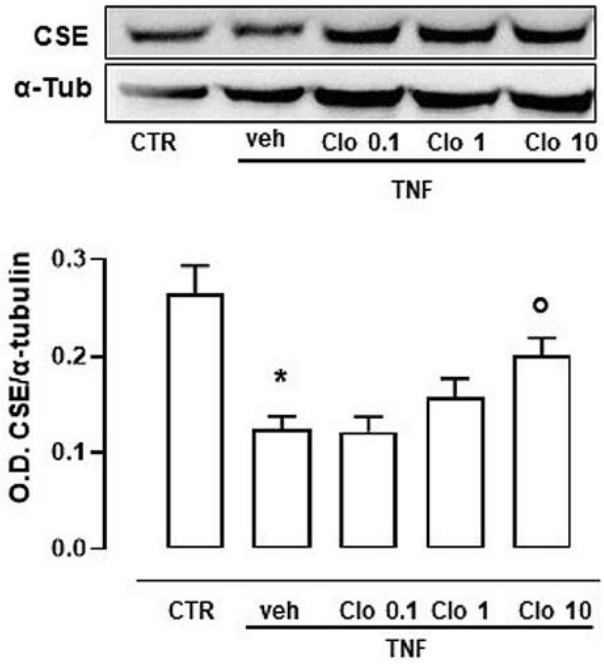


Fig.39 Clodronate effect on CSE expression in inflammatory condition (*p < 0.05 vs CTR; °p < 0.05 vs veh)

In addition, we evaluated the amount of H_2S, produced in K4IM, in presence of TNF-α or in combination with clodronate (0.1-10μM). Intracellular H_2S biosynthesis by CSE, following treatment with TNF-α, was significantly suppressed. However, clodronate reversed this effect (Fig. 40a). Similarly, H_2S levels in supernatants from TNF-α treated cells were reduced and clodronate was able to restore them (Fig. 40b).

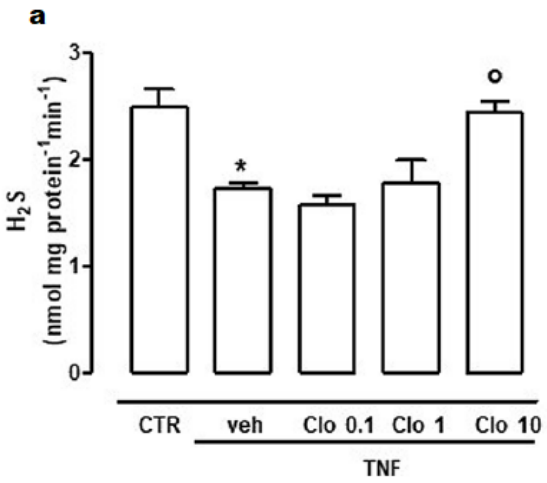


Fig.40a Clodronate effect on intracellular H_2S biosynthesis with TNF-α (*p < 0.05 vs CTR; °p < 0.05 vs veh)

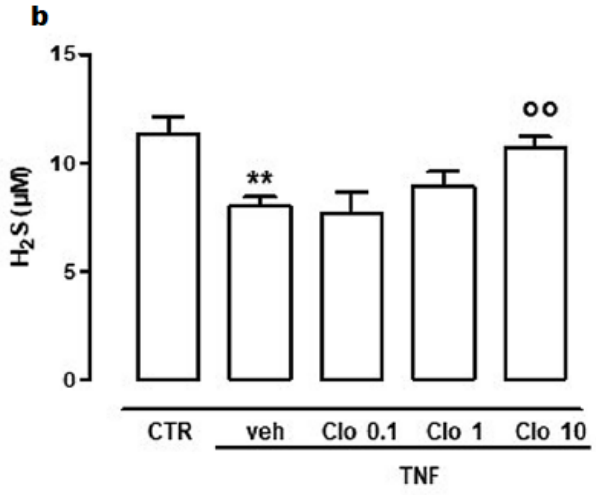


Fig.40b Clodronate effect on extracellular H_2S levels with TNF-α (**p < 0.01 vs CTR; °°p < 0.01 vs veh)

3.3. The role of H_2S in IBVD

To corroborate our data *in vitro* and to evaluate the role of H_2S in a IBVD model, we used a common murine model (C57BL/6 mice) treated with TNF-α (500ng/mouse, 6h), alone or in combination with AP123 (10µg/mouse), to induce an inflammatory condition and vascular impairment. Aorta was collected and used to evaluate vascular reactivity in response to phenylephrine (PE, 1nM-3µM) or acetylcholine (Ach, 10nM-30µM) in isolated organ baths. Our *ex vivo* experiments showed that response of aorta rings to PE and Ach are reduced in TNF-treated mice compared to vehicle. Interestingly, exogenous H_2S rescued the vascular function in TNF-treated animals, thus restoring the normal response to both PE and Ach (Fig.41).

