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## Effects of chronic nitric oxide deprivation on endothelial cell behaviour


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# Summary

The endothelium explicates its physiological functions by producing active molecules, among which nitric oxide (NO) is particularly important. It is well known that endothelial dysfunction (ED), *i.e.* an impaired function of the endothelium coupled with a reduced release of NO, is a risk factor for atherosclerosis together with a list of conditions such as hypertension, hypercholesterolemia, smoking, diabetes, and the aging process itself. These conditions are also associated with a significant increase in Reactive Oxygen Species (ROS) in the vascular wall that may contribute to the establishment of ED and to the development of its late effect on cardiovascular system.

In the present study, the behavioural and molecular consequences deriving from chronic NO deprivation were investigated in human primary endothelial cells (human umbilical vein endothelial cells, HUVECs). To inhibit NO formation, endothelial nitric oxide synthase (eNOS) was chronically inhibited by treatment with L-$N^G$-Nitroarginine methyl ester (L-NAME), a structural analogue of L-arginine that competitively block the active site of the enzyme, or by transfection with a siRNA specific for eNOS.

We observed that a 48-h L-NAME treatment induced in HUVECs a higher migratory capability (evaluated by chemotaxis assays in Boyden's chamber) which was independent of the reduced activity of the cyclic GMP/protein kinase G pathway present in chronically NO deprived HUVECs. In the attempt to explain the mechanism(s) through which NO deficiency enhances migration, we investigated if chronic L-NAME treatment affected the expression and production of Vascular Endothelial Growth Factor (VEGF) and of its receptor KDR. RT-qPCR analyses, accompanied by ELISA assays and western blot analyses, demonstrated that both VEGF and KDR mRNAs and proteins were significantly augmented in L-NAME treated cells, thus suggesting the establishment of an autocrine loop responsible for the increased migration.

Increased VEGF production and cell motility are typical events occurring in hypoxic cancer cells, due to the accumulation of hypoxia-inducible factor-1α (HIF-1α), which plays a major role in the transcriptional activation of genes encoding angiogenic factors. Similarly, induction of VEGF expression during hypoxia has been described in endothelial

cells (ECs). Interestingly, we observed a significant nuclear accumulation of HIF-1α in HUVECs chronically treated with L-NAME. Moreover, the transcriptional activity of HIF-1α was responsible for the increases in VEGF/KDR expression and migration since the transfection with ΔARNT (a dominant negative form of the HIF-1β subunit that maintains the capacity of forming an heterodimer but cannot bind DNA) is able to totally blunt both the effects in L-NAME treated HUVECs, thus confirming the involvement of an autocrine loop in the pro-migratory effect induced by NO deprivation. The dependence of HIF-1α stabilization from NO deficiency was confirmed by using the NO donor DETA/NO. Very low doses of DETA/NO reverted both the HIF-1α accumulation and the consequent increases in VEGF expression and cell motility induced by L-NAME treatment. Furthermore, to investigate whether the observed effects were due to the specific inhibitory effect of L-NAME on eNOS activity, we knocked-down the enzyme by using RNA interference methodology. In eNOS silenced cells, HIF-1α accumulated in the nucleus and VEGF production was enhanced thus confirming the dependence of the observed effects on eNOS inhibition. All these results suggest that basal release of NO may act as a negative controller of HIF-1α levels and cell motility in HUVECs with important consequences on ECs physiology.

In the attempt to unravel the pathway(s) linking NO deficiency to HIF-1α accumulation and activity, we focus our attention on ROS since their formation has been involved in HIF-1α stabilization in normoxia. We found that acute treatment with L-NAME induced in HUVECs an early and transient burst in ROS formation that was fully prevented by the presence of the antioxidant N-acetylcysteine (NAC). HIF-1α accumulation was reduced by 45% in the presence of NAC indicating that the peak of ROS was only partially involved in its stabilization. On the contrary, NAC did not affect the increase in cell migration in ECs chronically deprived of NO. At variance with acute treatment, chronic L-NAME exposure gave rise to an antioxidant environment characterized by a reduction in cellular ROS content accompanied by an increase in superoxide dismutase-2 (SOD-2) expression and activity. Importantly, this protective response was accompanied by the nuclear accumulation of the transcription factor NF-E2-related factor-2 (Nrf2) that was fully prevented in the presence of NAC. These results

suggest the establishment of an antioxidant status in HUVECs chronically deprived of NO in the attempt to neutralize any further cell damage induced by loss of NO.

In addition, since NO plays an important role in promoting mitochondrial biogenesis in different cell types and tissues, we analyzed the mitochondrial mass and function in HUVECs after NO deprivation. Long term L-NAME treatment induced a significant reduction in mitochondrial DNA (mtDNA) accompanied by decreases in the incorporation of the metabolic indicator MTS, in cellular ATP content, and in oxygen consumption. In agreement, the silencing of eNOS was able to decrease mtDNA and total cellular ATP levels thus confirming that loss of NO sustained the onset of mitochondrial dysfunction in HUVECs. Importantly, metabolic effects observed in chronically NO deprived ECs was independent of both HIF-1α activity and ROS generation.

In conclusion, we demonstrated that an endothelial deficit of NO, by mimicking the *in vivo* early phase of ED, induces important physiological modifications in human ECs. In particular, loss of NO leads to the accumulation and transcriptional activation of HIF-1α responsible for the enhanced VEGF/KDR expression and cell motility, and to the establishment of mitochondrial dysfunction. Importantly, most of the peculiar features shown by long term NO deprived HUVECs are independent of acute ROS generation, and must therefore depend on other pathways triggered by NO loss. On the contrary, ROS formation appears to be totally responsible for the Nrf2 accumulation that might account for the establishment of an adaptive antioxidant status in response to oxidative stress. Further experiments will be necessary to fully characterize our *in vitro* model of ED and to elucidate the molecular mechanism(s) involved in HIF-1α stabilization. Our model should however represents an useful system for the study and the identification of innovative pharmacological targets and markers for ED, thus contributing to a better knowledge of the endothelium behavior in the absence of NO and to an improved comprehension of the molecular mechanisms involved in the onset of cardiovascular pathologies.

# Introduction

## 1. The circulatory system

The circulatory system is an organ system constituted of hollow organs, which permits blood and lymph circulation to transport nutrients, oxygen, carbon dioxide, hormones, blood cells to and from cells in the body for nourishing it. Moreover, it plays a protective role against diseases and controls body temperature, pH stabilization, and homeostasis.

The circulatory system consists in an intricate network of blood vessels that are tubular organs made up of three distinct layer: *tunica intima*, *tunica media* and *tunica adventitia*. Depending on the type of the vessel, the composition of the *tunicae* vary slightly to better respond to different functional requirements of the districts, maintaining a common scheme. The *tunica intima* is the inner layer that is made up of a thin layer of simple squamous epithelium, called endothelium. It rests on a connective tissue membrane with many elastic and collagenous fibers. The *tunica media* is mainly constituted of connective tissue and elastic fibers with different proportion in relation to the function of the vessel itself. Finally, the *tunica adventitia*, the one more distant from the lumen, is made up mostly of connective tissue, where are located the nerves and, in the biggest vessels, the *vasa vasorum*. In particular, arteries are characterized by the presence of a *tunica media*, rich of muscular smooth fibers and a plenty of elastic fibers in the *tunica adventitia*. The presence of muscular and elastic fibers in the arteries permits the accumulation of the heart-derived energy. In fact, when the heart relaxes between two contractions, the energy accumulated from arteries is released to the blood column directed to the periphery of the body. In this way, the arteries transform the intermittent blood flow, derived from heart, in a continuous laminar flow that is essential to capillary exchange. Veins have instead less thick and more extensible walls, which minimized the resistance, a feature that permits the passing of a huge volume of blood. In the biggest veins, flap-like shaped valves are found that prevent the possible blood backflow. Finally, walls of capillaries are also composed of endothelium and form a semi-permeable layer through which substances in blood are exchanged with substances in

tissue fluids surrounding cells of the body. Capillary walls have thin slits where endothelial cells overlap. These slits have various size, affecting capillaries' permeability.

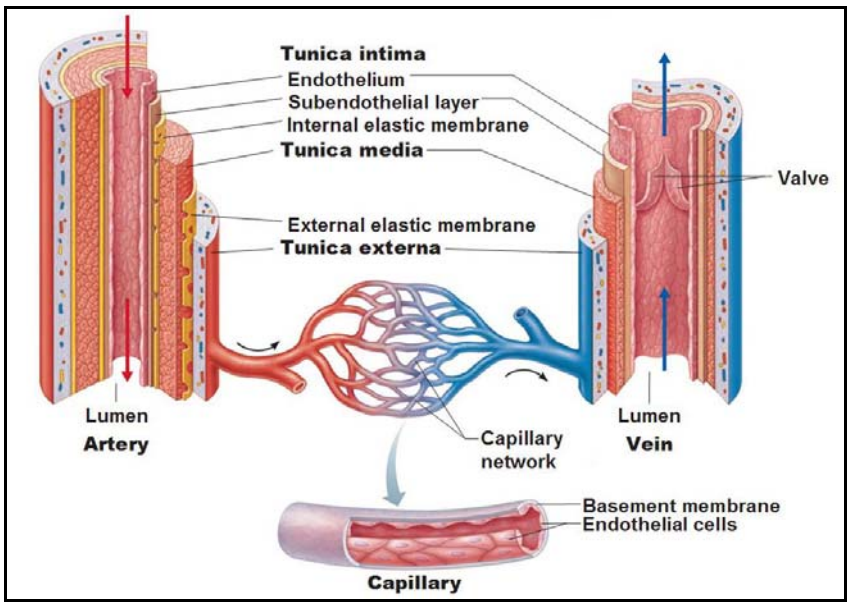



**Figure I.** Blood vessel structure

## 2. The endothelium

### 2.1. Structure

The endothelium forms the inner lining of a blood vessel and is constituted by a monolayer of thin squamous epithelial cells which are named endothelial cells (ECs) and arise from the splanchnopleuric mesoderm (Cines et al., 1998).

ECs are connected by two main types of intercellular junctions: tight junctions (also termed *zona occludens*) that are usually found at the apical region of the intercellular cleft, and adherens junctions (also termed *zona adherens*). Tight junctions form a continuous intercellular barrier that is required to separate tissue spaces and regulate selective movement of solutes across the ECs (the so-called "paracellular transport"). Moreover, tight junctions contribute to maintain cell polarity between the luminal and abluminal side of ECs. The junctional composition of intercellular clefts shows

a quite high variability along the vascular tree of the human body. For example, ECs composing large arteries display a well-developed system of tight junctions; on the contrary, at the level of microvasculature, junctions are tighter in arterioles compared with capillaries. Furthermore, the tight junctions are less organized in post-capillary venules, a feature that is related to the role of this blood vessels in mediating inflammation-induced extravasation of leukocytes and plasma constituents. Finally, the blood brain barrier is particularly rich in tight functions (Aird, 2007).

Endothelium may be continuous or discontinuous (Figure II) and the continuous one could be also fenestrated or non-fenestrated. Non-fenestrated continuous endothelium is found in arteries, veins, and capillaries of organs such as the brain, skin, heart, and lungs. Fenestrated continuous endothelium occurs in locations that are characterized by increased filtration or increased trans-endothelial transport. Typical examples are the capillaries of gastric and intestinal mucosa, exocrine and endocrine glands, glomeruli, choroid plexus and a subpopulation of renal tubules. Fenestrated endothelium due its name to "fenestrae", which are transcellular pores (about 70 nm in diameter) that extend themselves through the full "thickness" of the cell. The majority of fenestrae show a thin non-membranous diaphragm (5- to 6-nm) across their opening. Discontinuous endothelium is found in certain sinusoidal vascular beds, most notably in the liver. In contrast to fenestrated continuous endothelium, the discontinuous ones possess larger fenestrations (100 to 200 nm in diameter) lacking a diaphragm and containing gaps (or large circular pores) within individual cells. Besides, the underlying basement membrane is poorly formed.

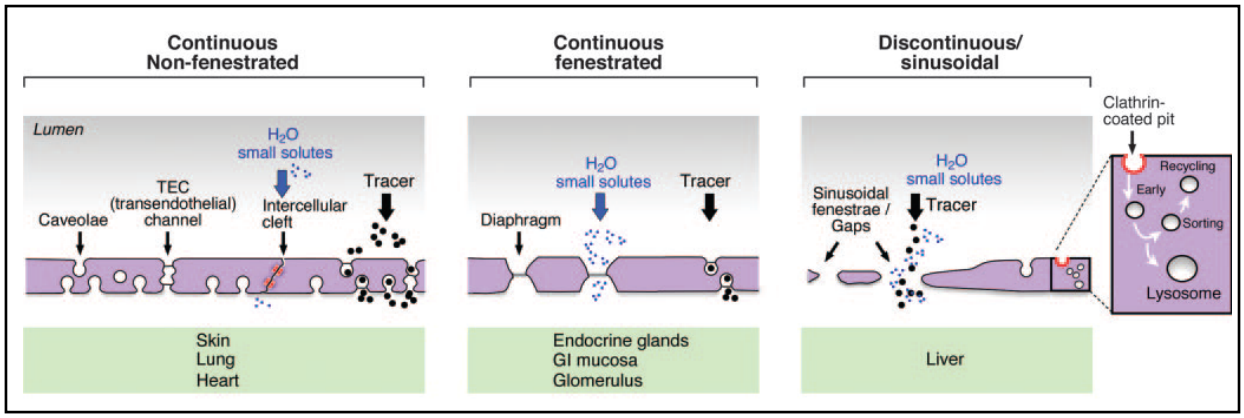



**Figure II.** Transports and permeability of capillaries in different tissues. Image modified from (Aird, 2007).

ECs (Figure III) are about 25–50 μm in length, 10–15 μm in width and their shape varies across the vascular tree. Although ECs are typically flat, they are plump or cuboidal in high endothelial venules. Endothelial cell thickness varies from less than 0.1 μm in capillaries and veins to 1 μm in the aorta. It should be also mentioned that endothelial cells (and their nuclei) are aligned in the direction of blood flow in straight segments of arteries but not at branch points. Thus, flow-dependent alignment of ECs represents a reversible endothelial structural remodelling in response to hemodynamic shear stress.

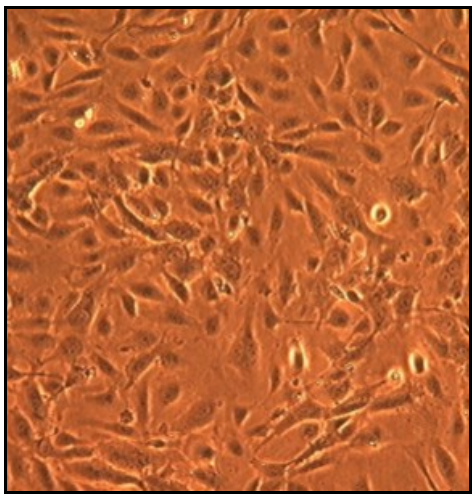


**Figure III.** Endothelial cells (ECs), 200X

ECs possess clathrin-coated vesicles, clathrin-coated pits, multivesicular bodies and lysosomes, which are the structural components of the endocytotic pathway. Endocytosis occurs either via receptor-dependent pathways or by a nonspecific (fluid-phase) process and directs macromolecules to the lysosomal compartment for degradation. The specific endocytotic pathway is mediated by so-called scavenger receptors, which are responsible for the uptake of transferrin, low-density lipoprotein (LDL), ceruloplasmin, albumin, and advanced glycosylation end products. Besides endocytosis, ECs actively play an important role in transcytosis, which regulates the transcellular transfer of molecules across the endothelium. This mechanism involves specialized structures, among which caveolae and vesiculo–vacuolar organelles (VVOs)

are essential. Caveolae are 70-nm membrane-bound, flask-shaped vesicles that usually open to the luminal or abluminal side but are occasionally free in the cytoplasm. In contrast to the clathrin-coated pits that have a thick electron-dense coat, caveolae have a smooth inner surface. In ECs caveolae are more numerous than clathrin-coated pits, except that in liver sinusoids. Furthermore, the density of caveolae is far greater in capillary endothelium (up to 10 000 per cell) compared with arteries, arterioles, veins, or venules. The number of caveolae is highest in continuous non-fenestrated endothelium, particularly in heart, lung, and skeletal muscle (Bendayan, 2002). A notable exception is the blood brain barrier, where caveolae are rare. However, VVOs which represent the focal collections of membrane-bound vesicles and vacuoles and are most commonly observed in venular endothelium, where the cytoplasm is thicker compared with capillaries (Dvorak & Feng, 2001; Feng, Nagy, Dvorak, & Dvorak, 2002). The complexity of venule VVOs varies according to the thickness of the endothelium.

### 2.2. Physiology

Healthy endothelium not only provides a structural barrier between the circulation and surrounding tissue, thus regulating the transfer of small and large molecules, but it is also able to respond to physical and chemical signals (Galley & Webster, 2004). In fact, the vascular endothelium serves as an important autocrine, paracrine and endocrine organ and maintains vascular homeostasis by modulating blood vessel tone, regulating extracellular matrix deposition and local cellular growth, and controlling homeostatic as well as inflammatory responses. Indeed, ECs produce a variety of vasculo-regulatory and vasculo-tropic molecules that act locally or at distant sites (Figure IV), thus influencing smooth muscle cells, platelets and peripheral leucocytes. Particularly important in endothelium stimulation are mechanical stimuli, *i.e.* shear and tensive stress. Shear stress is defined as the stress due to the friction produced by the blood flow on ECs and involves only the endothelium. On the contrary, the tensive stress involved the entire wall of the vessel (endothelial cells, fibroblasts and smooth muscle cells), because it is

caused by the hydrostatic pressure within the vessel. Shear stress activates ECs and promotes the release of vasodilatant mediators, whereas tensive stress stimulates directly smooth muscle cells, thus inducing their contraction and at the same time ECs stretching. Therefore, the net effect of mechanic stimuli on vascular tone results from the interactions between the pressure-induced myogenic contraction and the endothelium-dependent vasodilatation induced by blood flow.

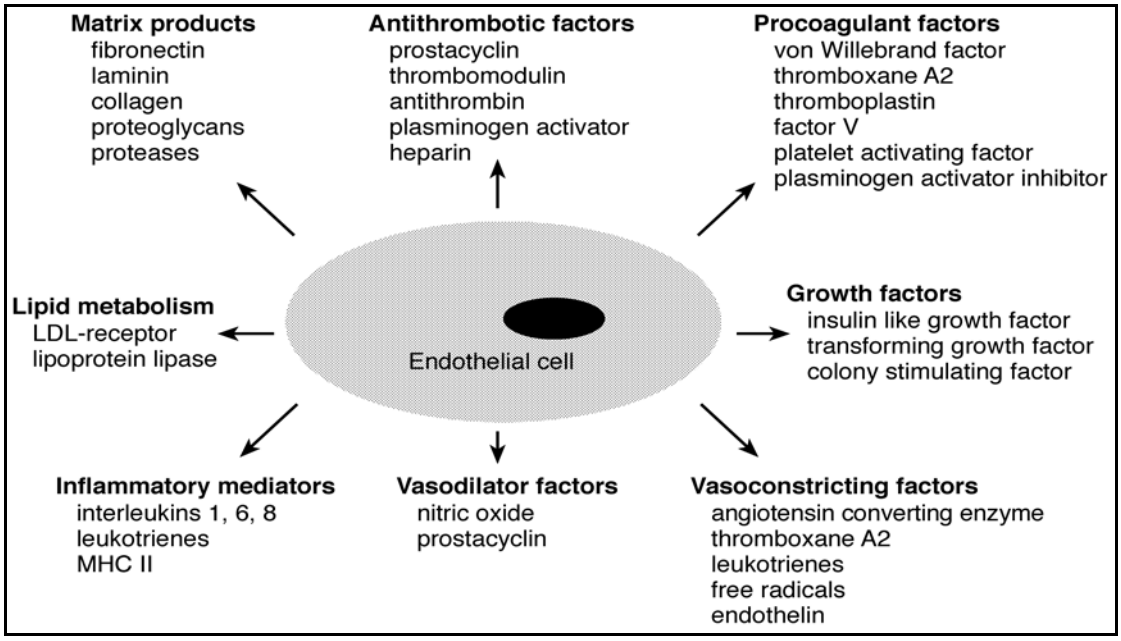



**Figure IV.** A schematic overview of mediators secreted by endothelial cells (Galley & Webster, 2004)

ECs contribute to the regulation of blood flow and pressure by releasing vasodilators such as nitric oxide (NO) and prostacyclin (PGI2), but also vasoconstrictors, including platelet-activating factor (PAF) and endothelin (ET). PGI2, ET and PAF are synthesized primarily in response to changes in the external environment. On the other hand, NO is constitutively secreted by ECs. Its production is however modulated by a number of exogenous chemical and physical stimuli.

Another crucial physiologic function of the endothelium is to provide an antithrombotic surface that inhibits platelet adhesion and clotting, thus facilitating blood flow. The anticoagulant activity of the endothelium is based on the deregulation of thrombin through the action of several factors. As an example, the action of two

anticoagulant enzymes, antithrombin III (Rosenberg, 1989)and heparin cofactor II can be potentiated by the glycocalyx glycosaminoglycans heparan sulfate and dermatan sulfate, respectively(Tollefsen & Pestka, 1985). Other anticoagulant factors produced by the endothelium are the Tissue Factor Pathway Inhibitor (TFPI), which inhibits the tissue factor-activated factor VII complex binding to activated factor X (Broze, 1995), and thrombomodulin (TM) that could be localized on the cell membrane or released into the circulation. The binding of TM to protein C favors the association with the cofactor protein S, which determines an increase of the anticoagulant activity of TM itself. When protein C is activated by its cofactors such as the endothelial protein C receptor (EPCR), it inhibits factors V and VIII. A homeostatic equilibrium is always present between anti- and pro-thrombotic factors. This dynamic equilibrium is however perturbed during injuries, or when the endothelium is exposed to physical forces (shear or tensive stress) or mediators such as thrombin, endotoxin, cytokines, hypoxia or oxidized lipids (Bevilacqua et al., 1986). In response to these conditions, ECs undergo programmatic biochemical changes that culminate in their transformation to a pro-thrombotic surface. Importantly, ECs can easily return to its unperturbed state once the given pro-coagulant stimulus has been dissipated.

In addition to the above-mentioned contribution of the endothelium to the regulation of blood coagulation, ECs also express cell surface-molecules that orchestrate the trafficking of circulating blood cells. These cell-associated molecules help directing the migration of leukocytes into specific organs under physiologic conditions and accelerate migration towards sites of inflammation. These pathways have been also implicated in platelet and erythrocyte adhesion in several common disorders associated with vascular occlusion (Cines et al., 1998).

The endothelium also plays a crucial role in the regenerative/reparative process and in neovascolarization. All these processes are based on angiogenesis, *i.e.* the development of new blood vessels/capillaries from pre-existing vessels (as opposed to

vasculogenesis which is the *de novo* formation of vessels during embryogenesis), which is an essential process in normal growth. However, in healthy adults, angiogenesis occurs only in selected phases of the female reproductive cycle and in processes of wound healing/tissue repair. Recent evidences suggests that angiogenic ECs arise not only from contiguous ECs but may also derived from bone marrow derived EC precursors. These precursor cells are identified by specific cell surface antigen expression and are present in the peripheral blood (Asahara et al., 1997). In particular, in an animal model of ischemia (Kalka et al., 2000; Murohara et al., 2000), it has been shown that these EC precursors serve as sites of angiogenesis and are directly involved in neovascularization.

## 3. Nitric oxide

Nitric oxide (NO) is one of the simplest biological molecules, but nevertheless it is involved in several important physiological processes by acting as a neurotransmitter, a vasodilator, and a cytotoxic agent (Alderton, Cooper, & Knowles, 2001).

NO merely consists of a single oxygen atom bonded to a nitrogen atom through a chemical bond that exhibits partial double and partial triple bond character resulting from the unpaired electron occupying the 2p$\pi*$ molecular orbital. NO is highly lipophilic and can therefore easily diffuse from its source to cross multiple cell membranes and reach its final targets often localized at some distance from the sites of NO synthesis. Furthermore, its free radical character confers to NO specific chemical reactivity properties and determines its tendency to interact with numerous *in vivo* targets (Dudzinski, Igarashi, Greif, & Michel, 2006).

One of the most important function of NO is the regulation of cardiovascular physiology (*e.g.* blood pressure levels) through its vasodilating properties. Moreover, NO inhibits platelet aggregation and inhibits the proliferation of vascular smooth muscle cells

(VSMCs). Finally, NO also plays a crucial role in angiogenesis by promoting EC proliferation, differentiation, and migration to the perivascular space.

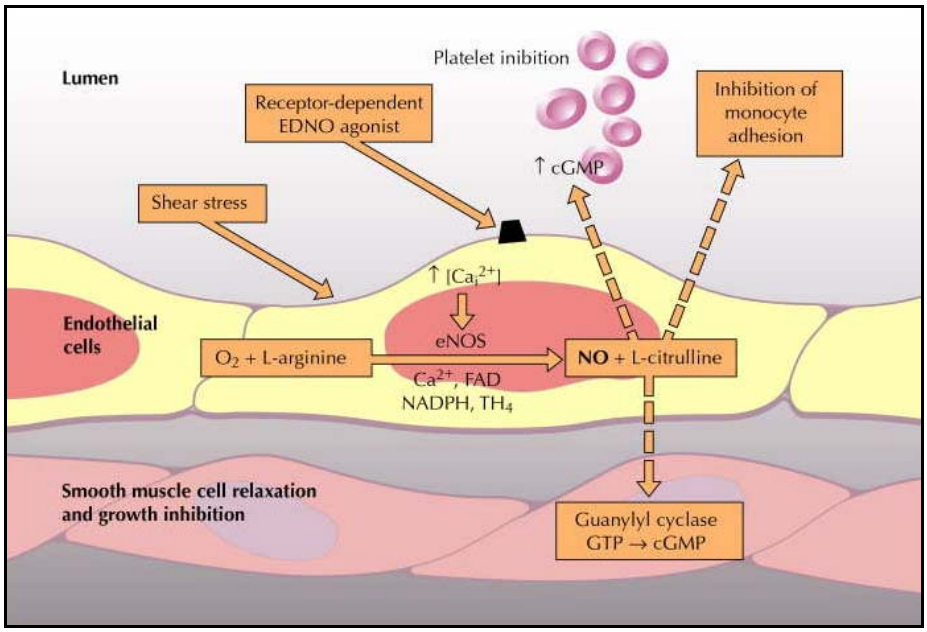



**Figure V.** Nitric oxide function in cardiovascular system

## 3.1. Nitric Oxide Synthase

NO is synthesized from L-arginine and $O_2$ by the catalytic action of dimeric enzymes termed NO Synthases (NOSs) (Dudzinski et al., 2006).

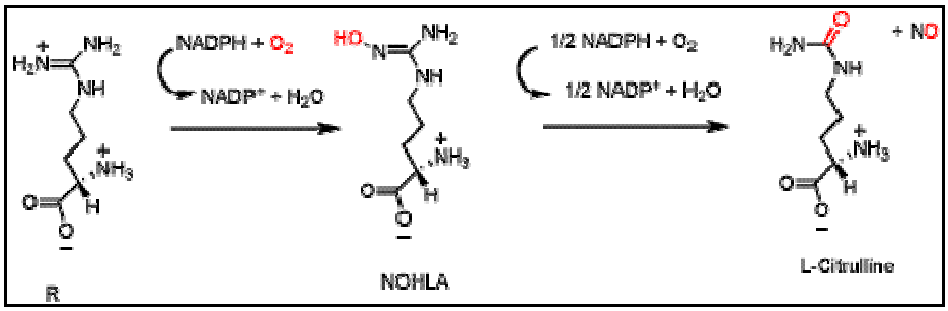



**Figure VI.** NO formation reaction

Three mammalian archetypal isoforms are distinguished: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). The three isoforms are the products of distinct genes but share approximately 50-60% sequence homology. Furthermore, they have distinctive catalytic and regulatory properties, although they share similar

enzymatic and chemical properties. The common structure of NOS show two main domains: (1) the N-terminus that exhibits homology with the other NOS isoforms and poor similarity versus the cytocrome P 450 monooxigenase enzymes; (2) the C terminus that shows homology with various cytocrome P450 reductases. The N- and C-terminal domains are linked by a short sequence that binds calmodulin, an allosteric effector essential to full NOS activity. The N-terminus presents two binding sites: one binds tetrahydrobiopterin (THB4) and heme, while the other one represents the active site where L-arginine binds to the enzyme. The L-arginine binding promotes the dimerization of the enzyme itself, a crucial step for acquiring catalytic activity. On the other hand, the C-terminal domain binds the co-factors NADPH, FAD and FMN, which are essential for NOS activity.

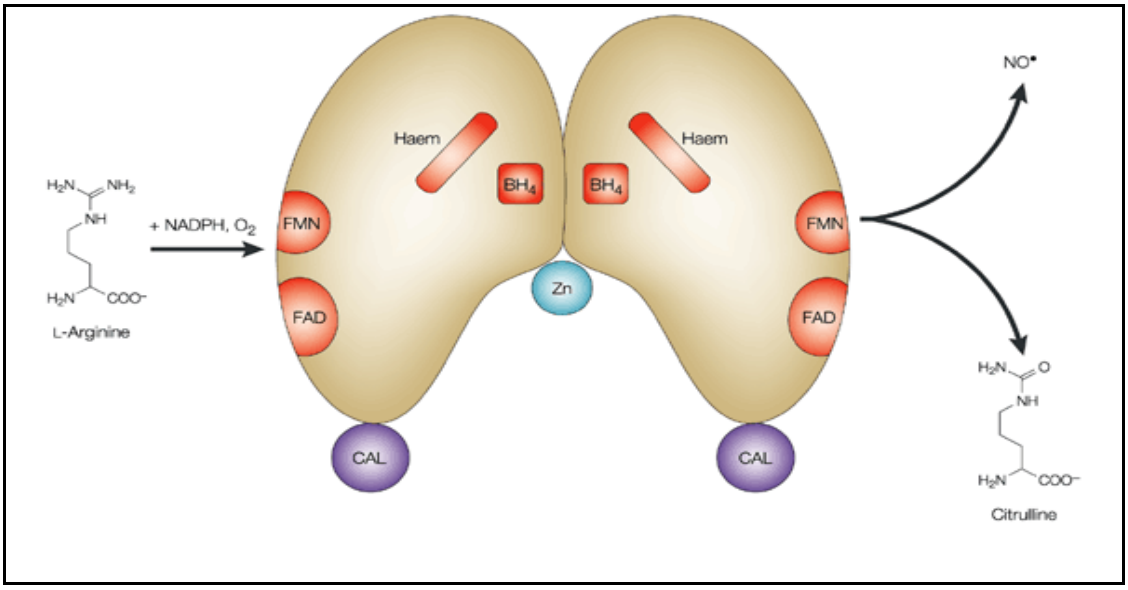



**Figure VII.** Nitric Oxide Synthase and its cofactors (Vallance & Leiper, 2002)

## 3.2. Regulation of NOS

NOS enzymatic activity is finely regulated to maintain NO homeostasis. In fact, the gas has *in vivo* an half-life of less than 5 seconds so it cannot be stored (Govers & Rabelink, 2001). The first regulation level of NOS consists in the tissue-specific expression of the different isoforms. nNOS is widely expressed in neurons of the central and peripheral nervous systems, but it is also found in skeletal muscle, the adventitial layer of some

blood vessels, pulmonary epithelium, the gastrointestinal system, and the genitourinary system. iNOS was first recognized in activated macrophages, but has been also identified in numerous activated cell types, including monocytes, neutrophils, eosinophils, hepatocytes, vascular smooth muscle cells, myocytes, osteoblasts, fibroblasts, epithelium, and endothelium. eNOS is expressed in vascular endothelium as well as in blood platelets and cardiomyocytes (Sessa, 2004).

