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REVIEW

The Mechanisms of L-Arginine Metabolism Disorder in Endothelial Cells

ENDOTHELIUM AND METABOLISM OF L-ARGININE

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Abbreviations: ADMA, asymmetric dimethyl arginine; BH_4, tetrahydrobiopterin; CaM, calmodulin; EC, endothelial cells; eNOS, endothelial nitric oxide synthase; Hsp90, heat shock protein 90; HUVEC, human umbilical vein endothelial cells; mTOR, mechanical target of rapamycin; NADP, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; ROS, reactive oxygen species.

Abstract—L-arginine is a key metabolite for nitric oxide production by endothelial cells, as well as signaling molecule of the mTOR signaling pathway. mTOR supports endothelial cells homeostasis and regulates activity of the L-arginine-metabolizing enzymes, endothelial nitric oxide synthase, and arginase II. Disruption of the L-arginine metabolism in endothelial cells leads to the development of endothelial dysfunction. Conflicting results of the use of L-arginine supplement to improve endothelial function reveals a controversial role of the amino acid in the endothelial cell biology. The review is aimed at analysis of the current data on the role of L-arginine metabolism in the development of endothelial dysfunction.

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INTRODUCTION

Endothelial nitric oxide synthase (eNOS) is an enzyme that catalyzes the reaction of nitric oxide (NO) synthesis from L-arginine. Nitric oxide (NO) controls blood vessels tone and permeability by activating guanylyl cyclase and increasing the level of cyclic 3′,5′-guanosine monophosphate (cGMP) in smooth muscle cells [1]. NO inhibits proliferation of the vascular smooth muscle cells, peroxidation of cell membranes and blood lipoproteins, aggregation of platelet, leukocyte adhesion, and regulates the blood gas transport properties [2]. The key role of NO in maintaining vascular homeostasis was proved in the studies of eNOS knockout mice that developed hypertension [3], demonstrated increased proliferation of vascular smooth muscle cells in response to damage [4], leukocyte adhesion to the endothelium [5], hypercoagulopathy [6], atherosclerosis exacerbation [7], and impaired angiogenesis regulation [8]. It was found that the L-arginine supplementation increased production of NO by endothelial cells (EC) [9, 10], hence, the use of the amino acid as a dietary supplement correcting endothelial function was studied in various pathologies in humans and in animal models [11-14]. However, a number of studies have shown that L-arginine supplementation has no positive effect [15], and in some cases even exacerbates endothelial dysfunction [16]. This indicates an ambiguous role of L-arginine in the regulation of eNOS function.

Tonic NO production by ECs is provided by a variety of processes that directly depend on the metabolism of L-arginine. L-arginine is not only a common substrate for eNOS and arginase which regulate each other [17], but also serves as a signaling molecule of mechanical target of rapamycin (mTOR) signaling pathway [18] closely related to the metabolism of the amino acid [19, 20]. All this makes L-arginine one of the key factors regulating vascular homeostasis. The review analyzes the literature data on the role of L-arginine metabolism disorder in the development of endothelial dysfunction.

eNOS ACTIVITY REGULATION

eNOS is constitutively expressed in ECs. Activity of the enzyme changes in response to hydrodynamic stress, coagulation, vasoconstriction, and hypoxia and is regulated by phosphorylation of its amino acid residues causing either activation (S615,