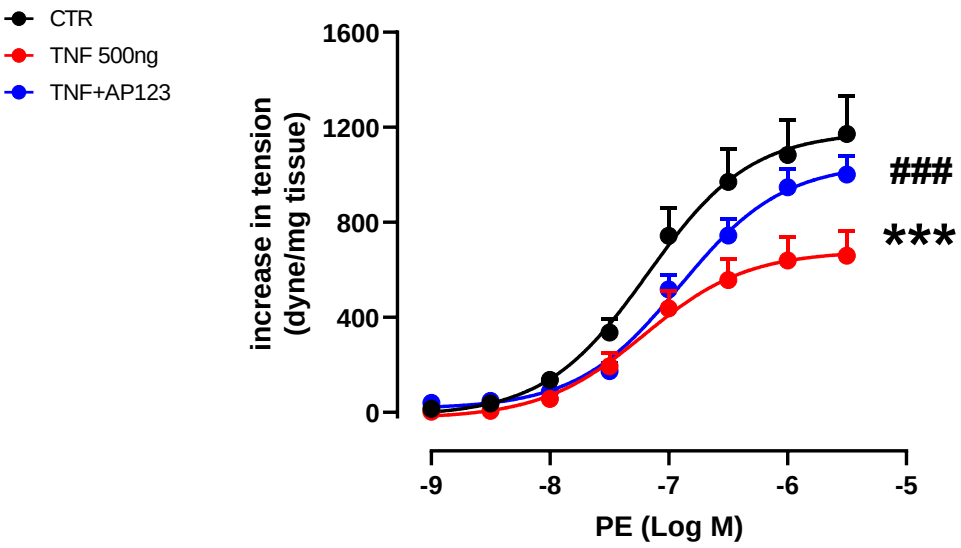


Fig.41a. The response of the aorta rings to PE in mice treated with TNF-α, alone or with AP123 (***p<0.001 vs CTR; ###p<0.001 vs TNF-α)

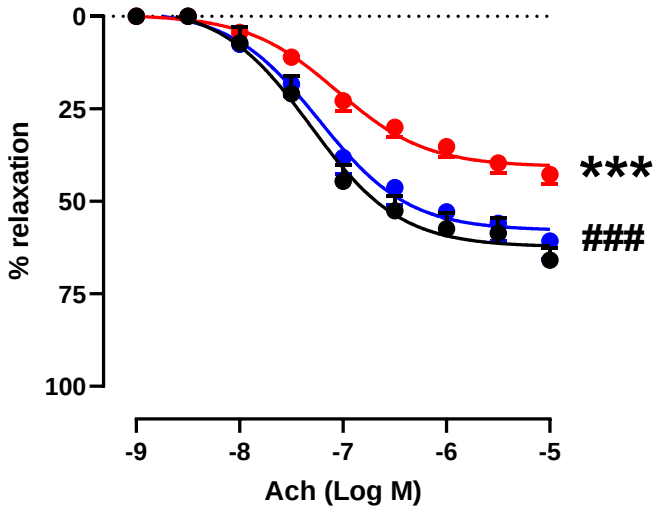


Fig.41b The response of the aorta rings to Ach in mice treated with TNF-α, alone or with AP123 (***p<0.001 vs CTR; ###p<0.001 vs TNF- α)

Thus, the effects of L-cysteine (L-Cys, 10nM-30μM) were tested in the aortic rings of mice treated. Of note, L-Cys induced vasodilation was abrogated by TNF-α administration and AP123 was able to recover normal vascular response to L-Cys (Fig.42).

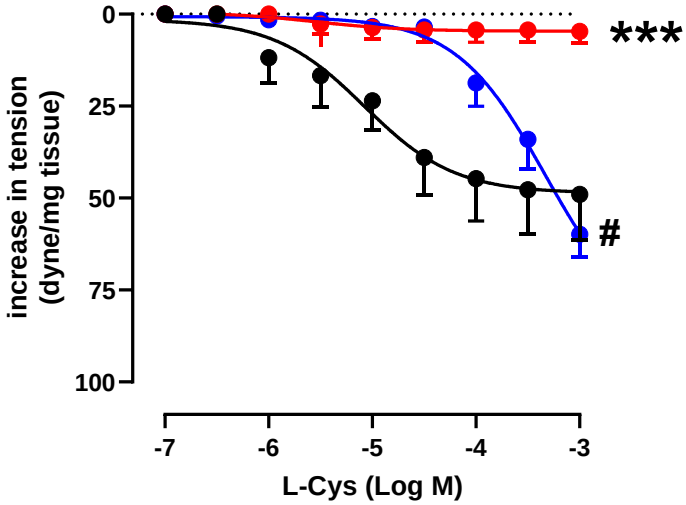


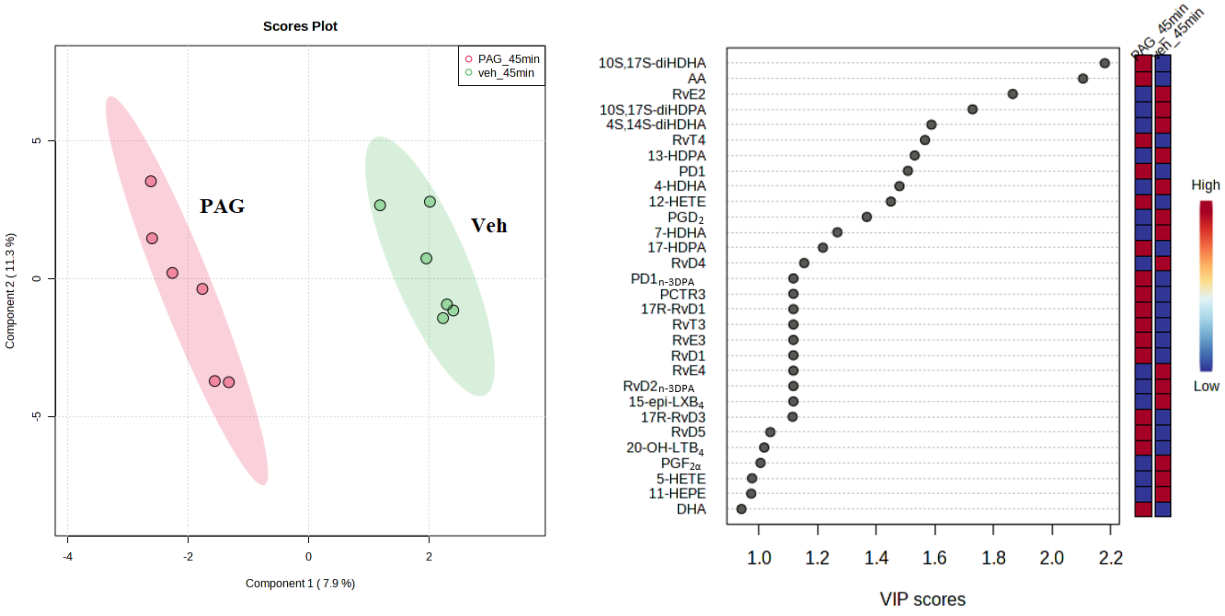
Fig.42 The effects of L-Cysteine on the aorta rings of mice treated with TNF-α, alone or with AP123 (***p<0.001 vs CTR; #<p<0.001 vs TNF- α)