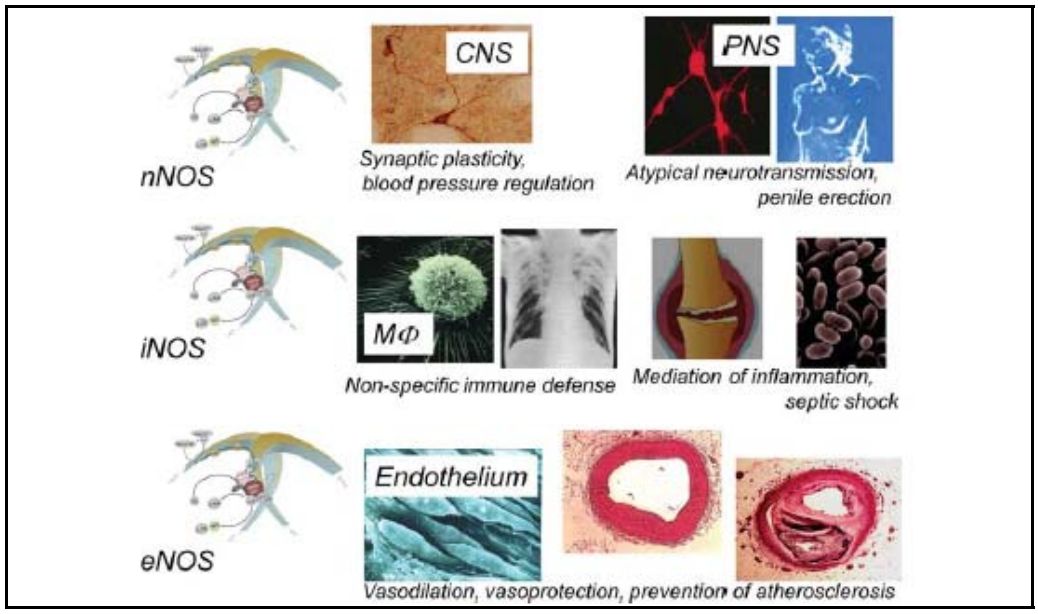



**Figure VIII.** Chief function of the different NOS isoforms: the importance of tissue-specific expression (Förstermann & Sessa, 2012)

All three isoforms are subject to transcriptional regulation. eNOS transcription can be for example actively modulated in response to laminar shear stress in ECs through the activation of the transcription factor KLF2 or by the action of the Rho/Rho kinase pathway. There is also a post-transcriptional level of regulation which acts on the NOS mRNA stability. For instance, although eNOS mRNA exhibits notable stability under normal physiological conditions, its stability is diminished in the presence of oxidized LDL, thrombin, inflammation, and hypoxia. This decrease in eNOS mRNA stability triggers a decrease in eNOS expression maintaining at the same time normal level of eNOS transcription. Nevertheless, the most important regulation of NOS activity occurs at post-transcriptional levels. For example the caveolar targeting of eNOS is entirely dependent on irreversible myristoylation at its N-terminal glycine (Gonzalez, Kou, Lin, Golan, &

Michel, 2002). Myristoylation also seems to initially target eNOS to the cell membrane, where the enzyme is doubly palmitoylated at N-terminal cysteine residues 15 and 26, a modification that further helps to anchor eNOS to caveolae membranes. However, this palmitoylation is reversible and is controlled by cell signalling, thus resulting in the dynamic regulation of eNOS localization. The recruitment of eNOS in caveolae is essential for its activity, because caveole are enriched in cholesterol and sphingolipids that decrease the fluidity of these membrane regions in comparison to the surrounding plasma membrane. In this way, eNOS is located in close physical proximity to other upstream signalling proteins that co-localized in caveolae, thus regulating its activity. Furthermore, in EC caveolae, eNOS can strongly and directly interact with caveolin-1. This protein-protein interaction inhibits eNOS activity by sterically occupy the calmodulin binding site. Calmodulin is a key-activator that specifically links NOS activity to cellular calcium levels. In fact, in the absence of bound calmodulin, the transfer of electrons from the reductase to the oxygenase domain is impeded and the catalytic activity is blunted. When intracellular calcium reaches appropriate levels, it promotes the dissociation of eNOS-caveolin-1 complex, permitting the association of eNOS with calmodulin.

Another fundamental post-transcriptional modification in all NOS isoforms is phosphorylation. eNOS is known to be phosphorylated at multiple sites by the action of the pathway phosphoinositide-3-kinase (PI3K)/Akt. This pathway can be activated by different factors such as insulin, bradykinin, Vascular Endothelial Growth Factor (VEGF), estrogens and shear stress. The most important phosphorylation site is Ser 1177. This residue is located in the reductase domain of the enzyme and its phosphorylation increase both calcium-sensibility and enzymatic activity. A further important stimulatory phosphorylation in response to shear stress occurs at Ser 635. Finally, phosphorylation at Ser 617 stabilizes the binding with calmodulin and supports the phosphorylation of the other sites. On the other hand, phosphorylation of Thr 495 and Ser 116 have an inhibitory role, preventing the binding of calmodulin. When ECs are stimulated to produce eNOS, this residues are dephosphorylated to permit full activation of the enzyme.

## 3.3. eNOS activators

### 3.3.1. VEGF

Vascular Endothelial Growth Factor (VEGF) is the most important and better characterized angiogenic factor. It is essential for proliferation, migration and survival of ECs (Ferrara & Davis-Smyth, 1997). Native VEGF is 45 kDa basic, heparin-binding and homodimeric glycoprotein, similar to Platelet-Derived Growth Factors (PDGFs). The VEGF gene family is composed by five different members: VEGF-A, VEGF-B, VEGF-C, VEGF-D e PIGF. Other two members were recently identified: VEGF-E (in virus) and VEGF-F (in some snake venom). In human, VEGF-A is the chief member and its gene is organized as eight exons separated by seven introns. Alternative exon splicing was initially shown to result in the generation of four different isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$), having 121, 165, 189 and 206 amino acids, respectively, after the cleavage signal sequence. $VEGF_{165}$ is the predominant isoform and lacks the residues encoded by exon 6, whereas $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Less frequent splice variants have been also reported, such as $VEGF_{145}$ and $VEGF_{183}$. The effects of VEGF isoforms on the vascular endothelium are schematically shown in table I.

VEGF receptors are a family of closely related tyrosine kinase receptors (RTKs) consisting of three members termed VEGFR-1, VEGFR-2 and VEGFR-3. These receptors are characterized by an extracellular portion consisting of 7 immunoglobulin-like domains, a single trans-membrane spanning region, and an intracellular portion containing a split tyrosine-kinase domain. When a member of the VEGF family binds a VEGFR on the cell surface, receptors dimerize and become activated through a transphosphorylation mechanism. Then, a cascade of reactions activate different pathways in the cells. In particular, VEGF may increases intracellular calcium levels in ECs, thus activating eNOS. In addition to these RTKs, VEGF interacts with a family of co-receptors called neuropilins.

| Ligand | Isoforms | Receptor | Biological activity |
|---|---|---|---|
| VEGF (VEGF-A) | VEGF-$A_{121}$<br>VEGF-$A_{165}$<br>VEGF-$A_{189}$<br>VEGF-$A_{206}$<br>(VEGF-$A_{138}$/$_{145}$/$_{162}$/$_{165b}$) | VEGFR-1 and R-2;<br>$VEGF_{165}$ binds to neuropilin-1 and -2;<br>$VEGF_{165}$ binds to neuropilin-1 | Vasculogenesis, angiogenesis, vascular homeostasis, vascular permeability, recruitment of bone marrow-derived cells |
| PIGF | $PIGF_{131}$ (PIGF-1)<br>$PIGF_{152}$ (PIGF-2)<br>$PIGF_{203}$ (PIGF-3) | VEGFR-1;<br>$PIGF_{152}$ binds to neuropilin-1 and -2 | Angiogenesis, monocyte migration, up-regulation of VEGF-A, recruitment of bone marrow-derived cells |
| VEGF-B | VEGF-$B_{167}$<br>VEGF-$B_{186}$ | VEGFR-1 and neuropilin-1 | Angiogenesis, recruitment of bone marrow-derived cells |
| VEGF-C (VEGF-2) | | VEGFR-2 and -3 and neuropilin-2 | Development of lymphatic system and lymphangiogenesis, angiogenesis |
| VEGF-D | | VEGFR-2 and -3 and neuropilin-2 | Lymphangiogenesis and angiogenesis |
| VEGF-E | | VEGFR-1 and neuropilin-1 | Angiogenesis |
| VEGF-F | | VEGFR-2 | Angiogenesis, and vascular permeability |

**Table I.** The different biological activity of VEGF isoforms

modified from (Ylä-Herttuala, Rissanen, Vajanto, & Hartikainen, 2007)

VEGF is essential for both physiological and pathological angiogenesis. Its synthesis is induced during hypoxia by the action of Hypoxia Inducible Factor 1α (HIF-1α), a transcriptional factor produced in response to hypoxic stress. The newly synthesized VEGF is secreted and acts on ECs close to the hypoxic site by stimulating new vessel formation to compensate for loss of $O_2$. This mechanism is also exploited by tumors that induce new vessel formation to compensate their high metabolic rate and grow more rapidly. Furthermore, angiogenesis is required for tumor metastatization. Anti-VEGF therapies based on monoclonal antibody or molecules inhibiting its receptor activity are indeed currently under study in the attempt to limit tumoral neo-vascularisation.

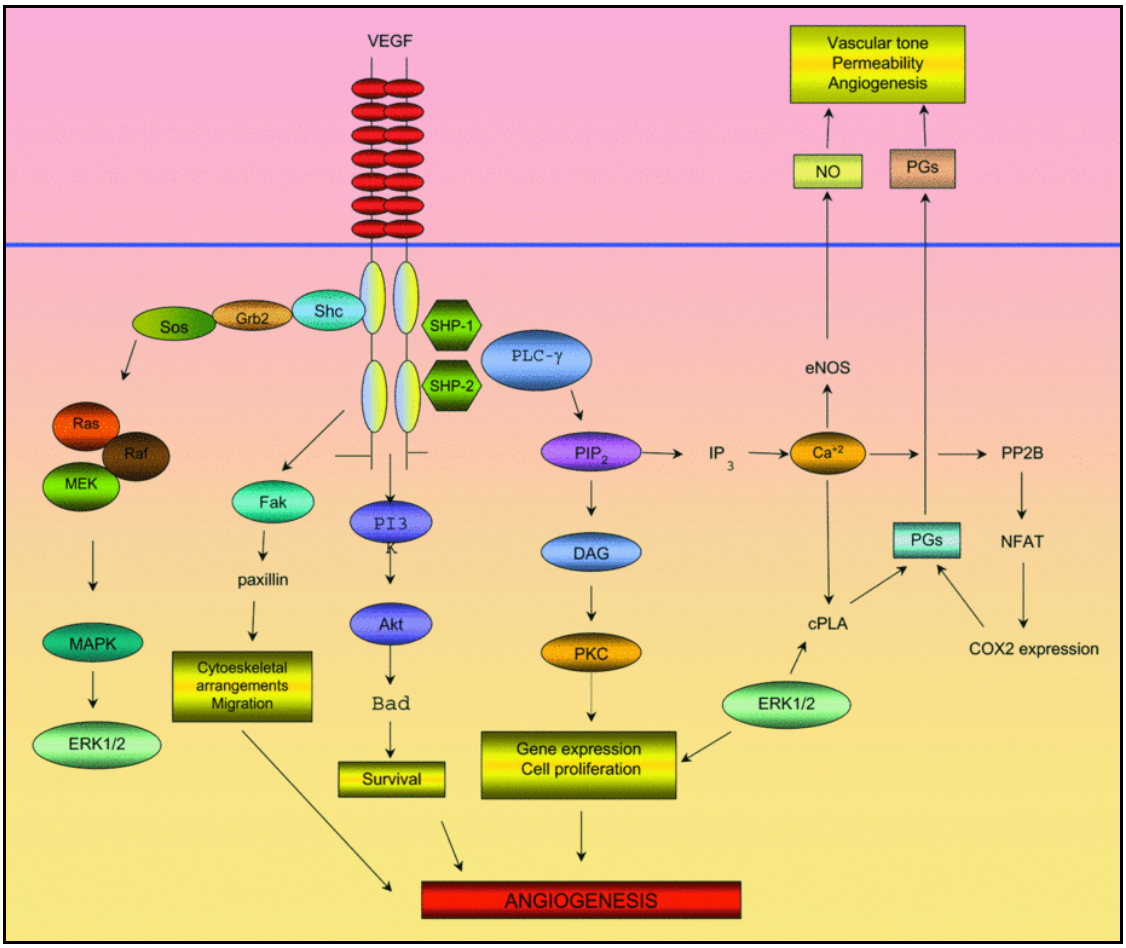



**Figure IX.** VEGF pathway: the role on angiogenesis (Romanque, Piguet, & Dufour, 2008)

### 3.3.2. Bradikinin

Bradikinin (BK) is a biologically active kinin. It is a short-lived peptide predominantly generated by the enzymatic action of kallikreins on kininogen precursors. BK is locally produced in tissue, often after a trauma, and acts as a potent endothelium-dependent vasodilator, leading to a drop in blood pressure. BK interacts with specific G-protein coupled receptor (GPCRs) at the EC surface, and this interaction leads to a rise in intracellular calcium through various mechanisms involving phospholipase C, prostaglandins, protein kinase and phospholipase $A_2$. The increase in intracellular calcium supports calcium-calmodulin complexes formation that together with the BK-induced PI3-K/AKT cascade stimulation, finally induces eNOS activation (Figure X).

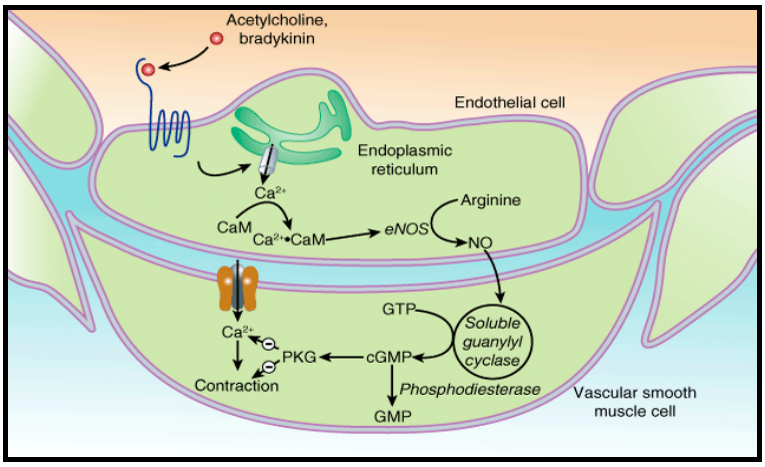



**Figure X.** Bradikinin pathway in ECs (Katzung et al., Basic & Clinical Pharmacology, 11th Edition)

### 3.3.3. Insulin

Insulin is an hormone produced by pancreatic beta cells crucially involved in the regulation of the carbohydrate and fat metabolism in the body. Insulin binds to a RTK that once activated phosphorylates its substrate IRS (Insulin Receptor Substrate). Activated IRS on its turn phosphorylates Grb2, an adaptor protein, which recruits Sos1 to finally activate the PI3-K/AKT pathway. In this way, the Ser1177 of eNOS become phosphorylated leading to enzyme activation and NO formation (Figure XI).

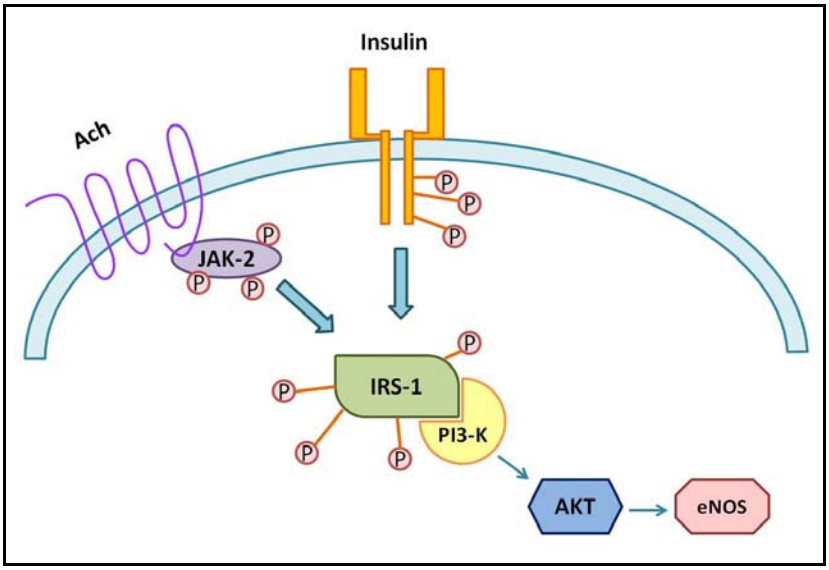



**Figure XI.** Insulin-mediated activation of eNOS

### 3.3.4. Shear stress

Vascular ECs are influenced *in vivo* by two distinct hemodynamic forces: cyclical strain due to vessel wall distension by transmural pressure, and shear stress that is the frictional force generated by blood flow. Shear stress acts at the apical cell surface to deform cells in the direction of blood flow, whereas wall distension tends to deform cells in all directions. Acute shear stress elicits *in vitro* rapid cytoskeletal remodelling and signaling cascades in ECs, with a consequent acute release of NO and prostacyclin. Transmural pressure acts instead in an opposite way. In fact, it induces the contraction of smooth muscle cells of the vessel wall by causing vasoconstriction. The net effect on the vessel wall depends on the combination of the two opposite forces.

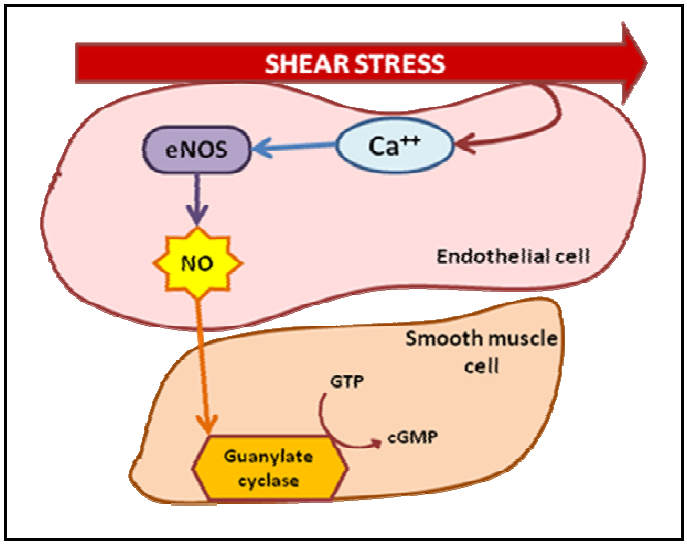



**Figure XII.** Shear stress and eNOS activation

## 3.4. eNOS inhibitor

### 3.4.1. ADMA

Asymmetric dimethylarginine (ADMA), an analogue of L-arginine, is a naturally occurring product of metabolism found in human circulation. ADMA derives from protein methylation catalyzed by a family of enzymes called S-adenosylmethionine protein N-methyltransferases (protein methylases I and II). The methyl groups transferred to

ADMA derive from the methyl group donor S-adenosylmethionine, an intermediate in the metabolism of homocysteine. When present at high levels, ADMA inhibits NO synthesis by competing with L-arginine for the active site of eNOS, thus impairing endothelial function.

ADMA can be easily measured in plasma and urine. It should be noticed that elevated ADMA levels have been found in plasma of patients with hypercholesterolemia, hypertension, chronic heart failure, chronic renal failure and other internal disorders. Recent prospective and cross-sectional studies seem to indicate that elevated ADMA levels are a risk factor for future cardiovascular events and mortality, suggesting for ADMA a diagnostic relevance as a novel cardiovascular risk marker (Siroen et al., 2006).

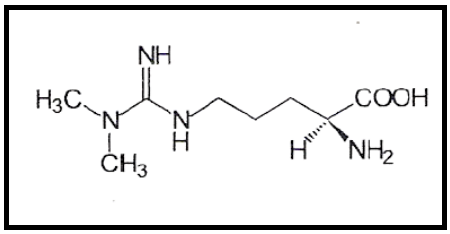



**Figure XIII.** Chemical structure of ADMA

3.4.2. L-NAME e L-NMMA

L-NAME (L-$N^G$-Nitroarginine methyl ester) and L-NMMA (L-$N^G$-monomethyl Arginine) are synthetic analogues of ADMA. They act as competitive inhibitor of all NOS isoforms in a dose-dependent manner.

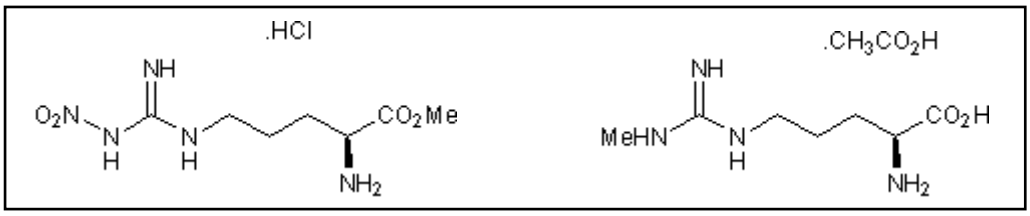



**Figure XIV.** Structures of L-NAME (left) and L-NMMA (right)

## 3.5. Physiological role of NO

In biological systems, NO acts as an important intra- and inter-cellular messenger and regulates many physiological functions. In particular, it crucially modulates the cardiovascular system through the ability of ECs of producing NO in response to various mechanic or chemical stimuli (Brennan et al., 2002). From ECs, NO diffuses in part into the haematic compartment, where it reduces platelet aggregation and leucocytes adhesion to the vessel walls, and in part into the vessel smooth musculature by inducing its relaxation. The resulting anti-aggregant, anti-inflammatory and anti-hypertensive effects are very important for the prevention of diseases such as atherosclerosis and thrombosis (Sumi & Ignarro, 2003). Vascular smooth muscle cell (VSMC) relaxation is mediated by the NO-dependent activation of soluble guanylate cyclase (sGC) and by the subsequent increase in intracellular cGMP levels (Figure XV).

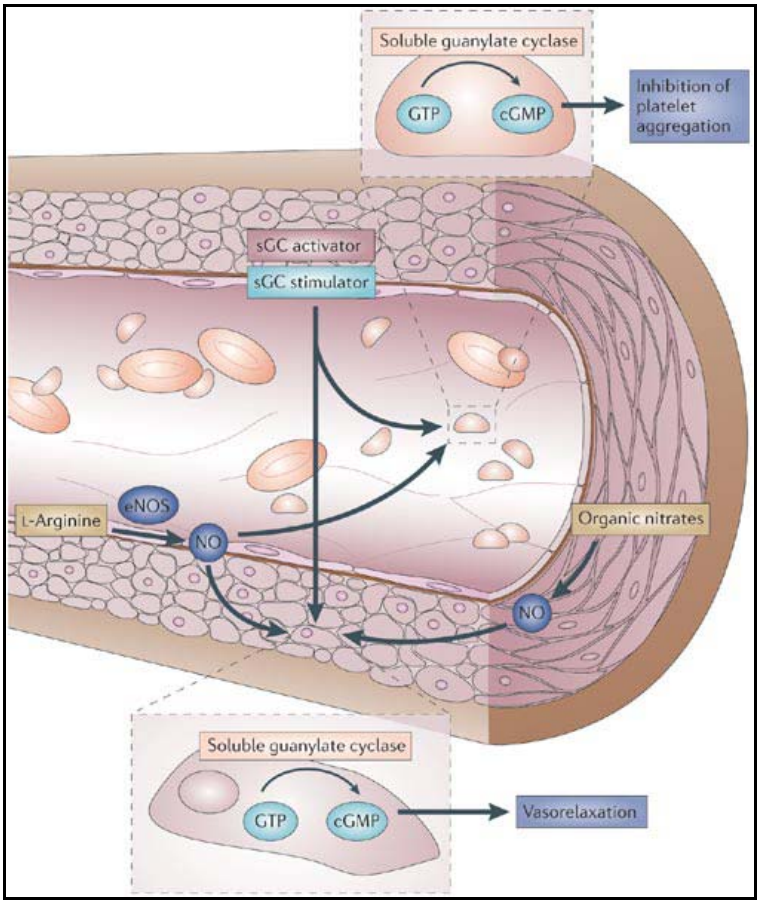



**Figure XV.** Physiological activity of NO in cardiovascular system (Evgenov et al., 2006)

NO is also involved in the immune response to pathogens. Indeed, phagocytes (monocytes, macrophages, and neutrophils) express inducible NOS (iNOS) and produce NO in response to iNOS activation by interferon-gamma (IFN-γ) and tumor necrosis factor (TNF). NO is secreted as free radical and is very toxic to bacteria, being able to induce DNA and plasma membrane damage, and degradation of iron-sulfur centers into iron-ions and iron-nitrosyl compounds. Moreover, in the presence of an increased oxidative stress, NO itself is transformed in peroxinitrite, a radicalic form which contributes to its toxicity (Channon & Jenkins, 1981). NO seems also to be involved in the stimulation of T and B lymphocyte proliferation that participate to the immune response.

NO may also act as neurotransmitter in the central nervous system, in the nor-adrenergic and nor-cholinergic peripheral nervous plexus of the bronchial tree (where shows a bronchodilator effect), and in the gastrointestinal tract.

In conclusion, NO plays a fundamental physiological role not only in cardiovascular system, but also in other system such as brain (learning and mnemonic control), gastrointestinal tract (secretion and motility), respiratory system (bronchial musculature tone), and kidney (blood flux autoregulation).

## 4. Endothelial dysfunction

Endothelial dysfunction (ED) is a systemic pathological state of the endothelium that can be defined as an imbalance between vasodilating and vasoconstricting substances produced by (or acting on) the endothelium (Endemann & Schiffrin, 2004; Furchgott & Zawadzki, 1980). ED is indeed characterized by a shift of the actions of the endothelium toward a reduced vasodilation, a pro-inflammatory state, and the acquirement of pro-thrombotic properties. It is associated with most of cardiovascular diseases (CVDs), such as hypertension, coronary artery disease, chronic heart failure, peripheral artery disease,

diabetes, and chronic renal failure. The patho-physiology of ED is complex and involves multiple mechanisms that include a reduced NO generation, an excess of oxidative species, and a reduced production of hyperpolarizing factor. Up-regulation of adhesion molecules, generation of chemokines such as macrophage chemoattractant peptide-1 (MCP-1), and production of plasminogen activator inhibitor-1 (PAI-1) participate to the inflammatory response and contribute to the establishment of a pro-thrombotic state. Vasoactive peptides such as angiotensin-II and endothelin-1, ADMA accumulation, hypercholesterolemia, hyperhomocysteinemia, an altered insulin signalling, and hyperglycemia also contribute to the generation of ED. Acting through these various mechanisms, ED represents one of the main cardiovascular risk factors predisposing to atherosclerosis, plaque instability and thrombosis, thus increasing the incidence of future clinical events (Widlansky, Gokce, Keaney, & Vita, 2003).

ED was first described in 1990 in the forearm vasculature of high-blood pressure suffering patients. Later, an impairment of vasodilation was described in type 1 and type 2 diabetes, coronary artery disease, congestive heart failure, and chronic renal failure (Figure XVI). Moreover, ED is not only associated with CVDs but also precede their development, as shown by a study on offspring of hypertensive patients. Importantly, ED is a functional reversible pathology. Early diagnosis is fundamental for treating ED to avoid its degeneration in more serious diseases (Hadi, Carr, & Al Suwaidi, 2005). Alterations of endothelial functions such as a diminished endothelium-dependent vasodilatation and an increased concentration of free oxygen-radical were found in potential pre-atherosclerotic situation such as hypercholesterolemia, arterial hypertension, diabetes, estrogenic deficiency and vascular aging. Currently, the deficiency of tetrahydrobiopterin (THB) has been added to the list of risk factors. In fact, THB is a NOS cofactor, and its decrease is associated to eNOS uncoupling and to the production of superoxide anions instead of NO.

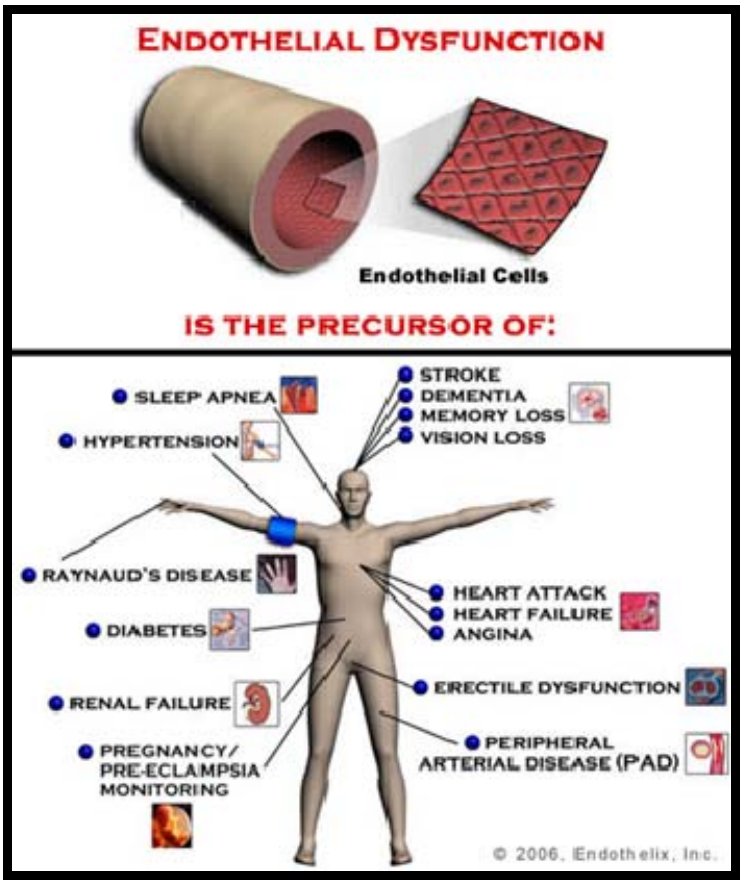



**Figure XVI.** A schematic overview of ED-related pathologies

The key player in ED is the diminished bioavailability of NO, due to a reduction in eNOS activity (Endemann & Schiffrin, 2004). The co-presence of an excess of reactive oxygen species (ROS) further contributes to NO depletion, since NO reacts with ROS to form peroxynitrites, cyto-oxidant molecules that alter cellular protein function through nitration. Peroxynitrites are also involved in the oxidation of Low Density Lipoproteins (LDLs), which in this way increase their pro-atherogenic effect and reduce NO bioavailability by inhibiting its synthesis. This blocking effect on eNOS seems to be due to the interaction of the oxidised LDL with inhibitory G-proteins (Gi). Oxidative stress is also associated with an increase in inflammatory processes and *thrombi* formation, and it is directly correlated to the decrease in endothelial-dependent vasorelaxation and to the rate of cardiovascular events. Finally, an important element involved in ED is Endothelin-1 (ET-1). ET-1 is the strongest vasoconstrictor molecule of endothelial origin, and acts through its binding to the specific receptors ETA and ETB. ETA receptors are localized only on VSMCs and cause vasoconstriction and cellular proliferation, while ETB receptors

are localized both on VSMCs and ECs and induce vasodilatation by stimulating NO production, which in turn acts as a negative feedback on ET-1 production. When NO bioavailability is reduced, the negative feedback is compromised and consequently the vasoconstrictor effect of ET-1 become predominant.

