Chapter 1

Endothelium at a Glance

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Additional information is available at the end of the chapter

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Abstract

Exposed to the blood milieu and variable hemodynamic forces, endothelial cells of different vessels exhibit significant heterogeneity, directing also the variety of endothelial functions. Endothelial cells are actively involved in many physiological processes, including vascular tone regulation, fluid filtration and reabsorption processes, maintenance of blood fluidity and proper hemostasis, leucocyte trafficking, tissue repair, and angiogenesis; accordingly, healthy endothelium is crucial for vascular homeostasis. On the other hand, many exo- and endogenous harmful factors can cause endothelial dysfunction, associated with inflammation, thrombosis, pathological vascular wall remodeling, and predisposing to the development of cardiovascular and other diseases. In order to design accurate clinical and pharmacological strategies to postpone or ameliorate endothelial dysfunction, endothelial dysfunction should firstly be recognized. Therefore, understanding endothelial physiology is crucial for clinical measures to be timely taken. The review briefly outlines some basic concepts of endothelial structure and function, focusing on endothelial barrier function and endothelium-dependent vasodilation, and addressing some potential therapeutic targets. Additional specific concepts of endothelial (dys)function, with particular emphasis on its involvement in inflammation, hemostasis, and its (mal)adaptation to environmental challenges are extensively described in the following book chapters.

Keywords: endothelium, endothelial cells, endothelial dysfunction, vascular tone regulation, nitric oxide, capillary permeability, cardiovascular diseases, oxidative stress

1. Introduction: general concepts

Endothelium, composed of approximately one trillion endothelial cells and extending over an area of 300 m^2 [1, 2], is a monolayer cell lining covering the luminal surface of blood and lymphatic vessels, and the heart. Its strategic position, being in a direct contact with the blood on one side and with the underlying tissues on the other, enables its communication with

blood elements and the adjacent cells. Developmentally, it arises from mesoderm via the differentiation of hemangioblasts and angioblasts [3]. However, other cell lineages, such as adipose lineage and mesenchymal cells, can transdifferentiate into endothelial cells even in adulthood [4, 5].

Besides presenting a mechanical barrier between the blood and the tissues, the endothelium is actively involved in various processes, including the regulation of vascular tone, maintaining blood fluidity, and enabling proper hemostasis when needed, in leucocyte trafficking, inflammation, wound repair, and angiogenesis, and, therefore, is of crucial importance for vascular homeostasis. As it metabolizes and releases many physiologically active substances that by acting in auto-, para-, and endocrine manner, govern the above physiological processes, it might justified be called an endocrine organ.

1.1. Endothelial heterogeneity

Although stemming from the same ontogenetic origin, to fulfill different metabolic and functional demands of tissues, the endothelial cells of different tissues exhibit distinct phenotypic and morphologic characteristics, accounting for its huge molecular and functional heterogeneity.

Endothelial heterogeneity could also be explained by the diversity of the vessel networks it is part of, namely arteries, veins, and capillaries which all serve different functions. While the endothelium of arteries and veins forms a continuous layer, it can be continuous, fenestrated, or discontinuous in capillaries, directing endothelial permeability and thus the degree of the filtration and reabsorption in the corresponding tissue. The representative tissues with the continuous non-fenestrated type of endothelium include the brain, the skin, the lung, and the heart; the continuous fenestrated type is found in tissues exhibiting extensive transcapillary transport: exo- and endocrine glands, the intestine mucosa, and the kidney glomeruli, whereas the prototype of the discontinuous endothelium are sinusoids in the liver and bone marrow vascular beds [1]. Endothelial cells of certain capillary beds, concomitantly with the adjacent tissue cells form specialized structures such as the blood-brain barrier composed of the brain capillary endothelial cells and the adjacent astrocytes, or special communications between maternal endothelial cells of the spiral arteries and fetal trophoblast cells in placenta [6].