3.4. The possible involvement of H_2S in pro-resolving lipid mediators (SPMs) activity

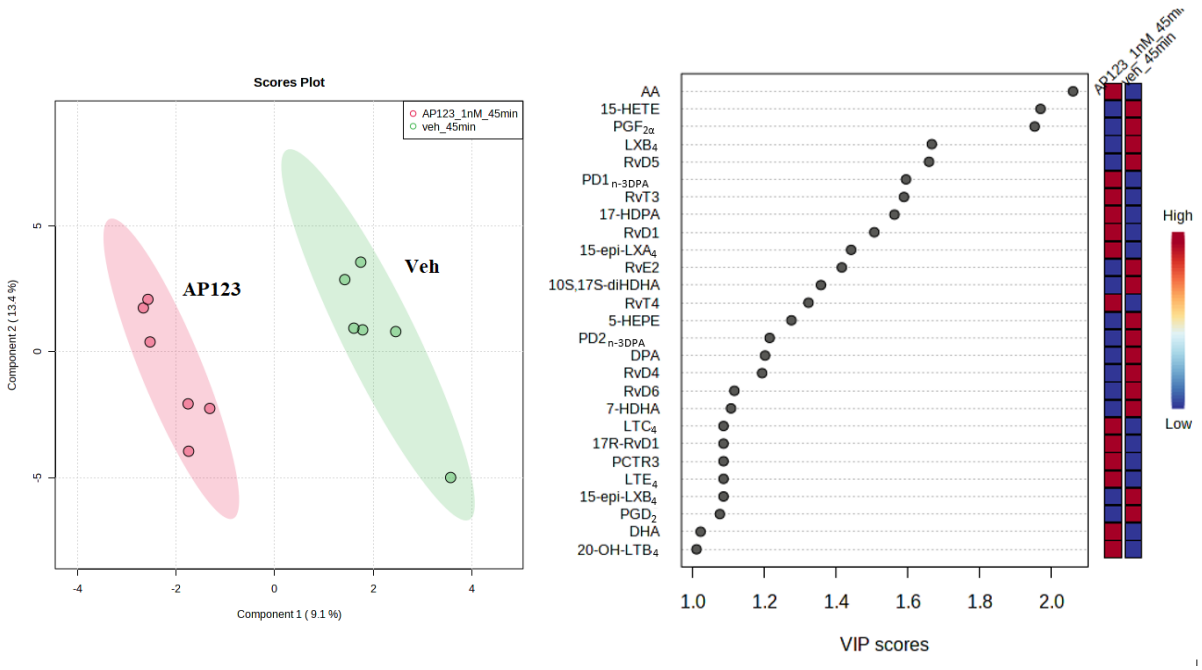
The anti-inflammatory role of lipid mediators has not been sufficiently delineated respect to the overall modulation of vascular function. At a similar extent, the possible relationship between H_2S and lipid mediators has not been highlighted at vascular level, as well as in immunity. Therefore, to check if other circulating mediators could possibly be involved in the mechanisms elucidated, we wanted test whether the novel H_2S donor molecule, AP123 (1nM-1μM, 45min and 24h), and a specific CSE inhibitor, PAG (10 mM, 45 min and 24h), could affect the changes to lipid mediators' activity, in an established model of *in vitro* inflammation on macrophages. To evaluate whether SPMs concentrations are differentially regulated in different conditions evaluated, we assessed a typical inflammatory condition, using M1 macrophages stimulated with LPS (1ng/mL), and the treatment with PAG or AP123. Using liquid chromatography tandem mass spectrometry (LC-MS/MS), we identified lipid mediators (LM) from all four bioactive metabolomes in each sample. We then assessed their relative levels using Partial Least Squares Discriminant analysis (PLS-DA), a dimensionality-reducing multivariate

analysis that creates a linear regression model accounting for multicolinearity and identifies the relationship between the two groups based on lipid mediators' concentrations between the two groups. Different LM clusters were observed between cells untreated and cell treated with PAG or AP123 (45min) as depicted in the score plot shown in fig. 43. Evaluation of the VIP scores, which identify the LM that most strongly contribute to the observed separation, identified several LM with a VIP score >1 (Fig.43).

