Review

Regulation of NOS expression in vascular diseases

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1. Abstract

Nitric oxide synthases (NOS) are the major sources of nitric oxide (NO), a small bioactive molecule involved in the regulation of many cellular processes. One of the most prominent functions of NO is regulation of vasodilatation and thereby control of blood pressure. Most important for vascular tone is NOS3. Endothelial NOS3-generated NO diffuses into the vascular smooth muscle cells, activates the soluble guanylate cyclase resulting in enhanced cGMP concentrations and smooth muscle cell relaxation. However, more and more evidence exist that also NOS1 and NOS2 contribute to vascular function. We summarize the current knowledge about the regulation of

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Table 1. Nomenclature of the different NOS isoforms.

Isoform	descriptive name	protein source	cDNA source
NOS1	bNOS (for brain NOS); cNOS (for constitutive or Ca^{2+}-regulated NOS); bcNOS (for brain constitutive NOS); nNOS (for neuronal NOS); ncNOS (for neuronal constitutive NOS)	rat cerebellum porcine cerebellum	human and rat brain
NOS2	iNOS (for inducible NOS); macNOS (for macrophage NOS); hepNOS (for hepatocyte NOS)	mouse RAW 264.7 macrophages, rat peritoneal macrophages, human DLD-1 adenocarcinoma cells	mouse macrophages, rat hepatocyte and liver, human A-172- and DLD-1 cells, hepatocytes and articular chondrocytes
NOS3	eNOS (for endothelial NOS); cNOS (for constitutive or Ca^{2+}-regulated NOS; overlap with nomenclature for NOS1); ecNOS (for endothelial constitutive NOS)	bovine lung endothelial cells	bovine and human endothelium

The descriptive names are used in the literature, the protein sources and the cDNA sources are described. Summary from [27].

NOS expression in the vasculature by transcriptional, post-transcriptional and post-translational mechanisms, in regard to inflammation and innate immune pathways.

2. Nitric oxide as bioactive molecule

Nitric oxide (NO), a small gas molecule, has been shown to act as bioactive substance. NO can be produced by a great number of organisms ranging from bacteria [1], yeast [2] and invertebrates [3] to mammals. NO as a simple gas molecule, controls important functions such as vascular tone, smooth muscle cell proliferation, platelet aggregation, leucocyte adhesion (see Fig. 1), and neurotransmission or the contraction of gastrointestinal organs. These broadly based regulation activities are performed mainly by the NO-dependent activation of soluble guanylyl cyclase [4]. Further, by activation or deactivation of transcription factors NO can affect gene transcription [5, 6] and mRNA translation (e.g., via iron-responsive elements) [7].

The NO radical reacts with multiple partners, *a*: the SH groups of cysteine in peptides or proteins, resulting in the formation of S-nitrosothiols. This modification is reversible and is important for the NO-related signaling functions in the immune system. *b*: superoxide anions ($O_2{}^-$) (a chemical reaction with one of the highest reaction speeds known) generating peroxynitrite ($ONOO^-$) able to modify proteins by tyrosine nitration. *c*: Fe^{2+}(in heme groups or iron-sulfur clusters) or Zn^{2+} (in zinc–sulfur clusters), important for the regulation of several enzymes and transcription factors. *d*: nucleic acids, resulting in deamination leading to mutations. *e*: to unsaturated lipids, producing nitrolipids [8].

At high concentrations NO is known to kill bacteria, parasites and certain tumor cells by inhibiting iron-containing enzymes [9], either by direct NO-DNA interactions [10, 11], or by post-translational modifications of proteins (for example S-nitrosothiol adduct formation [12] or ADP-ribosylation [13]). At these high NO concentrations (mostly formed by NOS2) reactive nitrogen species (RNS) are formed that harm cell membranes, the endoplasmic reticulum, mitochondria, nucleic acids and proteins/enzymes, which result in necrosis and cell death [14].

3. Nitric oxide synthases

In mammals, three isoforms of nitric oxide synthase (NOS) exist. The cDNA, protein structures and genomic DNA loci have been characterized in different species (see Table 1 [4, 15, 16]). NOS1, first discovered in *neurons* of rat and porcine cerebellum [17–19], and NOS3, originally described in *endothelial cells* [20], are Ca^{2+}-activated enzymes with relative low NO production whose physiological function is mainly signal transduction. NOS2, primarily detected in cytokine-induced macrophages [21, 22], is the high NO-producing isoform, able to produce toxic amounts of NO. Innate immune cells use this NO for their antimicrobial, antiparasitic and antineoplastic activities. NOS2 activity is mostly (human) or completely (mouse and rat) Ca^{2+}-independent. All NOS enzymes are homodimers that oxidize a guanidino nitrogen of L-arginine, using molecular oxygen and NADPH as co-substrates, to produce NO. Therefore, limiting the substrate arginine by other arginine utilizing enzymes, as by arginase I or II, or modulation of arginine transport [23, 24] is able to regulate the activity of all three isoforms. For example, in several cardiovascular diseases the consumption of arginine by arginase leads to the dysfunction of the NOS3 enzyme (NOS3 uncoupling) converting it to a superoxide-producing enzyme, resulting in NOS3-dependent superoxide production [25]. All NOS isoforms contain the prosthetic groups FAD, FMN and heme iron and depend on BH_4 as cofactor. Suboptimal concentrations of the essential cofactor BH_4 result in NOS3 uncoupling and superoxide production by the enzyme. NOS1 and NOS2 are mostly soluble enzymes. In