S633, and S1177) or inhibition (S114, T495, Y656) of the enzyme [21]. Ca^{2+}/calmodulin (CaM) mediated eNOS activation is caused by bradykinin, estradiol, and vascular endothelial growth factor (VEGF) [21]. Serotonin induces the enzyme activation, when platelets are activated and coagulation processes are enhanced [22]. The increase in the concentration of intracellular calcium leads to the CaM activation, which interacts with the CaM-binding site of eNOS, and blocks T495 – the enzyme inhibiting phosphorylation site. Simultaneously, CaM-mediated phosphorylation of calcium/Calmodulin-dependent protein Kinase II (CaMKII) leads to phosphorylation of the eNOS activating phosphorylation site (S1177) [23]. Shear stress, production of reactive oxygen species (ROS), and growth factors induce Akt1-mediated phosphorylation of eNOS at two sites S615 and S1177 [24]. Increased concentrations of AMP and intracellular calcium, as well as nutrient deficiency activate AMPK, induce eNOS phosphorylation at S1177, and NO production [25]. In studies on animals and EC cultures, activation of cAMP-dependent protein kinase A has been shown to restore endothelial barrier function by phosphorylating eNOS at S1177 [21, 26]. According to the recent data, the metabolic regulator mTOR, which integrates signals from Akt, AMPK, and CAMKII, also activates eNOS by direct phosphorylation at S1177 [19]. Under shear stress, Pp60src and PYK2 kinases regulate eNOS activity by phosphorylating at Y81 and Y657, respectively [26-28]. When exposed to damaging factors, such as oxidative, heat, or shear stress, the expression of heat shock protein 90 (Hsp90) increases in cells. The main function of Hsp90 is to stabilize conformational folding of proteins. In ECs, Hsp90 stabilizes the structure and supports the function of eNOS by binding to the enzyme oxygenase domain. In the study by Förstermann et al., Hsp90 was shown to increase the affinity of eNOS for L-arginine, CaM, and NADPH, which in turn increased the enzyme activity [29].

Located in the Ca^{2+}/CaM-binding site of eNOS, T495 is the main site that negatively regulates activity of the enzyme [26]. T495 phosphorylation mediated by PKC, Rho kinases [30] and AMPK [31] prevents formation of CaM complex with eNOS. Also, Rho kinase suppresses eNOS activity in an indirect way, through inhibition of CaM [32], Akt, and AMPK signaling molecules [30, 33]. Localization of eNOS in caveolae makes negative regulation of the enzyme activity by caveolin-1 [33] possible, which prevents CaM binding to the enzyme at low concentrations of intracellular calcium [21, 27].

THE ROLE OF ARGININE DEFICIENCY IN eNOS REGULATION

In its fully functional state, eNOS is a homodimer with two domains in each of monomers: the oxygenase domain that interacts with heme, L-arginine, and tetrahydrobiopterin (BH_4) and the reductase domain that interacts with NADPH synthase and CaM (Fig. 1).

eNOS performs sequential electron transfer from the NADPH reductase domain of one monomer, via flavin adenine nucleotide (FAD) and flavin mononucleotide (FMN), to the heme oxygenase domain of the other monomer, where L-arginine is oxidized with BH_4 and oxygen to form L-citrulline and NO [34, 35]. Under the effect of proinflammatory factors, oxidative stress, and L-arginine deficiency, the dimeric structure of eNOS is disrupted [36]. Due to the fact that electron transport is carried out in the trans-position from the reductase domain of one subunit to the oxygenase domain of the other eNOS subunit, the enzyme dissociation leads to separation of NADPH oxidation processes and a decrease in NO synthesis. Suppression of tonic production of NO leads to increased inflammation and thrombosis in blood vessels [37]. These processes are enhanced under the conditions of substrate deficiency, since oxygen becomes the terminal electron acceptor instead of L-arginine, hence, eNOS synthesizes the superoxide anion radical – $O_2^{\bullet-}$ in large amounts. The interaction of $O_2^{\bullet-}$ with NO leads to formation of peroxynitrite ($ONOO^-$) oxidizing BH_4 into BH_2, which, in turn, causes eNOS uncoupling and amplification of the process. Hyperproduction of $O_2^{\bullet-}$ and $ONOO^-$ results in ECs activation, induces expression of adhesion molecules and development of inflammation [36]. High ROS concentration has cytotoxic and mutagenic effects, causes ECs senescence and apoptosis, increases the risk of atherosclerosis and hypertension development [38]. Thus, phosphorylation of dissociated eNOS at activating sites is seen as one of the factors bringing about oxidative stress, inflammation, coagulation, and vascular tone deregulation. Therefore, under conditions of the substrate deficiency, eNOS activity should to be inhibited. However, a decrease in NO production with activation of the enzyme is often observed in the pathologies associated with endothelial dysfunction [39].