a



b



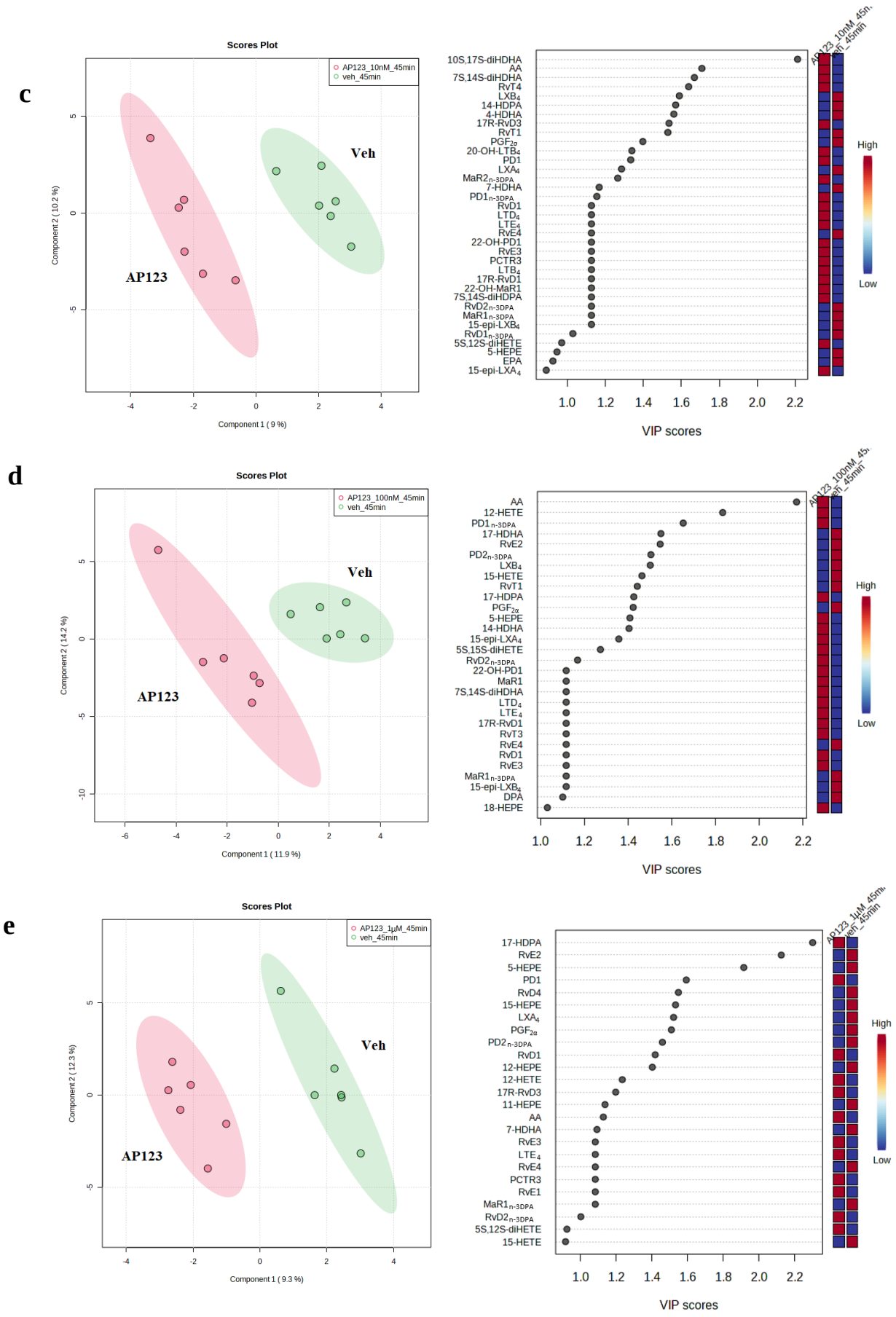
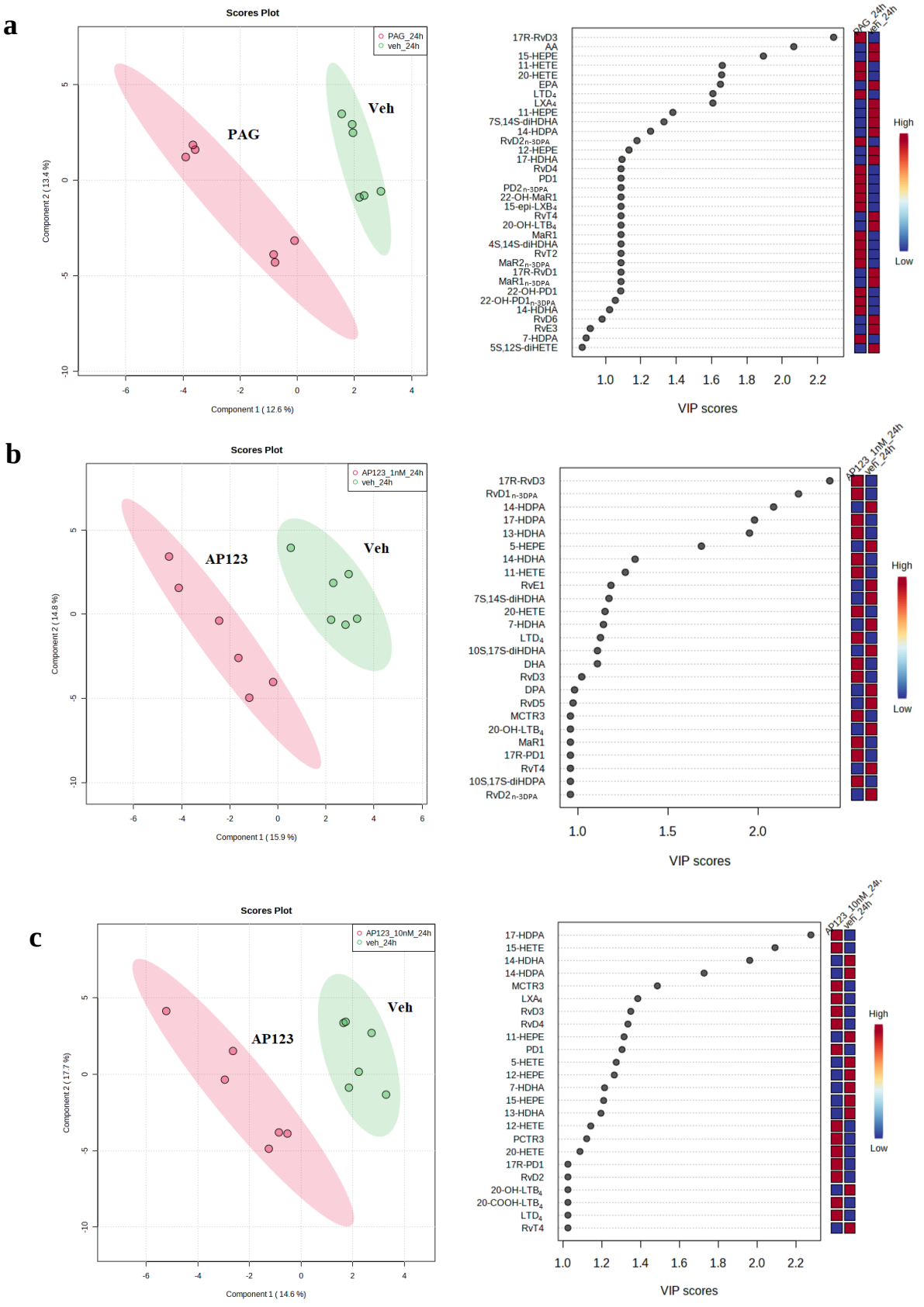