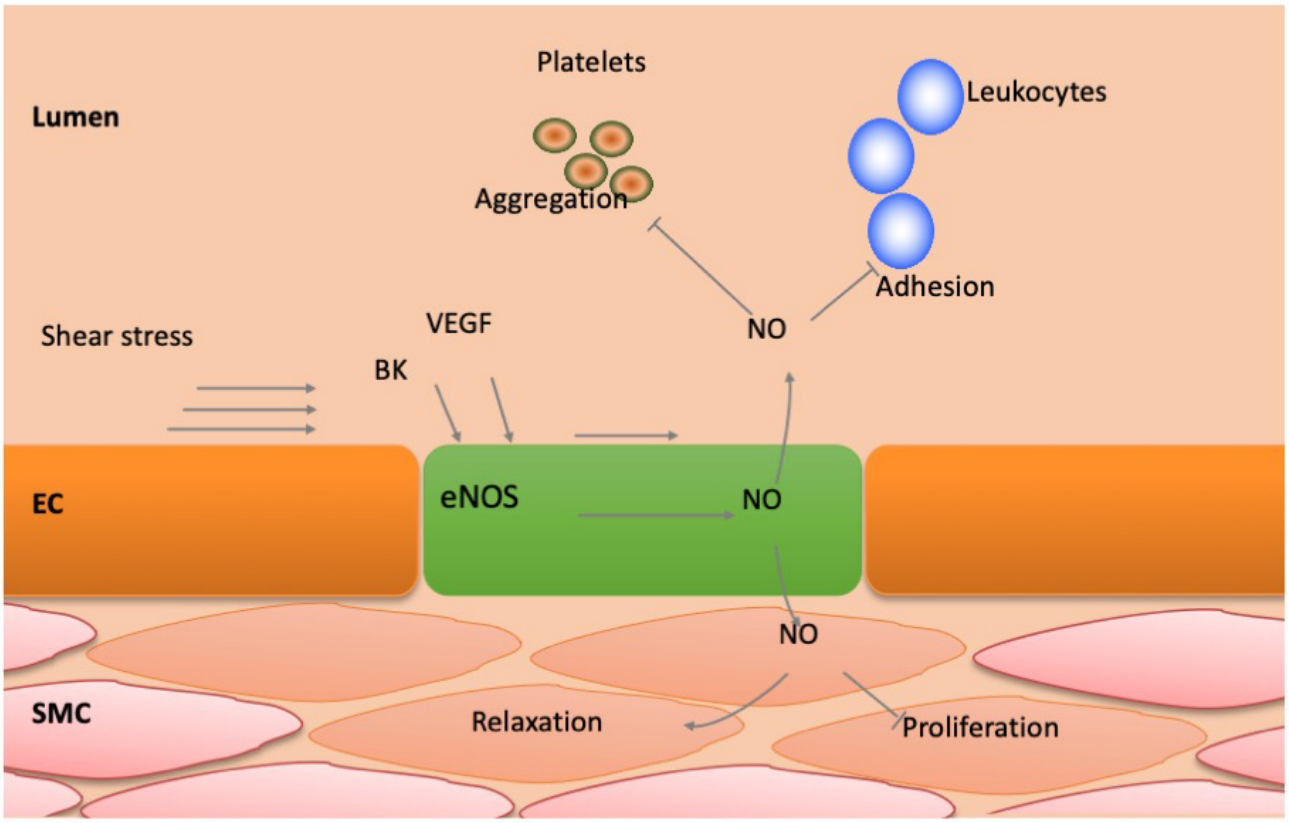

Fig. 1. Antihypertensive, antithrombotic, and antiatherosclerotic effects of endothelial NOS3. NOS3 enzyme activity in endothelial cells (EC) can be stimulated by shear stress or several agonists, like bradykinin (BK) and vascular endothelial growth factor (VEGF). The EC-synthesized NO diffuses into the blood stream and inhibits platelet aggregation and adhesion. In addition, the EC related NO inhibits leukocyte adhesion to the vascular endothelium and leukocyte migration into the vascular wall. NO also diffuses into smooth muscle cells (SMCs). In SMCs NO stimulates vasodilation and prevents SMC proliferation. Reprinted by permission from Springer Science + Business Media New York: Li H, Xia N, Förstermann U. Chapter 16-Nitric Oxide Synthesis in Vascular Physiology and Pathophysiology. In "Endothelial Signaling in Development and Disease" Eds. Schmidt MH, Liebner S. COPYRIGHT 2015.

contrast, NOS3 due to its amino-terminal fatty acylation by myristic and palmitic acid is membrane bound [4, 26].

4. NOS genes

In humans, three different genes located on chromosomes 12, 17 and 7, respectively, encode the NOS isoforms 1, 2 and 3. Deduced from the cloned cDNAs, the amino acid sequences of the three human isozymes show less than 59% identity. Across tested mammalian species, amino acid sequences are more than 90% conserved for NOS1 and 3, and greater 80% identical for NOS2.

5. NOS isoform expression

In contrast to the often-used descriptive names, researches have shown by immunohistochemical and western blotting methods that NOS1 and NOS3 are expressed in a large number of different cell types. NOS1 is expressed for example in skeletal myocytes, in endothelial-, smooth muscle-, or epithelial cells (see [16, 28, 29] for reviews) as well as unprimed macrophages [30]. NOS3 is expressed in different cell types like endothelial cells, epithelial cells, neuronal cells, T cells, erythrocytes, perivascular adipose tissue and platelets (see [16, 31] for reviews). NOS1 and 3 are believed to be constitutively expressed. However, also expression of NOS1 and 3 is regulated by external stimuli [28]. For example estrogens (for NOS1 and 3), shear stress, TGF-ß1, and in certain endothelial cells high glucose (for NOS3) enhanced the expression of these enzymes. The expressional regulation of the "constitutive" NOS (as well of NOS2) is mediated by different mechanisms. These include changes in chromatin packaging, mediated by histone methylation/acetylation, and/or effects of long non-coding RNAs (ncRNAs), activation/inhibition of transcription factors and usage of different promoters (modulation of transcription), regulation of mRNA-splicing, -localization and -stability (post-transcriptional regulation by RNA-binding proteins-RNA-BP, or micro-RNAs-miRNAs) and modulation of protein-stability.

5.1 Transcriptional regulation of the NOS1 gene

For the human and rodent NOS1 gene, tissue-specific or developmentally regulated NOS1 mRNA transcripts (at least 12 different human NOS1 mRNA isoforms) have been reported. These different NOS1 mRNAs are produced by alternative promoter usage, alternative splicing (see below), and/or the usage of alternate polyadenylation signals [32–38]. The different promoters display a multitude of potential transcription factor binding sites [37], but their functionality has been tested only for a small number. For example, the cAMP-depending enhancement of NOS1 expression (mRNAs containing the exons ex1f and g) has been shown to depend on a CRE site in the respective promoter sequence [37].

5.2 Transcriptional regulation of the NOS3 gene

To analyze the differential activity pattern of chromatin-versus episome-based human NOS3 promoter Chan *et al.* examined the methylation status of 5'-regulatory sequences of the human NOS3 gene. The authors observed huge differences in the DNA methylation of the NOS3 promoter sequence between endothelial and non-endothelial cell types, like vascular smooth muscle cells (VSMCs). The same cell type-specific methylation pattern was observed at the native murine NOS3 promoter *in vivo* in endothelial cells (EC) and VSMCs of the mouse aorta. Transient transfection analyses showed that that methylated NOS3 promoter sequences exhibited a marked decrease in the action of Sp1, Sp3, and Ets1 on NOS3 promoter activity, an effect enhanced by methyl-CpG-binding protein 2 (MECP2). In addition, ChIP analyses showed the binding of Sp1, Sp3, and Ets1 to the NOS3 promoter in ECs but not VSMCs. Finally, NOS3 mRNA expression could be induced in non-ECs by inhibition of DNA methyltransferase activity with 5-azacytidine [39]. As described by Miao *et al.* the LEENE lncRNA stimulates the binding of RNA polymerase II to the NOS3 promoter upregulating NOS3 nascent RNA synthesis [40]. The nuclear located lncRNA spliced-transcript endothelial-enriched lncRNA (STEEL) also enhances RNA polymerase II loading at the proximal promoter of the NOS3 gene and enhances NOS3 transcription [41]. The placenta-specific expression of a placenta NOS3 mRNA isoform is described to be related to usage of an alternative Herv-LTR10A-related promoter upstream of the classical promoter sequences [31, 42, 43] used in non-placental tissues. The placenta-restricted expression was also determined to be associated with placenta-specific hypomethylation of the LTR10A element [44]. Analysis of the human NOS3 promoter revealed the functional importance of binding sequences for several transcriptional factors like the AP-1-, AP-2-, Elf-1-, Erg-, Ets1-, GATA-, HIF-, KLF2-, MAZ-, MZF-, NF-1-, p53-like, PEA3-, Smad2-, Sp1-, Sp1/Sp3-like, YY1-like-binding site. Also, acute phase reactant-, sterol-, and shear stress-regulated elements have been described (see [31, 45] for reviews). Also, the LTR10A-derived NOS3 promoter element important for placenta-specific NOS3 expression, contains several putative transcription factor binding sites, for example C/EBPdelta, FOXO4, NF-Y, and Sox-5 [44], but the functionality of these sequences have not been proved yet.