## 5. Clinical highlights

Strong evidences demonstrate that ED is predictive for stroke and heart attacks due to the inability of arteries to fully dilate. Furthermore, it has also been shown that ED foregoes the development of artherosclerosis, a chronic disease characterized by loss of elasticity of the arterial walls due to abnormal thickening and hardening. Notably, artherosclerosis itself may cause a stroke or heart attack.

### 5.1. Diagnosis of ED

Since the evaluation of endothelial functions is operatively troublesome, the measure of endogenous NO activity is taken as representative of its overall functionality (Hirata et al., 2010). In the clinical practice, the golden standard technique used to evaluate endothelial function is the "acetylcholine endothelial function and adenosine coronary flow reserve" test that consists in two step:

1. adenosine, which normally causes the dilatation of small vessels of the heart, is injected into one of the coronary arteries and the amount of blood flow is measured;
2. acetylcholine, which normally causes dilation in the large arteries, is then injected and the blood flow is measured again.

If at least one of these two steps shows a decrease in the blood flow to the heart, physicians diagnose the presence of ED and microvascular disease.

Currently, some non-invasive procedures are available to check artery's health and elasticity:

- Carotid Duplex Ultrasound: this test is performed to evaluate symptoms including stroke, loss of memory, dizziness, loss of control of muscles and other findings that might result from blockage or narrowing of one or both carotid arteries;
- Pulse Wave Velocity (PWV) that evaluates, via ultrasound, the flow of blood from the carotid to the femoral artery. Imaging specialists can establish if any blockage exists by estimating the time of travel of the pulse wave;
- Peripheral Arterial Tonometry (PAT) that is performed using a blood pressure cuff combined with a graphic computer display, and measures diastolic, systolic and mean artery pressure. It has been demonstrated that a PAT ratio (hyperemic response) at 90-120 seconds after 5-minute forearm cuff occlusion correlates with cardiovascular risk factors (Framingham Heart Study).

Some new putative biomarkers of ED are currently under study and are listed in table II.

| **New Biomarkers of Vascular Endothelial Dysfunction** |
|---|
| Insulin resistance |
| Homocystinemia |
| Lipoprotein (a) |
| ADMA |
| Adiponectin |
| Inflammatory factors (CRP IL-1, IL-6, TNF-, MCP-1) |
| Endothelial progenitor cells (EPS) |
| Vasodilators (nitrite and nitrate, 6-keto PGF1) |
| Vasoconstrictors (endothelin, tromboxan A2, ROS) |
| Adhesion molecules (VCAM-1, ICAM-1, P & E-selectin) |
| Thrombotic hemostatic factors (PAI-1, TPA, von Willebrand factor. thrombomodulin) |

**Table II.** New Biomarkers of ED modified from (Hirata et al., 2010)

## 5.2. Treatment of ED

ED is positively influenced by healthful diet and exercise. These two devices are both necessary to control the patient's weight that is one of the potential risk factor of developing diseases such as type-2 diabetes and high blood pressure. In particular, a moderate aerobic exercise contributes to maintaining a healthy pumping heart and improves breathing, resulting in a more efficient delivery of oxygen-carrying blood cells. Strength and flexibility work-out could also have beneficial effects. On the other hand, pharmacological treatment of ED is based on the assumption of different drugs with the aim of reducing ED symptoms and restoring the physiological condition (Puddu, 2000; Radenković, Stojanović, Potpara, & Prostran, 2013). The most widely used drugs are summarized in Table III.

| **Drug** | **Timing** |
|---|---|
| Tetrahydrobiopterin | Acute |
| Glutathion | Acute |
| ACE inhibitors | Acute and chronic |
| Calcium antagonists | Acute and chronic |
| Antioxidants | Acute and chronic |
| L-Arginine, D-Arginine | Acute and chronic |
| Renin inhibitors | Chronic |
| Statins | Chronic |
| Insulin-resistance improving drugs | Chronic |
| Erythropoietin | Chronic |
| Treatments that affect the number of EPC | Chronic |
| β-blockers | Chronic |
| Extrogens | Chronic |
| Nitrate | Chronic |

**Table III.** A schematic overview of the drugs most widely used in ED's treatment

## 6. HIF-1

Hypoxia Inducible Factor 1 (HIF-1) is an hypoxia-induced transcriptional factor able to induce or inhibit the transcription of different genes involved in cellular homeostasis (Brahimi-Horn & Pouysségur, 2009). In particular, HIF-1 regulates the expression of genes codifying for proteins involved in the adaptation and survival of cells under stress conditions, especially in the presence of low oxygen concentrations. In these conditions, HIF-1 acts as a protective factor against pathological conditions. Its activity is however also involved in the hypoxic adaptive response of tumor cells that supports their growth and invasiveness. Hypoxia is indeed the major cause of HIF-1 accumulation both in growing tumor and in stromal cells, and immunohistochemical analyses of human tumor reveal increased HIF-1 levels in most primary tumor and their metastasis. HIF-1 also accumulate is the presence of reactive oxygen species (ROS) or nitrogen species able to inhibit the degradation of the factor. Finally, the transcriptional activation of HIF-1 is essential during the embryonic development.

From the molecular point of view, HIF-1 is an heterodimeric protein composed by a constitutive (HIF-1β) and a regulatory subunit that exist in different isoforms (HIF-1α, HIF-2α, and HIF-3α in humans), among which HIF-1α is the best characterized (Wang, Jiang, Rue, & Semenza, 1995; Wang & Semenza, 1995). HIF-2α shows a high sequence homology with HIF-1α, and is regulated by similar mechanisms. In fact, HIF-2a forms an heterodimer with HIF-1β and activates some of the same genes activated by HIF-1α (Lau, Tian, Raval, Ratcliffe, & Pugh, 2007). Finally, HIF-3α acts as an HIF-1α inhibitor. Its transcription is activated by HIF-1α itself and operates as a negative feedback regulation mechanism (Makino et al., 2007).

All three HIF-1α isoforms are regulated by a post-transcriptional hydroxylation of a proline residue in the Oxygen-Dependent Degradation Domain (ODDD) (Schofield & Ratcliffe, 2004). The hydroxylation of this residue permits the interaction between HIF

and the von Hippel-Lindau factor (VHL), which is part of an ubiquitination domain called VBC (VHL/Elongin B/Elongin C). This complex mediates the covalent binding of HIF to an ubiquitin chain that allows the interaction of HIF with the proteolytic complex for ubiquitinated protein (Kaelin & Ratcliffe, 2008) and the degradation of the factor. On the other hand, the hydroxylation of an asparagine in the extreme C-terminus of the subunits HIF-1α and HIF-2α inhibits their interaction with the co-activator p300 and CBP (CREB-Binding Protein) (Figure XVII).

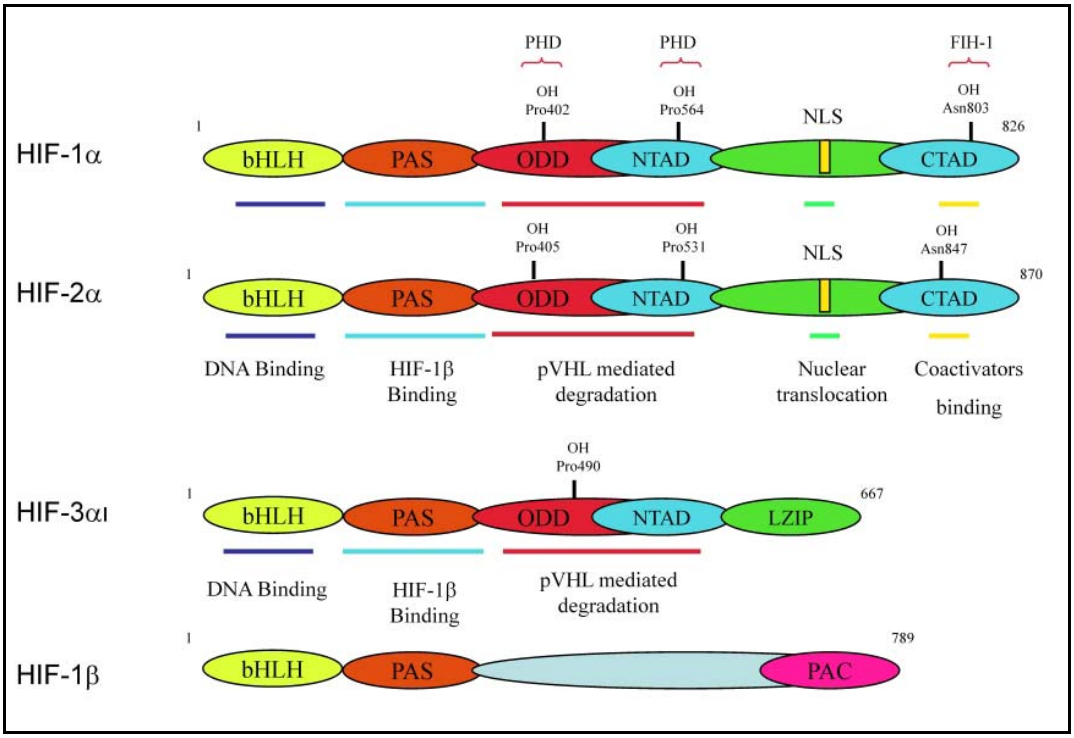



**Figure XVII.** Domain structures of HIF-1 isoforms (Kenneth & Rocha, 2008)

The above-described hydroxylation reactions are catalyzed by two different enzymes, the Prolyl Hydroxylase Domain Protein (PHD) and the Factor Inhibiting HIF (FIH). Both enzymes are di-oxygenases and their activities are dependent on oxygen, $Fe^{2+}$ and 2-oxoglutarate, an intermediate product of the Krebs cycle (Peet & Linke, 2006). During hypoxia, PHD and FIH are deactivated as a consequence of the lack of oxygen and 2-oxoglutarate required for the hydroxylation reactions. Therefore, in hypoxic conditions, HIF-1α is stabilized and immediately transferred to the nucleus via its C-terminus sequence NLS (Nuclear Localisation Signal) that binds to nuclear pores and guides its transfer (Kallio et al., 1998). Once in the nucleus, HIF-1α dimerizes with HIF-

1β, recruits the co-factors p300 and CBP, and by binding to the HRE (hypoxia-responsive element) sequences contained in its target genes finally induces their transcription.

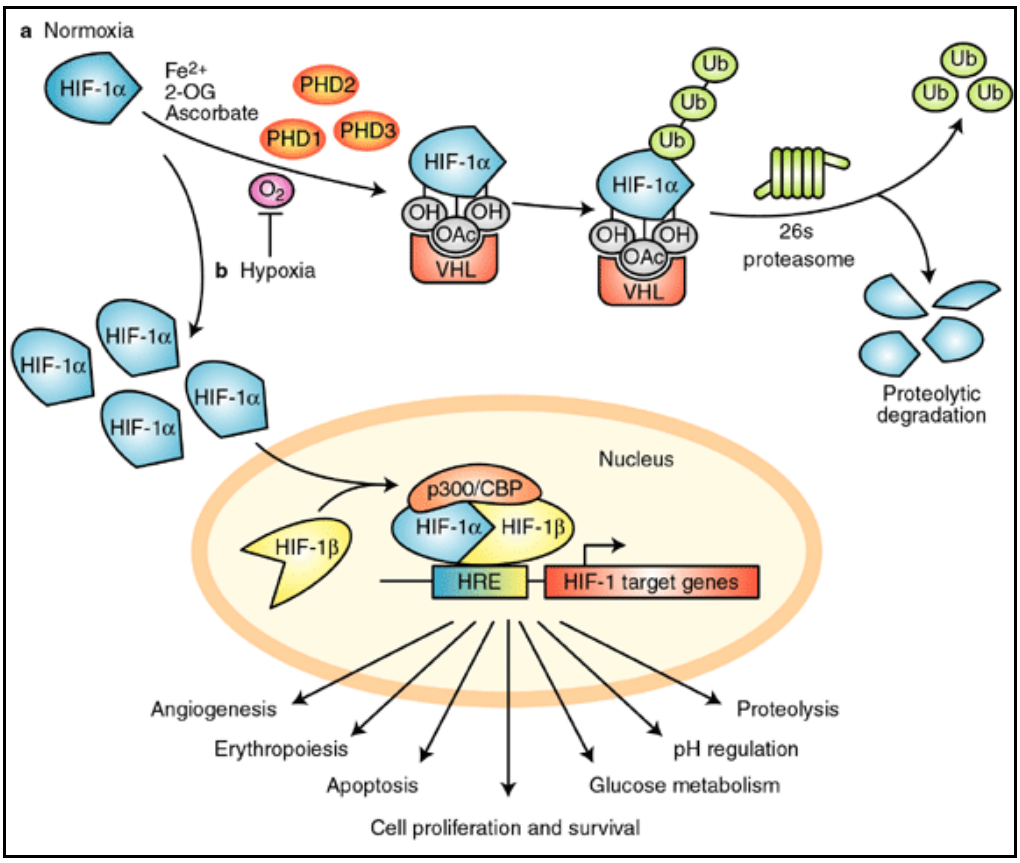



**Figure XVIII.** HIF-1α regulation by proline hydroxilation (Carroll & Ashcroft, 2005)

HIF-1 has also been shown to be activated in response to non-hypoxic stimuli. Interestingly, it has been demonstrated that ROS regulate HIF-1 stability and transcriptional activity in both oxygenated and hypoxic cells. Consequently, ROS are involved in the regulation of HIF-dependent pathways under normal and pathological conditions. Several hypotheses have been proposed about the mechanisms of interaction between ROS and the HIF-1 pathway (Guzy & Schumacker, 2006; Pouysségur & Mechta-Grigoriou, 2006). One hypothesis regards the oxidation by hydrogen peroxide of ferrous iron ($Fe^{2-}$) to ferric form ($Fe^{3-}$) that could consequently hinder the binding of the ferrous iron to PHD (Pan et al., 2007). Another possibility is the enrolment of ascorbate as a free radical scavenger. In such a way, ascorbate could not reduce ferric iron and/or directly bind to PHD. Moreover, free radicals and mitochondrial dysfunction can alter the concentration of 2-oxoglutarate (2-OG) and succinate (SC), which are both involved in

HIF-1α hydroxylation (Gottlieb & Tomlinson, 2005; Kozhukhar, Yasinska, & Sumbayev, 2006). Furthermore, ROS could also affect the HIF-1 pathway by influencing the availability of oxygen or by modifying PHD phosphorylation (Qutub & Popel, 2008). Finally, it has been recently demonstrated that low concentrations of peroxide are able to rapidly oxidized FIH on the catalytic iron centre and/or on other susceptible sites thus reducing the enzyme activity. FIH oxidation can be prevented by pre-treatment with iron chelators, whereas the restoring of co-factors is not able to re-establish FIH activity (Masson et al., 2012).

## 7. ROS

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. ROS originates as a by-product of the oxygen metabolism and possess important roles in cell signaling and homeostasis.

The most important ROS in physiological and pathophysiological conditions are $O_2^{\bullet-}$, $^{\bullet}OH$ and $H_2O_2$.

$O_2^{\bullet-}$ is the result of univalent reduction of triplet state molecular oxygen and can be formed enzimatically by the action of NADPH-oxidase (NOX) (Babior, 1999; Vignais, 2002), xanthine-oxidase and other enzymes, by auto-oxidation reactions (Cadenas, 1989; Cadenas & Davies, 2000), or non-enzymatically by the intervention of redox components such as the semi-ubiquinone compound of the mitochondrial electron transport chain. $O_2^{\bullet-}$ has a poor ability to cross biological membranes and it is also a relatively unreactive molecule in physiological condition. Indeed, superoxide become a very reactive intermediate only when it reacts with NO to give peroxinitrite or when it is converted into $H_2O_2$ by superoxide-dismutase (SOD) isoforms.

In contrast of $O_2^{\bullet-}$ , $H_2O_2$ can easily diffuse across biological membranes. It is a non-radical potent oxidizing agent that can oxidize or reduce several inorganic ions in aqueous solutions. $H_2O_2$ is usually removed by either catalase or glutathione peroxidise,

and can be transform in $^{\bullet}OH$ by the interaction with $O_2^{\bullet-}$ (Haber-Weiss reaction) or in the presence of divalent metal ions such as iron (when $Fe^{2+}$ is present, the reaction is called Fenton's reaction) and copper. $H_2O_2$ is also used by the myeloperoxidase of phagocytic cells to form hypochlorite (HOCl), a highly reactive compound able to oxidize thiol groups, amino groups and methionine in proteins.

$^{\bullet}OH$ is a three-electron reduction state of $O_2$ and despite its very short half-life ($10^{-9}$ s), it is the most reactive molecules among ROS. $^{\bullet}OH$ does not diffuse from the site of generation and can rapidly damage any surrounding molecules, such as amino acids (potentially leading to protein inactivation/denaturation), carbohydrates (degradation), lipids (lipid peroxidation) and nucleic acids (formation of adducts with deoxyguanidine and, potentially, mutations).

Finally, we should also mention the Reactive Nitrogen Species (RNS). The most important is peroxynitrite ($ONOO^-$) that form from the rapid reaction of NO with $O_2$. $ONOO^-$ is a strong oxidant able to react directly with thiol groups, iron-sulphur centres and -SH groups present in the active site of tyrosine phosphatases. In physiological conditions, the production of $ONOO^-$ is quite low and oxidative injury is minimized by endogenous antioxidant defences. When increased in pathological conditions, $ONOO^-$ can act either as a direct oxidising species or indirectly by decomposing into highly reactive radicals. When $ONOO^-$ acts as an oxidant, it produces nitrite and a hydroxide ion rather than isomerising to nitrate and can react with proteins (tyrosine nitration or direct reactions with specific amino acids), lipids (lipid peroxidation) and nucleic acids (oxidative modifications in nucleobases). It should be noticed that $ONOO^-$ can interact with mitochondria, reaching them from extra-mithocondrial compartments or being locally produced through the interaction of NO (generated by the mitochondrial NOS) and $O_2^{\bullet-}$. Mitochondrial toxicity of $ONOO^-$ results from its direct oxidative reactions with the principal components of the respiratory chain or from free radical-mediated damage. Persistent generation of significant levels of $ONOO^-$ can lead to apoptotic or necrotic cell death.

## 7.1. Intracellular sources of ROS

Cellular production of ROS occurs from both enzymatic and non-enzymatic sources. Any enzymatic system or electron-transferring protein result in the formation of ROS as by-products of the electron transfer reaction. In mitochondria, the unintentional ROS generation involves about 1–2% of total $O_2$ consumption under reducing conditions. Indeed, approximately about 1,5% of electrons flowing through the electron transport chain can be redirected to form $O_2^{\bullet-}$ at the levels of complex I (NADH/ubiquinone oxidoreductase) and complex III (ubiquinol/cytochrome c oxidoreductase). $O^{2\bullet-}$ is then usually transformed by mitochondrial SOD into $H_2O_2$, which can go through mitochondrial membranes to the cytoplasm, where it is processed by Catalase. Another important intracellular source of ROS is NOX, that is expressed in both phagocytic (macrophages, neutrophils, and eosinophils) and non-phagocytic cells and plays a crucial role in different diseases (Babior, 1999; Lambeth, 2007). NOX catalyzes the reduction of $O_2$ to $O_2^{\bullet-}$ in a NADPH-dependent reaction through the activity of the trans-membrane protein cytochrome *b*558. Finally, ROS can also be formed by the activity of 5-Lipoxygenase (5-LOX), an oxidase involved in the synthesis of leukotrienes from arachidonic acid in response to growth factors and cytokines.

ROS can also be generated in many subcellular compartments by several enzymes, such as oxidases, mono- and di-oxygenases, peroxidases and isoforms of the cytochrome P450 superfamily. Several enzymes i.e. nitric oxide synthase, xanthine oxidase (Pritsos, 2000), cyclooxygenase and other NAD(P)H dependent oxido-reductases, are all able to generate primarily $O_2^{\bullet-}$. In the same way, peroxisomal oxidases (Rojkind, Domínguez-Rosales, Nieto, & Greenwel, 2002) can generate $H_2O_2$ during the metabolization of different substrates. Another important source of intracellular ROS, mainly $O_2^{\bullet-}$ production, is the auto-oxidation of small molecules such as epinephrine, dopamine, hydroquinones and flavins.

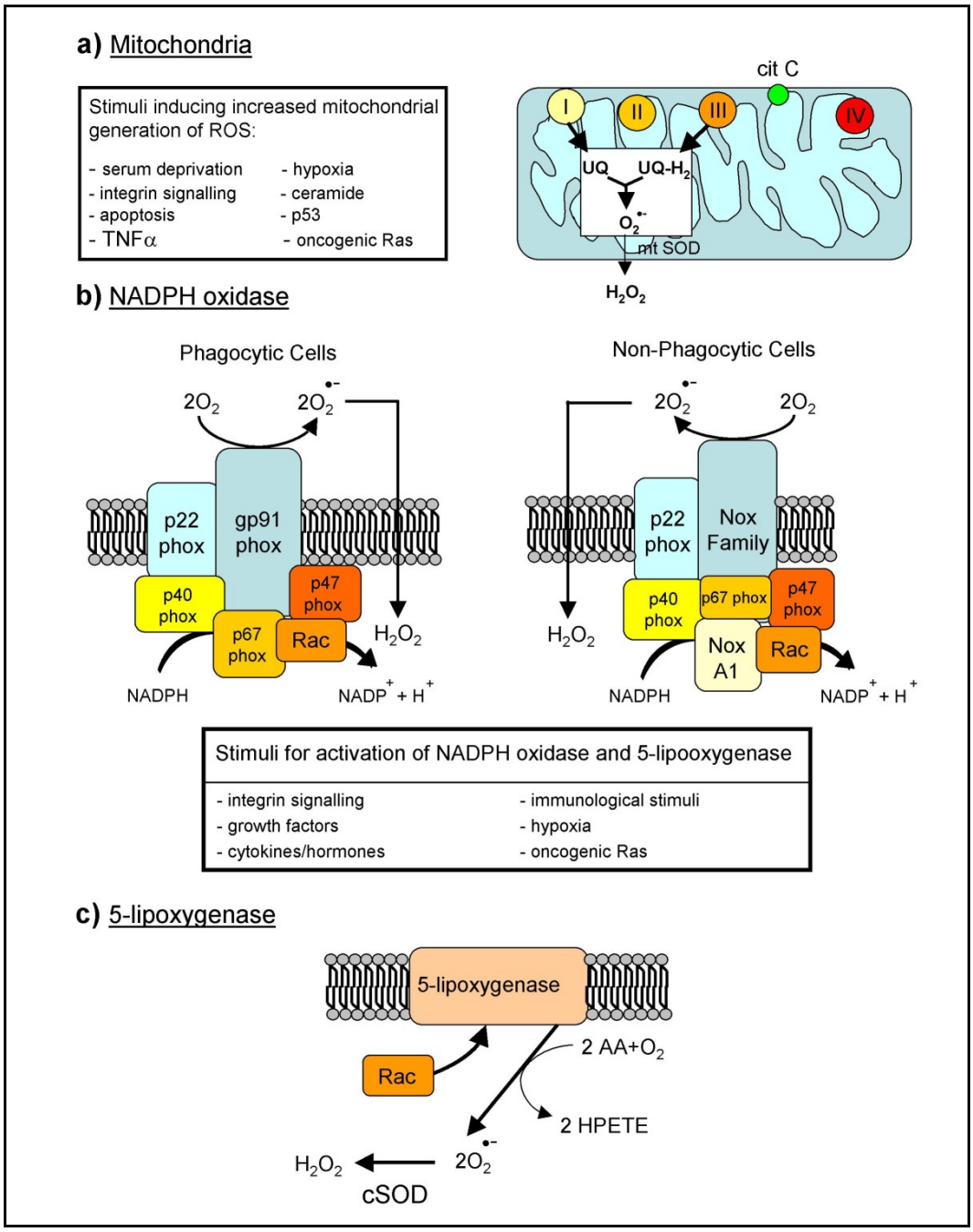



**Figure XIX.** Intracellular sources of ROS (Novo & Parola, 2008)

## 7.2. Cellular ROS content: use and damage

ROS do not have only a damaging activity in cells. Indeed, as summarized in table IV, a variety of cytokines and growth factors are able to generate ROS in non-phagocytic cells by acting through their specific receptors. The main effects of ROS signalling relate to the control of cell proliferation and differentiation, but they are also involved in angiogenesis, particularly in tumor development and metastasis.

| Ligand | Reactive Species | Enzymatic Source(s) | Cell or Tissue | Functional (or Pathophysiological) Effects |
|---|---|---|---|---|
| | | | *Cytokines* | |
| TNF-α | $O_2^-\cdot$, $H_2O_2$ | NAD(P)H oxidase | Fibroblasts | Unknown |
| | $O_2^-\cdot$ | Unknown | Endothelial cells | Unknown |
| | $O_2^-\cdot$ | NADPH oxidase | Mesangial cells | MCP-1, CSF-1 expression |
| | $H_2O_2$ | Flavoprotein oxidase | Chondrocytes | Mitogenesis |
| | Unspecified | Mitochondria | Fibrosarcoma | NF-κB activation, IL-6 induction Cytotoxicity |
| | Unspecified | Unknown | Breast carcinoma cells | HSP27 phosphorylation |
| + IL-1 | Unspecified | Flavoprotein oxidase | Rat mesangial cells | COX-2 expression |
| + IFN-γ | Unspecified | Unknown | Microglial cells | Unknown |
| IL-1 | $O_2^-\cdot$, $H_2O_2$ | NAD(P)H oxidase | Fibroblasts | Unknown |
| | $O_2^-\cdot$ | Unknown | Endothelial cells | Unknown |
| | $O_2^-\cdot$ | Unknown | Dog myocardium | Myocardial dysfunction |
| IFN-γ | $O_2^-\cdot$ | Unknown | Endothelial cells | Unknown |
| | $H_2O_2$ | Cyclooxygenase | Hepatocytes | Antibacterial action |
| | | | *Receptor tyrosine kinases* | |
| PDGF | $H_2O_2$ | Unknown | BALB/3T3 cells | "Competence factor" for cell growth |
| | $H_2O_2$ | Flavoprotein oxidase | Smooth muscle cells | Mitogenesis, MAPK activation |
| | $O_2^-\cdot$ | Unknown | NIH/3T3 cells | NOS expression, $PGE_2$ release |
| | $O_2^-\cdot$ | Flavoenzyme | Smooth muscle cells | NF-κB-dependent MCP-1 induction |
| EGF | $H_2O_2$ | Unknown | Epidermoid carcinoma | Tyrosine phosphorylation, cell growth |
| | Unspecified | Unknown | HaCaT cell line | JNK activation |
| | $H_2O_2$ | Unknown | Hepatocytes | Carcinogenesis |
| | Unspecified | Flavoprotein oxidase | Human keratinocytes | $PLA_2$, MAPK activation |
| | $O_2^-\cdot$ | Lipoxygenase | PC12 | Cell growth |
| | $H_2O_2$ | Unknown | Rat mammary CA cells | Enhanced metastasis |
| | $H_2O_2$ | Unknown | Mouse epidermal cells | $p70^{S6k}$ activation |
| HB-EGF | $H_2O_2$ | Unknown | Smooth muscle cells | HB-EGF autoinduction |
| FGF-2 | $H_2O_2$ | Flavoprotein oxidase | Chondrocytes | Induction of c-Fos |
| | $O_2^-\cdot$ | Unknown | Lung fibroblasts | Mitogenesis |
| IGF-I | $H_2O_2$ | Plasma membrane oxidase | 3T3 L1-preadipocytes | Adipocyte differentiation |
| HGF | $H_2O_2$ | Unknown | Sarcoma cells | Apoptosis |
| | | | *Receptor serine/threonine kinases* | |
| TGF-β1 | $H_2O_2$ | Unknown | Mouse osteoblasts | Growth inhibition; erg-1 induction |
| | $H_2O_2$ | Unknown | Endothelial cells | Growth inhibition |
| | $H_2O_2$ | Suppression of AOE | Rat hepatocytes | Growth inhibition |
| | $H_2O_2$ | NADH oxidase | Lung fibroblasts | Unknown |
| | $H_2O_2$ | Unknown | Hepatocytes | Apoptosis |
| | $H_2O_2$ | Suppression of AOE | Pancreatic beta cells | Apoptosis |
| | $H_2O_2$ | Unknown | Hepatic stellate cells | $\alpha_1$(I) procollagen, TGF-β induction |
| | | | *G protein-coupled receptors* | |
| Angiotensin II | $O_2^-\cdot$, $H_2O_2$ | NAD(P)H oxidase, $p22^{phox}$ | Smooth muscle cells | Cell hypertrophy, p38 activation |
| | $O_2^-\cdot$ | Unknown | Proximal tubule cells | $p27^{Kip1}$, cell hypertrophy |
| | $O_2^-\cdot$ | Flavoprotein oxidase | Mesangial cells | Cell hypertrophy |
| | $H_2O_2$ | PLD-dependent oxidase | Smooth muscle cells | Proliferation/hypertrophy |
| Serotonin | $O_2^-\cdot$ | NAD(P)H oxidase | Smooth muscle cells | ERK activation, proliferation |
| | $OH\cdot$ | 5-HT transporter dependent | Rat striatal tissue | 5-HT depletion |
| Bradykinin | $O_2^-\cdot$, $H_2O_2$ | Cyclooxygenase | Endothelial cells | Unknown |
| | $O_2^-\cdot$ | Unknown, $Ca^{2+}$ dependent | Human keratinocytes | Unknown |
| Thrombin | $O_2^-\cdot$, $H_2O_2$ | NAD(P)H-like enzyme | Endothelial cells | Unknown |
| | $O_2^-\cdot$, $H_2O_2$ | NAD(P)H oxidase, $p47^{phox}$ | Smooth muscle cells | Cell growth, p38 activation |
| Endothelin | $H_2O_2$ | Unknown | Cardiac myocytes | c-Fos induction |
| | | | *Ion channel-linked receptors* | |
| Glutamate | $H_2O_2$ | Unknown | Rat cerebral cortex | Unknown |
| | $O_2^-\cdot$ | Mitochondria, $Zn^{2+}$ dependent | Neurons | Unknown |
| Acetylcholine | $H_2O_2$ | Mitochondria | Cardiomyocytes | Preconditioning |

**Table IV.** Ligand-mediated generation of ROS in non-phagocytic cells (Thannickal & Fanburg, 2000)

Nevertheless, the effects of ROS are commonly negative for cells. In particular, the high chemical reactivity of these molecules makes ROS very unstable and able to induce lipid peroxidation by acting on hydrocarburic chains of unsaturated fat acids,

oxidation of amino acidic residues in proteins, and damage of carbohydrate and nucleotides in the DNA. ROS-induced damages could alter and compromise the function of macromolecules and cellular structures, thus inducing cell death.

Lipid peroxidation consists in a chain of reaction which progressively transforms unsaturated fat acids of membrane phospholipids in lipid radicals ($L^{\bullet}$), peroxide radicals ($LOO^{\bullet}$) and lipid peroxides (LOOH). Lipid peroxides can easily break down in different compounds, such as lipid alkoxides ($LO^{\bullet}$), aldehydes (malonyldialdehyde, MDA), alkanes, lipid epoxides and alcohol, which can also be generated from cholesterol modifications. These modification imply an increase in membrane rigidity that causes the waste of selective permeability (Spiteller, 2006).