Fig.43. LM concentrations in cells samples treated with PAG (10nM) (a), AP123 1nM (b), AP123 10nM (c), AP123 100nM (d) and AP123 1μM (e) for 45min

Thus, to evaluate whether SPM concentrations are differentially regulated in cells treated with PAG or AP123 for 24h, we assessed LM profiles in treated M1 macrophages and compared these with LM profiles obtained from untreated cells (Fig.44).



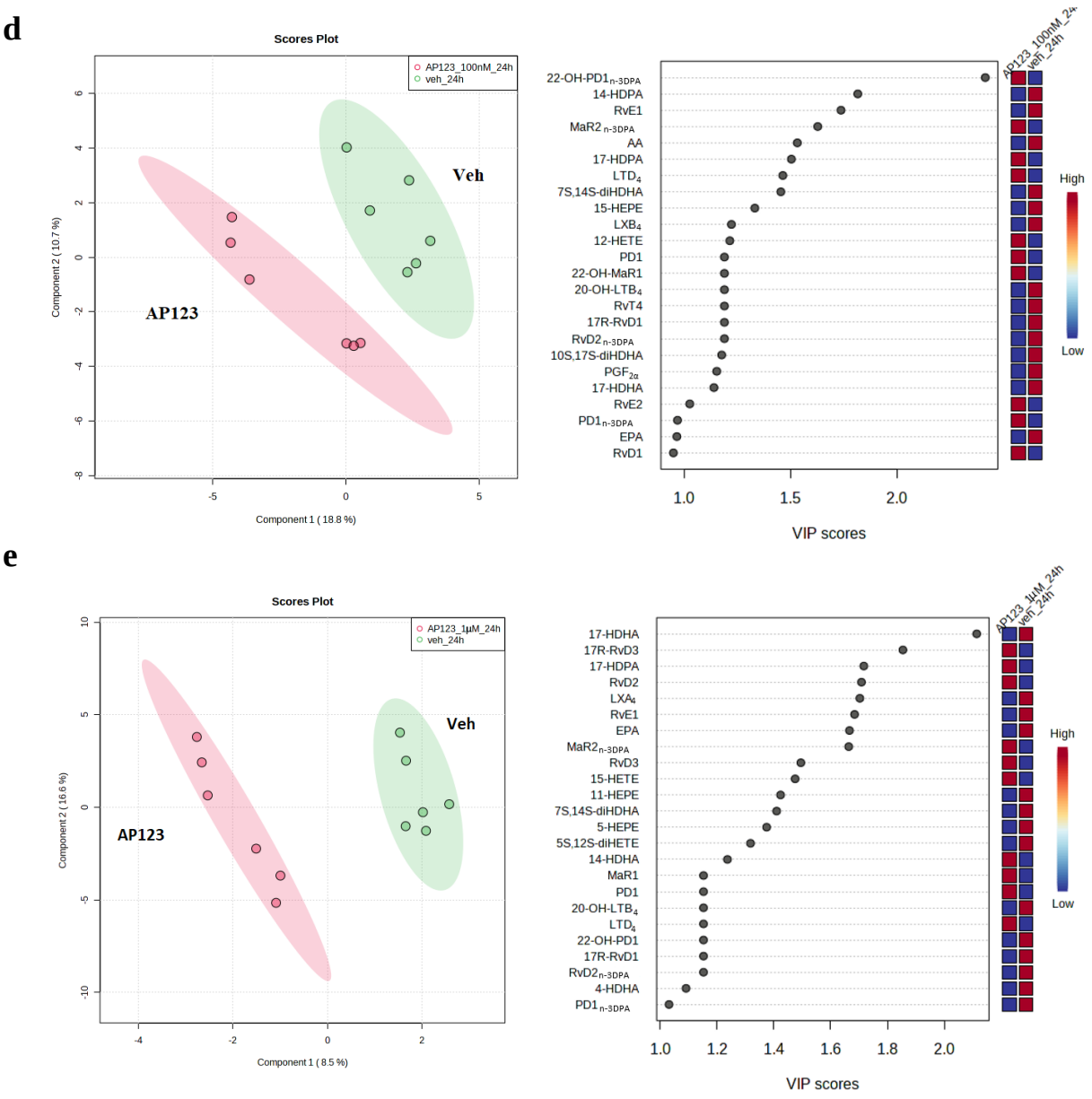


Fig.44 LM concentrations in cells samples treated with PAG (10nM) (a), AP123 1nM (b), AP123 10nM (c), AP123 100nM (d) and AP123 1μM (e) for 24h

4. Discussion

4.1. A confirmation of cross-talk between NO and H_2S in IBVD

All the pathologies included in the vascular diseases are characterized from two crucial factors, oxidative stress and inflammation, defining the concept of inflammation-based vascular diseases (IBVD). Indeed, unresolved and exacerbated inflammation is central to the pathophysiology of vascular diseases, because an aggressive inflammation can lead to tissue destruction. In normal conditions, the resolution of vascular inflammation, a key step in the inflammatory response, is so an important phase for vessel wall remodelling and their functional recovery. Usually, the modulation of this phenomenon is controlled by endogenous anti-inflammatory molecules such as nitric oxide (NO) and hydrogen sulfide (H_2S). Although there are several evidences that show NO and H_2S pathways are able to interact, however such a cross-talk has not been sufficiently delineated in vascular inflammation. In this study, using an *in vitro* model of hyperglycaemia on endothelial cells, we evaluated the NO/H_2S cross-talk in diabetes-mimicking conditions, an environment characterized from adverse vascular profile. First of all, we evaluated whether the administration of two slow-releasing H_2S donors, AP39 and AP123, restored nitrate/nitrite (NOx) levels as a function of the concentration or as a function of the administration time. In both cases, the incubation of ECs in HG environment significantly reduced NOx levels and both donors restored NOx levels in a similar fashion and independently from time of treatment. However, about the concentration-response effects for AP39 and AP123, the data showed that both donors modulated NOx levels, but in a different manner, being AP123 more efficient in restoring eNOS/NO signalling in hyperglycaemic conditions. Therefore, the results suggest that AP39 is not effective as AP123, despite it induced an "*on-off like*" effect. Conversely, AP123 restored NO biosynthesis in a concentration-dependent manner. Indeed, the data showed that AP123 modulated NOx levels by causing a transcriptional activation of CREB with a subsequent rescue of eNOS expression/activation. In particular, AP123 exerts its effect by modifying CREB phosphorylation in a PI3K dependent manner, also confirmed from our *ex vivo* experiments. Indeed, following the administration of the irreversible inhibitor of phosphatidylinositol 3 kinase (PI3K), wortmannin, these results showed that the vascular reactivity of the aortic rings of mice was compromised compared to the beneficial condition induced by AP123. Therefore, the AP39 inability to modulate eNOS expression