ABSOLUTE L-ARGININE DEFICIENCY

A number of studies on cell cultures and laboratory animals have shown that the

eNOS activity depends on the concentration of extracellular L-arginine [9, 10]. The synthesis of NO in human umbilical vein endothelial cells (HUVECs) depends on the concentration of L-arginine in the culture medium. It was shown that under conditions of L-arginine deficiency, NO production induced by bFGF was completely inhibited. L-arginine supplementation restored the production of the metabolite [40]. In experiments on the EA.hy926 EC line, it was shown that partial removal of the L-arginine transporter CAT-1 resulted in the decrease in NO production [41]. The condition of cardiovascular system of a person suffering a rare genetic L-arginine metabolism disorder has been described in the literature. Concentration of L-arginine in the plasma of this patient was reduced by 79% compared to the reference values due to mutation in the SLC7A7 gene encoding the amino acid transporter y + LAT-1. The consequences of L-arginine deficiency included development of myocardial ischemia, disruption of vasomotor vascular function, decrease in the absolute number of platelets, increase in the concentration of the thrombin-antithrombin III complex and the products of fibrin degradation in plasma [42]. The results obtained in this study raise many questions. In particular, absence of the amino acid transporter y + LAT-1 can lead to disruption of the transport of not only L-arginine, but a number of other amino acids. Therefore, the changes detected in this patient may be caused by various reasons. On the other hand, the changes expected for the blood from a patient with L-arginine deficiency were not detected indicating the existence of a compensatory mechanism. Despite the impossibility of unambiguous interpretation, this single observation provides important information about the role of L-arginine and NO in maintenance of vascular homeostasis.

Given that eukaryotic cells predominantly exist in a microenvironment with an excess of nutrients, it seems unlikely that the vascular endothelium should be deprived of one conditionally essential amino acid. However, a decrease in the blood L-arginine concentration as a result of arginine-metabolizing enzymes (arginases and iNOS) activity has been described in hemolysis [43], sepsis [44], surgical injuries [45], pulmonary arterial hypertension [46], as well as in the model of Alzheimer's disease in mice [47], and is considered as one of the possible causes of endothelial dysfunction [48].

RELATIVE L-ARGININE DEFICIENCY

It has been observed that in spite of high concentration of the intracellular L-arginine sufficient to meet eNOS needs, L-arginine supplement increases NO production in ECs. This shows that eNOS has a limited access to the intracellular L-arginine and uses predominantly extracellular pool of the amino acid for NO synthesis. This phenomenon was termed as “arginine paradox” [41]. To explain the “arginine paradox”, two hypotheses were suggested for a mechanism that regulates the substrate’s bioavailability for eNOS [41].

Asymmetric dimethylarginine (ADMA) is an endogenous eNOS inhibitor that restricts access of the enzyme to L-arginine and NO production, even if L-arginine concentration in ECs is sufficient [49]. ADMA suppresses the eNOS activity by competing with L-arginine for binding to the enzyme active site [50] and amino acid transporter CAT, decreasing L-arginine transport into endothelial cells [51]. Inhibition of NOS by ADMA can only be overcome by a relative excess of L-arginine [49]. An increase in ADMA concentration is observed in various pathologies associated with endothelial dysfunction (preeclampsia, diabetes mellitus, hypertension) [52, 53]. ADMA production is also enhanced in apoptotic and senescent ECs [54].

ADMA is an endogenous product of methylated proteins degradation. Methylation of L-arginine residues in proteins is catalyzed by a family of enzymes, protein-arginine methyltransferases (PRMTs), that use S-adenosyl-L-methionine as a source of methyl groups [55]. An increase in expression of PRMTs genes in ECs occurs under the effect of oxidized low-density lipoproteins (LDL) [56]. Hyperproduction of NO in inflammation leads to S-nitrosylation of cysteine in the active center of dimethyl arginine dimethyl amino hydrolase (DDAG), an enzyme that catalyzes ADMA degradation to L-citrulline and dimethylamine [57]. Inhibition of DDAG not only inhibits ADMA catabolism [58], but also reduces the production of L-citrulline, which can be used by arginine succinate synthases (ASS) and arginine succinate lyases (ASL) as a precursor for L-arginine resynthesis [59]. eNOS colocalization in caveola with ASS and ASL facilitates access of the enzyme to resynthesized L-arginine.