Regarding proteins, oxidative reactions caused by free radicals may lead to an aberrant proteolysis or aggregation as a consequence of alterations in the chemical and physical properties of the protein itself with variation of the iso-electric point and alteration of the molecular weight. These changes finally induce the activation of specific proteolytic enzymes and the degradation of the protein (Fulle et al., 2004).

At the level of nucleic acids, oxidative alterations irreversibly inhibit the processes of transcription, translation, and DNA replication, leading to premature senescence and cell death (Dean, Gieseg, & Davies, 1993; Spiteller, 2006).

Another important effect of ROS is the reduction of NO bioavailability through three main mechanisms:

1. by directly reacting with NO, thus forming peroxinitrites;
2. through the oxidation of the co-factor $BH_4$ and the consequent uncoupling of eNOS (Li & Förstermann, 2013; Mueller, Laude, McNally, & Harrison, 2005);
3. by a decrease in intracellular thiol levels that results in a reduced production of S-nitrosothiols and in the subsequent decrease of the expression and activity of guanylate ciclase, the main downstream effectors of NO.

## 8. Antioxidant defense

Human cells have several efficient mechanisms to neutralize ROS and defend themselves against oxidative chemical species. Antioxidants could be classified in primary or enzymatic antioxidants and secondary or non-enzymatic antioxidants. The definition of primary and secondary is based on temporal parameters, related to the sequence of action of the antioxidant mechanisms, and not to their importance.

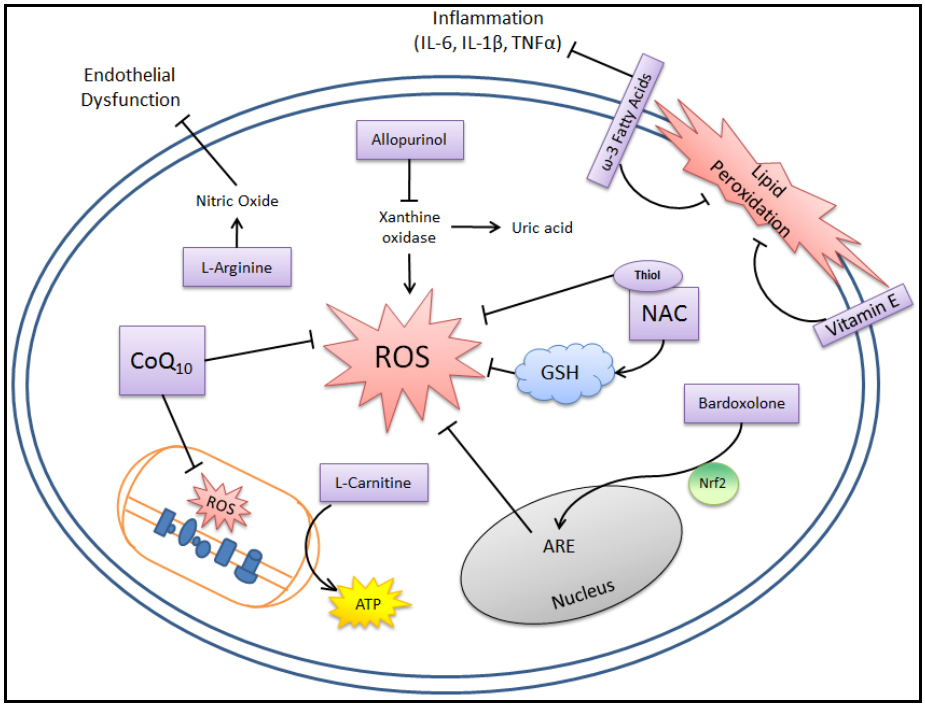



**Figure XX.** Intracellular antioxidant mechanisms (Small & Gobe, 2013, Oxidative Stress and Antioxidant Therapy in Chronic Kidney and Cardiovascular Disease, Oxidative Stress and Chronic Degenerative Diseases - A Role for Antioxidants, Dr. JA Morales-Gonzalez (Ed.), ISBN: 978-953-51-1123-8)

### 8.1. Primary antioxidants

The main enzymatic antioxidants are: Superoxide Dismutase (SOD), Catalase, Glutathione Peroxidase (GSHPx), Glutathione-S-transferase (GST-transferase), Glutathione Reductase (GSSG-Red) and Thioredoxin.

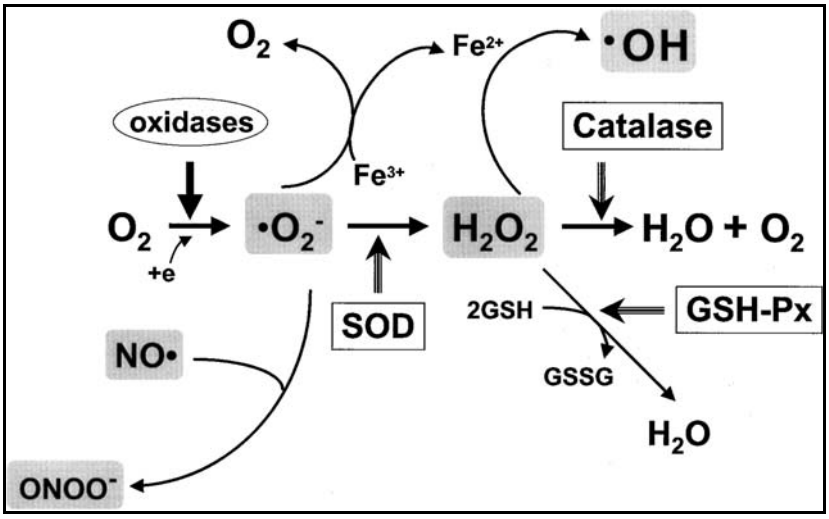



**Figure XXI.** Primary antioxidants (Griendling & FitzGerald, 2003)

8.1.1. Superoxide Dismutase

SOD is the main enzyme of primary defense against ROS. In particular, it catalyzes the dismutation of superoxide radical ($O_2^{\bullet -}$) that is reduced to hydrogen peroxide ( $H_2O_2$ ) and molecular oxygen ($O_2$). The SOD action is particularly important to avoid the peroxidation of nitrate to form ONOO, thus preventing cellular damage and the consequent endothelial and mitochondrial dysfunction. Three mammalian archetypal isoforms are distinguished: SOD-1, SOD-2 and SOD-3, which are codified by different genes, located on chromosomes 21, 6 e 4, respectively (21q22.1, 6q25.3 e 4p15.3-p15.1). All SOD isoforms catalyze the same reaction, but are characterized by their specific localization and by a different transition metal acting as cofactor in the redox reaction (Fukai & Ushio-Fukai, 2011). Indeed, the metal core is necessary for the cyclic reaction of reduction and oxidation through which the enzyme carries out its function. This core can modulate SOD activity and consists of copper (Cu) in SOD-1 and SOD-3, and manganese (Mn) in SOD-2.

SOD-1 is a homodimer of 154 kDa, composed by two identical subunit of 154 amino acids containing a $Cu^{2+}$ and a $Zn^{2+}$ ions coupled by weak, non-covalent interactions. SOD-1 is located in the cytoplasm and in mitochondrial intermembrane

space (Okado-Matsumoto & Fridovich, 2001). SOD-3 is a homotetramer of 135 kDa, composed by two identical dimer that contains $Cu^{2+}$ and $Zn^{2+}$ linked by disulfide bonds. SOD-3 is secreted, thus it can be found in the extra-cellular matrix, particularly close to blood vessels, where it is synthesized by fibroblast and smooth muscle cells, heart, lungs, uterus and liver. The dismutation reaction catalyzed by these two isoforms is based on two half-reaction with equal rate:

$SOD\text{-}Cu^{2+} + O^{2-} \rightarrow SOD\text{-}Cu^{+} + O_2$ (1)

$SOD\text{-}Cu^{+} + O_2^{-} + 2H^{+} \rightarrow SOD\text{-}Cu^{2+} + H_2O_2$ (2)

with the resulting reaction: $2O^{2-} + 2H^{+} \rightarrow O_2 + H_2O_2$

In this reaction, the oxidation state of the metal cation oscillates between 1+ and 2+ and the net result is the transformation of superoxide in oxygen and hydrogen peroxide. The activity of Cu/Zn-SOD is directly correlated to the amount of $Cu^{2+}$ ions, which represent the catalytic site of the enzyme and cannot be substituted by any other metal ions, whereas $Zn^{2+}$ ion seems to have only a structural role and can be substituted with $Co^{2+}$ or $Cu^{2+}$ (Fukai & Ushio-Fukai, 2011). In physiological condition, the intracellular levels of free $Cu^{2+}$ are very low. Indeed, it is directly transferred to its specific target by the chaperone CCS, thus requiring the modification of CCS to transfer $Cu^{2+}$ to SOD and activate it.

Mn-SOD is located in the mitochondrial matrix and, in respect to the Cu/Zn-SOD, its structure has not been yet well characterized. It is known that Mn-SOD is a tetramer formed by four identical subunits of 23 kDa containing manganese. The mechanism of action is similar to that of Cu/Zn-SOD and involves the redox couple $Mn^{3+}/Mn^{2+}$. However, its catalytic function remains still unclear. The catalytic efficiency of SOD-2 is lower than that of Cu/Zn-SOD, probably because SOD-2 activity is sensitive to the inhibitory action of single charge anions and to neutral pH ranges.

Since the dismutase reaction produces hydrogen peroxide, the antioxidant function of Cu/Zn-SOD is strictly linked to the action of other antioxidant enzymes *i.e.* catalase and GSH-Px.

### 8.1.2. Catalase

Catalase is an high molecular weight tetrameric enzyme that is formed by 4 polypeptidic chains. It contains four porphyrin heme (iron) groups that react with the hydrogen peroxide. Indeed, the main function of catalase is to eliminate the hydrogen peroxide through the catalyzation of its dismutation in molecular oxygen and water by means of two sequential reactions:

$H_2O_2 + Fe^{3+}\text{-CAT} \rightarrow H_2O + O{=}Fe^{4+}\text{-CAT}$

$H_2O_2 + O{=}Fe^{4+}\text{-CAT} \rightarrow H_2O + Fe^{3+}\text{-CAT} + O_2$

with the resulting reaction: $2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$

### 8.1.3. Glutathione Peroxidase, Glutathione-S-Transferase and Glutathione Reductase

Glutathione Peroxidase (GPX) is a selenium-dependent enzyme that has a detoxifying action on hydrogen peroxide and organic hydroperoxides by converting them in water and alcohol (ROH), respectively. The mechanism of action involved the oxidation of reduced Glutathione (GSH) to oxidized Glutathione (GSSG).

The dismutation reaction of $H_2O_2$ (1) and of organic hydroperoxides (2) are:

$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$ (1)

$2\ GSH + 2\ ROOH \rightarrow GSSG + ROH + H_2O$ (2)

In mammals five different GPX isoforms have been identified (GPX 1 to 5), showing different tissue and cellular localization. Whereas the GPX catalyzed the same reaction, they have specific substrates (Brigelius-Flohé, 1999). For instance, GPX-4 is associated to the membranes and specifically acts on hydroperoxides of membrane phospholipids (Ursini, Maiorino, & Gregolin, 1986).

As described above, the Glutathione-S-Transferase (GST) catalyzes the direct conjugation of GSH to oxidized substrates, which are eliminated from the organism at a later stage. In mammals, seven different GST classes have been identified and

characterized, which are all members of a family of soluble cytosolic enzyme. Recently, a mitochondrial and a microsomal families have been also identified (MAEPEG) (Hayes, Flanagan, & Jowsey, 2005).

The activity of GPXs and GSTs reduces the intracellular levels of total GHS with a consequent increase in GSSG content. At the aim to maintain a stable GHS/GSSG ratio, the GSSG is released in the extracellular matrix where is degraded, and GHS is regenerated by Glutathione Reductase (GR), a flavoprotein that reduces the oxidized form using NADPH, or by *ex-novo* synthesis. The *ex-novo* synthesis takes place through two sequential ATP-dependent reaction, which are catalyzed by γ-Glutamylcysteine Synthetase (γGCS) and Glutathione Synthetase, respectively.

### 8.1.4. Thioredoxin

Thioredoxin-1 (Trx-1) is a protein of 105 aminoacids, known to be present in all organisms, which is located in the cytoplasm. It possesses two cysteine residues in the active site essential to its catalytic function, *i.e.* reducing other proteins. In fact, this enzyme acts as antioxidant by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange, thus reducing the disulfide bonds of proteins, which have been oxidized by ROS. The thioredoxins' catalytic site is kept in the reduced state by the flavor-enzyme Thioredoxin Reductase-1 (TxR1) through to a NADPH-dependent reaction that regenerates the redox state of oxidized cysteines.

## 8.2. Secondary antioxidants

This class of non-enzymatic antioxidants is the responsible for the implementation of antioxidant defenses through the prevention of the formation of alkoxy- and hydroxyl-radicals by decomposition of hydroperoxide (ROOH). They can directly act as scavengers or by restoring the thiol pool. The most important secondary antioxidants is Glutathione

(GSH), a tripeptide that is ubiquitously present in all cell types. In addition to being a substrate for GPX and GST, GSH can directly act as a free radical scavenger during the process of detoxification of hydrogen peroxide and lipid hydroperoxides. A further non-enzymatic antioxidant is the Coenzyme-Q, which carries out its action at the level of the mitochondrial electron transport chain. Some vitamins such as vitamin A, E and C are also considered non-enzymatic antioxidants.

## 8.3. Chemical antioxidants

Over the years, several chemical antioxidants have been synthesized to be employ in both laboratory and therapy.

N-acetylcysteine (NAC) is one of the main chemical antioxidants, constituted by a modified version of the sulfur-containing aminoacid cysteine. NAC is able to replenish intracellular levels of GSH, helping to restore cells' defense to damages from ROS. NAC is therapeutically use for the treatment of paracetamol overdose. The restoration of GSH reserves indeed inactivates N-acetyl-p-benzoquinone imine (NAPQI), a toxic metabolite that by reacting with key hepatic enzymes induces severe hepatocyte damage, finally leading to severe liver damage and possibly to death by fulminant hepatic failure. NAC is also used to prevent the disease progression in the interstitial lung disease, and in the treatment of different psychiatric disorders such as schizophrenia, acute mood episodes, autism, obsessive-compulsive disorder and drug addiction. Although its efficacy is still debated, NAC is also use for the prevention of radiocontrast-induced nephropathy (a form of acute renal failure).

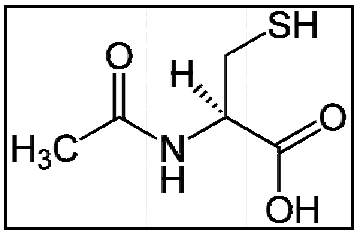



**Figure XXII.** Chemical structure of NAC

Another important chemical antioxidant is Tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl), a small cell-permeable molecule mimicking SOD. Tempol has been shown to possess neuroprotective effects in PC-12 cells and to restore the oxidative stress and cardiac dysfunction induced by TNF-α. Furthermore, it has been described its ability to decrease sympathetic nerve activity and blood pressure in DOCA-salt rat studies.

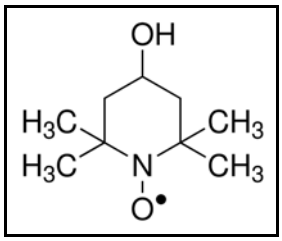



**Figure XXIII.** Chemical structure of Tempol

8.4. Nuclear factor-erythroid-2-related factor 2

The Nuclear factor-erythroid-2-related factor 2 (Nrf2) is a basic leucine zipper transcriptional factor which control the expression of several genes encoding for detoxifying enzymes and antioxidant proteins (Hayes et al., 2005). Therefore, Nrf2 is fundamental for the expression of both primary and secondary antioxidants. The on/off switch of Nrf2 activity contributes to cell protection against ROS damage and prevents apoptosis, thus promoting cell survival.

Nrf2 and its inhibitor Keap1 (or INrf2) are ubiquitously express in many cells and tissues and together act as cellular sensors of oxidative and electrophilic stress induced by chemicals and radiations. Nrf2 protein is composed by 5 domains as depicted in Figure XXIV: (1) an hydrophobic domain; (2) a transcriptional activation domain; (3) a Cap "n" Collar (CNC) domain; (4) a basic region, and (5) a leucine zipper. Keap1 is also composed by 5 domains: a N-terminal region (NTR), the broad complex region (BTB) that is the binding domain for Cul3-Rbx, the linker region (IVR), the Kelch domain that binds Nrf2, and the C-terminal region (CTR) (Figure XXIV).

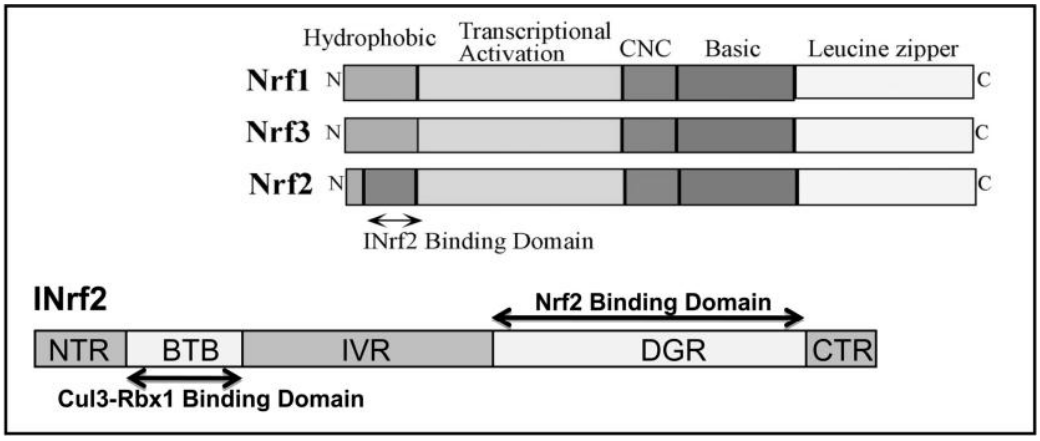



**Figure XXIV.** Domain structures of Nrf2 isoforms (Kaspar, Niture, & Jaiswal, 2009)

In physiological conditions, Nrf2 is bound to Keap1 and located in the cytoplasm. This binding prevents Nrf2 nuclear translocation and induces its degradation through the interaction between Keap1 and the complex Cul3-Rbx1 (cullin3/ring box1 E3 ubiquitin ligase complex). Cul3 acts as a scaffold protein which complexes E3 ligase with Rbx1 and recruits the E3 enzyme. The complex Nrf2-Keap1-Cul3 induces the ubitiquination and degradation of Nrf2 through the proteasome 26S. This mechanism prevents the nuclear translocation and transcriptional activation of Nrf2. Under stressing conditions, the complex Nrf2-Keap1 is modified by the action of different protein-kinases such as PKC, ERK, MAPK and p38, thus stabilizing Nrf2 that can translocate to the nucleus. The modifications required for the disruption of the complex involve both Keap1 and Nrf2 phosphorylations. Keap1 is phosphorylated on Cys151, thus causing a conformational change of BTB domain with the consequent ubiquitination and degradation of Keap1. The phosphorylation of Nrf2 on Ser40 by PKC is required to its release, whereas the phosphorylation of three other different cysteine residues (Cys151, Cys273 and Cys 288) is necessary to Nrf2 nuclear translocation and activation. Once in the nucleus, Nrf2 binds Antioxidant Response Elements (ARE) on DNA (sequence 5'-TGCTGAGTCAC-3') through its basic region (Kaspar et al., 2009). To be fully activated, Nrf2 also requires heterodimerization with another bZIP protein, such as Jun and Maf. ARE is an enhancer sequence located in the promoter region of several genes, codifying for cytoprotective enzyme. ARE has also structural and biological features that characterized its

responsiveness to oxidative stress. In fact, ARE sequences are activated by alteration in the cellular redox state as a consequence of high intracellular levels of ROS or electrophilic species and/or a reduced antioxidant ability (*i.e.* Glutathione depletion) (Kobayashi et al., 2004). Another important control mechanism of Nrf2 activity is the phosphorylation of its Thr568 through the action of Fyn, a nuclear tyrosine kinase. The action of Fyn causes the export of Nrf2 from the nucleus and its binding with Keap1, thus finally promoting Nrf2 degradation.

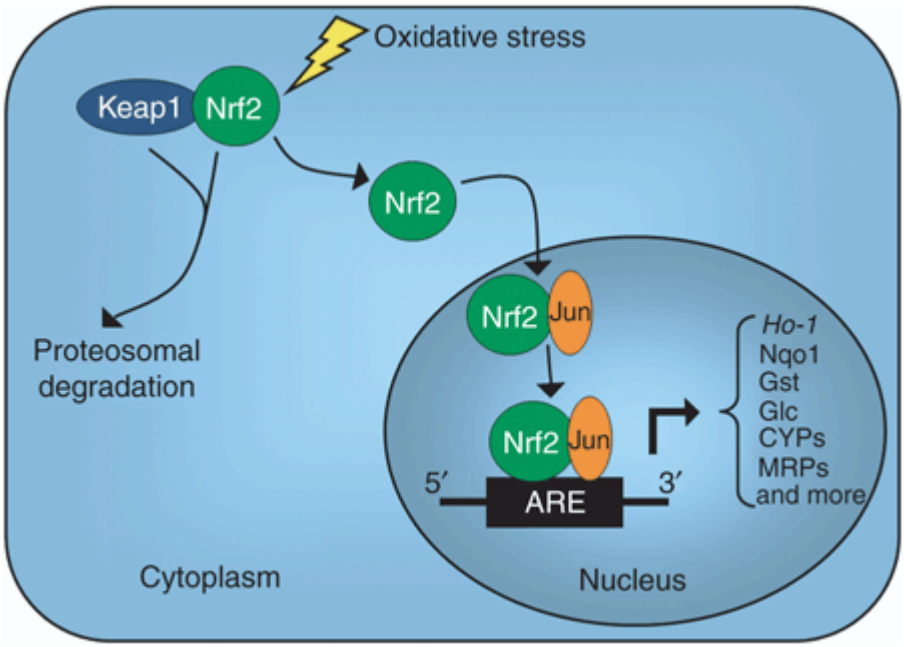



**Figure XXV.** A schematic representation of the mechanism of action of Nrf2 (Bataille & Manautou, 2012)

## 9. Mitochondria

Mitochondria are cytoplasmatic organelles surrounded by a double membrane with a small intermembrane space in between. The outermost membrane is smooth while the inner membrane typically presents a lot of folds, called cristae, that enhance the available surface area, thus increasing cellular respiration. The number, dimension and form of mitochondria are related to the energy needs of the cells. Indeed, mitochondria are generally considered as ”cellular power plants” because they produce most of ATP supply.

As described above, mitochondria are enclosed by two membranes. The outer membrane is rich in protein-based pores that are big enough to permit the passage of ions, molecules, and small proteins. On the other hand, the inner membrane is more similar to the plasma membrane with a more restricted permeability. This membrane contains also the proteins that form the electron transport chain (ETC) responsible for ATP synthesis (Figure XXVI). The mitochondrial matrix is located inside the inner membrane, where the citric acid cycle takes place by producing the electrons that travel from one protein complex to the next one in the ETC. The latter consists in a series of protein complexes through which electrons captured from donor molecules are transferred to release the energy required to pump protons into the intermembrane space. In this way, a strong electrochemical gradient across the inner membrane is established, which is used to synthesize ATP from ADP and inorganic phosphate (Pi) through a specific enzyme, the complex of ATP-synthase.

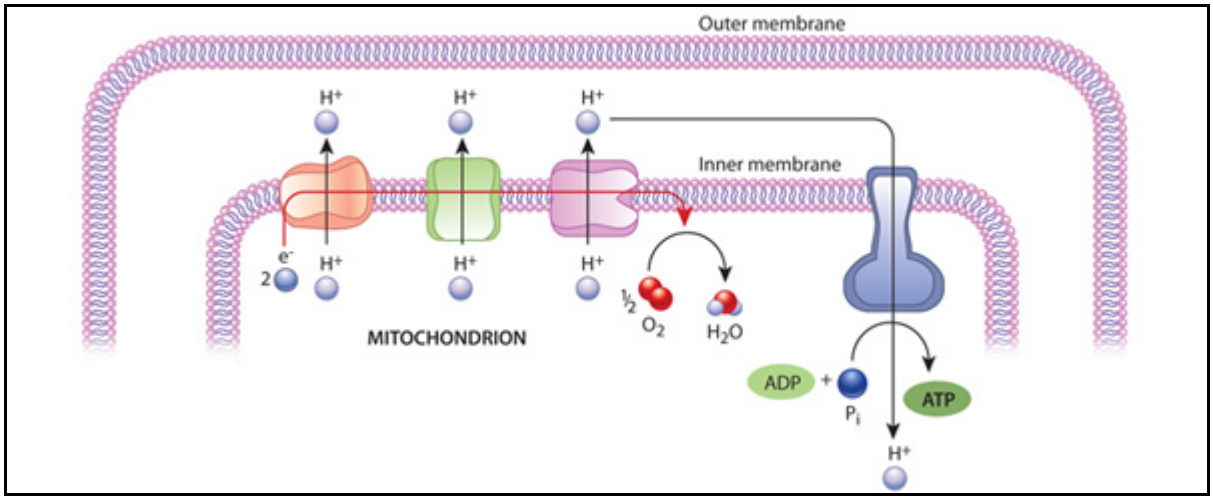



**Figure XXVI.** The electrochemical proton gradient and ATP synthase (©2010 Nature Education)

## 9.1. Mitochondria and ROS

In addition to ATP production, mitochondria are also involved in the regulation of the cellular redox state. Indeed, mitochondria are considered the main intracellular source of ROS, in particular of superoxide anion. Their production is triggered by a leakage of electrons from the mitochondrial respiratory chain and their consequent reaction with $O_2$

(Figure XXVII). To counteract ROS production, both the mitochondrial matrix MnSOD (SOD2) and the mitochondrial intramembrane space Cu/ZnSOD (SOD1) are recruited, thus converting superoxide anion to hydrogen peroxide, which can be subsequently transformed into harmless $H_2O$ through the action of catalase.

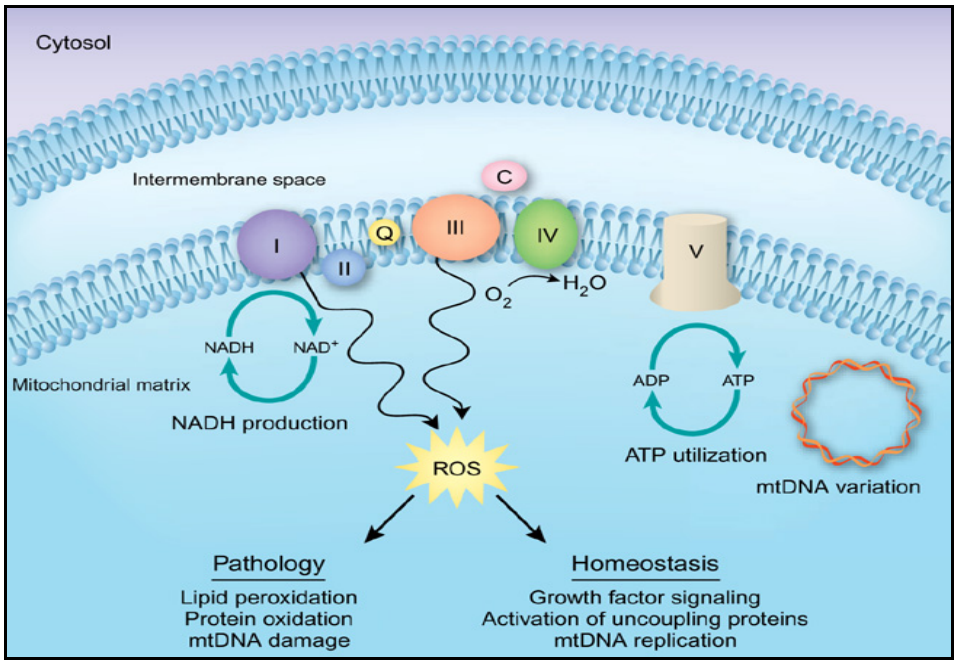



**Figure XXVII.** The generation and effects of ROS in mitochondria (Baughman & Mootha, 2006)

9.2. Mitochondria and NO

Mitochondria biogenesis and function can be regulated by several factors, among which NO is particularly important. Indeed, NO can influence mitochondria at several levels. First of all, it can indirectly regulate the affluence of respiratory substrates to mitochondria through its action on blood flow regulation. Furthermore, NO directly regulates oxyhemoglobin dissociation curve (Wolzt et al., 1999), thus influencing the supply of $O_2$ to mitochondria. In addition, NO can also directly interact with mitochondria at the level of cytochrome c oxidase, the terminal enzyme of the ETC, by competing with $O_2$ (Brown & Cooper, 1994; Cleeter, Cooper, Darley-Usmar, Moncada, & Schapira, 1994; Clementi, Brown, Feelisch, & Moncada, 1998; Clementi, Brown, Foxwell, & Moncada, 1999). As a result, the activity of cytochrome c oxidase is inhibited, thus regulating in a negative manner the mitochondrial oxidative phosphorylation, especially in tissues where

$O_2$ concentrations is usually low (Clementi et al., 1999). The competitive mechanism between NO and $O_2$ is also responsible for $O_2$ redistribution to neighboring cells (Hagen, Taylor, Lam, & Moncada, 2003). Another strong evidence of the importance of NO in mitochondria is the observation that eNOS is bound to the outer mitochondrial membrane in endothelial cells and neurons (Gao et al., 2004), thus indicating a possible reciprocal regulation of eNOS and mitochondrial function. It has been indeed discovered a mitochondrial NOS isoform, called mtNOS, which suggests that NO can directly regulate mitochondrial functions (Ghafourifar & Cadenas, 2005).

In addition to the described functional effects, NO is also crucially involved in mitochondria biogenesis. In fact, it has been demonstrated that the treatment of various cell types with NO donors increases their mtDNA content. On the contrary, the removal of NO by the scavenger oxyhemoglobin shows an opposite effect (Nisoli et al., 2003). The promoting effect of NO on mitochondrial biogenesis occurs through an increased expressions of PGC-1, *i.e.* the principal regulator of mitochondrial biogenesis, NRF-1, NRF-2 and Tfam (Kelly & Scarpulla, 2004). Moreover, it depends on GC activity and cGMP formation, the main downstream NO effectors. The crucial role of NO in mitochondrial biogenesis was confirmed in $eNOS^{-/-}$ mice (Nisoli et al., 2003). Furthermore, eNOS deletion is known to reduce mitochondrial mass in many tissues such as brain, muscle, liver and heart. The reduction in mitochondrial mass is accompanied by a reduction in basal $O_2$ consumption and steady-state ATP levels.

### 9.3. Mitophagy

One of the main mechanisms responsible for cellular homeostasis is autophagy. It consists in the regulated degradation of cellular components through their engulfment into autophagosomes. These intracytoplasmic vacuoles fuse with lysosomes where hydrolytic enzymes cause the breakdown of cellular components recruited in the organelle. Autophagy plays a key role during starvation, by recycling components into

more urgently needed molecules, but it also represents a fundamental system for maintaining quality control by turning over organelles and degrading protein aggregates.