could indicate an exclusive mechanism of "scavenging", where H_2S released could target to the reactive oxygen species (ROS) released during hyperglycaemia and so protect NO from degradation, preserving its levels. These studies suggest that restoring H_2S levels can positively influence NO levels, confirming a crosstalk between these two gaseous mediators at vascular level in a condition linked to an inflammatory process.

4.2. The role of pro-resolving AnxA1/FPR2 axis at vascular level and the cross-talk with NO/H_2S pathway

It is widely accepted that endogenous pro-resolving mediators released during an inflammatory response play a critical role in effective recovery from inflammation. Indeed, resolution of inflammation is a critical orchestrated process, involving other specific endogenous molecules such as formyl-peptide receptor 2 (FPR2) and annexin A1 (AnxA1), that "limit" inflammation through the reduction of leukocyte migration and the facilitation of immune cells efferocytosis. Nonetheless, although these elements are involved in the process of resolution of inflammation, currently there are many evidences with respect to pro-resolution in immune cells, but there are few details and information about the role of AnxA1/FPR2 axis within endothelial cells, the first cells of vessels wall. Our aim was to define the molecular mechanisms underlying the role of AnxA1 and FPR2 in endothelium, using an *in vitro* model of inflammation on endothelial cells, obtained by administration of a flogogen agent, TNF-α. The exposure of ECs to TNF-α induced a significant expression of AnxA1 and FPR2. However, the administration of the slow-releasing H_2S donor, in inflammatory condition, positively modulated their expression, showed an interesting ability to influence this pathway in an inflammatory process. To confirm the influence of NO/H_2S interplay on the AnxA1/FPR2 axis in vascular function, we evaluated CSE expression, the main H_2S-producing enzyme at vascular level, through the administration of TNF-α and Ac2.26, a FPRs pan agonist. Although the use of TNF-α can lead to a decrease of the expression of CSE and eNOS, therefore FPR pan agonist Ac2.26 rescued their physiological levels. However, the resolution pathway associated to the axis AnxA1/FPR2 is positively modulated by H_2S donor AP123, indicating a crucial role of H_2S, with NO, in the control of inflammation. Indeed, these data suggest that, in endothelial cells exposed to TNF-α, AnxA1/FPR2 axis cooperate with activity of CSE or

eNOS in the regulation of vascular function and H_2S pathway is affected by lack of functional AnxA1.