5.3 Post-transcriptional regulation of the NOS1 expression

The different 5'-UTRs of the multiple NOS1-mRNA isoforms (see above) are likely to regulate the translatability of these different NOS1 mRNAs [16]. Several miRNAs have been shown to directly [46–51] or indirectly [52] modulate human NOS1 expression.

5.4 Post-transcriptional regulation of the NOS3 expression

Lorenz *et al.* detected three splice variants (NOS3-13A, NOS3-13B, and NOS3-13C) of the NOS3 mRNA in HUVEC with novel 3' splice sites within intron 13. All variants use the same polyadenylation site located at the end of the novel exon, and all these NOS3 mRNA isoforms code for inactive NOS3 proteins. These mRNA isoforms are expressed in endothelial cells and various human tissues. By formation of heterodimers, expression of the full-length NOS3 with NOS3-13A diminished NOS3 enzyme activity in COS-7 cells [53].

Beside promoter activity regulation, TNF-α reduces NOS3 expression in endothelial cells of different species by destabilization of its mRNA [54–59]. By RNA-protein interaction analyses different RNA-BPs (translation elongation factor 1-alpha 1 - eEF1A1 and polypyrimidine tract-binding protein 1 - PTB1) were found to bind to the 3'-UTR of the NOS3 mRNA [56–58]. In addition, TNF-α-dependent post-transcriptional regulation of NOS3 expression by different miRNAs binding to its mRNA has been described [59–61].

5.5 Post-translational regulation of the NOS1 protein

Several post-translational modifications, such as phosphorylation, ubiquitination, and sumoylation, of the NOS1 protein have been described [62]. NOS1 localization, enzymatic activity and protein stability is also regulated by protein-protein interactions with calmodulin (CaM), heat shock proteins (hsp90/hsp70), PDZ-domain containing proteins (syntrophin, PSD-95, or PSD-93), the Carboxy-Terminal Postsynaptic Density-95/Discs Large/Zona Occludens-1 Ligand of NOS1 (CAPON) (also named Nitric Oxide Synthase 1 Adaptor Protein - NOS1AP) [62] and PIN, a protein inhibitor of NOS1 acting by dissociation of NOS1 dimers into monomers [63, 64].

5.6 Post-translational regulation of the NOS3 protein

Post-translational modification of NOS3 has been shown to include acetylation (decreasing its activity), acylation (membrane targeting), glutathionylation (uncoupling, resulting in superoxide production), phosphorylation (reg-

ulation of enzyme activity) or S-nitrosylation (reducing its activity) [26]. Especially phosphorylation of different amino acids (Y81, S615, S633, S1177 activating; S114, T495, Y657 deactivating) by multiple kinases (Akt, AMPK, CaM-K-II, PKA, PKC, PKG, pp60src, PYK) modulates NOS3 activity by different signaling pathways [65].

Beside post-translational modifications there are numerous reports demonstrating the importance of proteins interacting with NOS3 and thereby stimulating or inhibiting NOS3 function. In addition to CaM, several proteins like caveolin-1, cell division cycle 37 (Cdc37), C-terminal hsp70-interacting protein (CHIP), connexin 37 and 40 (Cx37/40), G-protein-coupled receptor (GPCR) kinase interactor-1 (GIT1), hemoglobin alpha (Hbα), heat shock protein 90 (Hsp90), integrin-linked kinase (ILK), NOS3 interacting protein (NOSIP) and NOS3 traffic inducer (NOSTRIN), proviral integration site for Moloney murine leukemia virus 1 (Pim1), prolyl isomerase (Pin) 1, and stromal cell-derived factor 2 (SDF2), have been shown to interact and regulate NOS3 (see [66] for a recent review). In addition, the plasminogen activator inhibitor-1 (PAI-1) interacts with NOS3 and inhibits its activity [67].

Cytokine-dependent regulation of NOS1 and NOS3 by microbial products have been reported also. The differentiation and activity of immune cells *in vitro* is affected by NOS1 or 3. In addition, modulation of immune responses and inflammatory processes *in vivo* have been described [8].

5.7 Regulation of NOS2 expression

A "constitutive" expression of NOS2 has been described for epithelial cells of the colon and lungs, which is likely "induced" by the microbiota in these organs, and spinal tissue of the brain and for different human cancer cells (see [28] for a review).

NOS2 is mainly regulated at the expressional level (Fig. 2). LPS, cytokines, and several other compounds (mostly secreted by the innate immune system) are able to induce NOS2 synthesis in many cell types (see [68] for a review). Pathways involved in the NOS2 promoter activation seem to vary in different cells. However, activation of the transcription factors NF-κB and STAT-1α are believed as essential steps for NOS2 transcription in most cells. Beside transcriptional control NOS2 expression is intensively controlled by post-transcriptional regulation of NOS2 mRNA stability [68].

5.8 Transcriptional regulation of the NOS2 gene

Buzzo *et al.* demonstrated that NOS2 expression in murine peritoneal macrophages, induced by purified flagellin from Bacillus subtillis, involves caspase-1 mediated cleavage of the chromatin regulator Poly [ADP-ribose] polymerase 1 (PARP1) to enhance the chromatin accessibility of the NF-κB binding sites located in the NOS2 promoter [69] (Fig. 2). In sharp contrast to murine macrophages, LPS- and IFN-γ-treated human alveolar macrophages express no NOS2 mRNA or protein. This unresponsiveness is related to epigenetic gene silencing (chromatin compaction, CpG methylation and histone modifications) [70].

In macrophages from Leishmania amazonensis patients binding of the inhibitory NF-κB p50/50 monomer leads to a recruitment of histone deacetylase 1 (HDAC1) to the human NOS2 promoter, preventing histone acetyltransferase (CBP/p300) binding to the NOS2 promoter and further acetylation of H3K9 [71] (Fig. 2).

LPS/cytokine induced NOS2 expression in the murine system depends on a promoter sequence with around 1000 bp [72, 73]. In sharp contrast, the 1000 bp human NOS2 promoter displays only basal activity not induced by cytokine stimulation [74–76]. Only if much longer DNA promoter fragments (up to 16 kb) are used in transfection experiments (transient or stable) with human A549, AKN or DLD-1 cells, a clear promoter induction (8-10-fold) was detected (see [68] for a review). Analyzation of the 16 kb human NOS2 promoter sequence with bioinformatic tools revealed a multitude of putative transcription factor binding sites. However, only a few of these binding sites have been shown to be functional important. The human 16 kb promoter contains a TATA-box and binding sites for AP-1, CAR, C/EBPβ, EGFR-STAT3, FKHRL1, HIF-1α, HMGA1, KLF6, NF-κB, NRF, Oct-1, RAR/RXR, PXR, STAT-1α, Tcf-4, TCF11/MafG, and YY1 (all proven to be functional at least in transfection experiments) [68, 77].