While the thin capillary walls (of 0.2 μm order of magnitude) are composed only of endothelial cells anchored in their basal lamina (and surrounded by pericytes), in larger vessels, endothelium is part of the much thicker vascular wall. The latter comprises also the beneath lying vascular smooth muscle cells (VSMC) of arteries and veins, respectively, and the perivascular cells including macrophages and mesenchymal cells from the vessel adventitia.

It has been appreciated that the endothelial lining of arteriolar vascular beds primarily affects vascular tone and thus vascular resistance regulation, adjusted to tissue demands. Capillary endothelium in turn mainly determines water and solute passage into the tissues, whereas the one in the postcapillary venules directs vascular permeability and blood cell trafficking, being more involved in immune and inflammatory processes governing tissue repair and angiogenesis [7]. Therefore, apart from inter-endothelial cell junctions influencing capillary permeability, mutual communication of endothelial cells with other elements of the vascular wall importantly contributes to vascular homeostasis [8].

The vast heterogeneity of the endothelium of arteries and veins could additionally be explained by significantly different physiological and physical conditions to which endothelial cells of various vascular beds are exposed, such as blood pressure, shear stress, and pulsatility. Hemodynamic forces significantly impact endothelial structure and function: compared to ellipsoid morphology and coaxial alignment under the conditions of laminar flow, cell morphology and alignment change drastically in the settings of turbulent flow and at vessel bifurcations, all predisposing to atherogenicity [9, 10].

Endothelial cells therefore exhibit a wide range of plasticity, from alterations in the expression of various membrane receptors and adhesion molecules, changes in their morphology and shape, their mitogenic potential and even their potential to migrate or transit into different cell types (endothelial-to-mesenchymal transition) [3, 11].

1.2. Endothelial dysfunction

Being directly exposed to intravascular milieu, it is obvious that the composition of blood and the (patho)physiological conditions strongly affect endothelial cells, in terms of mediating signals which directly target their surface and activate numerous intracellular signaling pathways. During our life span, we are exposed to a variety of risk factors and toxic and noxious stimuli from the external environment (including air pollutants, tobacco smoke, chemicals from food, radiation, different eating habits in terms of high salt, sugar or saturated fatty acids intake, etc.) which strongly impact endothelial cells and their functions. As such, endothelial cells are constantly being challenged by changing internal environment to which they adapt more or less successfully. As long as their adaptive capacity in terms of maintaining homeostasis between vasoconstrictors and vasodilators reflecting vascular tone regulation; anti- and procoagulant activity reflecting hemostatic processes; anti- and pro-inflammatory mediators affecting the inflammatory response, and pro- and antiangiogenic factors affecting new vessel formation, remains in physiological limits, one might consider the endothelium to be healthy. However, the delineation between health and disease is not easy to define. When the maladaptive patterns overweigh, endothelial dysfunction issues what leads to disease [12].

Although the mostly exposed clinical sign of endothelial dysfunction is impaired endothelium-dependent vasodilation, endothelial dysfunction on a broader scale encompasses a pro-inflammatory, proadhesive, procoagulant, and proliferative state predisposing to atherosclerosis [13]. Multiple mechanisms have been involved in the development of endothelial dysfunction, connected to alterations in glucose and lipid metabolism, insulin resistance, obesity, dyslipidemia, hyperhomocysteinemia, altered hormone and cytokine secretion, imbalance in the autonomic nervous system activity, arterial hypertension, etc. Oxidative stress is acknowledged to play a central role in the development of endothelial dysfunction; moreover, oxidative stress has been appreciated as one of the main factors involved in normal aging, which imminently is also associated with endothelial dysfunction [14]. Although the severity of endothelial dysfunction might differ between vascular beds, independent studies have shown correlations between endothelial dysfunction in different vascular beds [13, 15]. Therefore, endothelial dysfunction can be regarded as a systemic disorder: as tissues and organs depend on proper vascularization and an adequate supply of nutrients and removal of waste products, the dysfunctional endothelium not only predisposes to the development of cardiovascular diseases (atherosclerosis, hypertension, peripheral arterial disease, stroke,

etc.) but also to a wide range of other diseases, including metabolic (diabetes, obesity), inflammatory, rheumatoid, oncology, and degenerative diseases, and in the worst scenario, culminates in organ failure.