4.3. Involvement of hydrogen sulfide in therapeutical effects associated to clodronate therapy and its wide role in the inflammation control

H_2S has been shown to have anti-inflammatory effects in different pharmacological models. Inflammation is also a crucial core for several diseases, in particular for patients suffering osteoarticular alterations, very common in arthritis or other bone disease like Paget's syndrome. Bisphosphonates are commonly used in osteoporosis as they prevent the loss of bone density, and this class is divided into nitrogen-containing BP (NBP) and nitrogen free (BP). Clodronate (Clo) is one of the most known BP that is till used in therapy. However, Clo also shows anti-inflammatory and analgesic activities, although these aspects are unclear in literature. Therefore, we decided to investigate whether Clo can supply additional beneficial effects and if these effects were somehow related to H_2S. To evaluate the molecular mechanisms underlying the anti-inflammatory role of H_2S in the clodronate activity, we used an *in vitro* model of inflammation on fibroblast-like synoviocyte (FLS) cells, obtained by administration of TNF-α. In order to pursue our aim, we evaluated the expression of CSE and then H_2S levels. Our data demonstrated that TNF-α administration induced a slight reduction of CSE enzyme. While, the treatment with clodronate restored CSE expression. At the same way, enzymatic biosynthesis of intracellular H_2S by CSE and the levels of H_2S in supernatants seem suppressed by TNF-α. In addition, clodronate was able to revert this effect, restoring H_2S levels to control values. Therefore, this evidence highlights that the anti-inflammatory effects of clodronate could be associated to the modulation of H_2S pathway in ongoing inflammation. Although different investigations are needed to better explore the underlying mechanism of the interplay between clodronate and hydrogen sulfide, however our data demonstrated that clodronate is able to regulate levels of H_2S. Moreover, these data confirmed that H_2S could have an anti-inflammatory action independently from the experimental setting used, confirming its critical role in the resolution of inflammation.

4.4. From cells to tissues: inflammation and vascular impairment

By analogy to other endogenous gaseous molecules, such as nitric oxide (NO) and carbon monoxide (CO), H_2S was hypothesized to have a physiological activity in regulating cardiovascular functions. Indeed, several studies showed H_2S role at vasculature. In the first report on this subject, it was demonstrated that H_2S relaxed rat aortic tissues *in vitro* (Hosoki et al., 1997). Moreover, accumulating evidence has shown vascular potassium channels activation is involved in H_2S-induced vasodilation (Liu et al., 2022). The use of a common mouse model, such as C57BL/6, allowed us to propose an inflammatory condition, through TNF-α administration, and to evaluate H_2S effect on vascular reactivity associated to vascular impairment. We first verified the effects of TNF-α on the aorta rings, through the incubation with phenylephrine (PE) and acetylcholine (Ach), which usually cause vasoconstriction and vasodilation, respectively. Our data showed that the response of the aorta rings seems reduced in mice treated with TNF-α compared to untreated mice. Conversely, the administration of slow-releasing H_2S donor in the animals restored the vascular function of TNF-treated animals. At the same time, the usual relaxation induced by L-cysteine (L-Cys) was abrogated by the TNF-α administration. However, the administration of AP123 triggered vasodilation, restoring normal vascular response to L-Cys. Therefore, our data demonstrated that the administration of exogenous H_2S could restore vascular function in a condition of vascular impairment, typical of inflammation, confirming its potential role such as a pro-resolving mediator at vascular level.

4.5. H_2S interplay with resolution pathways and specialized pro-resolving lipid mediators

Therefore, we demonstrated that H_2S could modulate and affect the vascular integrity, restoring the vascular function, which usually is compromised following the process during, typical of IBVD. Although NO/H_2S pathway is able to interact with AnxA1/FPR2 axis, determining small changes could have big impact on tissue homeostasis, however the resolution of inflammation is a complex process that involves other several factors, such as pro-resolving lipid mediators (SPMs). Indeed, lipid mediators play an active role in stimulating endogenous anti-inflammatory and pro-resolving pathways. Therefore, SPMs could represent therapeutic agents in the treatment on several diseases, based on inflammatory component such as vascular inflammatory diseases. The promotion of an active program of inflammation resolution by SPMs is therapeutically attractive because the aim is to restore tissue homeostasis without significantly affecting key components of the inflammatory response (i.e., cytokines). Furthermore, lipid mediators such as lipoxins, PD1 and Rvs are rapidly generated in response to stimuli, act locally and then rapidly inactivated by metabolic enzymes (i.e. LAX4/PGE 13, 14-reductase/LTB_4 12-hydroxydehydrogenase) (Anderson and Delgado, 2008). Therefore, no side effect should be expected for them. Finally, SPMs are small molecules. Consequently, they have crucial permeability properties that permit rapid access to the site of inflammation as well as demonstrating high-affinity binding to specific receptors. For this reason, our aim was to investigate whether the role of H_2S influenced the activity of other pro-resolving mediators. Indeed, we expected to find some kind of interconnection between them all to define a series of possible targets to control vascular alterations driven by inflammatory processes. However, the possible interplay between hydrogen sulfide and lipid mediators has not been carefully investigated in immunity. In the present study, we used an *in vitro* model of inflammation on macrophages, treated with a specific CSE inhibitor (PAG) and a H_2S donor (AP123). These preliminary data showed that the majority of mediators found are differentially regulated between two treatments, thus involving pro- and anti-inflammatory mediators. Lipid mediators are involved in both the initiation and perpetuation of inflammatory events as well as in the termination of acute inflammation. Moreover, it is important the role of each mediator involved in the inflammatory response. Therefore, the comparison of two treatments at different time suggested that the

administration of AP123 (1nM, 45 min) mainly displays an involvement of anti-inflammatory lipid mediators. However, our preliminary results demonstrated that the administration of PAG (10nM, 45 min) can lead a decrease of the levels of a particular lipid mediator, referred to as $RvD2_{n\text{-}3DPA}$, compared to vehicle. $RvD2_{n\text{-}3DPA}$ is a specialized pro-resolving mediator biosynthesized from the omega-3 fatty acid n-3 docosapentaenoic acid. This mediator is able to promote degradation of bacterial and fungal particles, reduce TNF-α induced chemotaxis and adhesion of isolated human neutrophils (Reinertsen et al., 2021).