5.9 Post-transcriptional regulation of NOS2 expression

We and others have shown that the post-transcriptional regulation of the mammalian (especially human) NOS2 expression is quite complex (Fig. 2). Translational efficacy and non-sense mediated mRNA decay [78, 79] of the human NOS2 mRNA is regulated by a short μORF located in exon 1 of the human NOS2 gene [80]. Several RNA-BP have been shown to bind to the 3'-UTR of the human NOS2 mRNA and regulate mRNA-stability [81–88]. In addition, different miRNAs (miR-26a, miR-146a miR-939) directly bind to the human NOS2 mRNA and regulate its translatability and stability [8, 59, 89]. In murine and rat cells natural antisense RNAs (NATs) are transcribed from the 3'-UTR of the NOS2 gene, which stabilize NOS2 mRNA by interacting with the mRNA 3'-UTR [90]. No such NATs were detected in the human system.

5.10 Post-translational regulation of NOS2 expression and activity

Post-translational modification of NOS2 seem to be important for NOS2 activity and intracellular localization (Fig. 2). Palmitoylation of NOS2 at the amino acid Cys-3 is essential for NO synthesis and intracellular localization [91]. In muscle of septic patients, tyrosine-nitration of NOS2 has been described, which re-

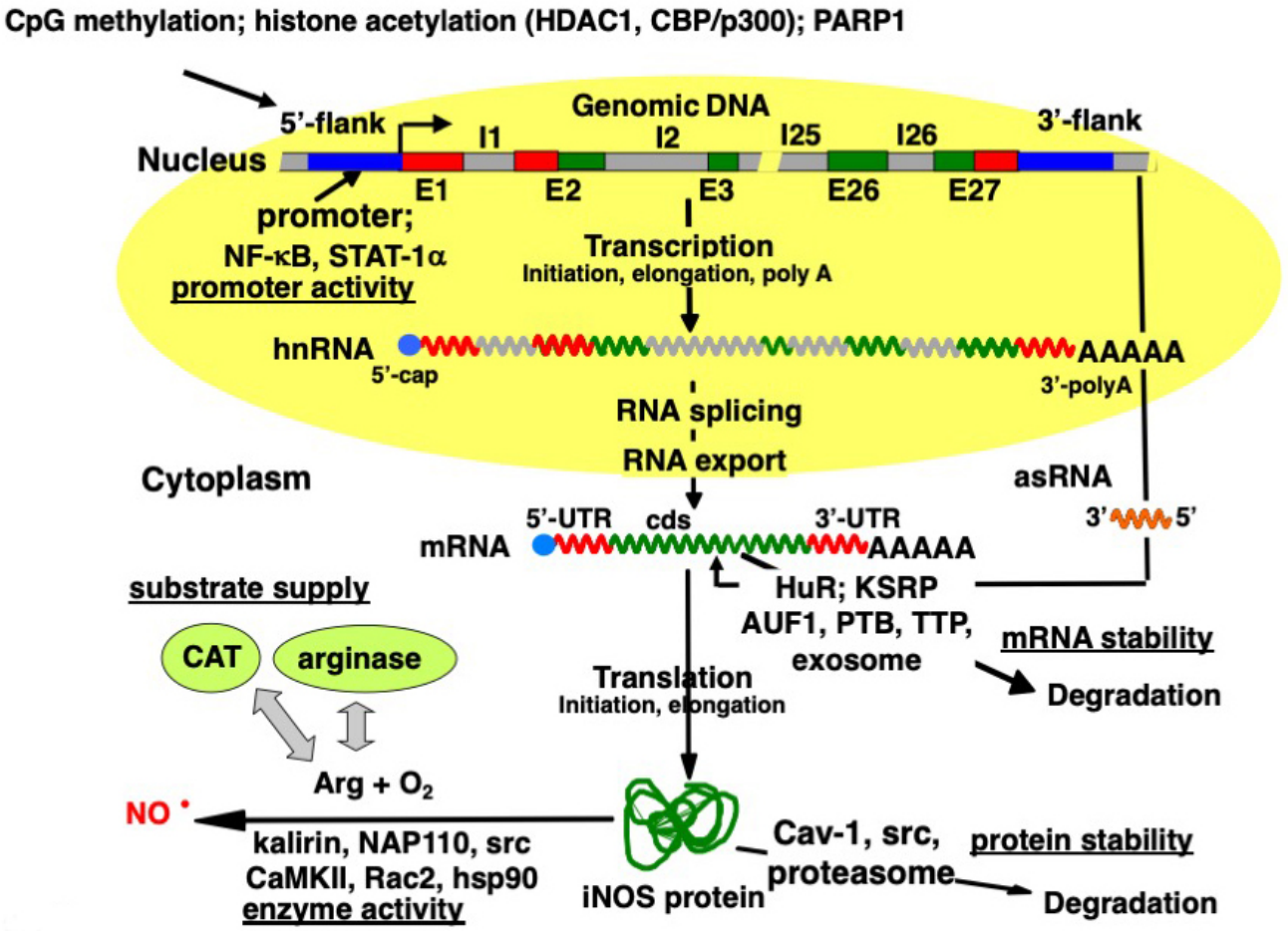

Fig. 2. Regulation of (human) NOS2 expression and NOS2-mediated NO production. NOS2 is mainly regulated by modulation of NOS2 expression. NOS2 promoter activity is regulated by modulation of the accessibility of the chromatin (CpG-Methylation, histone acetylation) and binding of transcription factors (NF-κB, STAT-1α). Also modulation of NOS2 mRNA stability is a major regulatory mechanism. RNA-BP (AUF1, HuR, KSRP, PTB, TTP) bind to the NOS2 mRNA and regulate stability by interaction with the exosome. In rodents, modulation of NOS2 mRNA stability by interaction with anti-NOS2-asRNA has been described. NOS2 protein stability is regulated by interacting proteins (Cav1, src) and the proteasome. Finally, enzyme activity of the NOS2 protein is modulated by interaction with several proteins (kalirin, NAP110, src, CaMKII, Rac2, hsp90). NOS2-mediated NO production depends on arginine (Arg) supply. Therefore, proteins that also use arginine as substrate (arginase) may regulate NOS2 activity by substrate competition. Membrane transporters important for arginine uptake into cells (cationic amino acid transporter, CAT) may have a role in the regulation of NOS2-dependent NO production.

duces enzymatic activity [92]. Also, for the NOS2 protein several protein-protein-interactions have been published that enhance or reduce the activity of the NOS2 enzyme (α-actinin-4, ezrin/radizin/moesin-binding phosphoprotein 50-EBP50, kinase suppressor of Ras-1-Ksr1-adaptor-or scaffold-proteins; Hsp90, Rac2-allosteric activators; kalirin-dimerization inhibitor). Other protein interactions lead to proteasomal degradation of the NOS2 protein (e.g., Rpn13/ARDM1/NAP110, UCH37) (see [8] for a review).

6. Structure of the healthy vessel wall

Normal blood vessels are made of the tunica intima, the tunica media and the adventitia surrounded by the perivascular adipose tissue (PVAT) [93].