Arginase catalyzes hydrolysis of L-arginine producing urea and L-ornithine. eNOS affinity to the substrate is 1000 times higher than that of arginases (K_m = 2 μM vs. K_m = 2 mM). However, the maximum reaction rate (V_{max}) of arginases is 1000 times higher than that of eNOS [60]. Therefore, when intracellular L-arginine is

deficient, due to the higher reaction rate (Vmax) carried out by arginase II, the equilibrium shifts towards polyamine production, and NO production decreases [17, 41]. Inhibition of eNOS is further enhanced by spermin, an arginase metabolism intermediate product, which inhibits calcium ions release from mitochondria and CAMKII-mediated eNOS activation [61].

The second hypothesis of the "different intracellular pools of arginine" proposed to explain the arginine paradox is based on the following facts. It is assumed that the extracellular pool of L-arginine is mainly used by eNOS for NO production, while the intracellular pool is equally available for both eNOS and arginase. Therefore, increases in arginase activity results in a relative substrate deficiency for eNOS and a decrease in NO production. The hypothesis of "different intracellular pools of arginine" explains both the arginine paradox and the competitive suppression of eNOS activity by arginase [17, 60].

Indeed, the activity of arginase and eNOS are regulated reciprocally [61]. As shown on human senescent ECs and aortic endothelium of elderly mice, the increased gene expression, protein synthesis, and activity of arginase-II lead to dissociation of eNOS, promote ECs senesce with increased adhesive molecules expression. In contrast, shRNA-mediated silencing of arginase-II or destruction of the arginase-II gene restores eNOS function and reduces expression of the key markers of ECs senescence. Induction of the arginase-II gene expression in the culture of "young" ECs triggers their senescence and emergence of the phenotype, characteristic of this state [62].

Human ECs express two isoforms of arginases with different localization. Arginase I is a cytosolic form, and arginase II is localized in mitochondria. Latter is the predominant isoform in ECs [60, 63]. Arginase activation is induced by damaging and proinflammatory factors – oxidized low-density lipoproteins, peroxynitrite [64], lipopolysaccharide, cytokines (TNF-α, IFN-γ), 8-bromo-cGMP, thrombin [65], and hypoxia [66]. Often, the development of endothelial dysfunction is accompanied by a simultaneous increase in arginase activity and ADMA production [26, 67]. ADMA increases arginase activity by releasing the intracellular pool L-arginine and reducing the production of N-hydroxy-L-arginine, an intermediate eNOS metabolite that suppresses arginase activity [61].

THE ROLE OF INTRACELLULAR L-ARGININE SENSORS IN REGULATION OF eNOS ACTIVITY

Amino acids are structural monomers of proteins, so their bioavailability affects anabolic processes in cells. Currently, an active search for intracellular amino acid sensors and study of signaling pathways that integrate and convert signals from these sensors are underway. Of great interest are the sensors within the serine/threonine protein kinase mTOR pathway, which regulates the processes of anabolism, cell growth, proliferation [68], and arginine metabolizing enzymes activity [19, 20]. mTOR is in the composition of two separate multi-protein complexes mTORC1 and mTORC2, which perform different but partially overlapping functions [68]. A large amount of data has been accumulated in the literature proving the dependence of mTORC1 activation on L-arginine bioavailability [69]. Amino acid signaling sensors SLC38A9 and CASTOR1 were detected within mTORC1 [18, 69]. It is noteworthy that while CASTOR1 is a signaling molecule, SLC38A9 is a transceptor, i.e., it is both an L-arginine receptor and a lysosomal transporter [70]. Recent studies show that mTORC1 activity critically depends on the L-arginine bioavailability, at least in undifferentiated stem cells of human embryos (hESC) and immortal HeLa, MEF, HEK293T, U2OS, MRC5 cell lines [18]. Targeted studies of the effect of the amino acid deficiency on the mTOR regulation in ECs have not been conducted yet. In the literature, there are only indirect data indicating a possible role of L-arginine in the regulation of mTORC1 and EC function. It was shown that L-arginine deficiency reduced EC proliferation, adhesion [71, 72], migration [73, 74], suppressed tube formation on Matrigel *in vitro* [75], and angiogenesis *in vivo* [40, 75]. The possibility of positive mTOR regulation by L-arginine indicates the important role of this amino acid in maintaining endothelial cell homeostasis.