Accordingly, understanding the physiology of the endothelium is of crucial clinical importance, and there is an ongoing search for potential strategies to postpone or at least ameliorate endothelial dysfunction and disease progression, either in terms of drugs application or avoiding the known risk factors; even more, when timely treated, endothelial dysfunction might be reversible [13]. To this end, additional methodological tools have to be accomplished in order to timely detect potential endothelial dysfunction. In spite of huge effort put in mechanistic studies performed on animal models and cell cultures, the exact tool to specifically reveal endothelial dysfunction clinically is missing and, at the moment, is far from being optimal. Various molecules have been used as surrogate markers of endothelial function, including soluble vascular adhesion molecule-1 (sVCAM-1), von Willebrand factor (vWF), angiopoietin-2 [16], adipocytokines [17], microparticles [18], and several more; yet, they mostly lack the sensitivity and specificity, and are often too robust to detect subtle preclinical changes. Also functional studies to assess endothelium, such as measurement of arterial intima-media thickness or flow-mediated dilation (FMD), offer just a raw estimation of endothelial function. As proper organ functioning strongly depends on microcirculation, particular attention should be given to this vascular bed also in the clinics. An interesting observation when tracing the microcirculation *in vivo* is that it exhibits typical oscillations of distinct frequencies [19, 20]. It has been suggested that each of them could reflect particular aspect of vascular tone regulation: low frequency oscillations have shown good correlations with the endothelial component of vascular tone regulation and therefore could be used as a marker of microvascular endothelial function [19].

Endothelium represents a therapeutic potential: many newer drugs targeting endothelium—either its surface and the corresponding membrane receptors, or intracellular targets affecting various signaling or metabolic pathways or directly its genome, are being developed and tested on the level of preclinical and clinical studies. In addition, independent studies have also shown that many classical cardiovascular drugs, such as angiotensin receptor antagonists, calcium (Ca^{2+}) antagonists, angiotensin converting enzyme (ACE) inhibitors, and beta adrenergic blockers, and other drugs (antidiabetic agents, sulfonylurea, etc.) apart from acting via their established mechanisms also exert positive actions connected directly or indirectly to endothelium [21–23]. Yet, keeping in mind the huge heterogeneity of endothelial cell phenotype, one must not forget that therapy affecting endothelium should be targeted to specific vascular beds. In the last time, endothelium-targeted nanomedicine has evolved as a promising new model to deliver drugs directly into the endothelial cells [24]. Last but not least, exercise has long been appreciated as one of the most efficient measures to improve endothelial (dys)function in various ways: increasing nitric oxide (NO) bioavailability; induction of reactive oxygen species (ROS) scavenging enzymes [25]; affecting the sympathetic nervous system; and increasing the number of endothelial progenitor cells (EPCs) to list just a few.

In the subsequent sections of the chapter, basic concepts of endothelial (patho)physiology, relevant for the book, will briefly be addressed. More detailed information on endothelial (dys)

function, with particular emphasis on its genetic background and senescence, its involvement in inflammation and hemostasis, and its adaptation to various environmental challenges are extensively given in the following book chapters.