The tunica intima is composed of an EC monolayer attached to a basement membrane filled with extracellular matrix. EC are exposed to shear stress resulting from the blood flow [94]. Laminar shear stress up-regulates in EC the expression of vasculoprotective transcription factors such as KLF2 and Nrf2, which orchestrated the anti-inflammatory and antioxidant EC phenotype. However, disturbed shear stress induces the pleiotropic transcription factor NF-κB, leading to a pro-inflammatory and proatherogenic EC phenotype. As natural barrier of the blood vessel ECs prevent toxic molecules from penetration into the arterial vessel wall and inhibit platelet- and leukocyte adhesion. ECs are able to regulate the vascular tone by secretion of vasoactive substances, such as endothelium-derived hyperpolarizing factor (EDHF), NO, and prostaglandin I_2 (PGI_2), which are vasodilators. EC-derived NO is also regarded as a

major anti-inflammatory factor in the vasculature [95]. On the other hand, EC are able to secrete endothelium-derived contracting factors (EDCFs) such as angiotensin II (Ang II), endothelin 1 (ET-1), thromboxane A_2 (TXA_2), and uridine adenosine tetra-phosphate (UP4A). Healthy EC promote the balance of pro- and anti-thrombotic mechanism by releasing anti- or pro-thrombotic substances and also regulate VSMC proliferation [96].

The tunica media contains a layer of smooth muscle cells (SMC), which secrete elastic and collagen fibers, and pericytes. Mature SMCs contain a unique set of contractile proteins (e.g., α-smooth muscle actin (αSMA) or smooth muscle myosin heavy chain (SM-MHC)), ion channels, and specific signaling molecules that are required for their contractile functions [97]. SMCs produce different components of the extracellular matrix (ECMs), namely cadherins, collagen, elastin, integrins, and proteoglycans that build up a major portion of the blood vessel mass [98].

The adventitia consists of fibroblasts, mesenchymal stem cells (MSCs), vasa vasorum, nerves and a small number of immune cells in connective tissue [99].

In addition, most vessels (e.g., aorta and coronary arteries) are embedded by perivascular adipose tissue (PVAT), which is an active endocrine tissue affecting the vasculature by secreting different mediators [100]. In addition, also cells of the immune system (like macrophages, T cells), fibroblasts and capillary EC are found in the PVAT [95, 101].

The above-described blood vessel structure is mainly preserved throughout the body. However, the vasculature in the different parts of the human body has unique functions depending on the needs of the different organs and tissues. For instance, the resistance vessels (arteries and arterioles), are in contact with shear stress resulting from the high pressure [102]. Towards the veins, the blood pressure and shear stress are stepwise reduced. Veins are exposed to a nearly 70-fold less pressure than arteries. As a result of this high pressure, arteries and arterioles possess a thick media layer with copious SMCs that provide elastic support. In sharp contrast, capillaries display only an intima layer covered with a basement membrane and are supported by pericytes.

7. NOS isoforms expressed in the healthy vasculature

The expression of the different isoforms of NOS1-3 has been published for nearly all cell types of the healthy vasculature.

NOS1 is expressed in vascular smooth muscle cells [103, 104] and vascular endothelium [105, 106]. This was shown by immunohistochemistry or western blot using isoform-specific antibodies. Research, often done in NOS3 deficient mice, showed a physiologically relevant role of NOS1 in modulating cardiac function [107], systemic arterial pressure [108], myogenic tone [109], and cerebral blood flow [110]. Also, inactivation of the NOS1 gene resulted in reduced acetylcholine-induced vasodilation [111] in the mouse aorta. There are clear data that NOS1-generated H_2O_2 [112] has an important impact on the regulation of the vascular tone.

By immunohistochemistry NOS2 protein expression has been described in normal aortas in the surrounding adventitia. NOS2 protein was detected also in neutrophils and monocytes enclosed in thrombi surrounding these vessels [113].

NOS3 expression in the vasculature has been shown for the EC (see [31] for a review) and the PVAT [114–116] by immunohistochemistry and western blot. Although NOS3 is mainly believed to be a constitutively expressed gene there are several reports showing induction of NOS3 expression. NOS3 expression has been described to be upregulated by fluid shear stress [117] and cyclic stretch [118] in cultured EC (see Fig. 1). This has been also observed in animals after exercise [119, 120].

In normal vessel NO synthesized by NOS3 is believed to be a major regulator of vascular tone and to be the most important anti-inflammatory mediator in the vessel (see Fig. 1).

By post-translational acylation NOS3 is localized to biological membranes such as the Golgi apparatus or plasmalemma caveolae. This subcellular localization permits optimal regulation by shear stress, calcium ions and kinases. Therefore, agonists enhancing intracellular calcium concentrations (e.g., bradykinin, histamine, VEGF), or modulating pathways leading to increased CaM binding or reduced CaM dissociation are able to activate NOS3-dependent NO release [121].

8. Innate immunity

In higher vertebrates the immune system is made up by two components: the non-specific innate immunity and the adaptive immunity, which is highly specific. As first level of reaction against anything foreign, the innate immune system have evolved conserved strategies to defend the body against a pathogen. These defense mechanisms comprise a magnitude of structures and mediators like the skin barrier, saliva, tears, various cytokines, complement proteins, lysozyme, bacterial flora, and numerous cells including neutrophils, basophils, eosinophils, monocytes, macrophages, reticuloendothelial system, natural killer cells (NK cells), epithelial cells, endothelial cells, red blood cells, and platelets.

The adaptive immune system (B- and T lymphocytes and their products) depends on antigen receptors, which are somatically generated and clonally selected. In contrast, the innate immune system senses pathogens by highly conserved, relatively invariant structural motifs. The “danger theory” published by Polly Matzinger in 1994

[122] described that the innate immune system responds to endogenous or exogenous "danger signals". Pathogen-associated molecular patterns (PAMPs) are exogenous danger signals and consist of highly conserved motifs in microbial organisms. Endogenous danger signals, also named danger-associated molecular patterns (DAMPs), are proteins, cytokines, chemokines, and other molecules from distressed and injured cells. PAMPs and DAMPs stimulate innate immune cells by binding to pattern recognition receptors (PRRs), which then activate signaling pathways (e.g. MAPK-pathways), which result in the activation of transcription factors, like AP1, CREB, c/EBP, IRFs, NF-κB, and STATs, or RNA-BPs, involved in the regulation of mRNA-stability and translatability like HuR, and modulate ncRNA expression (miRNAs and lncRNAs) to initiate a wide array of responses against cell damage [123–128]. Aberrant activation of innate immune signaling cascades can lead to a failure to regulate inflammatory events, resulting in considerable damage to host tissues and is involved in the pathophysiology of cardiometabolic diseases [129].

Innate immune cells (phagocytes) use NOS2-generated NO and NADPH Oxidase 2 (NOX2)-generated superoxide to kill invading microorganisms. A patient with genetic deficiency of NOS2 died by a fatal cytomegalovirus infection [130], demonstrating the importance of NOS2 for anti-viral innate immune processes. NOS2 expression in innate immune cells resulted in the modulation of cell-intrinsic capabilities and phenotypes, and regulatory effects on neighboring (immune) cells. For example, NOS2-generated NO modulates different important immune-relevant mechanisms like antigen presentation, cytokine production, expression of MHC class II and costimulatory molecules, phagocytosis, and survival as well as apoptosis of myeloid cells [8].