However, numerous literature data show that mTOR pathway activation accompanies many pathologies associated with endothelial dysfunction (table) [76]. As shown for the HUVECs *in vitro* and in the culture of aging rats aorta *ex vivo*, mTOR activation contributes to ECs senescence and eNOS uncoupling with increased ROS, and decreased NO production [77]. Inhibition of this signaling pathway, on the contrary, produces a vasoprotective effect observed in patients with kidney transplantation. The use of rapamycin, specific inhibitor of mTOR, for the purpose of immunosuppression, reduced cases of hypertension in this pathology [78, 79]. Inhibition of mTORC1 *in vivo* by rapamycin administration enhanced NO production and vasodilation [80, 81]. It was

shown that rapamycin has cardioprotective properties [76], reduces expression of mRNA of the endothelial activation markers, VCAM-1, and E-selectin in HUVECs [82]. The use of rapamycin and resveratrol in the senescent EC culture and aorta of old rats restored eNOS function [62]. The aortic EC senescent phenotype in C56BL/6J mice with induced obesity was due to Akt/mTOR activation [77]. Rapamycin-induced Akt/mTOR inhibition restored EC proliferation, sprouting, eNOS activity, and vasodilation. *In vivo,* this correlated with enhanced angiogenesis, restored blood flow and increased capillary density after lower limb ischemia [77]. Inhibition of mTORC1 by rapamycin under shear stress increased the basal level of carotid artery eNOS expression in mice in dose-dependent manner [77].

It is remarkable that mTORC1 activates both eNOS and arginase, despite the fact that these enzymes reciprocally regulate each other (Fig. 2). Activation of mTOR leads to eNOS phosphorylation at S1177 [19]. This enhances interaction of eNOS with Ca^{2+}-binding proteins and makes activity of the enzyme less dependent on Ca^{2+} concentration [98]. It is also assumed that the Ca^{2+}/calmodulin complex can serve as a structure that stabilizes the interaction of eNOS with mTORC1 [98]. It is important to emphasize that eNOS phosphorylation at the activating sites does not necessarily increases NO production and restores endothelial function. On the contrary, phosphorylation of uncoupling eNOS at S1177 can increase ROS production, oxidative stress, and induce inflammation associated with cell aging [40].

An additional factor contributing to eNOS dysfunction could be formation of a positive feedback loop between arginase II and mTORC1 [99]. mTOR/S6K1 hyperactivation leads to an increase in arginase-II mRNA synthesis and stabilization [99]. It was found in the recent studies on the mouse AML12 hepatoma cells that arginase II can also increase mTORC1 activity, and this does not depend on L-arginine hydrolysis [20]. Induction of arginase-II expression in hepatoma cells led to mTORC1 activation due to its association with lysosomes mediated by non-conventional class I myosin (Myo1b) [20]. It was established that interaction of Arg-II-Myo1b-mTORC1-S6K1 triggered human vascular smooth muscle cells (HUVSMC) senescence and apoptosis [20]. Arginase II activated mTOR/S6K1 signaling pathway in isolated mesenteric arteries in mice [77]. It is assumed that the synergy of arginase and mTOR activation is necessary for synchronization of glycolysis and activation of the urea cycle enzymes [100]. These data cast doubts on the dependence of mTOR on L-arginine bioavailability in endothelial cells. Otherwise, arginase activation should lead to mTOR inhibition by creating deficiency of intracellular L-arginine.