A specific form of autophagy is mitophagy *i.e.* the degradation of mitochondria through specifically targeted autophagic processes (see Figure XXVIII). In fact, several recent findings suggest that mitophagy can selectively degrade damaged mitochondria, *e.g.* in laser irradiated hepatocytes where defective mitochondria are selectively removed by mitophagy (Kim, Rodriguez-Enriquez, & Lemasters, 2007). Mitophagy has been proposed as an important homeostatic mechanism acting in a variety of conditions such as hypoxia and oxidative stress (Ashrafi & Schwarz, 2013; Lee, Giordano, & Zhang, 2012; Liu et al., 2012). Moreover, autophagy is also regulated by NO availability (Sarkar et al., 2011). The first step in mitophagy is nucleation, which consists in the formation of a phagophore through the formation of a complex among Beclin-I and two vacuolar protein sorting (VPS), VPS34 and VPS15. Later, two ubiquitin-like conjugation systems represented by the microtubule-associated protein 1 light chain 3 (LC3) and the autophagy protein (ATG) 12-ATG5 mediate the expansion of phagophore's membrane, thus promoting the conjugation of LC3 with phosphatidylethanolamine and the assembly of the ATG16L complex (Kabeya et al., 2004; Mizushima, Noda, & Ohsumi, 1999). The ATG16L complex promotes the transformation of the cytoplasmic LC3-I to the membrane-bound LC3-II form. Despite a higher molecular weight, LC3-II migrates faster than LC3-I in SDS-PAGE because of its hydrophobicity and therefore displays a lower apparent molecular weight. For this reason, the LC3-II/LC3-I ratio can be used as a marker of mitophagy. During the second step of mitophagy, the phagophore expands until its edges are fused around its mitochondrial target(s) thus forming a typical double-membrane structure: the autophagosome. Finally, the autophagosome fuses with a lysosome and the contents are degraded by lysosomal enzymes (Mizushima, Levine, Cuervo, & Klionsky, 2008).

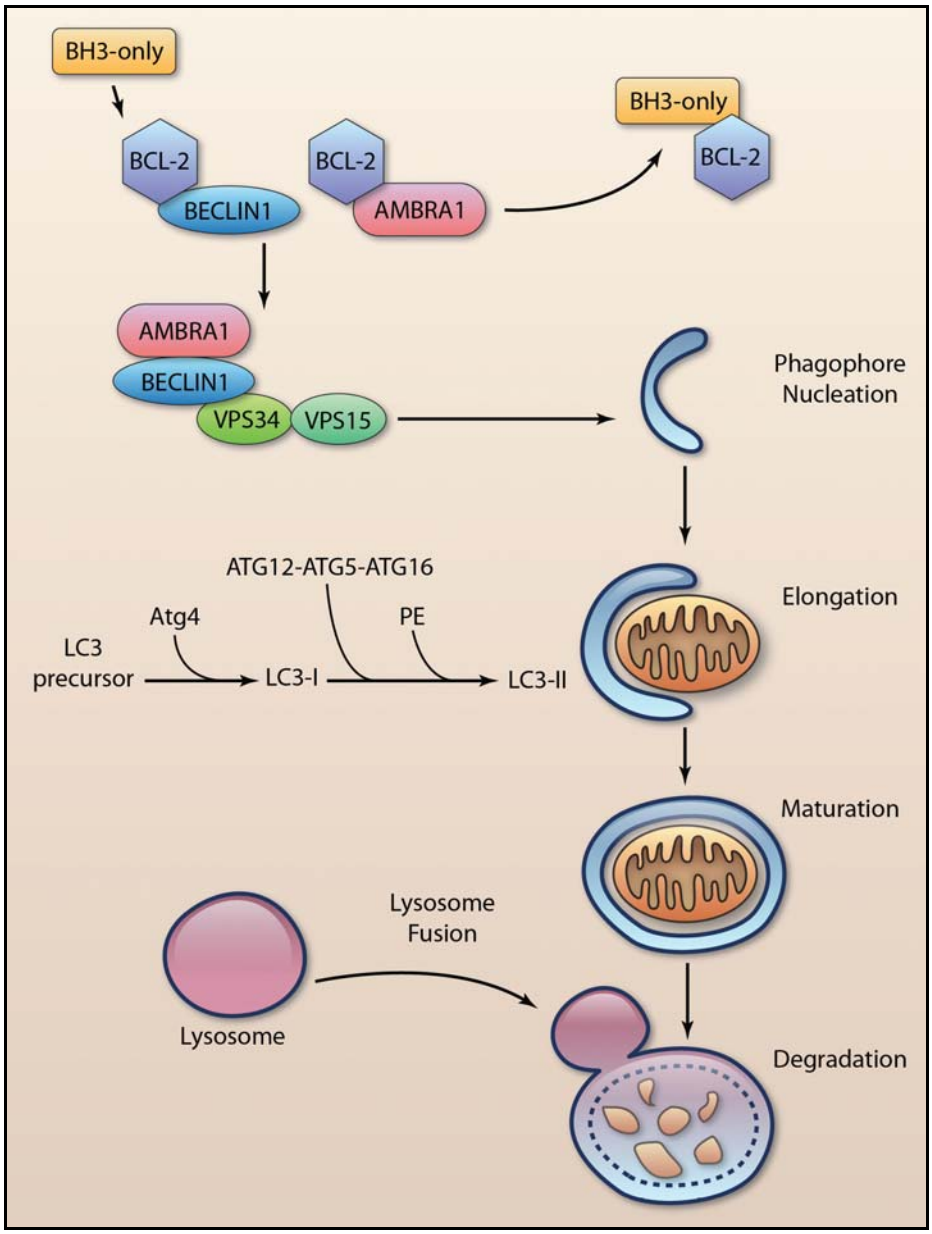



**Figure XXVIII.** Autophagosome formation (Kubli & Gustafsson, 2012)

Mitophagy can take place via both an ubiquitin-dependent or independent pathway. In the ubiquitin-dependent pathway, ubiquitin is used as a signal for autophagic degradation of mitochondria (Kirkin et al., 2009; Pankiv et al., 2007). It involves the action of Parkin that recognizes the dysfunctional mitochondria and ubiquitinates specific protein substrates present on the outer membranes. The autophagy adaptor protein p62 has a fundamental role in the engulfment of ubitiquitinated mitochondria in the phagophores. Indeed, p62 binds to LC3 on the phagophore via its LC3-interacting region, and to ubiquitinated proteins via its ubiquitin-associated domain 110 (Figure XXIX). Given its importance in the mitophagic process, p62 is used as a marker of mitophagy

along with LC3-II/LC3-I ratio. On the other hand, the ubiquitin-independent pathway involves the direct binding of a series of ATG8 family proteins to specific autophagy receptors on the mitochondria (Figure XXIX). In mammalian cells, the main mitophagic receptors are NIX/BNIP3L and BNIP3 (Hanna et al., 2012; Novak et al., 2010; Schwarten et al., 2009). In conclusion, both the ubiquitin-dependent and independent pathways flow into the recruitment of LC3, that leads to autophagosome formation, thus permitting the degradation of dysfunctional and/or damaged mitochondria.

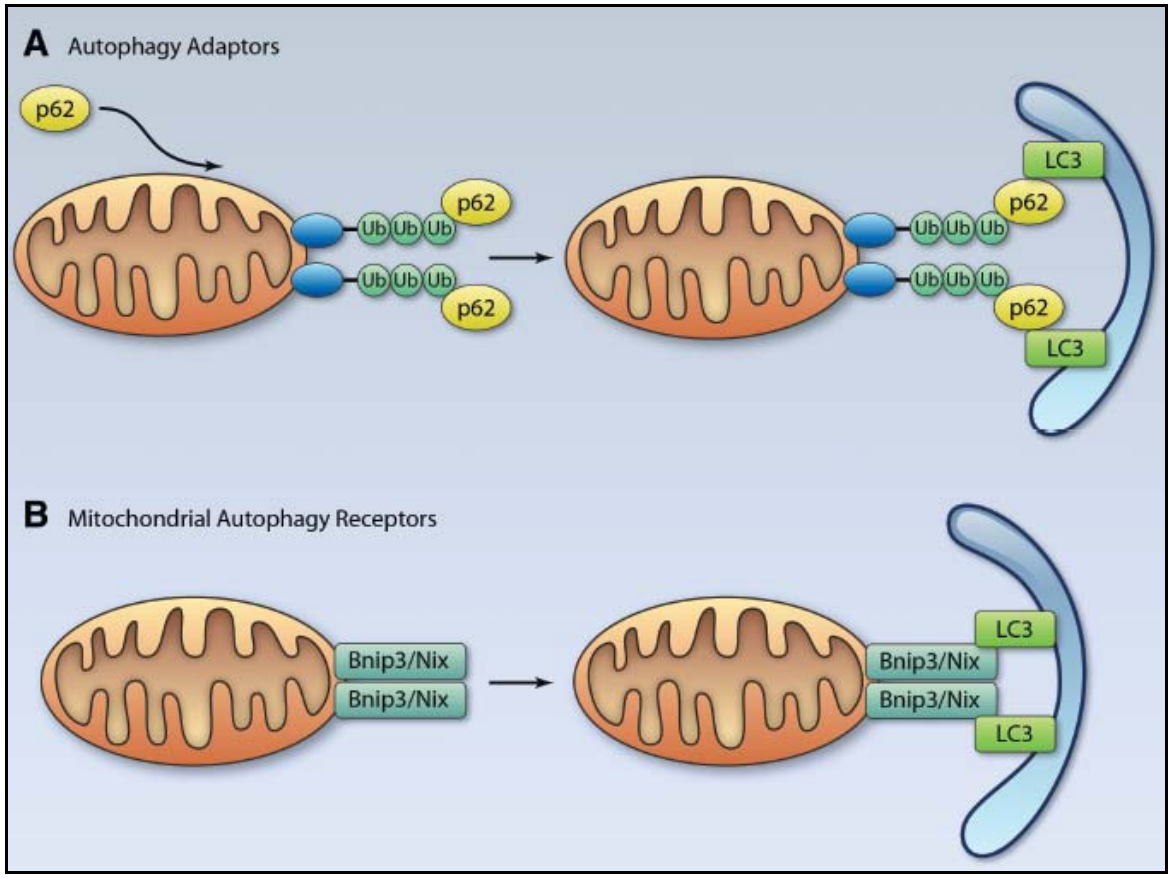



**Figure XXIX.** Mitophagy mediated by LC3 and Bnip3/Nix (Kubli & Gustafsson, 2012)

# Aims of the project

Integrity of endothelial cells is crucial for the maintenance of vascular homeostasis. The endothelium explicates its physiological functions by producing active molecules, among which nitric oxide (NO) is particularly important. A tonic basal NO release controls blood pressure levels and maintains the endothelium in an anti-atherogenic state. Indeed, NO loss is associated with endothelial dysfunction (ED) typical of atherosclerosis, diabetes and senescence.

In the present study, we investigated in primary cultures of human endothelial cells the behavioral and molecular consequences induced by a chronic NO deprivation. To this aim, we set up an *in vitro* model of ED, using human umbilical vein endothelial cells (HUVECs) chronically deprived of NO through two different approaches: a pharmacological approach, by treating HUVECs with $N^G$-Nitro-L-Arginine Methyl Ester (L-NAME, 5 mM for 48 h), a structural analogue of L-arginine that competitively block the active site of endothelial nitric oxide synthase (eNOS), the enzyme responsible for endothelial NO formation; and a genetic approach, by silencing eNOS expression with RNA-interference.

The characterization of our *in vitro* model, that mimics the early phases of ED, might have important implications for understanding the consequences of NO deprivation on endothelium behavior, and finally in the onset of cardiovascular diseases. The availability of this model could help the identification of innovative pharmacological targets and markers useful for ED diagnosis and treatment, thus preventing its degeneration in most serious cardiovascular diseases.

# Materials and Methods

**Cell cultures**

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly derived umbilical cords essentially as described by Jaffe et al. (Jaffe, Nachman, Becker, & Minick, 1973). Cords were anonymously donated after informed consent according to national ethical legislation, and gently collected by gynecologists and nurses of the Macedonio Melloni Hospital. Umbilical cord vein was cannulated with a butterfly needle (0,8 mm) and then washed with abundant sterile saline solution (0.9% NaCl) to remove blood clots. Afterwards, the vein was sealed at one end with a clamp and filled with a collagenase solution (0.125%) solubilised in $Ca^{2+}$/$Mg^{2+}$ phosphate-buffered saline (PBS) buffer (prepared by mixing 4 volumes of part A containing NaCl 140 mM, KCl 3.9 mM, $KH_2PO_4$ 2.1 mM, $Na_2HPO_4$ 8.1 mM with 2 volumes of part B containing $CaCl_2$ 1.3 mM and $MgCl_2$ 0.93 mM). After 10 minutes of incubation at 37°C, the collagenase solution was collected in a 50 ml tube with a equal volume of 199 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were finally harvested by centrifugation and plated in 25 $cm^2$ flasks. HUVECs were routinely grown in 199 medium supplemented with 20% FBS, 100 μg/ml endothelial cell growth supplement (ECGS) and 50 μg/ml heparin, and used at passages 2-7.

**Cell treatment**

Where indicated, HUVECs were treated with 5 mM $N^G$-Nitro-L-arginine methyl ester (L-NAME) in 199 medium containing 10% FBS for 48 h preceding the experiments. The concentration of L-NAME was chosen according to Papapetropoulos et al. (Papapetropoulos, García-Cardeña, Madri, & Sessa, 1997).

**Crystal violet**

Cell proliferation was evaluated on HUVECs plated at a density of 1.5-2x$10^4$ cells/well in 96-well microplates by crystal violet staining. Briefly, after a fixation with 100% methanol, cells were stained with a 0.1% crystal violet solution. The dye was washed several times with deionized water and then solubilized in 10% acetic acid solution. The

absorbance was measured at 595 nm using a multiplate spectrophotometer (Victor™, PerkinElmer, Waltham, MA, USA).

**Evaluation of apoptosis by FACS analysis**

Quantification of both apoptosis and necrosis was performed by Annexin V-FITC conjugate and propidium iodide (PI) staining (Abcam, Cambridge, UK) followed by fluorescence activated cell sorting (FACS) performed with a FACScalibur flow cytometer equipped with a 488 nm argon laser (Becton Dickinson, San José, CA, USA). The collected data were evaluated by Cell Quest software. The degree of apoptosis was calculated as apoptotic index considering cells both in early and late apoptosis.

**Immunoblot and immunoprecipitation analyses**

For immunoblot analysis, HUVECs plated in 35-mm diameter Petri dishes were washed with PBS ($NaH_2PO_4 \cdot H_2O$ 3,3 mM, $Na_2HPO_4$ 6,7 mM, EDTA 0,2 mM, NaCl 130 mM), and then directly lysed in SDS-PAGE sample buffer (62 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 0.04% bromophenol blue). After SDS-PAGE electrophoresis, proteins were transferred onto nitrocellulose membranes that were blocked with 5% (w/v) non fat dried milk in Tris-buffered saline (TBS: Tris-HCl 10 mM pH 8, NaCl 150 mM) containing 0.05% Tween-20 (TBS-T). Filters were firstly incubated overnight with the indicated primary antibodies, and then for 1 h with the appropriate peroxidase-conjugated secondary antibody (DAKO, Denmark). The immunoreactive bands were visualized by chemiluminescence (LiteAblot Plus, EuroClone, Italy).

For KDR immunoprecipitation, HUVECs were washed with PBS and lysed for 10 min on ice with RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM β-glycerophosphate, 1 mM sodium fluoride, 1 mM sodium orthovanadate) supplemented with protease inhibitors. Aliquots of cleared cell lysates (250 µg protein/sample) were incubated with an anti KDR antibodies,

followed by incubation with Protein A Sepharose. After washes with lysis buffer, the immune complexes were eluted by boiling in sample buffer, and analyzed by SDS-PAGE. Densitometric analyses of the immunoblots were performed using the National Institute of Health (NIH) Image J program.

**Cell migration assays**

HUVEC migration was evaluated by chemotaxis experiments in a 48-well modified Boyden chamber (Bulotta et al., 2009; Cattaneo, Chini, & Vicentini, 2008). Shortly, Nuclepore polyvinylpyrrolidine-free polycarbonate filters coated with 10 μg/ml of type IV collagen were placed over a bottom chamber containing 25 ng/ml VEGF as attractant factor. The cells were suspended in 199 media containing 2% FBS and then inoculated in the upper chamber at a density of $5.0x10^4$ cells/well. After 6 h of incubation at 37°C, the cells that had migrated to the lower side of the filter were stained with Diff-Quick stain (VWR Scientific Products, Bridgeport, NJ, USA). The assays were run in triplicate and 5 unit fields per filter were counted by a scorer blind to the experimental conditions using a Zeiss microscope.

**Determination of cGMP accumulation**

HUVECs were cultured in 60-mm Petri dishes and treated for 48 h with L-NAME or ODQ. 30 minutes before the end of treatment, phosphodiesterases were inhibited by the administration of 1 mM isobutylmethylxanthine (IBMX). cGMP was extracted in 500 µl of 0.1 N HCl, and its quantification was performed by an enzyme immunoassay (EIA) kit (Enzo Life Sciences, Vinci-Biochem, Vinci, Firenze, Italy) following manufacturer's instructions for the acetylated assay procedure.

**Total RNA extraction for reverse transcription and quantitative real time PCR (RT-qPCR)**

Total RNA was extracted using the RNeasy® Mini Kit and accompanying QIAshredder™ (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A 15 min on-

column incubation was performed with DNase I (Qiagen), thus avoiding DNA contamination of samples. Reverse transcription was carried out using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen), again following the manufacturer's instructions. For quantitative analysis of gene expression, we used the ABI Prism 7000 Sequence Detection System, SDS software version 1.2.3 (Applied Biosystems, CA, USA) with the following TaqMan® Primer and Probe assays: human VEGF-A (Hs99999070_m1), KDR (Hs00176676_m1), eNOS (NOS3, Hs00167166_m1), HIF-1α (Hs00153153_m1) and the endogenous control 18S (Hs99999901_s1). For SOD analysis, RT-qPCR reactions were run with the iQ SybrGreenI SuperMix (Bio-Rad, Segrate, Italy) on an iCycler iQ Real-Time PCR detection system (Bio-Rad). In both cases, sequences were amplified from 50 ng of cDNA. For calculation of results, the $2^{-\Delta\Delta Ct}$ method was used allowing normalization to 18S and to the calibrator which is set to a value of 1.

**ELISA determination of VEGF levels**

VEGF measurements were performed on cell supernatants collected from HUVECs plated in 35-mm Petri dishes, using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) following manufacturer's instructions. VEGF levels were expressed relative to total cell protein (pg/mg of total protein) evaluated by the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL, USA).

**Preparation of nuclear extracts**

HUVECs, cultured in 100-mm diameter Petri dishes, were washed with PBS and collected by scraping. Cells were then lysed for 10 min on ice in buffer A (10 mM HEPES pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.05% Nonidet P-40, 1 mM sodium orthovanadate) supplemented with protease inhibitors. After a 10 min of centrifugation at 2,500*g* at 4°C, crude nuclei were washed with buffer A before lysis in buffer C (20 mM HEPES pH 8.0, 1.5 mM $MgCl_2$, 420 mM NaCl, 1.0 mM DTT, 0.2 mM EDTA, 1 mM sodium orthovanadate, supplemented with protease inhibitors) for 30 min

on ice. The nuclear extracts were clarified by centrifugation, and loaded on a 10% SDS-PAGE.

**Transient transfection**

HUVECs, plated in 35-mm Petri dishes, were transfected with the expression vector pcDNA3ARNTdelta_b (ΔARNT), coding for a dominant negative mutant form of the HIF-1β ARNT subunit, and the void vector pcDNA3 using the Pep Mute transfection reagent (Signa Gen Laboratories, USA). Six hours after transfection, the culture medium was replaced by fresh medium, supplemented or not with L-NAME for the following 48 h.

**Small interfering RNA (siRNA) transfection**

Validated Stealth™ RNAi duplexes against human eNOS (GC content 48%) were provided from Invitrogen. As control RNA, we utilized a Stealth™ RNAi negative control duplex (Medium GC Duplex, Invitrogen) with a 48% GC content, suitable for use as a control with Stealth™ RNAi duplexes containing 45-55% of GC. All sets of RNAi molecules were transfected individually into HUVECs at a 30 nM concentration using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). The ability of the RNAi molecules to knockdown eNOS expression was analyzed 48 h after transfection by western blot analysis.

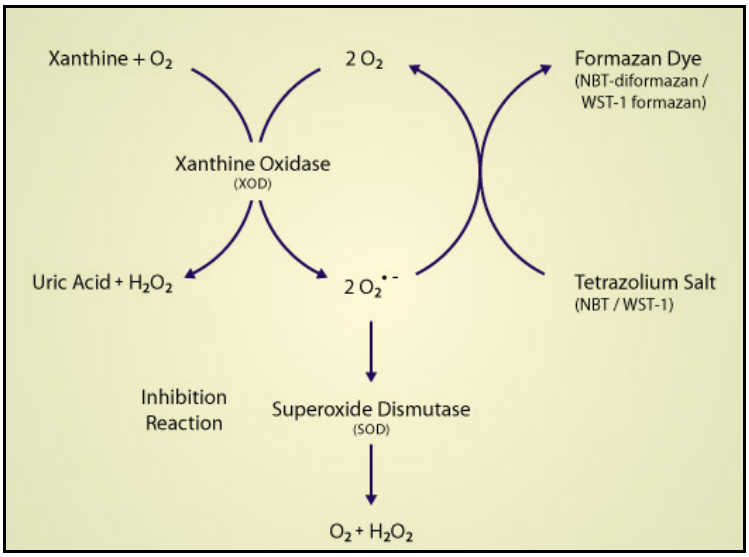
**Determination of ROS levels**

HUVECs, plated at a density of $1.5x10^4$ cells/well in a 96-well black microplate, were loaded for 30 min at 37°C in the dark with the fluorescent dye 5(6)-Carboxy-2'7'-dichlorofluorescein diacetate (CM-DCFA, 10 μM, Sigma Chemicals, St. Louis, MO, USA) in HBSS buffer (Hepes 25 mM pH 7.4, NaCl 120 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 25 mM, glucose 15 mM) containing 1% FBS. Afterwards, cells were exposed to L-NAME (5 mM in HBSS), and fluorescence was assessed by means of a multiplate reader with excitation and emission wavelengths of 485 nm and 530 nm, respectively (Victor™, PerkinElmer, Waltham, MA, USA). Where indicated, cells were pre-treated for 1h with N-

Acetyl-L-Cysteine (NAC, 5 mM) in 199 medium containing 10% FBS, and CM-DFCA was added 30 minutes after NAC administration. For the determination of ROS content in HUVECs chronically treated with L-NAME, cells were loaded as described with CM-DCFA during the last 30 min of treatment.

**SOD activity**

HUVECs, plated in 25-cm$^2$ flasks, were washed with PBS and collected by scraping at 4°C in a lysis buffer containing Hepes 20 mM pH 7.4, 1 mM EGTA, 210 mM Mannitolo, 70 mM Saccarose. After sonication on ice and a 5-min centrifugation at 1,500*g* at 4°C, SOD activity was measured in the surnatant by using a commercially available Superoxide Dismutase Assay Kit (Cayman Chemical Company, Ann Arbor, MI) following manufacturer's instructions. This assay kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Optical density at 460 nm were measured using a multiplate spectrophotometer (Victor™, PerkinElmer, Waltham, MA, USA).




**HPLC detection of MDA levels**

MDA levels were measured following the procedure described by Seljeskog et al (Seljeskog, Hervig, & Mansoor, 2006). Briefly, samples (50 µl) were mixed with 150 µl

of 0.1 N perchloric acid and 150 µl of 40 mM 2-thiobarbituric acid, and incubated at 96°C for 1 h. After cooling at -20°C for 20 min, 300 µL of methanol and 100 µL of 20% trichloroacetic acid were added to the samples, and mixed for 10 s. The samples were centrifuged at 10,000×g for 10 min, and 20 µL of the supernatant were subjected to HPLC analysis on a Supelcosil C18 3,5 µm (150 x 4,6 mm ID) equipped with a guard column (Supelcosil C18). Mobile phase consisted of 25 mM $KH_2PO_4$ pH 6.2–methanol (75:25, by vol). Buffer flow rate was of 1.0 mL/min, and spectrofluorimetric detector wavelengths were set at 525 nm (excitation) and 560 nm (emission).

**Evaluation of mitochondrial DNA (mtDNA)**

Total DNA was extracted with QIAamp DNA extraction kit (Qiagen, Hilden, Germany). Then, mtDNA levels were amplified by quantitative real time PCR (RT-qPCR) reactions, which were run with the iQ SybrGreenI SuperMix (Bio-Rad, Segrate, Italy) on an iCycler iQ Real-Time PCR detection system (Bio-Rad) using 50 ng of total DNA. The primers used to detect mtDNA were specific for the mitochondrial cytochrome *b* gene (CytB: F, 5'–CTTCGCTTTCCACTTCATCTTACC-3' and R, 5'-TTGGGTTGTTTGATCCTGTTTCG-3') and normalized to genomic DNA by amplification of the rRNA 18S nuclear gene (18S: F, 5'-CTGCCCTATCAACTTTCGATGGTAG-3' and R, 5'-CCGTTTCTCAGGCTCCCTCTC-3'). Calculations were performed with the $2^{-\Delta\Delta Ct}$ methods using 18S rRNA as an internal control.

**Cell metabolism assays**

Cell metabolism was evaluated on HUVECs plated at a density of 1.5-2x$10^4$ cells/well in 96-well microplates using a Cell Titer 96® Aqueous ONE Solution Reagent colorimetric assay (MTS, Promega, Madison, WI, USA), and the total cellular ATP content by means of a CellTiter-Glo® Luminescent Assay (Promega). Both assays were performed according to the manufacturer's instructions. Optical density at 490 nm (for MTS) and luminescence (for ATP) were measured using a multiplate spectrophotometer (Victor™, PerkinElmer, Waltham, MA, USA).

**Oxygen consumption**

Cellular oxygen consumption was measured as previously described (Clementi et al., 1998). Briefly, HUVECs were re-suspended in respiration buffer (0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM $K_2PO_4$, pH 7.4) (Barrientos, Fontanesi, & Díaz, 2009) at a density of $3.0x10^6$/ml, and analyzed at 37°C in a gas-tight vessel equipped with a Clark-type oxygen electrode (Rank Brothers Ltd., Cambridge, UK) connected to a chart recorder. The oxygen electrode was calibrated assuming the concentration of oxygen in the incubation medium at 37°C to be 200 μM. Protein content in cell samples was estimated by the BCA protein assay.

**Statistical procedures**

All data were expressed as mean ± s.e.m. Statistical analysis was carried out using one-way or two-ways analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test, or Student's t-tests, where applicable.

In Two-way ANOVA analyses, (Figures 10A, 11B-C-F, 13B), we considered as factors the treatment with NAC, and the treatment with L-NAME. P-values of $<0.05$ were considered significant.

**Reagents and antibodies**

All tissue culture reagents were from Sigma Chemicals (St. Louis, MO, USA). The following reagents were purchased as indicated: human $VEGF_{165}$ from Calbiochem (Darmstadt, Germany); collagen type IV from BD Bioscience (Bedford, MA, USA); L-NAME, ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), DETA-NO (2,2'(hydroxynitrosohydrazono)bis-ethanimine) and NAC (N-acetylcysteine) from Sigma Chemicals (St. Louis, MO, USA).

Antibodies used were: rabbit polyclonals anti caspase-3, anti LC3, anti p62, anti p-Akt and anti p-eNOS (Cell Signaling Technology, Danvers, MA, USA), anti Bax, anti KRD and anti Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse monoclonals anti

Bcl-2, anti Lamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti total eNOS, anti HIF-1α, anti total Akt (BD Transduction Laboratories, Franklin Lakes, NJ, USA) and anti β-actin (Sigma Chemicals, St. Louis, MO, USA).

# Results

**Chronic treatment with L-NAME did not affect HUVEC vitality**

To characterize our model of endothelial dysfunction, we firstly analyzed the effects of chronic NO deprivation on the vitality of HUVECs. As shown in Figure 1A, we did not observe any variation in cell number and vitality evaluated by cell counting with the vital dye Trypan Blue. These results were confirmed by a crystal violet assay that did not show any significant difference between the number of control and chronically L-NAME treated cells (Figure 1B). In addition, long term L-NAME treatment did not induce caspase-3 cleavage (Figure 1C) which instead occurred when human ECs were exposed to high glucose (30 mM for 48 h), a condition known to be apoptotic for HUVECs (Baumgartner-Parzer et al., 1995). Also the expression of Bcl-2 and Bax, two well-known proteins involved in the regulation of apoptosis endowed with anti-apoptotic and pro-apoptotic activity respectively, was not affected by L-NAME treatment (Figure 1D). Finally, apoptotic index and necrotic cell percentage quantified by annexin V-conjugated FITC and PI staining followed by FACS analysis did not show any difference between control and L-NAME treated cells (Table 1). We should therefore conclude that long term NO deprivation does not induce neither apoptosis nor loss of vitality in HUVECs.

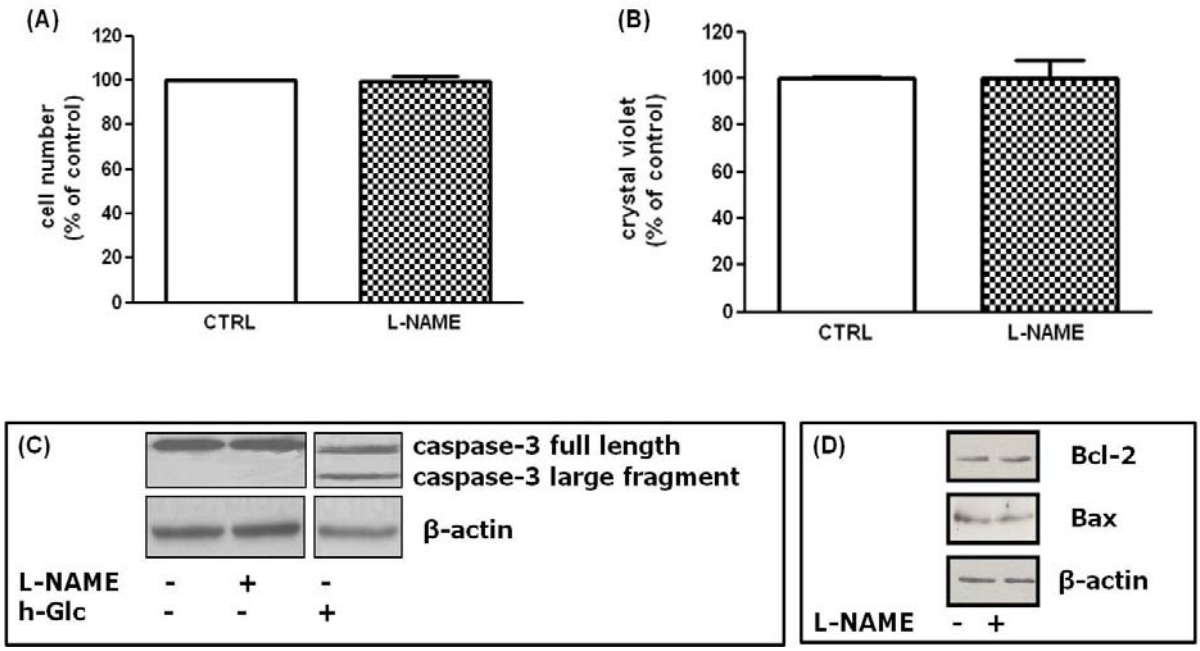



**Figure 1. Effects of chronic treatment with L-NAME on HUVEC vitality. (A)** The vitality and number of control (CTRL, set at 100%) and L-NAME treated cells was evaluated by cell counting with the vital dye Trypan Blue; n=25. **(B)** The total number of control (CTRL, set at 100%) and L-NAME treated cells was measured by crystal violet staining; n=7. **(C)** Lysates of HUVECs treated for 48 h with 5 mM L-NAME or 30 mM glucose (high glucose, h-Glc) were separated by 12% SDS-PAGE and immunoblotted with an anti-caspase 3 antibody which recognized full length caspase-3 (35 kDa) and its large fragment resulting from cleavage (17 kDa). β-actin was used as a loading control. Shown is a representative blot of 2 comparable experiments. **(D)** Total cell lysates prepared as described in (C) were separated by SDS-PAGE and immunoblotted with anti Bcl–2 or anti Bax antibodies. β-actin was used as a loading control. Shown is a representative blot of 2 comparable experiments.

| | **Control** | **48h L-NAME** |
|---|---|---|
| **Apoptotic index** | 0.16±0.03 | 0.15±0.05 |
| **Necrotic cell %** | 8.3±0.26% | 4.1±0.21% |

**Table 1. Effects of chronic treatment with L-NAME on HUVEC apoptosis and necrosis.** Apoptotic index and necrotic cell percentage were quantified by annexin V-conjugated FITC and PI staining respectively, followed by FACS analysis in control and L-NAME treated HUVECs.