Beside classic innate immune cells (monocytes, macrophages, neutrophils, dendritic cells, and natural killer cells) other non-immune cells like cardiomyocytes, endothelial cells, and fibroblasts express these receptors and can actively contribute to immune response via PRR signaling [131, 132].

ECs can exert some innate immune functions that macrophages can also perform, for example cytokine secretion, phagocytic function, antigen presentation, pro-inflammatory immune-enhancing as well as anti-inflammatory and immunosuppressive actions. Therefore, Shao *et al.* have introduced ECs as multifunctional innate immune cells [133].

9. Vascular inflammation

Vascular inflammation can be induced by a multitude of stimuli. In microbial infections, the increased concentrations of pro-inflammatory cytokines and chemokines result in vascular inflammation. Also, alterations in blood flow and shear stress, hypoxia, metabolic dysregulation like increase of the low-density lipoprotein (LDL)-, fatty acid- or blood glucose-concentration as well as cardiovascular diseases like hypertension induce (and often result from) vascular inflammation [134–139]. As in infections also in cardiometabolic diseases the important involvement of several cytokines, chemokines and adipokines (including IL-6, IL-1β, TNF-α, MCP1, and leptin) in the pathophysiologic process has been described [140]. In vascular inflammation circulating leukocytes (monocytes/macrophages as well as neutrophils, cells of the innate immune system) are allured to the site of injury and transmigrate into the intima. Their task is to clear the tissue from the source of inflammation and dead cells and ultimately resolve the inflammation. However, if the inflammation cannot be stopped and develops into a chronic state, this leads to pathologic situations through the development of vascular diseases like atherosclerosis. In these processes, enhanced generation of reactive oxygen/nitrogen species (ROS/RNS) by innate immune cells is central to the pathological mechanisms [139]. Since blood vessels play an important role in the maintenance of homeostasis, the dysregulation of vascular function in inflammation is central to numerous disorders such as atherosclerosis [134] and related complications (ischemia, myocardial infarction, stroke, and thrombosis [141]), as well as age-related cognitive decline [142], cancer [143], and neurodegeneration [144].

10. The intima in atherosclerosis and vascular inflammation

Endothelial dysfunction (ED) is the most important step in the development of atherosclerosis. Cardiovascular risk factors, such as aging, diabetes mellitus, hyperlipidemia, hypertension, obesity, and smoking induce endothelial cell damage, resulting in ED [145]. In contrast to the healthy situation, dysfunctional EC accelerate the generation of ROS and potentiate vascular inflammation [146]. The defect of the endothelium causes a disturbance of the balance between vasoconstriction and vasodilation. The increased EDCFs (especially ET-1) and reduced EDRFs (mainly NO) initiate pathophysiologic changes that stimulate or fortify atherosclerosis, like increased vascular permeability to lipoproteins and enhanced leukocyte adhesion, platelet aggregation, and generation of cytokines [147]. In addition, the enhanced concentrations of pro-inflammatory cytokines, (TNF-α, IL-1β, and IL-6), result in the endothelial expression of adhesion molecules (VCAM, ICAM), as well as MCP-1 and other chemokines, transforming it to an "inflamed endothelium". This also leads to enhanced adherence and migration of monocytes [148–151]. After immigration into the intima the monocytes develop to tissue macrophages with enhanced expression of scavenger receptor (SR) and increased internalization of (ROS)-modified lipoproteins [152]. In the end these cells become foam cells (FCs), a highlight of an early atherosclerotic lesion

[153]. Atherosclerotic plaque rupture leads to an imbalance of thrombotic and anti-thrombotic substances. Here, EC-dysfunction leads to an increase of thrombotic substances (vWF, TXA2) and to reduced concentrations of antithrombotic substances. These effects result in thrombosis, causing devastating consequences [154].

11. The media in atherosclerosis and vascular inflammation

In the inflamed vessels SMCs have been shown to be crucially involved in the pathophysiological process of atherosclerosis [155]. In this process, SMCs migrate to the intima, proliferate, synthesize extracellular matrix (ECM) and deposit lipids. This facilitates arterial wall fibrosis and thickening and leads to luminal stenosis. Normally, SMC proliferation is inhibited by NO (and other factors) but, as described above, NO concentration decline in the inflamed vessel. Some of the ECMs released by SMCs contribute to stabilization of the fibrous cap of the atherosclerotic plaque and thereby help to protect against plaque rupture and thrombosis [156]. Several cytokines are produced by SMC (PDGF, TGF-β1, MIF, IFN-γ and MCP-1) which are involved in the inflammatory response to lipids [157].

12. The adventitia in atherosclerosis and vascular inflammation

Several data show that the adventitia displays an important role in the pathogenesis of atherosclerosis. Mainly activated by TGF-β1, fibroblasts in the adventitia could differentiate into myofibroblasts [158], resulting in increased expression of inflammatory cytokines and growth factors [159, 160]. In addition, the NAPDH oxidase (NOX)-generated ROS in adventitial fibroblasts has been described as sensors and messengers for the development of vascular diseases [161].

Also, lymphocytes (T and B cells) accumulate in the adventitia, the major site of inflammation in the arterial wall. These processes are related to lymphocyte infiltration in atherosclerotic arteries [162]. T helper 1 (Th1) cells, secreting proinflammatory cytokines such as IL-2, TNF-α, and IFN-γ, are believed to be proatherogenic cells. In contrast, by releasing anti-inflammatory cytokines (e.g., IL-4, IL-5, IL-9, IL-10, and IL-13) regulatory T (Treg) cells are atheroprotective. Th2 cells are mainly proatherogenic whereas Th17 cells are predominantly atheroprotective. Although the exact mechanisms are unclear, natural killer T (NKT) cells are regarded as proatherogenic cells. B-1 cells, commonly found in peripheral sites and not in spleen or lymphnodes, are involved in antibody response during an infection or vaccination. They exert anti-atherogenic activities via secreting IgM, inhibiting the formation of FCs. B-2 cells (also named as common B cells) stimulate Th1 cells and dendritic cells (DCs) to play a proatherogenic role. By secreting GM-CSF (acts on DCs), innate responsive activator (IRA) play proatherogenic roles [163].

13. The PVAT in atherosclerosis and vascular inflammation

PVAT acts as modulator of the vessel function by releasing adipokines, such as leptin, adiponectin, visfatin, resistin, and cytokines/chemokines, such as TNF-α, IL-6, IL-8, MCP-1, and other factors like plasminogen activator inhibitor 1 (PAI-1). Altogether, these factors contribute to SMC migration and proliferation [164], enhance neointimal formation and hyperplasia [165, 166], stimulate inflammation responses and oxidative stress [167], and regulate vascular tone [168]. All these factors exert important roles in atherosclerosis.