CONCLUSION

Numerous experimental data show that disruption of the arginine-metabolizing enzymes balance with predominant activation of arginases causes eNOS dissociation, which results in an increase in production of superoxide anion radical instead of vasoprotective NO. This mechanism underlies the development of vascular dysfunction in various pathologies and aging. Experiments aimed at inhibiting mTOR in endothelial dysfunction demonstrate that this intracellular signaling pathway is the central hub responsible for disrupting the balance of L-arginine metabolism in ECs. In the case of mTORC1 hyperactivation, a loop of mutual positive regulation is formed between mTORC1 and arginase, which finally leads to the eNOS uncoupling. The functional state of eNOS in ECs is determined by a variety of factors, including signaling molecules, substrate bioavailability, and enzyme conformation. The data currently accumulated in this area are to a large degree contradictory. It remains unclear how arginase can cause substrate deficiency for eNOS if the latter primarily uses extracellular arginine. Paradoxically, the mTOR pathway can synergistically activate eNOS and arginase, although these enzymes reciprocally regulate activity of each other. Another contradiction is that L-arginine deficiency caused by arginase suppresses eNOS activity, but increases activation of the L-arginine-dependent mTOR pathway. These issues require clarification to provide a more comprehensive understanding of the endothelial dysfunction mechanisms.

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Changes in activity of the arginine-dependent enzymes and production of their metabolites in the course of different pathologies and aging

Conditions	ADMA level	Arginase activity	eNOS uncoupling	mTOR	NO production	ROS production
Atherosclerosis	↑ [83]	↑ [84]	↑ [85]	↑ [86]	↓ [87]	↑ [87]
Obesity	↑ [26]	↑ [88]	↑ [20]	↑ [89]	↓ [90]	↑ [90]
Diabetes mellitus (type II)	↑ [26]	↑ [83]	↑ [91]	↑ [18, 91]	↓ [91]	↑ [91]
Hypertension	↑ [92]	↑ [93]	↑ [94]	↑ [95]	↓ [96]	↑ [97]
Aging	↑ [26]	↑ [92]	↑ [26]	↑ [18]	↓ [96]	↑ [97]