2. Endothelial function depends on its structural and molecular characteristics

Electron microscopy combined with various labeling techniques has enabled an insight into the endothelial structure. Along the arterial tree, the shape of endothelial cells varies from predominantly flat in arteries and capillaries to even cuboidal in high endothelial venules, a special type of postcapillary venules [1, 7]. As endothelial cells are challenged by diverse extracellular stimuli from their environment on a spatial and temporal basis, various signaling pathways finally culminate in (post)transcriptional modifications and thus marked alterations of the phenotype. In addition, some site-specific properties of endothelium are epigenetically determined. Using DNA microarray analysis, distinct gene patterns between large vessel and microvascular endothelial cells, as well as between blood and lymphatic endothelial cells have been shown [26]. While large vessels express several genes involved in the biosynthesis and remodeling of extracellular matrix (fibronectin, various collagens, osteonectin), microvascular endothelial cells abundantly express genes coding for basement membrane proteins (laminin, various integrins, CD36). In addition, microvascular endothelial cells were shown to express higher levels of transcripts encoding proteins involved in trafficking of circulating blood cells and pathogens which enables pathogens and immune cells to migrate to target tissues [26]. Interestingly, data suggest that the artery-vein identities of endothelial cells are established before blood circulation begins [26]. Recent findings on the organotypical differentiation of microvascular beds are extensively presented elsewhere [3, 27].

2.1. On endothelial permeability, inter-endothelial cell junctions, and caveolae

Given the pivotal role of the microvascular endothelium in supplying tissues with nutrients and oxygen, endothelial integrity is crucial for tissue-fluid homeostasis. While basal permeability mainly governs water and solutes transport across the capillary wall of a healthy endothelium, the term inducible (or induced) permeability refers to alterations in endothelial permeability associated with inflammation and occurs predominantly at the site of postcapillary venules [1]. The latter occurs as a consequence of endothelial cell retraction and intercellular gap formation by a variety of agonists (histamine, serotonin, bradykinin, substance P, vascular endothelial growth factor (VEGF)) [28], or according to more recent speculations, due to transcellular vascular leakage of macromolecules [29].

In general, microvascular endothelial structure directs the capillary dynamics: fluids and small solutes (less than 3 nm) move passively across the endothelium mainly via paracellular transport mechanisms or transcellularly by simple diffusion (nonpolar substances and gases) whereas larger macromolecules are transported by transcellular mechanisms, including receptor-dependent and receptor-independent transcytosis [30].

Paracellular transport pathways across the capillary wall depend on endothelial permeability. Permeability is mainly determined by the integrity of endothelial glycocalyx and two types of inter-endothelial cell junctions, the distribution of which depends on the capillary network: tight junctions (TJs) and adherens junctions (AJs). The main component of AJs are cadherins (cadherin-VE being the most abundant), which belong to adhesion molecules and are also connected to actin cytoskeleton [31, 32]. Changes in VE-cadherin dynamics at AJs can lead to disassembly of AJs, increasing the junctional permeability; chronic vascular leakage that occurs in tumor vessels is associated with downregulation of VE-cadherin expression [33]. Interestingly, endothelial NO synthase (eNOS) hyperactivation in response to vascular endothelial growth factor (VEGF) or platelet aggregating factor (PAF) triggers S-nitrosylation of VE-cadherin, thereby causing destabilization of AJs, and leading to endothelial hyperpermeability [34]. In addition, ROS have been implicated in junctions disorganization [35]. As altered Ca^{2+} signaling has been implicated in inducing endothelial hyperpermeability, activation of transient receptor potential (TRP) channels which regulate Ca^{2+} entry has been associated with hyperpermeability states and transendothelial migration of leukocytes [31]. In addition, the integrity of AJs depends on small RhoGTPases that have been intensively investigated as potential drug targets [31]. Interestingly, statins (simvastatin) have been shown to prevent vascular injury associated with hyperpermeability and inflammation by affecting the RhoA- and Rac1-mediated cytoskeletal arrangements [36].

Transcellular transport mechanisms are accomplished by membrane-bound vesicular carriers, such as caveolae and vesiculo-vacuolar organelles. Caveolae are plasma-membrane invaginations composed of lipids and caveolins, scaffolding proteins that are involved in transmembrane signaling. Caveolin has been used as molecular marker for the isolation of caveolae-enriched membranes from *in vitro* cultured cells [37].