PVAT plays an essential role in the inflammatory response to atherosclerosis. For example, analyzing the EC-dependent, NO-mediated vasodilator response to acetylcholine in aortas isolated from high-fat diet treated male C57BL/6J mice, Xia *et al.* described normal vasodilation in PVAT-free samples. In sharp contrast, a decent reduction in the acetylcholine-induced vasodilator response was observed in aortas from obese mice with intact PVAT. By immunohistochemistry, the authors demonstrate that adipocytes in PVAT express NOS3. High-fat diet did not change NOS3 expression but resulted in reduced NO production due to NOS3-uncoupling. This was related to arginase induction and l-arginine deficiency observed in PVAT [169]. In addition, locally elevated levels of leptin in the PVAT seems to promote neointimal formation [166]. Finally, endovascular injury-induced neointimal formation is associated with a rapid phenotypic modification of PVAT with proinflammatory adipocytokines being upregulated, and adiponectin downregulated. TNF-α has been shown to play a central role in these changes in the PVAT [165].

14. Changes in NOS expression and activity in atherosclerosis and vascular inflammation

As stated above, in vascular cells expression of all NOS isoforms (1-3) is regulated by a number of different stimuli (e.g., cytokines, ROS, miRNAs). The mode of regulation is complex and comprises multiple epigenetic, transcriptional, post-transcriptional post-translational mechanisms as well as protein-protein-interactions.

14.1 Changes in NOS1 expression/activity

Both in early and advanced human atherosclerotic lesions NOS1 expression is up-regulated in ECs, macrophages and in the neointima [113]. As demonstrated in NOS1 knockout mice the inactivation of the NOS1 gene results in a worsening of neointimal formation and constrictive vascular remodeling [170]. In line with that,

NOS1/apoE double knockout mice, compared to apoE-ko animals, displayed an accelerated atherosclerotic vascular lesion formation [171]. These data imply that NOS1 may also suppress atherosclerotic vascular lesion formation [172]. The upregulation of NOS1 expression is likely to have a compensatory role in case of reduced NOS3 expression/activity, as present in inflammation and atherosclerosis, to maintain vascular homeostasis. In addition, there are reports using immunohistochemically methods or western blot showing enhanced vascular NOS1 expression after stimulation with inflammatory/proliferative stimuli (angiotensin II, interleukin-1β, and platelet-derived growth factor), hypoxia, hypertensive situation, and statin treatment [103, 173–176].

14.2 Changes in NOS2 expression/activity

In human atherosclerotic plaques, NOS2 expression was detected. Immunostaining and *in situ* hybridization localized NOS2 to (CD68-positive) macrophages, FC and VSMC [177]. In contrast to murine endothelial cells, cytokine incubation do not induce NOS2 expression in human endothelial cells (HUVEC). Dreger *et al.* indicated at least a partial role of the histone methyltransferase enhancer of zeste homolog 2 (Ezh2), which mediates trimethylation of histone 3 at lysine 27-H3K27me3, in the epigenetic suppression of NOS2 expression in human endothelial cells [178]. In septic patients high expression NOS2 is described in many organs or tissues, which results in an enhanced NO formation that are important for hypotension, vascular hyporeactivity to vasoconstrictors, organ injury, and organ dysfunction [179]. The marked hypotension in septic shock patients is attributed to the strong induction of NOS2 in the vessels as shown in different animal studies [180]. It seems that the major part of this enhancement could be attributed to enhanced NOS2 expression in VSMC [181].

14.3 Changes in NOS3 expression/activity

Regulation of NOS3 expression by treatment of EC with pro-inflammatory mediators activating the innate immune system or cytokines (like TNF-α) produced by these cells has been reported. In addition, hypoxia regulates NOS3 expression both on the transcriptional as well as on the post-transcriptional level.

TNF-α reduces human NOS3 promoter activity in pulmonary microvessel endothelial monolayers (PMEM) [182]. This decrease was related to TNF-α-induced modulation of the binding activity of the transcription factors GATA-4 and Sp3 to the promoter sequence.

Activators of the innate immune system like oxidized LDL (ox-LDL) [183] and LPS [184] as well as cytokines produced by innate immune cells like TNF-α [54–59, 117] as well as hypoxia [185, 186] have been described to regulate NOS3 mRNA levels post-transcriptionally. Analyzing the factors involved in the TNF-α-mediated reduction of NOS3 mRNA, the RNA-BPs translation elongation factor 1-alpha 1 (eEF1A1) and polypyrimidine tract-binding protein 1 (PTB1) were found to interact with the 3'-UTR of the NOS3 mRNA and thereby destabilizing the mRNA [56–58]. In addition to RNA-BPs, also miRNAs have been shown to be involved in the TNF-α-mediated reduction of NOS3 mRNA expression. In HUVEC, TNF-α increased the expression of miR-155, an important regulator of the innate immune system [187], which directly binds to the 3'-UTR of the NOS3 mRNA and destabilize it. In addition, in human internal mammary artery rings adenoviral overexpression of miR-155 decreased both NOS3 expression and acetylcholine-induced endothelium-dependent vasodilation [60]. As shown by Lee *et al.* NF-κB is important for the TNF-α-mediated upregulation of miR-155 expression and the post-transcriptional downregulation of NOS3 mRNA expression [61]. Kim *et al.* reported that the NF-κB-regulated miR-31-5p is up-regulated in sera from patients with pre-eclampsia and in HUVECs treated with TNF-α. miR-31-5p downregulated human NOS3 mRNA expression by post-transcriptional destabilization [59].

Hypoxia regulates NOS3 expression both transcriptionally and post-transcriptionally [185]. Analyzing the effects of hypoxia on the NOS3 expression in human EC (HUVEC and HMEC cells) Coulet *et al.* described hypoxia-induced NOS3 mRNA expression. In transfection experiments a hypoxia regulated element (HRE) was identified (located at position -5382/-5356) in the human NOS3 promoter. Binding of the transcription factors HIF-1α/1β and 2 to this element was shown by supershift experiments [188]. Hypoxia is known to induce endothelial dysfunction (ED), in part, by reduction of NOS3 in ECs. Fish *et al.* showed that hypoxia reduced NOS3 transcription with parallel decreased histone acetylation and H3 lysine 4 methylation on NOS3 proximal promoter histones. In addition, the authors demonstrate that histones are quickly removed from the proximal promoter NOS3 in hypoxia. Longer duration of hypoxia leads to reincorporation of histone, lacking substantial histone acetylation. After reoxygenation of the ECs the chromatin remodeler BRG1 is involved in the reactivation of NOS3 expression [186]. Hypoxia-mediated downregulation of NOS3 mRNA and protein expression enhances the expression of a natural antisense transcript (NAT) ncRNA sONE, also known as NOS3AS or APG9L2, in HUVEC or rat aorta. sONE displays antisense homology to the 3'-UTR and part of the coding sequence of the NOS3 mRNA. Downregulation of sONE by siRNAs diminished hypoxia-induced reduction of NOS3 expression indicating NOS3 expressional regulation by sONE [189]. Hypoxia upregulates the expression of miR-134 in rat cardiomyoblast H9c2 cells. As miR-134 directly targets NOS3 mRNA and reduce NOS3 protein expression this post-transcriptional mechanisms seem to be part of the hypoxia related downregulation of NOS3 expression [190].