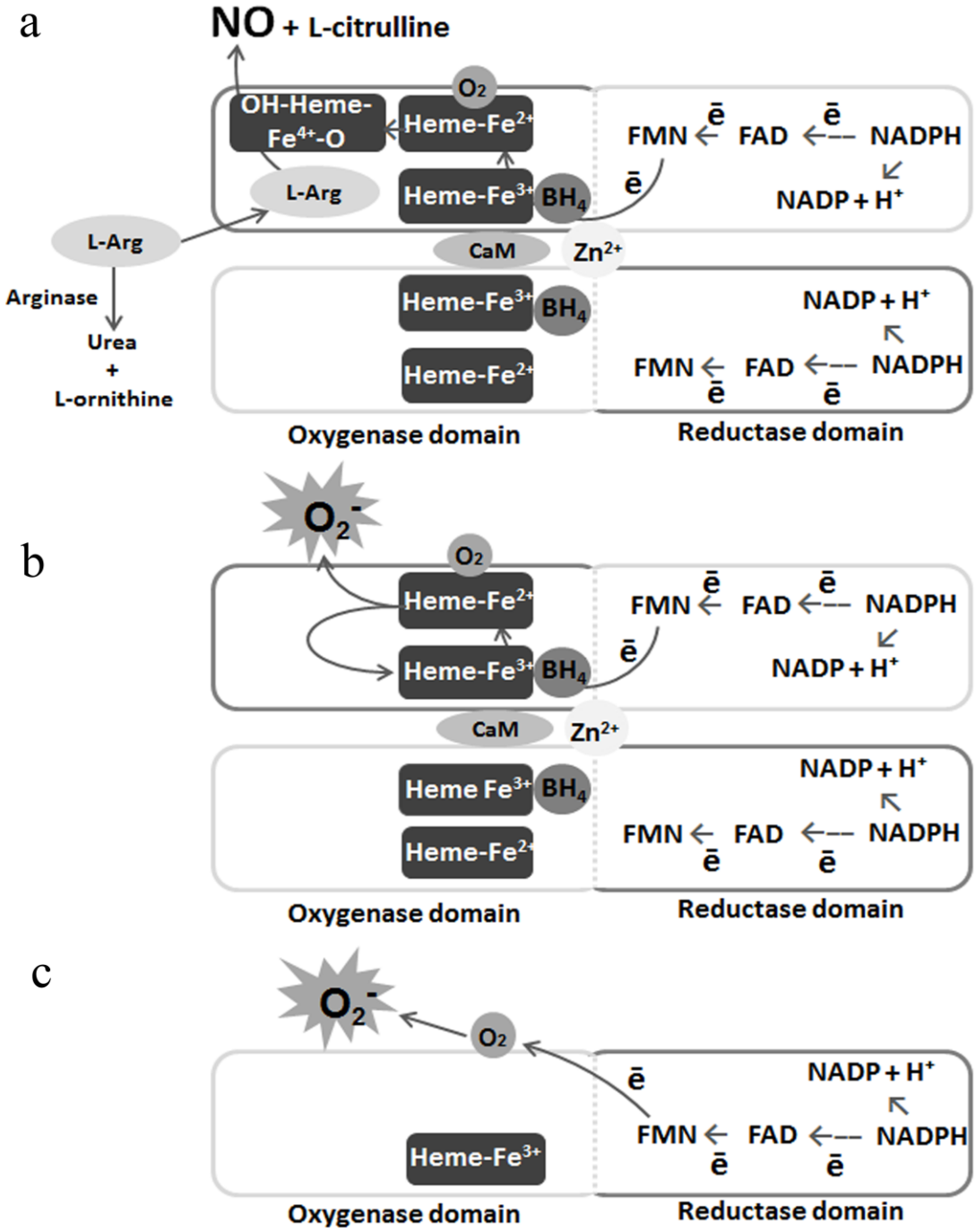

Fig. 1. Schematic illustration of eNOS functional activity in the presence or absence of the substrate. a) eNOS activity when sufficient amount of the substrate is present; b) eNOS activity when the substrate is deficient; c) eNOS activity when the enzyme dissociates.

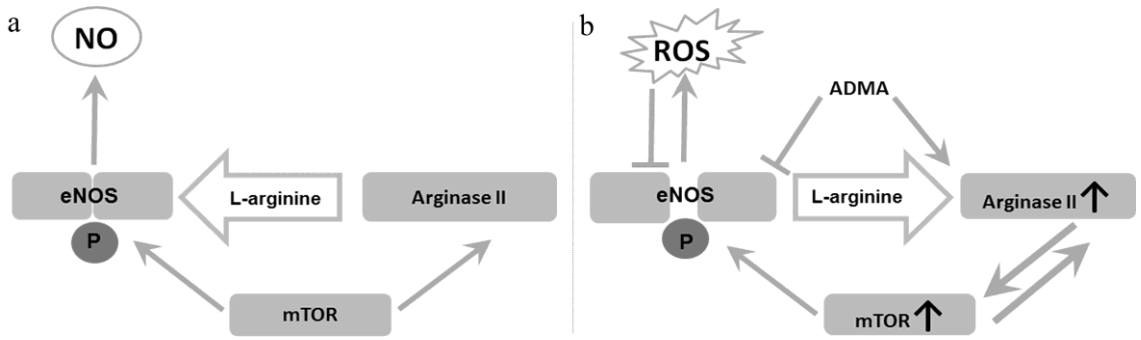

Fig. 2. Arginine metabolism and endothelial dysfunction. a) Physiological conditions. mTOR maintains the balance between arginase II and eNOS activities. b) Endothelial dysfunction. Under the effects of proinflammatory factors and oxidative stress, arginase II activity increases, and a loop of mutual activation between arginase and mTOR is formed. mTOR-mediated eNOS phosphorylation at S1117 amplifies ROS production. This, combined with increased ADMA production, leads to a relative substrate deficiency for eNOS, the enzyme uncoupling and production of ROS instead of NO.