**Migratory behavior of L-NAME treated HUVECs**

Since NO is strongly involved in angiogenic processes and EC migration, we investigated by chemotaxis assay the migratory properties of chronically NO deprived HUVECs. Long term treatment with L-NAME induced a significant increase in HUVEC migratory capability both in the absence and in the presence of chemotactic stimuli such as VEGF (25 ng/ml) (Figure 2A) and FBS (10%) (data not shown).

To investigate whether the increased migratory behavior was due to a deficiency in cyclic GMP (cGMP) accumulation as a consequence of NO deprivation, we evaluated the effect of a 48-h treatment with the guanylate cyclase inhibitor ODQ on HUVEC migration. Despite the ability of both L-NAME and ODQ to significantly reduce cGMP levels in HUVECs (Figure 2B), long-term treatment with ODQ did not affect the motility of either basal or VEGF-stimulated cells (Figure 2A). These results suggest that blunting of the cGMP signaling pathway is not involved in the pro-migratory effect induced by NO depletion.

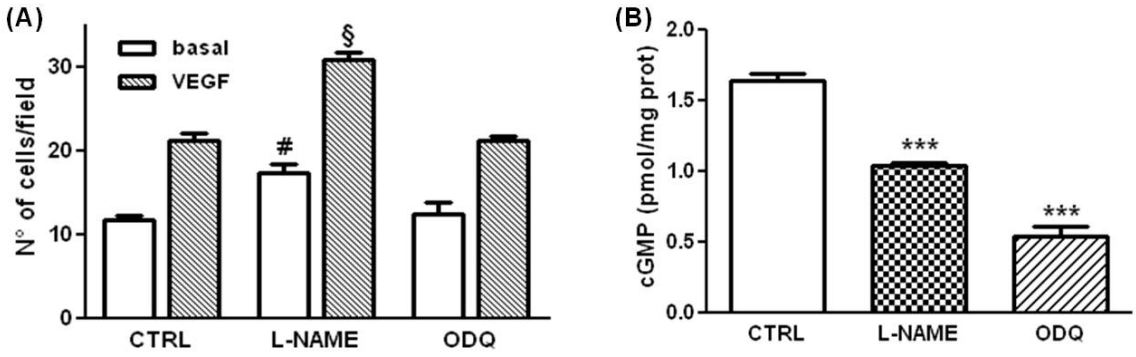



**Figure 2. The enhancement in HUVEC migration induced by L-NAME is independent of the cGMP pathway. (A)** HUVECs were treated for 48 h with 5 mM L-NAME or 1 mM ODQ, and chemotaxis experiments were then performed using 25 ng/ml VEGF as attractants. Results are expressed as the number of migrating cells in the different experimental conditions. #p<0.001 *vs* basal migration in control cells (CTRL); §p<0.001 *vs* VEGF-induced migration in control cells; no significant differences between control and ODQ treated cells; One-way ANOVA with Bonferroni's test, n = 3. **(B)** cGMP accumulation in HUVECs treated for 48 h with L-NAME or ODQ was evaluated by EIA and expressed as pmol of cGMP normalized to the cell protein content (pmol/mg protein). ***p<0.001; One-way ANOVA with Bonferroni's test; n = 3.

## Effects of chronic L-NAME treatment on eNOS, VEGF and KDR expression in HUVECs

To evaluate if a rebound increase in eNOS expression induced by chronic NO deficiency might be responsible for the enhanced motility observed in L-NAME treated cells, we analyzed by RT-qPCR and western blot the expression of eNOS mRNA and protein in control and L-NAME treated HUVECs. Surprisingly, we found a significant decrease in eNOS protein expression (by 48±5%) (Figure 3A) in NO deprived cells that was not however accompanied by a parallel reduction in mRNA expression (Figure 3B). These results suggest that chronic inhibition of eNOS might cause an increased degradation of the enzyme and/or impairment of the translation of its mRNA.

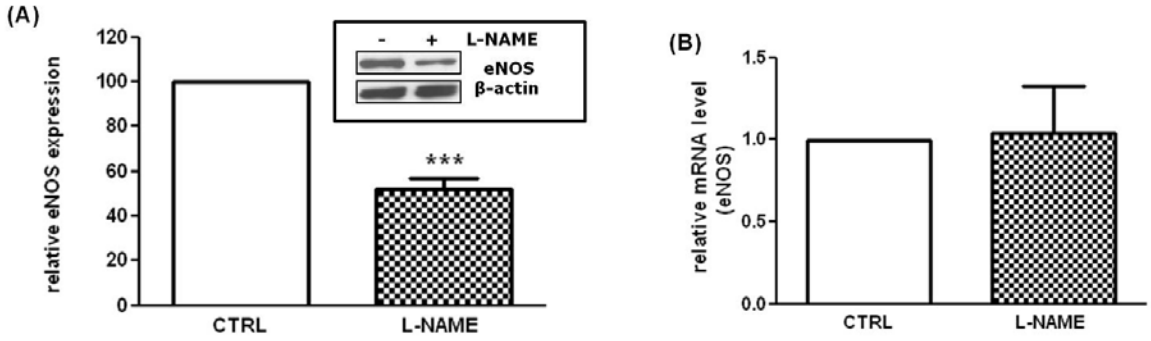



**Figure 3. Effect of L-NAME treatment on eNOS expression. (A)** Densitometric analysis of eNOS protein expression. ***p<0.001; t test; n = 11. Inset: a representative blot out of eleven is shown. Total eNOS protein was evaluated by western blotting on lysates prepared from control cells (lane 1) or from 48 h L-NAME treated cells (lane 2). β-actin was used as a loading control. **(B)** eNOS RNA levels were measured by RT-qPCR and normalized to the level of the housekeeping gene 18S. No significant differences between control and L-NAME treated cells; *t* test, n = 3.

VEGF through its tyrosine kinase receptor KDR is the main growth factor acting on ECs. Therefore, we investigated if chronic L-NAME treatment might affect the expression of VEGF and KDR, thus potentiating migration via an autocrine mechanism. RTqPCR analysis showed an increase in the expression of both VEGF and KDR mRNA (1.91±0.2 and 1.79±0.2 fold, respectively) in L-NAME treated cells compared to control cells (Figure 4A). The increased expression of VEGF and KDR mRNAs were also confirmed for the

corresponding proteins. The quantitative measurements of VEGF by means of an ELISA assay showed a 1.7-fold increase in the amount of VEGF secreted from L-NAME treated cells in comparison to untreated cells (Figure 4B). Similarly, a biochemical analysis on HUVEC lysates demonstrated a 1.8-fold increase in KDR protein expression in chronically NO-deprived HUVECs compared to untreated cells (Figure 4C). These findings are suggestive for the establishment of an autocrine loop sustained by the increased endogenous production of VEGF that through its receptor KDR could finally enhance cell migration.

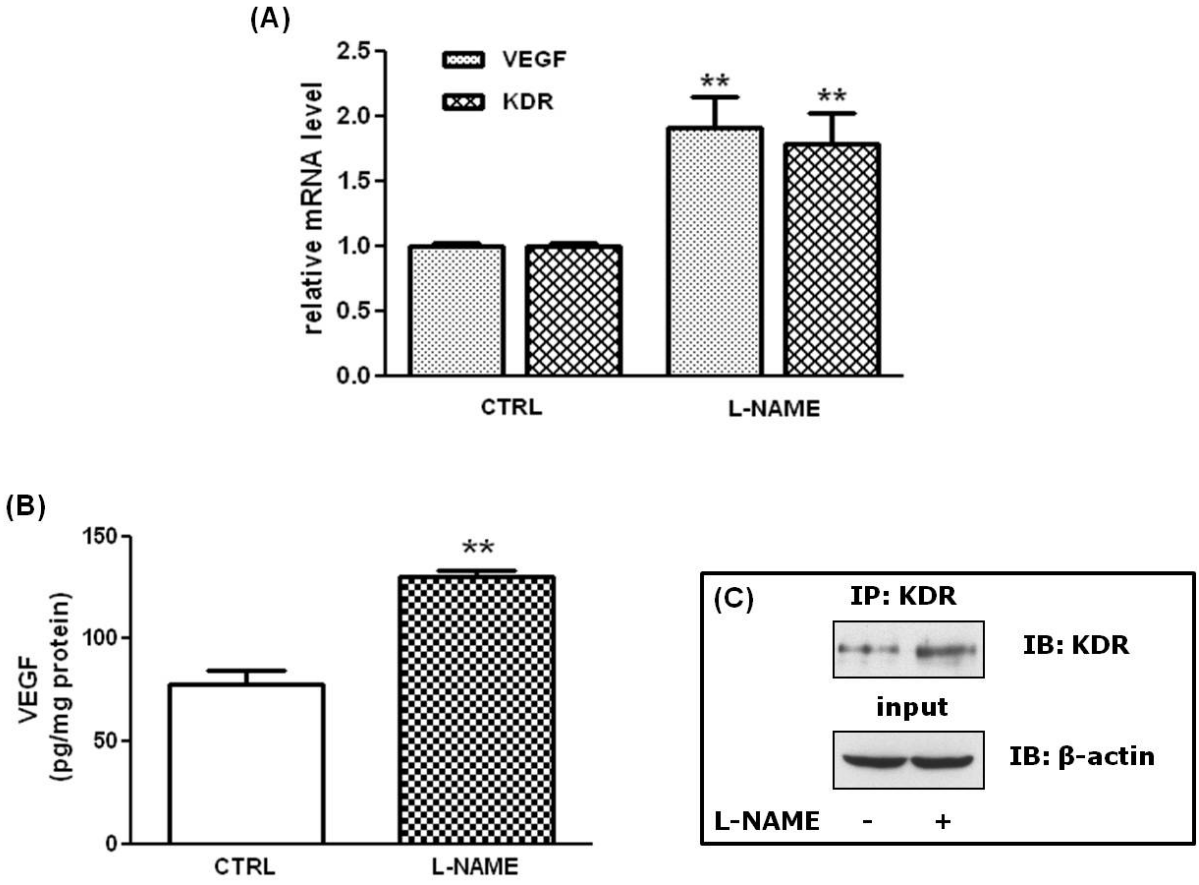



**Figure 4. Effects of L-NAME treatment on VEGF and KDR expression. (A)** VEGF and KDR mRNA levels were measured by RT-qPCR and normalized to the level of the housekeeping gene 18S. **p<0.01 vs control cells (CTRL); *t* test; n = 6-4 for VEGF and KDR, respectively. **(B)** VEGF protein levels were detected by ELISA measurement in conditioned media collected from control or 48 h L-NAME treated cells. Results are expressed as pg of VEGF normalized to the cell protein content (pg/mg protein). **p<0.01; *t* test; n = 3. **(C)** KDR protein was visualized by western blot after immunoprecipitation with KDR antibodies of HUVEC lysates obtained from control (lane 1) or from 48 h L-NAME treated cells (lane 2). An aliquot of total cell lysates was immunoblotted with β-actin antibodies as a control (input). Shown is a representative blot of 2 comparable experiments.

**Effects of chronic L-NAME treatment on VEGF-induced signaling pathways in HUVECs**

A major VEGF-induced signaling pathway involved in the control of HUVEC migration depends on the activation of phosphatidylinositide 3-kinase (PI3-K) that through the phosphorylation of its downstream kinase AKT activates eNOS by phosphorylation on Ser 1177. To verify if chronic NO deprivation might affect VEGF-induced PI3-K/AKT/eNOS pathway, the phosphorylation state of AKT and eNOS after a 5-min stimulation with VEGF (25 ng/ml) was assessed by western blot in control and L-NAME treated HUVECs. As shown in Figure 5, we observed an increase in eNOS and AKT phosphorylation in VEGF-treated control cells (lane 2), as expected. On the other hand, basal levels of eNOS and AKT phosphorylation were already increased in L-NAME treated cells (see lane 3 *vs* lane 1), and VEGF stimulation was not able to induce any further phosphorylation (lane 4). A densitometric analysis confirm that basal eNOS and AKT phosphorylation were 3.4±0.9 and 1.6±0.2 times greater respectively in L-NAME treated cells in comparison to untreated cells. In conclusion, these data support the hypothesis that L-NAME chronic treatment activates the VEGF/KDR system in HUVECs, thus enhancing basal and VEGF-stimulated migration.

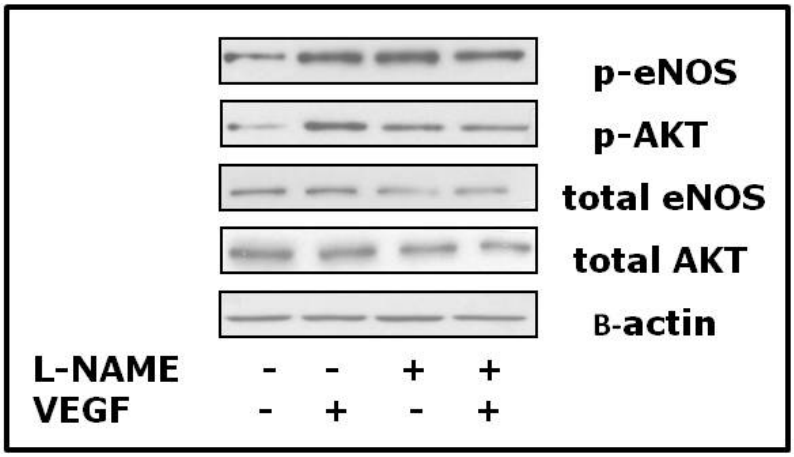



**Figure 5. Effects of chronic L-NAME treatment on VEGF-induced signaling pathways in HUVECs.** Control cells (lanes 1 and 2) or 48 h L-NAME treated cells (lanes 3 and 4) were stimulated for 5 min with 25 ng/ml VEGF. Aliquots of cell lysates were separated by 10% SDS-PAGE and immunoblotted with the indicated antibodies. β-Actin was used as a loading control. Shown is a representative blot of 4 comparable experiments.

## Chronic deprivation of NO induces nuclear accumulation of HIF-1α in HUVECs

The increase in VEGF production and cell motility is one of the typical events that occur in cancer cells during hypoxia, and are related to the nuclear accumulation and activation of the transcription factor Hypoxia Inducible Factor 1α (HIF-1α). HIF-1α also plays a major role in ECs where its activation crucially regulates the transcription of genes encoding for angiogenic factors such as VEGF (Namiki et al., 1995). We therefore analyzed by western blot HIF-1α levels in nuclei of 48-h L-NAME treated HUVECs. Interestingly, we observed a significant accumulation of nuclear HIF-1α (5.5±1.6 fold over the basal level) in NO deprived HUVECs compared to control cells (Figure 6A). Moreover, the nuclear accumulation of HIF-1α seemed to be dependent on a stabilization of the protein and not on an increased transcription. Indeed, RTqPCR experiments showed that L-NAME treatment did not induce any significant increase in the levels of HIF-1α mRNA (1.21±0.1 fold over control cells) (Figure 6B). All together, these findings suggest that chronic L-NAME treatment induces a pseudo-hypoxic state in HUVECs *i.e.* the nuclear accumulation of HIF-1α under normoxic conditions.

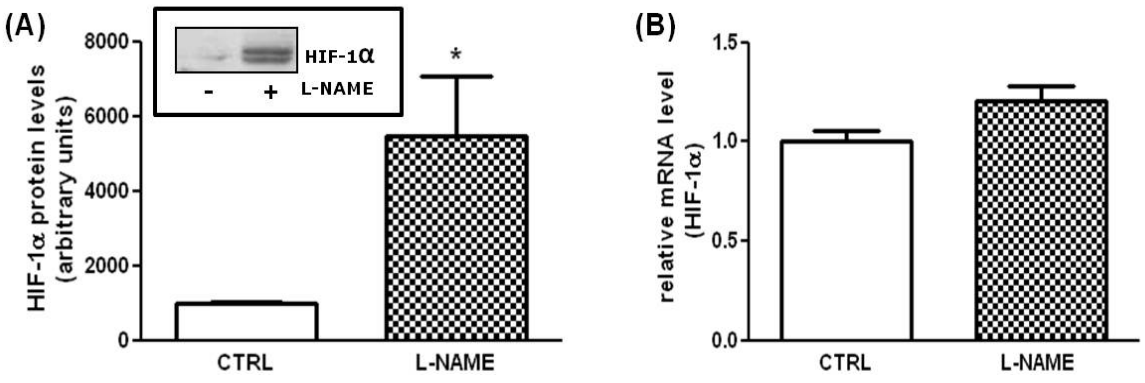



**Figure 6. L-NAME treatment induces HIF-1a nuclear accumulation in HUVECs. (A)** Densitometric analysis of nuclear HIF-1α protein levels. *p<0.05; *t* test; n = 4. Insert: a representative blot out of 4 is shown. HIF-1α protein levels were detected by western blotting of nuclear extracts from control HUVECs (lane 1) or from HUVECs treated with L-NAME for 48 h (lane 2). HIF-1α migrates as a doublet with apparent molecular weight of 118 and 120 kDa. **(B)** HIF-1α RNA levels were measured by RT-qPCR and normalized to the level of the housekeeping gene 18S. No significant differences between control and L-NAME treated cells; *t* test; n=3.

To directly correlate the nuclear accumulation and transcriptional activity of HIF-1α to the late effects observed in chronically deprived NO HUVECs *i.e.* increased VEGF production and cell motility, we transfected control and L-NAME-treated cells with a plasmid expressing a dominant negative form of the HIF-1β subunit (ΔARNT), which maintain the capacity of forming heterodimer but cannot bind DNA (Tacchini, De Ponti, Matteucci, Follis, & Desiderio, 2004; Tacchini, Gammella, De Ponti, Recalcati, & Cairo, 2008). As shown in Figures 7A and B, transfection with ΔARNT totally blunted both the increase in VEGF production and cell motility that we previously observed in L-NAME treated HUVECs. These results confirmed the central role of HIF-1α in the regulation of VEGF expression and of the consequent increased migration induced by long-term L-NAME treatment. Interestingly, the decrease in eNOS protein expression that we observed in L-NAME treated HUVECs (see Figure 3A) was maintained in the presence of ΔARNT (Figures 7C and D) demonstrating that this late effect induced by NO deprivation was independent of the transcriptional activity of HIF-1α.

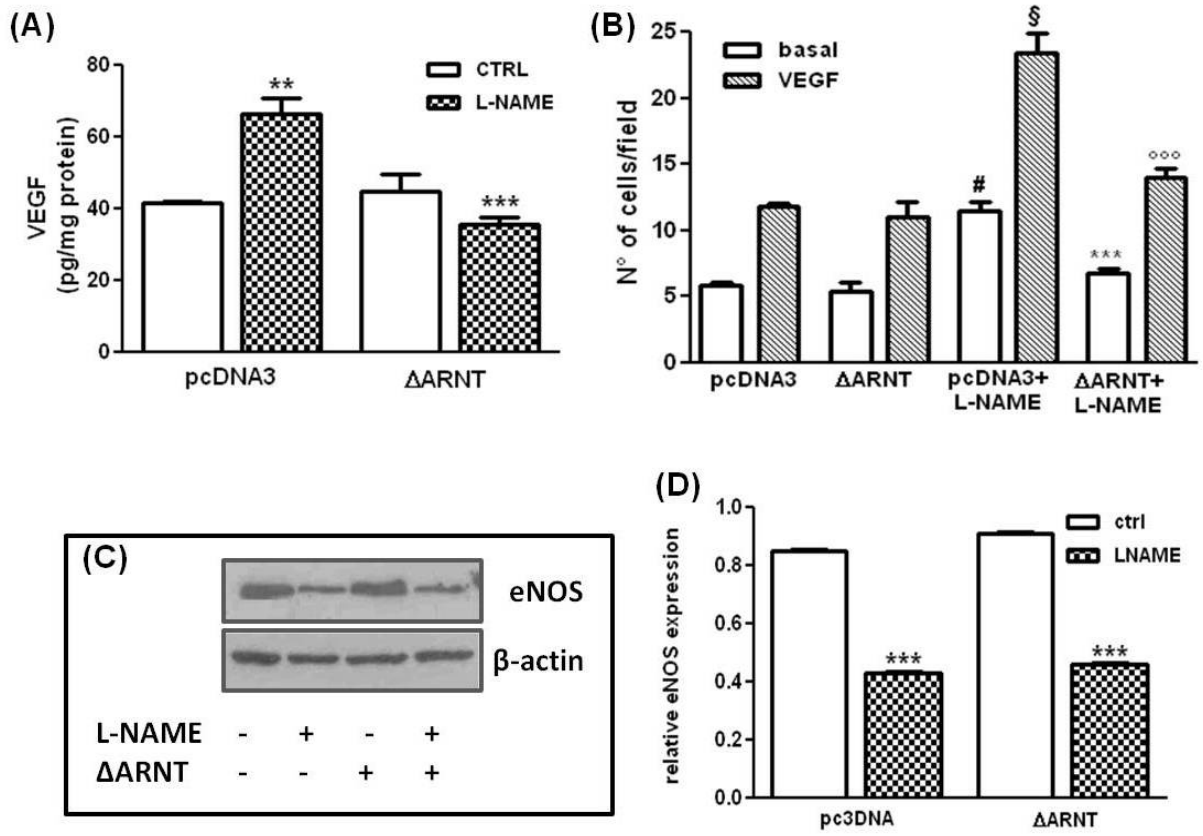



**Figure 7. The block of the transcriptional activity of HIF-1a reverts L-NAME-induced VEGF secretion and cell migration but not the decrease in eNOS protein expression. (A)** VEGF protein levels were detected by ELISA measurement in conditioned media collected from HUVECs transfected with the empty vector (pcDNA3) or with the expression vector ΔARNT, and treated with L-NAME for the 48 h following transfection. Results are expressed as pg of VEGF normalized to the cell protein content (pg/mg protein). **p<0.01 vs untreated cells transfected with pcDNA3; ***p<0.001 vs L-NAME treated cells transfected with pcDNA3; One-way ANOVA with Bonferroni's test; n=3. **(B)** HUVECs were transfected with pcDNA3 or ΔARNT, and treated with L-NAME for the 48 h following transfection, when indicated. Chemotaxis experiments were then performed using 25 ng/ml VEGF as attractant. Results are expressed as the number of migrating cells. #p<0.001 *vs* basal migration in untreated pcDNA3 cells; §p<0.001 *vs* VEGF-induced migration in untreated pcDNA3 cells; ***p<0.001 *vs* basal migration in pcDNA3 cells treated with L-NAME; °°°p<0.001 *vs* VEGF-induced migration in pcDNA3 cells treated with L-NAME; no significant differences between untreated pcDNA3 and ΔARNT transfected cells and between untreated and L-NAME treated ΔARNT tranfected cells; One-way ANOVA with Bonferroni's test, n=10. **(C)** Total cellular lysates obtained from HUVECs transfected with pcDNA3 or ΔARNT and treated with L-NAME for the following 48 h were separated by SDS-PAGE and immunoblotted with the indicated antibodies. **(D)** Densitometric analysis of eNOS protein levels normalized to β-actin which was used as a loading control. ***p<0.001 *vs* the corresponding untreated cells (CTRL); no significant differences between both untreated and L-NAME treated pcDNA3 and ΔARNT transfected cells; One-way ANOVA with Bonferroni's test; n=3.

**The NO-donor DETA-NO reverts the effects of L-NAME treatment on HIF-1α stabilization and VEGF expression**

To understand whether the effects observed in HUVECs after L-NAME treatment depend on chronic NO deprivation, we restored physiologic NO levels by using the long lasting NO donor DETA-NO (500 nM). The treatment with the donor was performed for the last 24 h of incubation. The results showed that DETA-NO completely reverted the effects of long-term L-NAME treatment on HIF-1α stabilization (Figure 8A) and on the consequent increase in VEGF mRNA expression and cell migration (Figures 8B and C). Moreover, DETA-NO also reverted the decrease in eNOS expression induced by the treatment with L-NAME (Figure 8A).

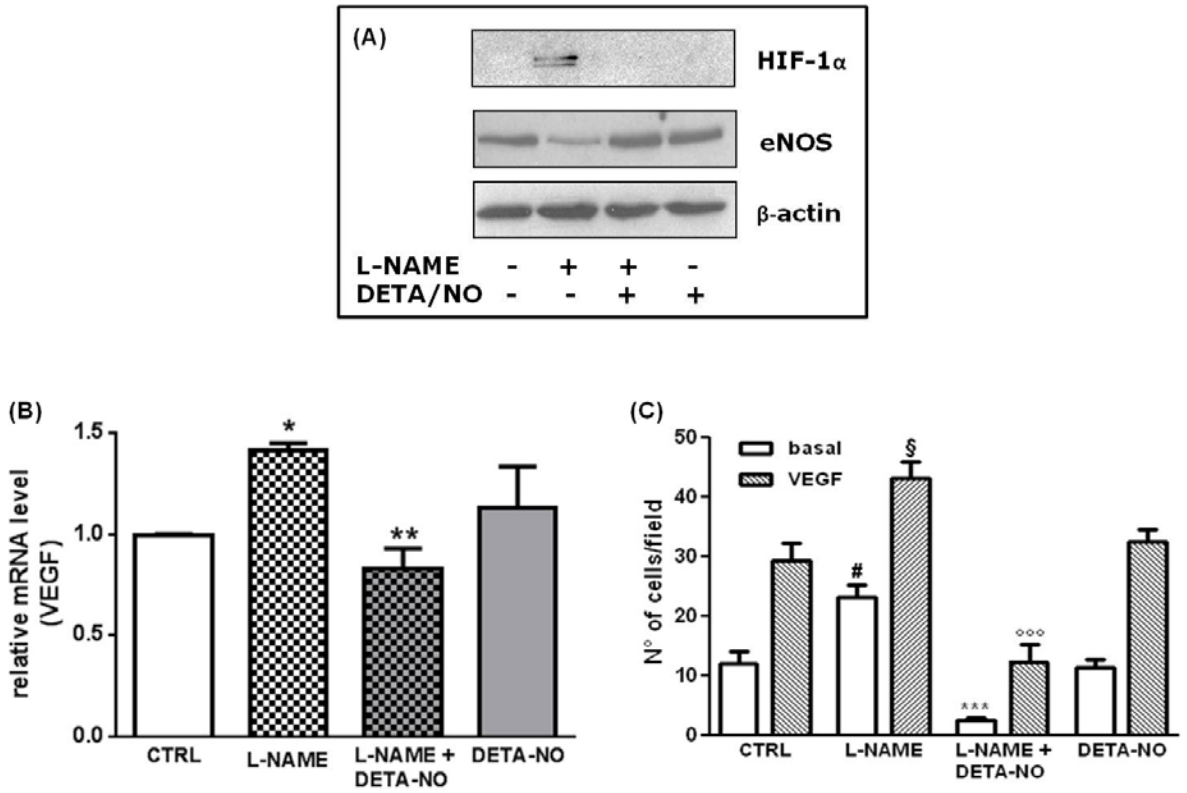



**Figure 8. The NO donor DETA-NO reverts the effects induced by L-NAME on HIF-1α stabilization, VEGF and eNOS expression, and cell migration. (A)** HIF-1α protein levels were detected by western blotting of nuclear extracts from HUVECs treated for 48 h with 5 mM L-NAME in the absence or in the presence of 500 nM DETA/NO for the last 24 h, as indicated. An aliquot of total cell lysates was immunoblotted with anti eNOS antibodies, and with anti β-actin antibodies as loading control. A representative blot of 3 comparable experiments is shown. **(B)** VEGF mRNA levels were measured by RT-qPCR and normalized to the level of the housekeeping gene 18S. *p<0.05 vs control cells (CTRL); **p<0.01 *vs* L-NAME treated cells; no significant differences between control and DETA/NO treated cells; One-way ANOVA with Bonferroni's test; n = 3. **(C)** HUVECs were treated with L-NAME and/or DETA/NO as described in (A). Chemotaxis experiments were then performed using 25 ng/ml VEGF as attractants. Results are expressed as the number of migrating cells. #p<0.001 *vs* basal migration in control cells (CTRL); §p<0.01 *vs* VEGF-induced migration in control cells; ***p<0.001 *vs* basal migration in L-NAME treated cells; °°°p<0.001 *vs* VEGF-induced migration in L-NAME treated cells; no significant differences between control and DETA/NO treated cells; One-way ANOVA with Bonferroni's test, n = 15.

**Silencing of eNOS mimics the effects of chronic L-NAME treatment in HUVECs**

To verify if the effects observed in L-NAME treated HUVECs were due to the specific inhibitory effect of the drug on enzyme activity, we silenced the expression of eNOS by RNA interference (siRNA). HUVEC transfection with eNOS siRNA induced a significant reduction in eNOS protein expression (by 70±0.1%) in comparison to cells transfected with control siRNA (Figure 9A). In agreement with the results obtained in L-NAME treated cells, eNOS silenced cells showed nuclear accumulation of HIF-1α (Figure 9B) and an increased production of VEGF protein (Figure 9C) that were instead absent in control siRNA transfected cells.