Cardiovascular diseases often are related to enhanced synthesis of reactive oxygen/nitrogen species (su-

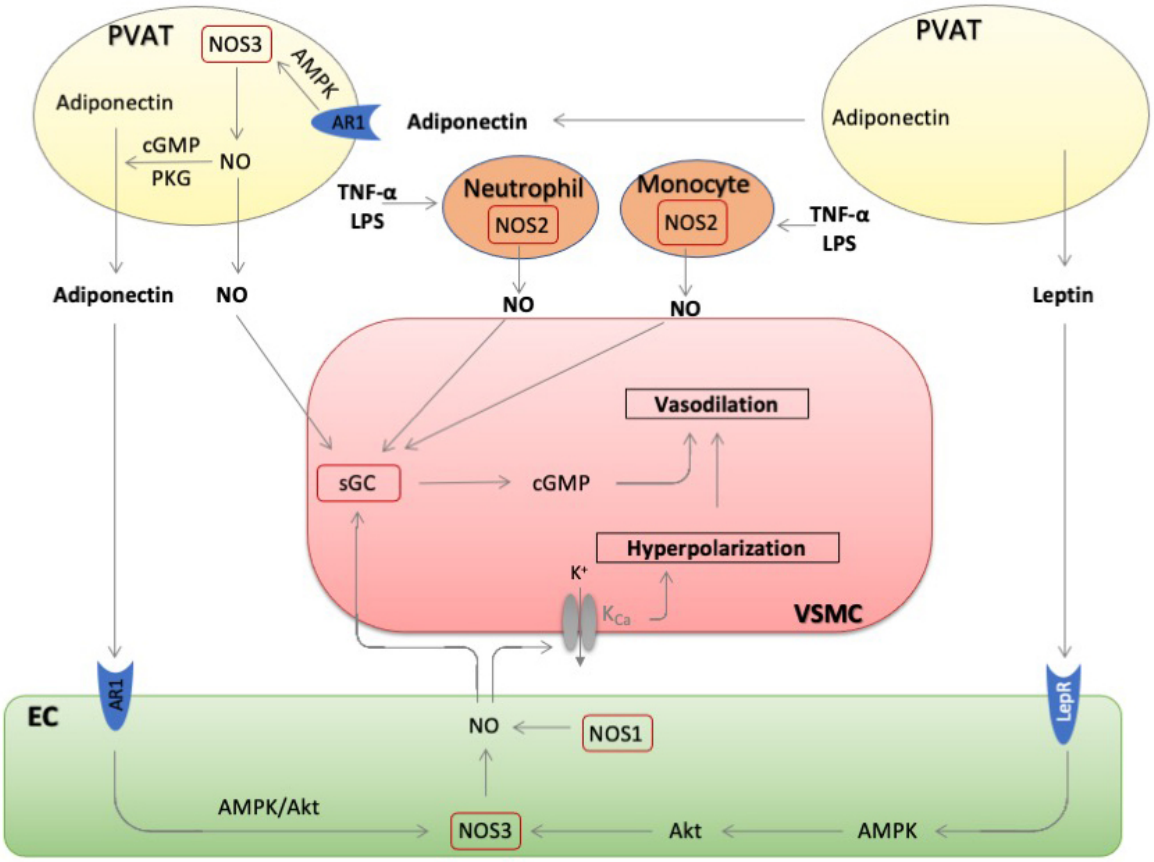

Fig. 3. PVAT-, EC- and immune cell-derived NO regulates vascular tone. Adiponectin stimulates NO production from PVAT and from endothelial cells (EC). By stimulating the leptin receptor (LepR) Leptin induces EC-dependent vasodilatation. The Leptin/LepR interaction results in NOS3 activation via the AMP-activated protein kinase (AMPK) and Akt pathway. PVAT- and EC-synthezised NO induce vasodilatation by activating soluble guanylate cyclase (sGC), leading to the synthesis of cyclic guanosine monophosphate (cGMP). NO from PVAT and EC can also induce/potentiate vascular smooth muscle cell (VSMC) hyperpolarization through K_{Ca} channels. Pro-inflammatory mediators, like LPS and TNF-α, can induce NOS2 expression in innate immune cells and thereby lead to the synthesis of high amounts of NO resulting in strong vasodilation. NOS1 expression has been detected in EC and VSCM and contribute to NO-mediated vasodilation. Modified from [116], an open access article under the terms of the Creative Commons Attribution-NonCommercial License.

peroxide, hydrogen peroxide) as well as peroxynitrite or hypochlorous acid. In addition, the detoxification of theses reactive molecules by low molecular weight antioxidants or ROS degrading enzymes is often reduced [139, 191–194]. As shown in several animal models and also in humans, the pathophysiology of vascular inflammation and ED depends on enhanced expression/activity of superoxide generating NOX enzymes resulting in enhanced production of ROS [193]. This excessive superoxide has been shown to react with NO to peroxynitrite and which in turn by oxidation of the essential NOS cofactor BH_4 leads to NOS3 uncoupling converting it into a superoxide-producing enzyme. Beside cellular and animal studies, NOS3 uncoupling in ED has also shown in patients with hypercholesterolemia [195], diabetes mellitus [196], or essential hypertension [197].

EC express arginase II and its expression can be enhanced by different factors leading to ED. As NOS3 and arginase II compete for the substrate l-arginine the enhanced arginase II expression/activity also contributes to vascular dysfunction [193].

The endogenous NOS inhibitor asymmetric dimethyl-L-arginine (ADMA) is synthesized by the enzyme arginine N-methyltransferase (PRMT) and degraded by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Both enzymes are redox-sensitive, and ROS have been shown to upregulate PRMT- and downregulate DDAH activity. As a result, ROS-induced ADMA-levels may reduce NOS3-mediated NO synthesis or even uncouple the enzyme [193].

As described above, in healthy situations there are post-translational regulatory mechanisms of NOS3 activity and localization, such as modulation by interacting proteins like calcium/calmodulin, caveolin, HSP90 as well as protein modifications like phosphorylation, palmitoylation, and myristoylation. Different kinases (like PKB/Akt, AMPK) perform the stimulating phosphorylation at Ser1177. In the inflamed vessel dysregulation of NOS3 activity is related to the synthesis of redox-active species

that initiate inhibitory phosphorylation by redox-active kinases at Thr495/Tyr657 (e.g., PKC and PYK-2), disruption of the zinc-sulfur-complex needed to stabilize the NOS3 dimer, S-glutathionylation, oxidative BH_4 depletion, and ADMA depletion (enhanced activity of PRMT and reduced activity of DDAH) (reviewed in [26, 139, 194, 198]).

In summary, NOS1 and NOS3 are vasoprotective (see Fig. 3) whereas NOS2 has detrimental effects in the vasculature. During sepsis, NOS2 induction represents a major cause of hypotension (see Fig. 3). In addition, NO produced by NOS2 in inflammatory cells contributes to atherogenesis. In contrast, NOS3-derived NO is diminished during atherosclerosis. The reduced level of endothelial NO is mainly attributable to NOS3 uncoupling, reduced NOS3 enzymatic activity and enhanced NO inactivation by superoxide.

15. Author contributions

AP and HK prepared the original draft. AP, HL and HK reviewed and edited the manuscript.

16. Ethics approval and consent to participate

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19. Conflict of interest

The authors declare no conflict of interest.

20. References

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