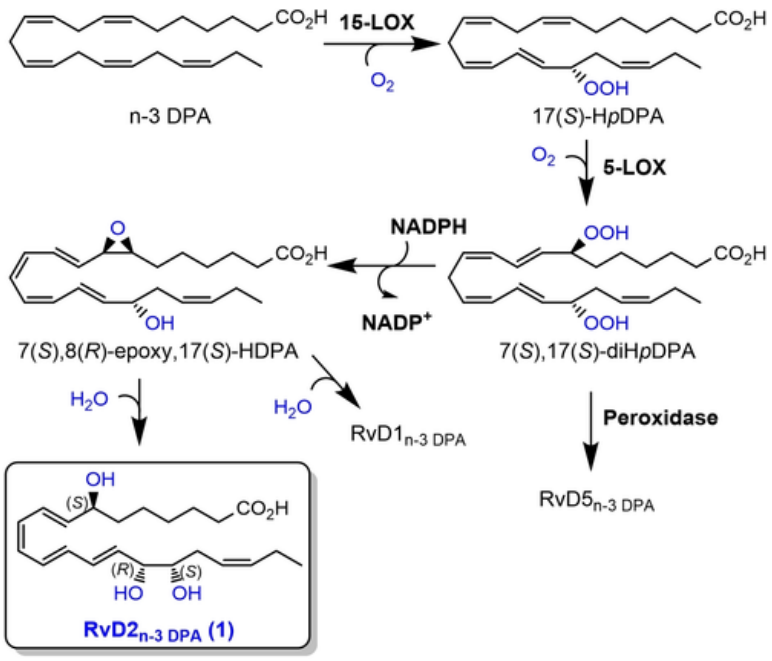


Fig.45 Biosynthesis of $RvD2_{n\text{-}3DPA}$ from n-3 DPA (Reinertsen et al., 2021)

Of note, AP123 (100nM and 1μM, 45min) was able to increase the concentrations of this LM. However, the exposure of the cells to AP123 for 24h determined a significant decrease of this mediator, demonstrating that the time of exposure to H_2S donor could be crucial. Nevertheless, more investigations need to be conducted to understand the pathways involved in this modulation.

5. Conclusion

5. Conclusion

The anti-inflammatory properties of NO/H_2S pathway have never been sufficiently delineated, mainly at vascular level, although there are evidence showing that NO and H_2S are able to somehow interact. Moreover, although the involvement of NO in inflammation-based vascular disease (IBVD) appears to be relatively clear, this is not the case for hydrogen sulfide. Therefore, for the first time, we showed and confirmed the potential anti-inflammatory activity of H_2S. Our study demonstrated that hydrogen sulfide can improve vascular function and control local inflammation. In particular, the studies performed in this project suggest that:

- restoring H_2S levels can positively influence NO levels;
- exogenous source of H_2S restores AnxA1/FPR2 expression and vascular function;
- AnxA1 restores CSE/H_2S pathway and H_2S pathway is affected by lack of functional AnxA1;
- exogenous H_2S could positively influence the anti-inflammatory activity of other pro-resolving mediators, such as $RvD2_{n3\text{-}DPA}$.

Overall, our studies suggest that H_2S is a crucial vasculoprotective mediator in IBVD at vascular level and H_2S-releasing molecules may be useful in preventing the harmful effects to the vasculature of "H_2S deficiency". Therefore, these findings may open new perspectives in the therapeutic scenario, where hydrogen sulfide may acquire relevance in the modulation of inflammation.

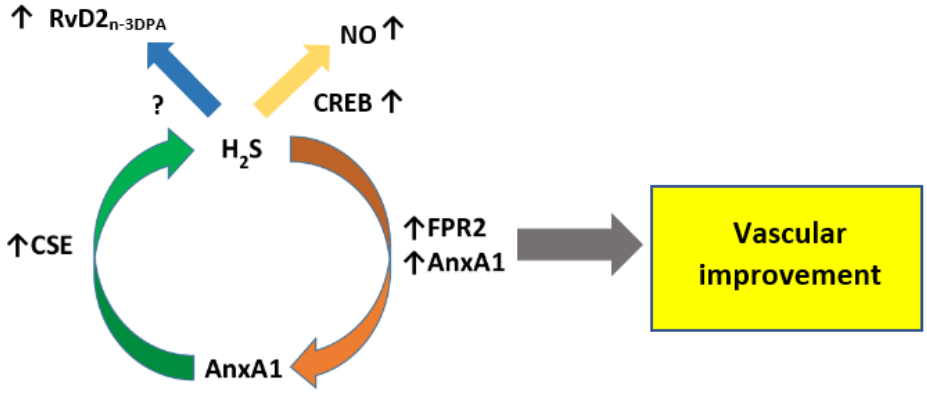


Fig.46 The beneficial effects of H_2S on several mediators involved at vascular level in inflammatory processes

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