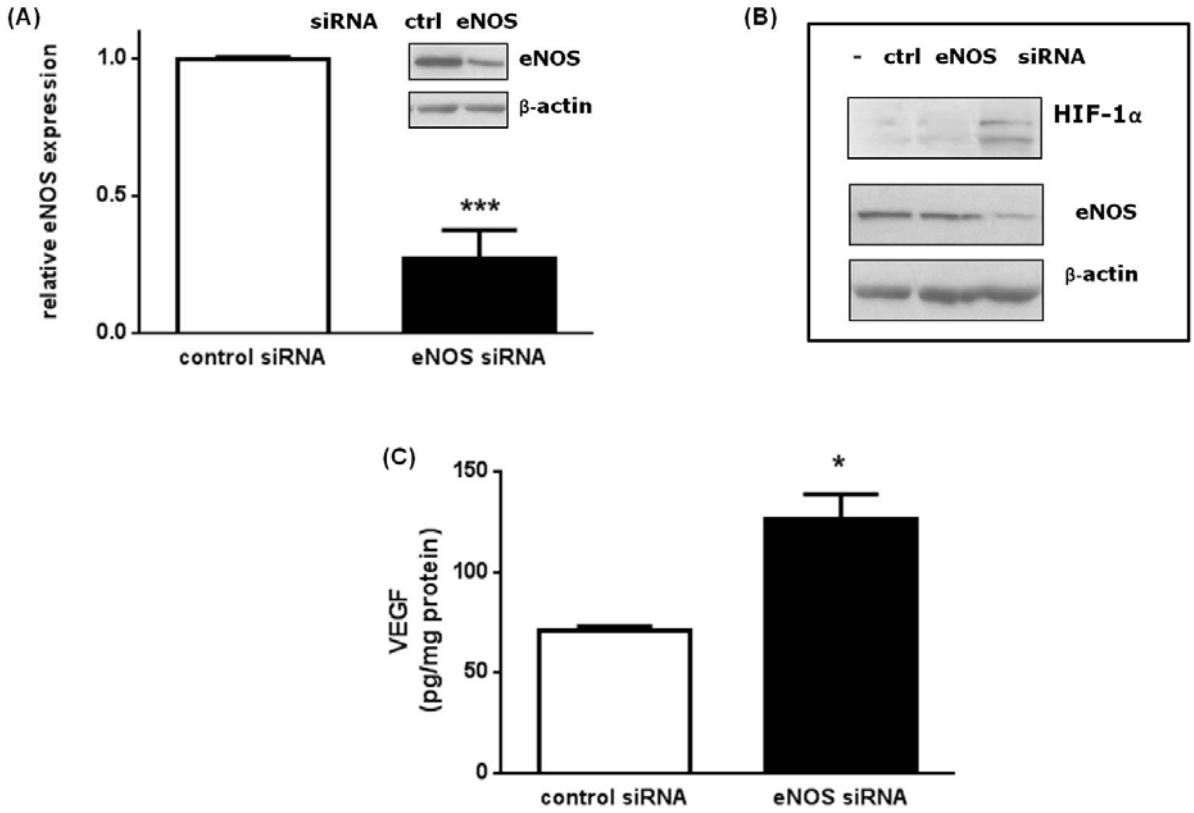



**Figure 9. Effect of eNOS silencing on HIF-1α accumulation and VEGF secretion. (A)** Characterization of HUVECs transfected with eNOS siRNA: densitometric analysis of eNOS protein expression where eNOS protein levels were normalized to β-actin protein. ***p<0.001; *t* test; n = 4. Inset: representative blots of eNOS protein in cells transfected with control (ctrl) or eNOS siRNA. **(B)** HUVECs were transfected with control (lane 2) or eNOS siRNA (lane 3), and HIF-1α protein was detected by western blotting on the corresponding nuclear extracts. In lane 1, nuclear extracts from untransfected cells. An aliquot of total cell lysates was immunoblotted with anti eNOS antibodies to check silencing, and with anti β-actin antibodies as loading control. A representative blot of 2 comparable experiments is shown. **(C)** VEGF protein levels were detected by ELISA measurement in conditioned media collected from HUVECs 48 h after transfection with control or eNOS siRNA. Results are expressed as pg of VEGF normalized to the cell protein content (pg/mg protein). *p<0.05; *t* test; n = 3.

**Acute treatment with L-NAME induces ROS generation in HUVECs**

In the attempt to elucidate the mechanism(s) responsible for HIF-1α stabilization in chronic NO-depleted HUVECs, we investigated the role of Reactive Oxygen Species (ROS). ROS were indeed proposed as one of the putative mechanism responsible for HIF-1α stabilization in normoxia through their ability to block the activity of PHDs, the enzyme family responsible for HIF-1α degradation (Pagé, Chan, Giaccia, Levine, & Richard, 2008; Patten et al., 2010). Furthermore, ROS are involved in the pathogenesis and progression of endothelial dysfunction and cardiovascular diseases. As a matter of fact, the acute exposure to L-NAME (5 mM) induced in HUVECs a rapid increase in cell-associated fluorescence (1.78±0.18 fold over the control) that was prevented by the pre-treatment with the well-known antioxidant N-Acetil-Cysteine (NAC, 5mM), as expected (Figure 10A). In the absence of L-NAME, the treatment with NAC did not significantly modify the intracellular amount of ROS. Importantly, the L-NAME-induced burst in ROS production was transient, and was followed by a fast decrease in cellular ROS content that returned to baseline levels about 2 hours after L-NAME addiction (Figure 10B).

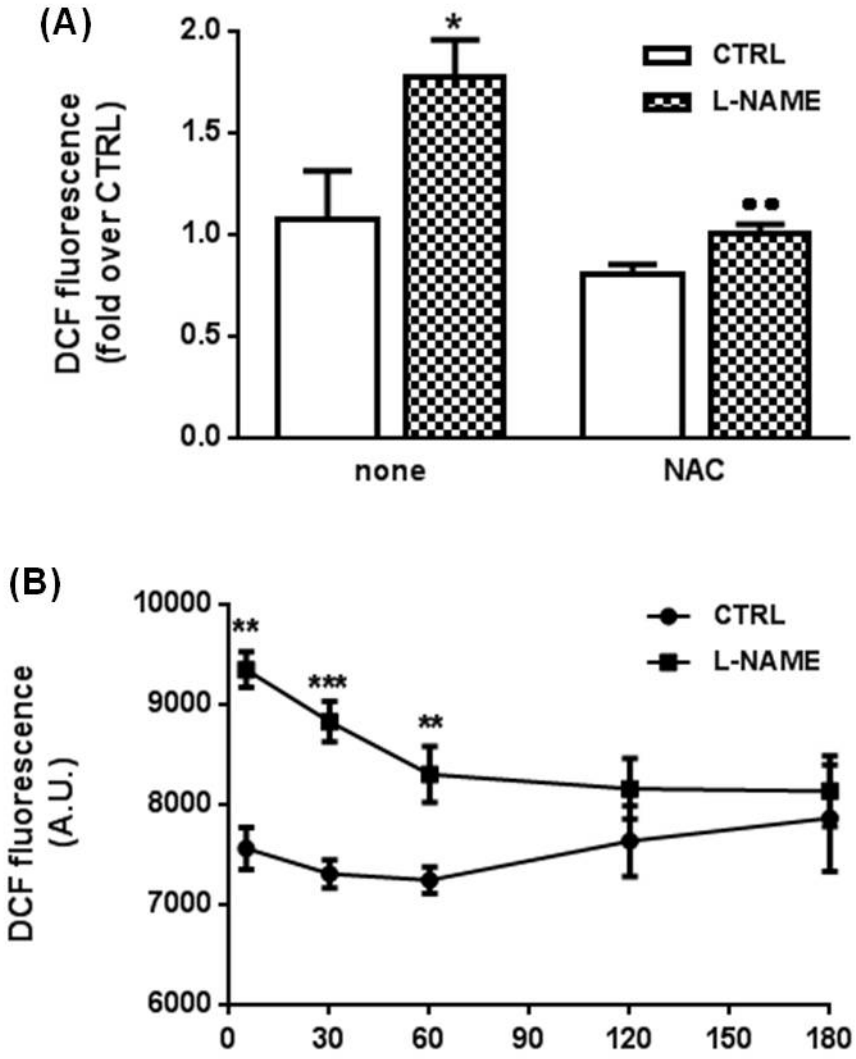



**Figure 10. L-NAME acutely induces ROS generation in HUVECs. (A)** HUVECs were loaded for 30 min with 10 μM CM-DCFA before treatment with 5 mM L-NAME. Where indicated, cells were pretreated for 1 h with 5 mM NAC. Fluorescence was detected 10 min after L-NAME addition. Results are expressed as arbitrary units of fluorescence (A.U.). The presence of NAC significantly reduced the L-NAME induced ROS generation. *p<0.05 *vs* control cells (CTRL) in the absence of NAC; ••p<0.01 *vs* L-NAME treated cells in the absence of NAC; no significant differences between untreated or NAC treated CTRL cells; Two-way ANOVA with Bonferroni's test, n=9–11. **(B)** HUVECs were loaded with CM-DCFA and treated with L-NAME as in (A), and fluorescence was recorded at the indicated times. Results are expressed as in (A). **p<0.01, ***p<0.001 *vs* the corresponding untreated cells (CTRL); *t* test, n=3–5.

**Relationship between L-NAME-induced acute ROS formation and L-NAME late effects in HUVECs**

Firstly, we focused our attention on the role of acute ROS production on the nuclear accumulation of HIF-1α. To this purpose, we analyzed by western blot nuclear extracts prepared from HUVECs chronically treated with L-NAME in the absence or in the presence of NAC. As shown in Figures 11A and B, the L-NAME-induced HIF-1α accumulation was only partially reduced (by 45±6.4%) in the presence of the antioxidant, suggesting that other still unknown mechanism(s) might participated to HIF-1α stabilization in chronically NO deprived HUVECs. Despite the significant decrease in HIF-1α nuclear levels, the treatment with NAC did not modify the enhanced migratory properties shown by L-NAME treated cells (Figure 11C), arguing against the involvement of ROS in the establishment of the pro-migratory phenotype induced by chronic NO deprivation. The independence of the L-NAME-induced increase in cell migration from acute ROS generation was supported by the observation that the drug was continuously required during the 48 h of treatment to exert its promoting effect on cell motility. Indeed, as shown in Figure 11D, migration was unaffected when the L-NAME containing medium was replaced after the first hour of treatment (corresponding to the time span where the drug-induced ROS levels remain significantly higher if compared to control values) with an L-NAME-free medium for the subsequent 47 h. Finally, also the reduction in eNOS protein expression observed in HUVECs chronically treated with L-NAME was not affected by the presence of NAC (Figures 11E and F).

All these results suggest that ROS generation does not play a crucial role in establishing the phenotype observed in chronically NO-deprived cells.

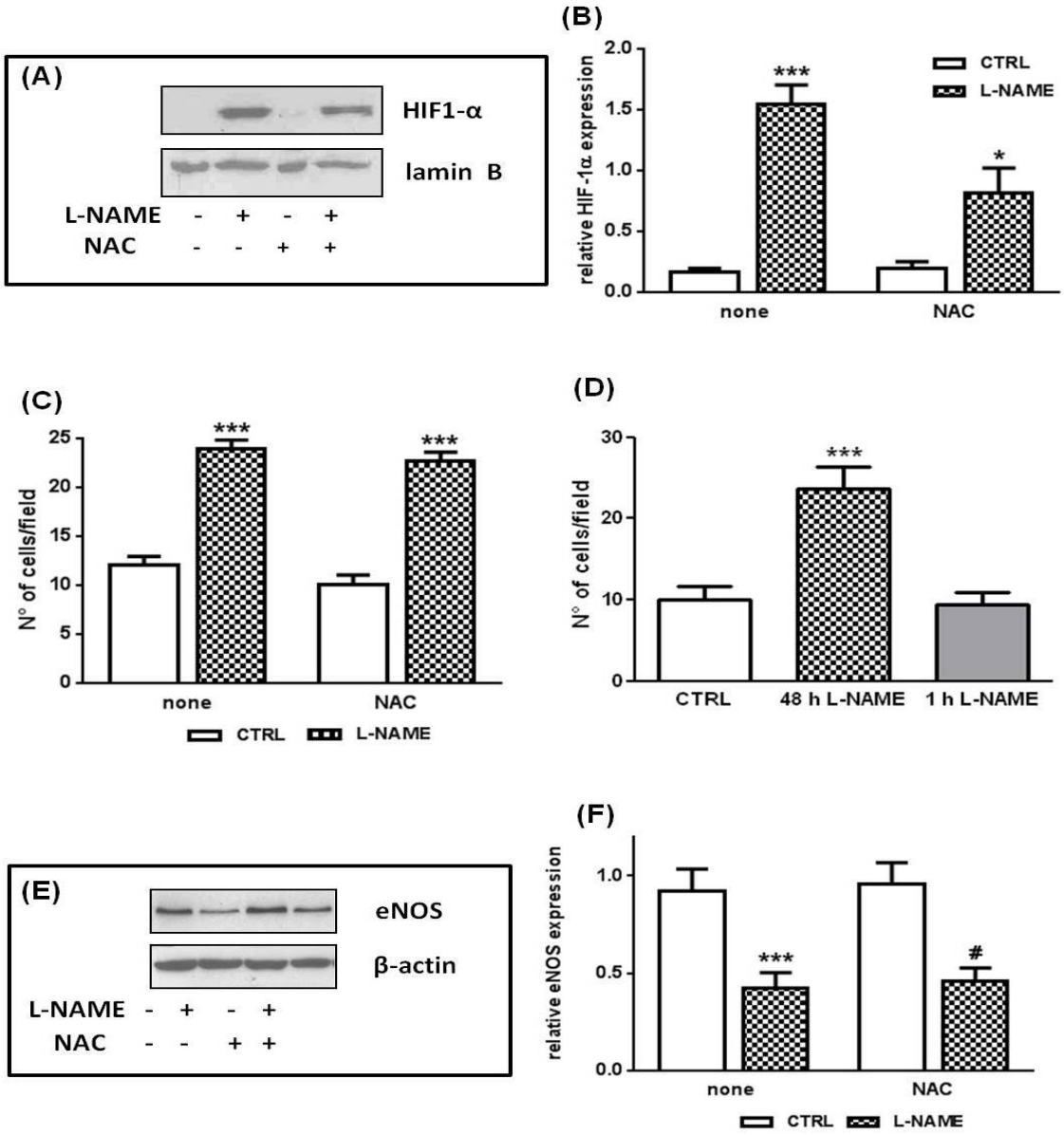



**Figure 11. Effects of NAC on late responses induced by L-NAME treatment. (A)** HIF-1α protein levels were detected by western blotting of nuclear extracts obtained from HUVECs treated for 48 h with 5 mM L-NAME in the absence (lane 2) or in the presence (lane 4) of 5 mM NAC. Lanes 1 and 3: untreated and NAC treated cells, respectively. The antioxidant was added 1 h before L-NAME treatment. **(B)** Densitometric analysis of nuclear HIF-1a protein levels normalized to lamin B which was used as a loading control. ***p<0.001 *vs* control cells (CTRL) in the absence of NAC; *p<0.05 *vs* L-NAME treated cells in the absence of NAC; no significant differences between untreated or NAC treated CTRL cells; Two-way ANOVA with Bonferroni's test, n = 3. **(C)** Chemotaxis experiments were performed using 10% FBS as attractant on HUVECs treated for 48 h with 5 mM L-NAME in the absence or in the presence of 5 mM NAC, as indicated. Results are expressed as the number of migrating cells. The presence of NAC did not significantly affect the L-NAME induced migratory response. ***p<0.001 *vs* control (CTRL) or NAC treated cells in the absence of L-NAME; no significant differences between untreated or NAC treated CTRL cells; Two-way ANOVA with Bonferroni's test, n = 10. **(D)** HUVECs were treated with L-NAME for 48 h (checked bar) or for 1 h only (grey bar), and chemotaxis

experiments were performed 47 h later as described in (B). ***p<0.001 *vs* CTRL; One way ANOVA with Bonferroni's test, n = 15. **(E)** Total cellular lysates obtained from HUVECs pretreated for 1 h with NAC (5 mM) before a 48-h incubation in the presence of L-LAME (5 mM) were separated by SDS-PAGE and immunoblotted with the indicated antibodies. **(F)** Densitometric analysis of eNOS protein levels normalized to β-actin which was used as a loading control. ***p<0.001 *vs* control cells (CTRL) in the absence of NAC; #p<0.001 *vs* NAC treated cells in the absence of L-NAME; no significant differences between untreated or NAC treated CTRL cells; Two-way ANOVA with Bonferroni's test, n = 3.

## Chronic treatment with L-NAME induces an adaptive antioxidant status in HUVECs

When ROS levels were assessed at the end-point of all experiments *i.e.* after 48 h of L-NAME treatment, we observed a significant decrease of about 50% in cellular ROS content in chronically NO-deprived HUVECs compared to control cells (Figure 12A). In the attempt to explain this observation, we focused our attention on the expression and activity of SOD-2 (Superoxide Dismutase-2), the major antioxidant defense system acting against ROS at the vascular level. RT-qPCR analysis demonstrated that the expression of SOD-2 mRNA was significantly increased in chronic L-NAME treated cells (Figure 12B) and was accompanied by an enhancement in its enzymatic activity (Figure 12C). Interestingly, also the expression of catalase, another well-known antioxidant enzyme, was increased by long term L-NAME treatment (2.6±0.2 fold). Catalase activity was not however modified by the treatment (3.6±0.3 and 3.6±0.6 mU/mg in control and L-NAME treated cells, respectively).

The absence of cell damage by oxidative stress was confirmed by the HPLC measurement of malondialdehyde (MDA) levels that were unaffected by L-NAME treatment at all times tested (Figure 12D).

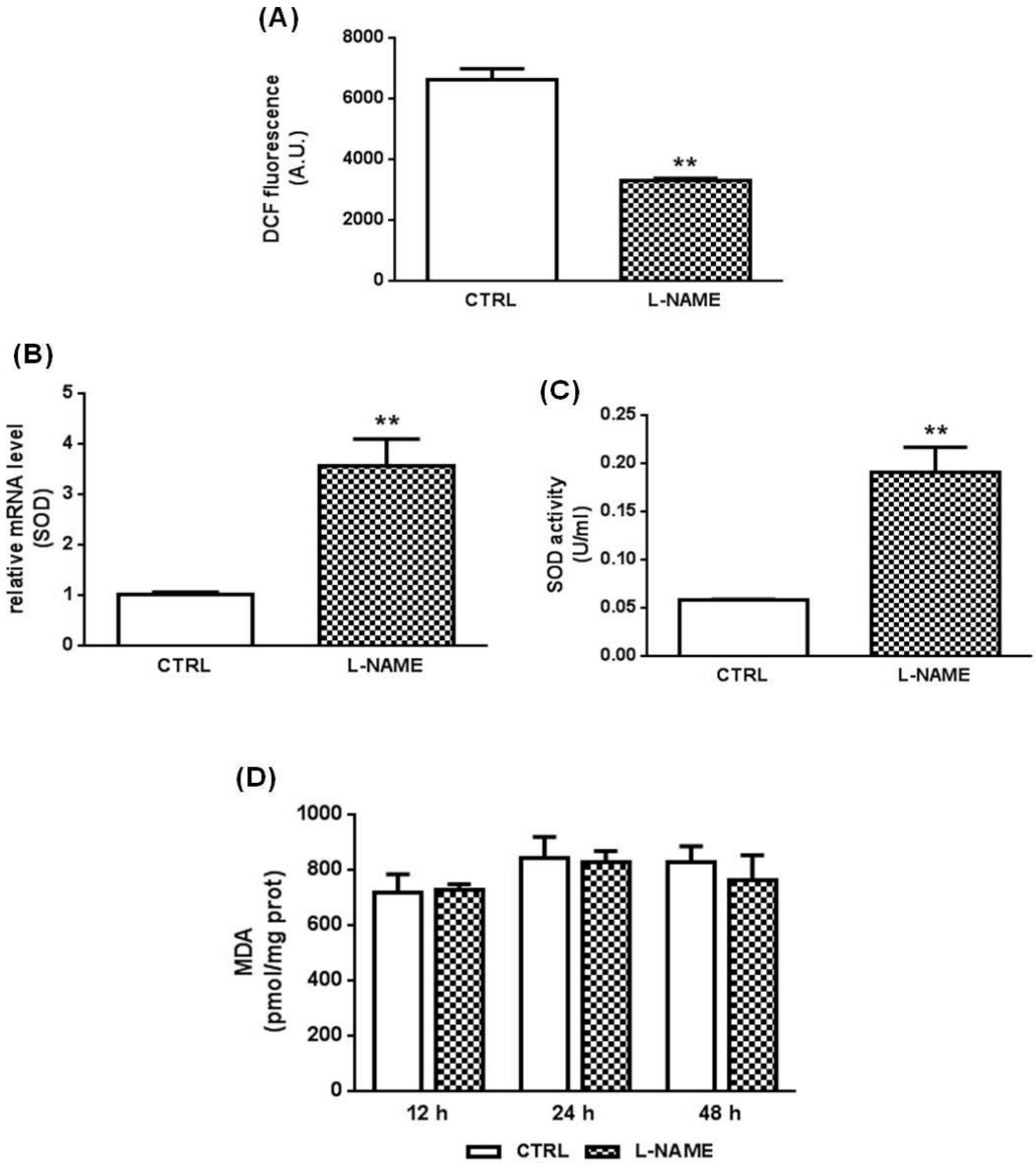



**Figure 12. Chronic treatment with L-NAME reduces ROS content and increases SOD-2 expression and activity. (A)** ROS content was evaluated by measurement of the cell-associated fluorescence in HUVECs treated for 48 h with L-NAME after loading with 10 μM CM-DCFA during the last 30 min of incubation. **p<0.01 *vs* control cells (CTRL) (*t* test, n = 3). **(B)** SOD-2 mRNA levels were measured by RT-qPCR and normalized to the level of the housekeeping gene 18S. **p<0.01 *vs* control cells (CTRL) (*t* test; n = 3). **(C)** SOD activity was evaluated on the total cell lysates by a commercial kit following the manufacturer's instructions. Results are expressed as U/ml. **p<0.01 *vs* control cells (CTRL) (*t* test, n = 3). **(D)** MDA levels were detected by HPLC of supernatants obtained from HUVECs treated for the indicated times with 5 mM L-NAME. Results are expressed as pmol of MDA fold mg of total protein. No significant differences between control and L-NAME treated cells (*t* test; n =3).

A crucial role in the cellular adaptive response to oxidative stress is played by the transcription factor NF-E2-related factor-2 (Nrf2) that through its binding to Antioxidant Responsive Elements (AREs) located within the regulatory region of target genes activates the transcription of antioxidant enzymes such as SOD-2 and catalase (Ma, 2013). To investigate the possible involvement of Nrf2 in the establishment of the antioxidant behavior observed in our model, we evaluated by western blot the amount of Nrf2 in nuclear extracts prepared from HUVECs chronically treated with L-NAME in the absence or in the presence of NAC. As shown in Figures 13A and B, we observed a significant nuclear accumulation of Nrf2 in L-NAME treated cells that was reduced in the presence of the antioxidant. It should be therefore possible to hypothesize a putative involvement of Nrf2 in the transcriptional activation of SOD-2 and catalase genes and in the maintenance of the redox homeostasis in chronically NO-deprived HUVECs.

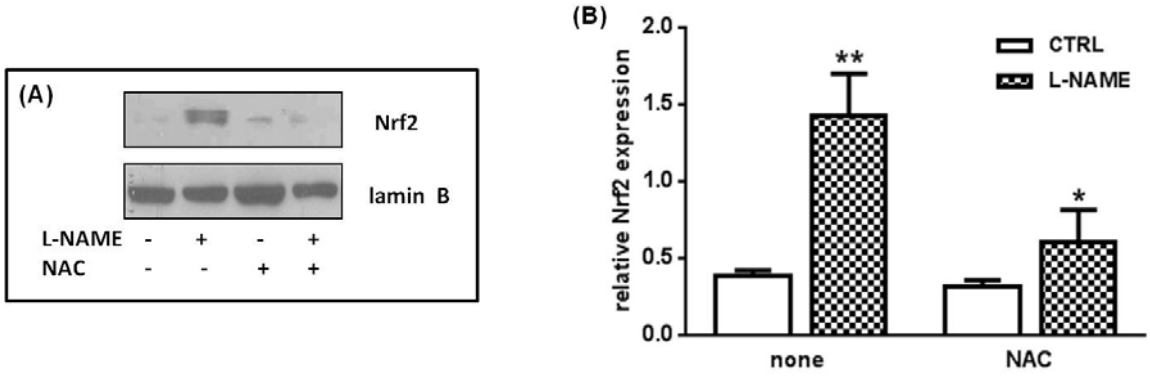



**Figure 13. Chronic treatment with L-NAME induces nuclear accumulation of Nrf2. (A)** Nrf2 protein levels were detected by western blotting of nuclear extracts obtained from HUVECs treated with 5 mM L-NAME for 48 h in the absence (lane 2) or in the presence (lane 4) of 5 mM NAC. Lanes 1 and 3: untreated and NAC treated cells, respectively. **(B)** Densitometric analysis of nuclear Nrf2 protein levels normalized to lamin B which was used as a loading control. **p<0.01 *vs* control cells (CTRL) in the absence of NAC; p*<0.05 *vs* L-NAME treated cells in the absence of NAC; no significant differences between untreated or NAC treated CTRL cells; Two-way ANOVA with Bonferroni's test, n = 3.

**Effects of chronic NO-deprivation on mitochondrial mass and activity in HUVECs**

It has been demonstrated that NO possesses a central role in mitochondrial biogenesis and function in various cell types (Nisoli et al., 2003; Nisoli et al., 2004). For this reason, we investigated the effects of chronic NO deprivation on HUVEC mitochondrial mass and activity. We found a reduced amount of mitochondrial DNA (mtDNA) (by 38±0.05%) in chronically L-NAME treated cells in respect to control cells (Figure 14A). Moreover, we also observed a decrease in the transformation of the metabolic indicator MTS (by 26±5%) (Figure 14B), suggestive of a reduced mitochondrial activity in NO-deficient cells. This observation was supported by the measurement of ATP levels and oxygen consumption that were both reduced (by 25±7% and 25±6%, respectively) in L-NAME treated cells (Figures 14C and D). Importantly, the decreases in mtDNA and ATP cellular levels were also observed in eNOS-silenced cells (Figure 14E and F). These results demonstrated for the first time the crucial role of NO in the maintenance of mitochondrial activities in ECs.

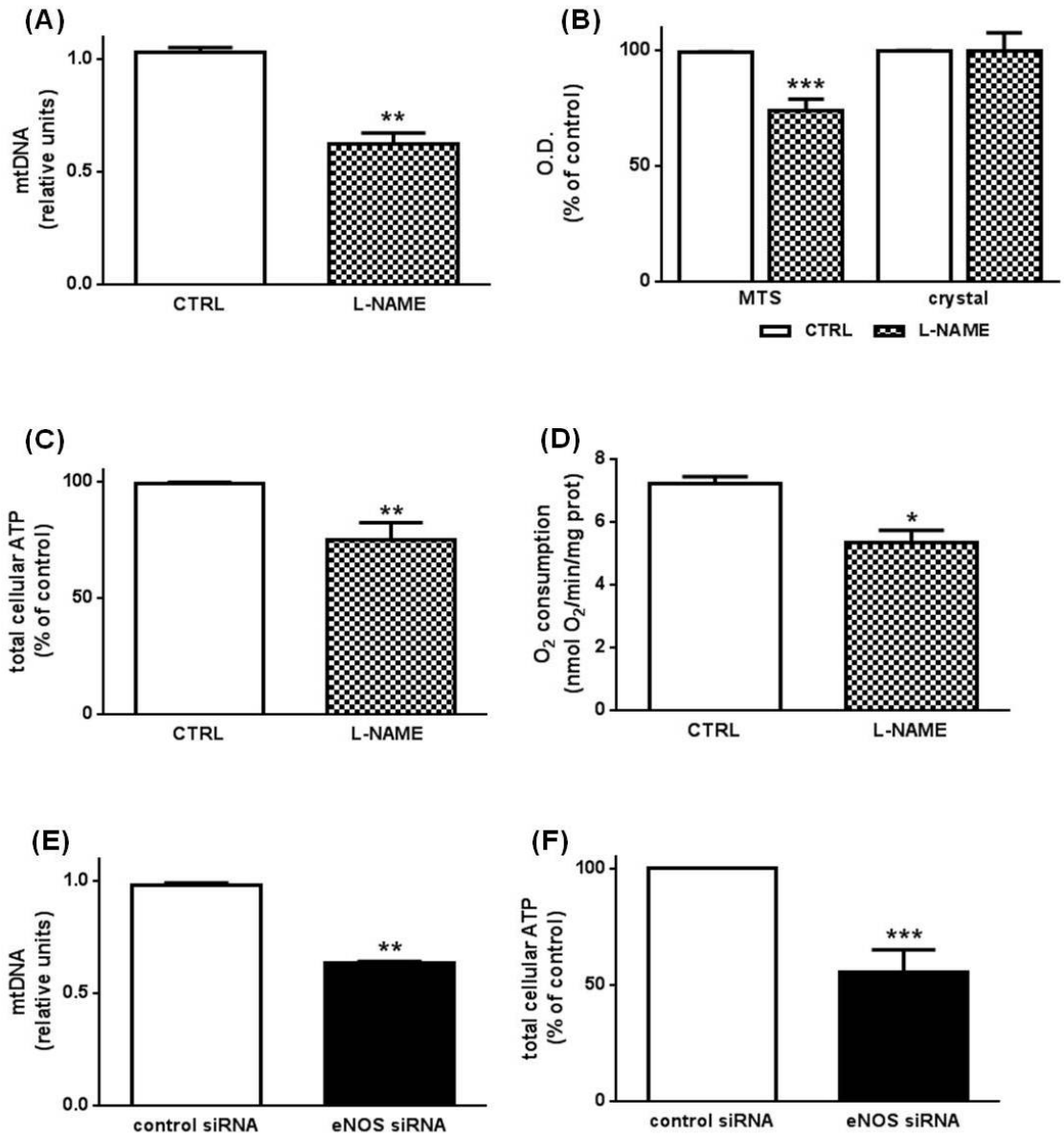



**Figure 14. Effect of chronic NO deprivation on HUVEC mitochondrial mass and function. (A)** Mitochondrial DNA (mtDNA) was quantified by RT-qPCR from control cells (CTRL) or from cells treated with L-NAME for 48 h, and normalized to the level of the housekeeping gene 18S. **p<0.01; *t* test; n = 3. **(B)** Mitochondrial activity of control and L-NAME treated cells was evaluated by means of MTS. In parallel samples, the total cell number was measured by crystal violet staining. ***p<0.001 *vs* control cells (CTRL, set at 100%); *t* test; n = 7. **(C)** Total cellular ATP levels were reduced by 25±7% after 48 h of 5 mM L-NAME treatment. **p<0.01 vs control cells (CTRL, set at 100%); *t* test; n = 8. **(D)** After L-NAME treatment, oxygen consumption was reduced by 25±6% in comparison to control cells. The values were normalized to the cell protein content. *p<0.05; *t* test, n = 3. **(E)** MtDNA was measured in HUVECs transfected for 48 h with control or eNOS siRNA. In silenced cells, mtDNA was reduced by 36±0.4%. **p<0.01; *t* test; n = 3. **(F)** Total cellular ATP content of HUVECs transfected for 48 h with control or eNOS siRNA was reduced by 45±9.7% in silenced cells. ***p<0.001; *t* test; n = 3.

We further investigated whether the reduction in mitochondrial activity could be dependent on the acutely L-NAME-induced ROS formation that we previously observed in HUVECs (Figure 10A). The treatment with NAC (5 mM) did not affect the reduction of ATP levels in chronically NO-deprived cells (Figure 15A), suggesting a lack of correlation between acute ROS generation and decreased ATP production. It was impossible to perform the MTS assay because of the interference of antioxidants with the mitochondrial succinate dehydrogenase activity on which the assay is based (Bruggisser, von Daeniken, Jundt, Schaffner, & Tullberg-Reinert, 2002).

Having excluded ROS requirement for the establishment of mitochondrial dysfunction resulting from NO deprivation, we studied the possible involvement of HIF-1α by evaluating MTS and ATP levels in HUVECs transfected with ΔARNT (the dominant negative form of HIF-1β subunit). The results shown in Figures 15B and C demonstrated that the decrease in mitochondrial activity and ATP levels were both maintained in ΔARNT transfected cells, suggesting that the effects of NO-deprivation on mitochondria does not depend on L-NAME induced HIF-1α transcriptional activity.

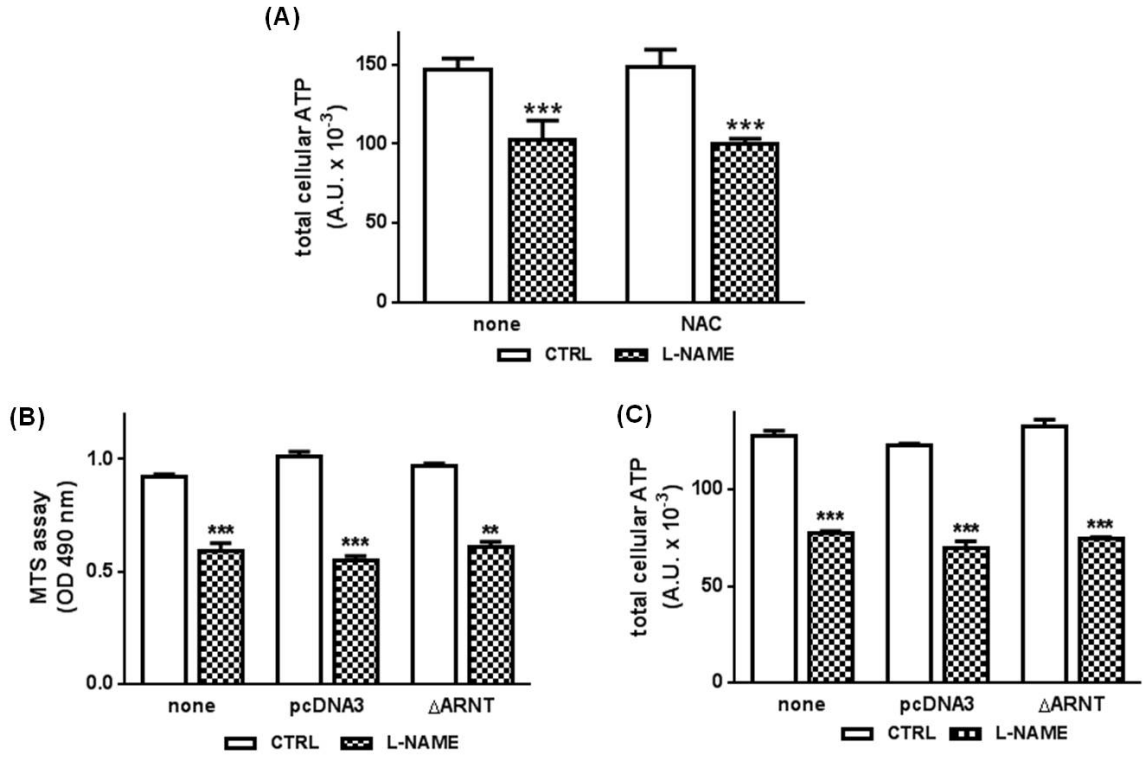



**Figure 15. L-NAME-induced metabolic dysfunction is independent of early ROS generation and HIF-1α activity. (A)** Total ATP content was measured in HUVECs treated for 48 h with L-NAME in the absence or in the presence of NAC (5 mM), as indicated. ***p<0.001 *vs* the corresponding untreated cells (CTRL); *t* test; n = 4. Mitochondrial activity evaluated by the MTS assay **(B)** and total cellular ATP content **(C)** were measured in HUVECs transfected with the empty vector pcDNA3 or with the expression vector ΔARNT, and treated with L-NAME for the 48 h following transfection. None: untransfected cells. ***p<0.001, **p<0.01 *vs* the corresponding untreated cells (CTRL); *t* test; n = 4.

**Chronic NO deprivation did not induce autophagy in HUVECs**

It is well known that oxidative stress and hypoxia are key regulators of mitophagy *i.e.* the mechanism responsible for the elimination of damaged organelles to maintain mitochondrial quality. Therefore, we tested the extent of autophagy in our cellular model characterized by NO deficiency, pseudo-hypoxia, ROS formation, and dysfunctional mitochondria. To this purpose, we evaluated the levels of LC3 (Light Chain 3)-II and p62, two proteins widely used as markers of autophagy. We did not observed neither a decrease in LC3-II nor a reduction in p62 levels in chronically L-NAME treated cells (Figures 16A and B), even in the presence of concanamycin, a perforin inhibitor capable of stabilizing autophagolysosomes and slowing down their degradation (data not shown). The same results was obtained in eNOS silenced cells (Figures 16C and D), thus confirming the absence of autophagy in HUVECs chronically deprived of NO. The ability of HUVECs to undergo autophagy was confirmed by using a positive control represented by amino-acid deprived cells where we observed a significant increase in both LC3-II protein levels and LC3-II/LC3-I ratio (Figure 16E and F), as expected.

We can therefore conclude that chronic deprivation of NO induced by pharmacological or genetic inhibition of eNOS does not activate autophagic processes in HUVECs.

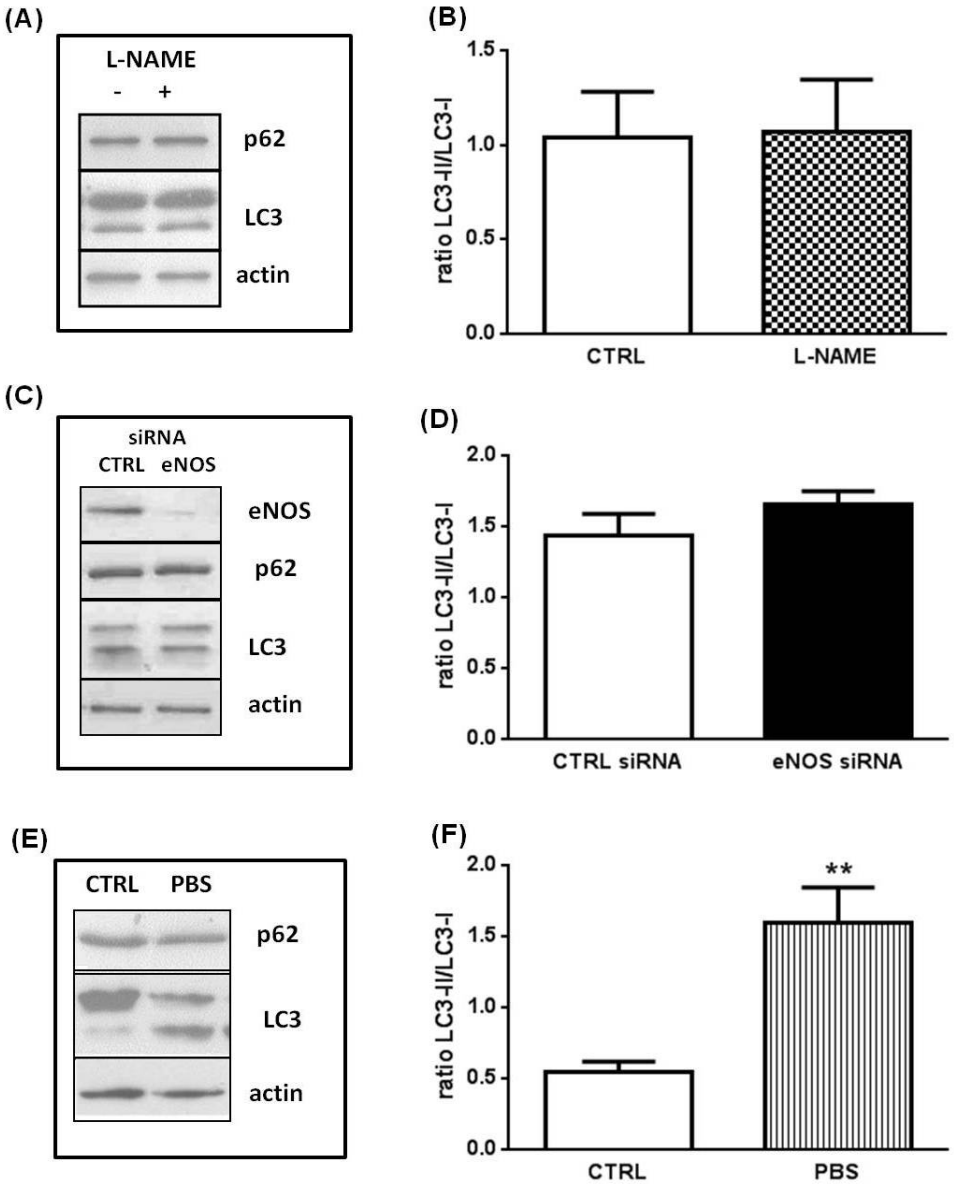



**Figure 16. L-NAME treatment does not induce autophagy in HUVECs.** Total cell lysates obtained from HUVECs treated for 48 h with L-NAME **(A)** or from HUVECs silenced for eNOS **(C)** were analyzed by western blot with anti-LC3 and anti-p62 antibodies. In **(E)**, HUVECs were starved for 2 h in PBS alone (lane 2). Lane 1: control cells (CTRL). β-Actin was used as a loading control. The LC3-I (18 kD) and LC3-II (16 kD) bands were quantified, and autophagy was reported as a variation in the ratio of LC3-II/LC3-I for each condition. No significant differences were detected between control (CTRL, open bar) and L-NAME treated cells (checked bar) **(B)** and between HUVECs transfected with control (CTRL, open bar) or eNOS siRNA (solid bar) **(D);** *t* test, n = 3. In **(F)**, the LC3-II/LC3-I ratio was significantly increased in PBS-treated HUVECs (vertical bar) in comparison to control cells (CTRL, open bar) **p<0.01; *t* test, n = 3.

# Discussion

Nitric oxide (NO) is a central regulator of cardiovascular physiology. As a matter of fact, NO crucially contributes to vessel homeostasis and blood pressure control by inhibiting vascular smooth muscle contraction, platelet aggregation, and leukocyte adhesion to the endothelium. The main source of NO are endothelial cells (ECs) that constitutively express eNOS, the enzyme responsible for NO synthesis from L-arginine. Lack of NO is associated with endothelial dysfunction (ED), a pre-pathological condition coupled with almost all risk factors for atherosclerosis and more in general for cardiovascular diseases (Pober, Min, & Bradley, 2009). To better characterize the molecular mechanisms underlying its development, we set up an *in vitro* model of ED using human ECs chronically deprived of NO, thus mimicking ED environment. Chronic deficiency of NO was reached through both a pharmacological and genetic approach by using the eNOS inhibitor L-NAME or by silencing the enzyme, respectively. We demonstrated that chronic L-NAME treatment did not induce neither apoptosis nor necrosis in our *in vitro* model, thus permitting to attribute the obtained results to NO deprivation, and not to a drug-induced toxicity.

A remarkable finding was the observation of a significant increase in the migratory capability of chronically NO-deprived HUVECs (Cattaneo et al., 2011). The enhancement in motility was present both in basal conditions *i.e.* in the absence of chemotactic stimuli, and in the presence of VEGF. It is well-known that VEGF is the main growth factor acting on ECs, thus stimulating their proliferation, migration, and finally angiogenesis (Morales-Ruiz et al., 2000; Papapetropoulos et al., 1997). The effects of VEGF are mediated through the binding of the growth factor to its specific tyrosine kinase receptor KDR, and induces the activation of multiple intracellular signaling pathways. Among these pathways, the PI3-K/AKT/eNOS pathway is crucially involved in the control of EC motility (Morales-Ruiz et al., 2000). When we analyzed the levels of VEGF and KDR in NO deprived HUVECs, we found a significant increase in their expression, both at mRNA and protein level. Furthermore, L-NAME treated cells showed high levels of basal AKT and eNOS phosphorylation that were not further enhanced by VEGF stimulation. Collectively,

the increase in VEGF and KDR expressions and the concurrent finding of a "constitutive" activation of the AKT/eNOS axis suggest the presence of an activated VEGF/KDR system in chronically NO-deprived HUVECs. The enhancement in the VEGF/KDR axis might be therefore responsible for the establishment of an autocrine loop between the growth factor and its receptor, leading to an improved cell motility.

Soluble guanylyl cyclase (sGC) is the main downstream effectors of NO and mediates a wide range of physiological effects through elevation of intracellular cGMP levels (Dudzinski et al., 2006). Chronic treatment with L-NAME and the subsequent loss of NO is associated with a significant decrease in cGMP accumulation that however was not involved in the pro-migratory effect induced by the drug. Cell motility was indeed unaffected when cGMP content was reduced by means of the GC inhibitor ODQ, thus excluding that the reduced activity of the pathway NO/cGMP is involved in the late effect of NO deprivation on cell migration.

An increased VEGF expression and cell motility are typically present in hypoxic cancer cells, due to the activation of the transcription factor HIF-1, that plays a central role in the transcriptional activation of genes encoding angiogenic factors (Rey & Semenza, 2010; Semenza, 2010). Similarly, induction of VEGF expression has been described in ECs during hypoxia (Namiki et al., 1995). In chronically NO deprived HUVECs, we observed a significant nuclear accumulation of HIF-1α, due to the stabilization of the protein and not to an increased transcription of its encoding gene. This observation is quite interesting and suggests that chronic NO-deprivation causes the instauration of a pseudo-hypoxic state, *i.e.* a nuclear accumulation of HIF-1α in normoxic condition, which might be responsible for an adaptation of gene expression leading to an up-regulation of the members of the VEGF/KDR system. The direct correlation between HIF-1 transcriptional activity and VEGF expression was demonstrated by the experiments performed on cells transfected with ΔARNT (a dominant negative form of HIF-1β subunit that blocks the transcriptional activity of the factor) where the L-NAME-induced increase

in VEGF expression was totally blunted. Importantly, the transfection with ΔARNT also reverted the increase in cell motility induced by long-term L-NAME treatment. These results confirm a direct correlation among HIF-1 transcriptional activity, VEGF production and increased migratory properties in NO-deprived cells. The dependence on HIF-1 transcriptional activity of both VEGF and migration enhancement further confirms the establishment of a pro-migratory autocrine loop involving the VEGF/KDR axis. However, some late effects observed in NO deprived HUVECs are not dependent on HIF-1 activity such as for example the reduction in eNOS protein expression and the metabolic effects of NO deprivation (that will be discussed later). All these results suggest that the behavior observed in ECs chronically devoid of NO is the result of a complex network of interactions involving not only HIF-1 but also other different and still unknown pathways triggered by NO loss.

Importantly, to elucidate whether HIF-1α nuclear accumulation was directly correlated to NO deficiency, we used the NO-donor DETA-NO to restore the physiological levels of NO in L-NAME treated cells. The treatment with DETA-NO reverted the effects on HIF-1α accumulation and the increase in VEGF expression and migration, as expected. Moreover, the genetic block of eNOS induced HIF-1α accumulation and VEGF protein expression as observed in L-NAME treated cells. We can therefore conclude that the effects described in chronically NO-deprived HUVECs are strictly due to NO deficiency, and not to possible side effects of L-NAME treatment.

The complex relationships between NO and HIF-1α are subject of many recent studies (Berchner-Pfannschmidt, Tug, Kirsch, & Fandrey, 2010). In particular, NO may play a dual role in regulating HIF-1α function. In fact, it has been observed a significant nuclear accumulation of HIF-1α in cells cultured in normoxic condition and in presence of a high concentrations of NO, being the latter induced by NO donors or by the expression of the inducible form of NOS (iNOS). On the contrary, under hypoxic conditions, *i.e.* when HIF-1a levels are already high, low physiological concentrations of NO seem to have the

opposite effect (Kimura et al., 2000; Mateo, García-Lecea, Cadenas, Hernández, & Moncada, 2003; Palmer, Gaston, & Johns, 2000; Sandau, Zhou, Kietzmann, & Brüne, 2001). The effects of the gas under the latter conditions are similar to those observed here. Further underscoring the similarity, the involvement of sGC and cGMP in the low NO-induced reduction of HIF-1a was, like in our migration experiments, excluded (Sandau et al., 2001; Takabuchi et al., 2004). However, differently from the previously reported results, in our experiments ECs were not subjected to hypoxic conditions. On the basis of these observation, we might suppose that: *(a)* in ECs an higher concentration of $O_2$ is necessary to prevent HIF-1α stabilization in comparison to other cell types; *(b)* basal NO production prevents the stabilization of HIF-1α which would otherwise occur under normoxic conditions. In this way, NO might act as a brake on migration, thus preventing inappropriate angiogenic response.

Searching for the molecular mechanism(s) connecting the lack of NO to the stabilization of HIF-1α, we focused our attention on Reactive Oxygen Species (ROS). ROS have been indeed proposed as one of the putative mechanism responsible for HIF-1α stabilization in normoxia through their ability to directly inactivates PHDs. Moreover, ROS are crucially involved in the pathogenesis and progression of ED as well as of other cardiovascular diseases. As a matter of fact, we observed a transient peak of ROS in HUVECs acutely treated with L-NAME, that was probably due to the eNOS uncoupling induced by the drug. This early increase in ROS levels is however only partially involved in HIF-1α nuclear accumulation and it is not required for the increased cell motility as demonstrated by the results obtained in the presence of NAC, an antioxidant that blocks ROS formation leaving unaffected cell motility. The involvement of acute effects of L-NAME treatment on migration was also excluded by the results observed in cells treated with the drug for 1 hour only (the duration of the ROS peak) that did not show any significant increase in migratory capacity demonstrating that the drug was continuously required during the 48 h of treatment to exert its promoting effect on motility. These results suggest that the stabilization of HIF-1α depends only in part by acute ROS

generation and that other mechanisms depending on still unknown pathways triggered by NO loss participate to its activation. It should be possible to hypothesize the involvement of co-factors such as iron, ascorbate, and the Krebs cycle intermediate 2-oxoglutarate (Pan et al., 2007), all required for the enzymatic activity of PHDs. Deficiency of NO might influence their intracellular availability, thus inhibiting PHD and causing HIF-1α accumulation. Obviously, we cannot exclude that other mechanisms, not directly related to PHD, might be involved in HIF-1α stabilization in normoxia during NO deficiency (Bilton & Booker, 2003). Importantly, the residual ROS-independent stabilization of HIF-1α is however enough to induce HIF-1α-dependent effects such as cell migration.

Interestingly, at the end-point of our experiments *i.e.* after 48 h of L-NAME treatment, we observed a significant decrease in cellular ROS content accompanied by an increase in both expression and activity of SOD-2 (Superoxide Dismutase-2), the main enzyme involved in redox homeostasis at the vascular level. These results suggest the establishment of an adaptive antioxidant status in NO-depleted HUVECs with the aim of protect cells from the concurrent presence of multiple risk factors *i.e.* NO loss and ROS generation. Several networks may lead to a cellular adaptive response to oxidative stress. One of the most important is the pathway dependent on serine/threonine AMP-activated protein kinase (AMPK) (Fisslthaler & Fleming, 2009). Activation of AMPK by phosphorylation of Thr172 stimulates the transcriptional activity of FOXO3, which on its turn leads to an increased expression of various antioxidant enzymes, including SOD-2 and catalase (Greer et al., 2007; Kops et al., 2002). In our *in vitro* model, we did not found however any significant increase in AMPK phosphorylation after both pharmacologically and genetically induced chronic NO deprivation (data not shown). We therefore focused our attention on the transcription factor NF-E2-related factor-2 (Nrf2) that through its binding to Antioxidant Responsive Elements (AREs) located within the regulatory region of target genes activates the transcription of antioxidant enzymes such as SOD-2 and catalase (Dong, Sulik, & Chen, 2008; Kaspar et al., 2009). As a matter of

fact, we found a significant Nrf2 accumulation in the nucleus of L-NAME-treated HUVECs that was totally prevented by the pre-treatment with NAC. We should therefore hypothesize a putative involvement of Nrf2 in the transcriptional activation of SOD-2 and catalase genes and in the maintenance of the redox homeostasis in chronically NO-deprived HUVECs. Importantly, this adaptive response seems to be dependent on the early burst in ROS formation acutely induced by L-NAME treatment.

The involvement of Nrf2 in the redox defense of ECs has been suggested by various recent papers. In particular, the protective role of Nrf2 has been proposed in different chemical, metabolic and physical stress conditions such as exposure to $H_2O_2$ and high glucose, glutathione depletion, and fluid shear stress (Chen et al., 2006; Heiss, Schachner, Werner, & Dirsch, 2009; Speciale et al., 2011). Further experiments in Nrf2 silenced HUVECs will be necessary to confirm the relationship between Nrf2 accumulation and the increase in SOD-2 and catalase expression observed in our *in vitro* model of ED. Moreover, it should be interesting to evaluate a possible correlation between HIF-1α and Nrf2 nuclear accumulation. The two transcription factors indeed share similar mechanisms of degradation dependent on PHD and Keap1 that address HIF-1α and Nrf2, respectively, to proteosomal degradation. We cannot therefore exclude that NO deprivation could affect the activity of the ubiquitin-proteosomal degradation system (UPS), thus favoring HIF-1α and Nrf2 nuclear accumulation (Chapple, Siow, & Mann, 2012; Dreger et al., 2010). A relationship among oxidative stress, mitochondrial dysfunction and UPS impairment has been for example described in neurodegenerative pathways leading to Parkinson's disease (Domingues et al., 2008). Moreover, a Nrf2-dependent up-regulation of antioxidant enzymes has been described in ECs treated with proteosomal inhibitors (Dreger et al., 2010).

Finally, we observed a consistent decrease in mitochondrial DNA and activity in chronically NO depleted HUVECs. The crucial role of NO in mitochondrial biogenesis and function has been demonstrated in different cell types and tissues (Nisoli et al., 2003;

Nisoli et al., 2004), and a decrease in mitochondrial mass have been previously described in the microvasculature of mice treated with L-NMMA for 1-2 months (Addabbo et al., 2009). To our knowledge, the effect of NO deprivation for shorter times on mitochondrial function in ECs had not yet investigated. Our work demonstrated that HUVECs already respond to NO deprivation after 48 h of treatment, making them a useful model for the investigation of the effects of the gas on mitochondrial biogenesis and function. Furthermore, it should be highlighted that various recently published papers focus on the crucial role of mitochondrial damage and dysfunction in the patho-physiology of cardiovascular diseases (Addabbo et al., 2009; Kluge, Fetterman, & Vita, 2013; Yu, Mercer, & Bennett, 2012). Therefore, the presence of a reduced mitochondrial mass and activity in our NO-depleted HUVECs further validate our model as a suitable model able to recapitulate *in vitro* most of the aspects that contribute *in vivo* to the establishment and maintenance of ED.

Mitochondria are also the main source of cellular ROS, which are synthesized as a by-product of the respiratory chain (Turrens, 2003). At the same time, mitochondria themselves are very sensitive to oxidative stress, since ROS are able to damage mitochondrial DNA and membranes and to induce oxidative modifications of mitochondrial lipids and proteins, finally altering cellular bioenergetics. Despite the existence of this strong relationship between ROS and mitochondria, our results seem to rule out a possible involvement of ROS in the establishment of mitochondrial dysfunction in chronically NO deprived HUVECs. Similarly, we demonstrated that mitochondrial dysfunction was also independent of HIF-1α transcriptional activity, being unaffected by transfection with ΔARNT. In addition, we considered the possibility that mitophagy *i.e.* a specialized form of autophagy that maintain mitochondrial quality by eliminating damaged organelles, might play a role in mitochondrial dysfunction. Mitophagy has been observed during hypoxia and oxidative stress, where it plays an important role as homeostatic mechanism (Ashrafi & Schwarz, 2013; Lee et al., 2012; Liu et al., 2012).

Furthermore, NO itself plays a complex role in the regulation of autophagy (Sarkar et al., 2011).

Despite the presence of NO deprivation, HIF-1α accumulation, transient ROS formation and mitochondrial dysfunction, we did not observe any modification in the levels of both LC3-II and p62 in cells deprived of NO. We cannot however exclude that a more extensive analysis of other and more specific markers for mitophagy and of mitochondrial shape and structure might reveal the execution of underestimated mitophagic processes in our cells.

In conclusion, our results show that NO deprivation induces complex modification in HUVECs' physiology and behavior, as summarized in Figure 17. Chronic NO deprivation induces a nuclear accumulation of HIF-1α, which is on its turn responsible for the increases in VEGF and KDR expression and cell motility. HIF-1α nuclear accumulation is only partially due to the acute peak of ROS production following the acute addition of L-NAME, while the increased cell migration is totally independent of early ROS generation suggesting that the residual ROS-independent HIF-1α stabilization might be sufficient to induce the increased migratory behavior. In addition, ROS formation was not required for the reduced eNOS expression and for the establishment of mitochondrial dysfunction. On the contrary, nuclear accumulation of Nrf2 depends on L-NAME-induced acute ROS formation and might account for the establishment of an antioxidant status in the attempt to neutralize any further cell damage induced by loss of NO.

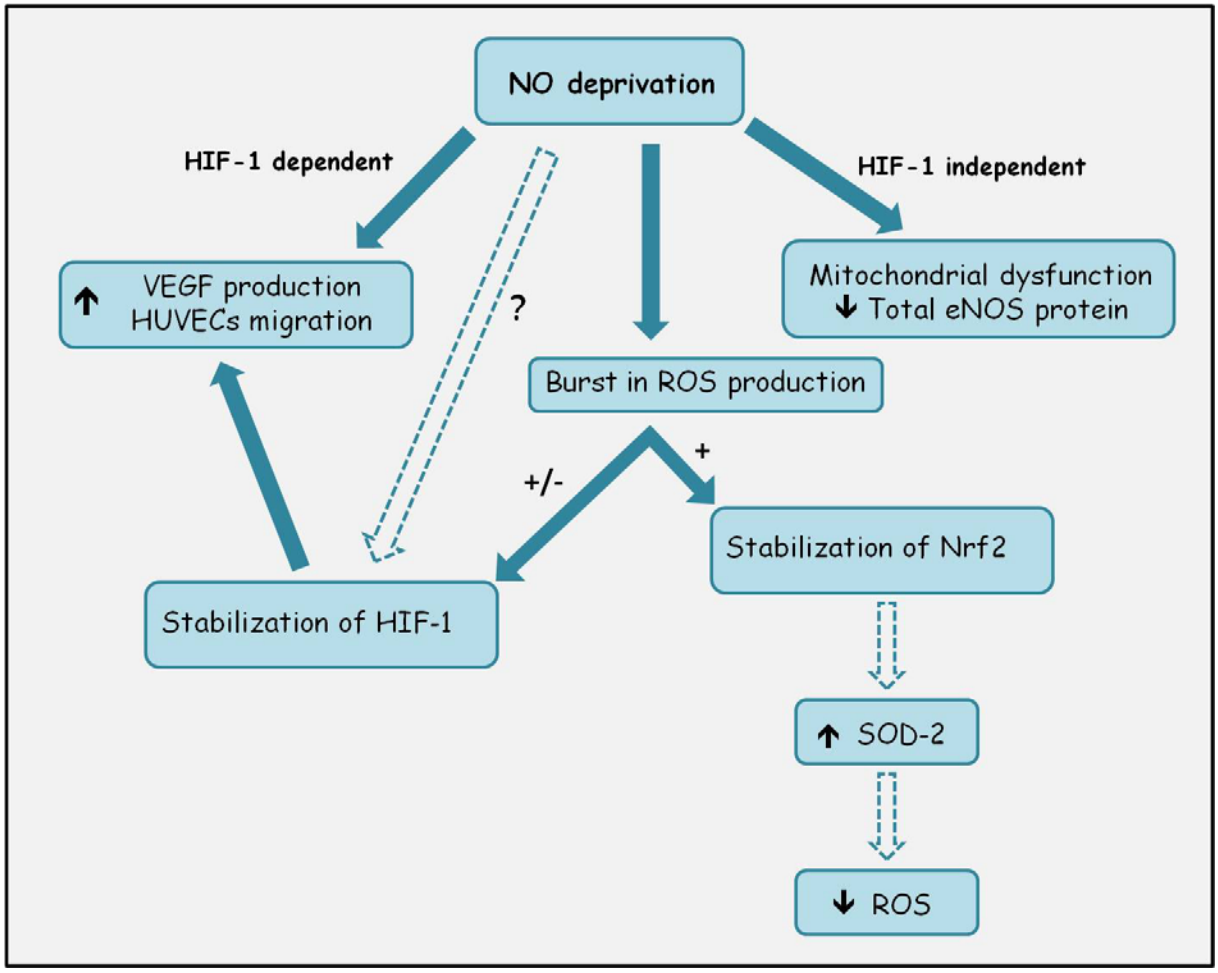



**Figure 17.** Summary of the events observed in NO-deprived HUVECs. The persistent pharmacological block of eNOS induces in HUVECs both HIF-1α- dependent and independent effects

Future experiments are still required to fully characterized our *in vitro* model of ED. Nevertheless, it represents an useful system to study the effects of NO deprivation on ECs and to reveal the complex molecular mechanisms underlying ED. Further studies will allow to identify new pharmacological targets and/or markers for the prevention or the treatment of ED, thus preventing its degeneration in most serious cardiovascular diseases such as atherosclerosis. To this purpose, the nuclear accumulation of HIF-1α might be considered an interesting pharmacological target. Indeed, it has been recently proposed a role for HIF-1α in cardiovascular diseases, based on the observation that HIF-1 and the consequent increase of VEGF expression might possess a defensive function in the acute response to cardiovascular injuries (Loor & Schumacker, 2008; Ockaili et al., 2005). On the contrary, a chronic stabilization of HIF-1 α might contribute to the establishment of the pro-inflammatory behavior accompanying ED development, as

shown in ApoE-/- mice, where an enforced expression of VEGF leads to a significant increase in plaque formation (Celletti et al., 2001).

Another remarkable result observed in our model is the nuclear stabilization of Nrf2. Nrf2 is now largely studied as an innovative therapeutic target both for its protective effect against oxidative stress (Dreger et al., 2009; Levonen et al., 2007) and for its ability to inhibit inflammatory responses via suppression of NF-kB pathway. In addition, Nrf2 might also prevent neointimal thickening by inhibiting proliferation and growth of smooth muscle cells (Hurttila et al., 2008) and up-regulate the expression and activity of heme-oxygenase 1 (HO-1) that mediates VEGF-induced tissue repair (Loboda et al., 2008). For these reasons, Nrf2 stimulation might be able to restore cellular redox homeostasis in cardiovascular diseases (stroke, atherosclerosis, but also diabetes) and in aging process, improving vitality and well-being of ECs. Currently, Nrf2-stimulating drugs such as Protadim (a mix of natural compound which could stimulate Nrf2 expression) are already employed, mainly to contrast aging. Despite promising results showing protective effects of Nrf2 in an *in vivo* model of ischemia/reperfusion (Calvert et al., 2009), there are however other studies describing expression-dependent properties of Nrf2 in a murine model of atherosclerotic plaque formation such as the $ApoE^{-/-}$ mice (Barajas et al., 2011; Sussan et al., 2008). These results show that in the double knock-out $ApoE^{-/-}/Nrf2^{-/-}$ mice the total absence of Nrf2 expression resulted in decreased atherosclerotic lesions, while the partial Nrf2 deficiency present in the $ApoE^{-/-}/Nrf2^{+/-}$mice did not influence atherogenesis. Very importantly, the reduced development of aortic plaques in $ApoE^{-/-}/Nrf2^{-/-}$ animals was significantly higher in male mice compared to female mice, thus showing a critical dependence on sex of the observed effects. Interestingly, it should be mentioned that preliminary results suggest that male and female HUVECs differently respond to chronic NO deprivation in our *in vitro* model of ED. These remarkable findings are beyond the aims of the present PhD project and will be subject of further investigations in the lab.

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