With reference to endothelial heterogeneity, there are significant differences in the number of caveolae in endothelium along the vascular tree reaching up to 10,000 per cell in the capillaries [1]. Besides being involved in transcellular trafficking, caveolar network in endothelial cells affects many other endothelial functions. Caveolin can modulate signal transduction: G-protein-coupled receptors and receptor tyrosine kinases are localized into caveolae and interact with caveolin [37]. Caveolins have also been involved in cell migration, angiogenesis, and cancer [38]. VEGF is an important proangiogenic factor that promotes angiogenesis by increasing vascular permeability, endothelial cell migration, and the release of proteolytic matrix metalloproteinases (MMPs). VEGF receptors have been localized in caveolae and interact with caveolin-1 [39]. VEGF disrupts the interaction between the VEGF receptor (VEGFR)-2 and caveolin-1, thereby activating the downstream signaling [39]. Silencing caveolin-1 has been shown to affect MMPs activity and VEGF-induced angiogenesis [40]; moreover, it induced morphological alterations of endothelial cells, and reduced cell migration and tubulogenesis induced by VEGF, pointing to an important role of caveolae in angiogenesis. Additional evidence on the involvement of caveolae in vasculogenesis comes from studies showing that the recruitment of EPCs [41], which are now widely recognized as one of the key mechanisms in adult vasculogenesis, to the peripheral blood is dependent on the expression of VEGFR2 [42]. Finally, one should not forget one of the main effects of caveolin, namely inhibition of eNOS [43, 44]. As caveolin also interferes with adenosine receptor trafficking and the role of adenosine has been implicated in ischemia and inflammation, caveolar network may represent a potential therapeutic target [45].

In addition, being part of the mechanosensory complex, caveolae and AJs are involved in mechanosensing [31, 46]: exposure of endothelial cells to shear stress has been shown to affect the number and distribution of caveolae [47, 48].

2.2. On endothelial barrier and mechanosensing

Mechanosensing is accomplished by a complex mechanism involving parts of endothelial cells, which convey the mechanic signals sensed by surface cellular elements in the transduction intracellular signaling pathways, finally adjusting the endothelial response to alterations of shear-stress. Mechanosensing complex, located in AJs, is composed of endothelial glycocalyx and endothelial cilium, VE-cadherins, VEGFR2 and platelet endothelial cell adhesion molecules (PECAM-1 or CD31), and various ion channels: the role of Ca^{2+}-dependent potassium channels (K^{+}_{Ca}) and TRP channels has extensively been investigated [10, 48]. However, certain predilection sites are prone to the generation of characteristic spatiotemporal shear-stress patterns favoring atherogenesis [9, 10, 48, 49].

Glycocalyx, a 50–100 μm thick structure anchored in the endothelial plasma membrane and composed of carbohydrate-rich proteoglycans, is located at the luminal surface of the endothelium [2, 30, 50]. It is importantly involved in mechanosensing, in the regulation of permeability, and inflammatory response [32, 48, 50] and has also been investigated as a potential therapeutic target [51, 52]. It prevents leucocyte and platelet adhesion [53] and mediates shear-stress-induced NO release, thus exerting vasculoprotective effects and contributing to vascular homeostasis [10, 54]. Besides being susceptible to pathological alterations in blood flow, other environmental alterations affect its composition and thus function, e.g., high glucose has been shown to be involved in glycocalyx degradation contributing to lower NO bioavailability in hyperglycemia [55, 56]. Recent concept has confirmed the existence of an additional thicker (0.5–1 μm) layer on the luminal endothelial surface (termed endothelial surface layer, ESL) which significantly impacts hemodynamic conditions, oxygen transport, vascular control, coagulation, inflammation, and atherosclerosis [2].

Endothelial cilium is a single hair-shaped projection from the cellular surface of endothelial cells. Besides being involved in sensing hemodynamic forces, it coordinates cell migration and division [30, 57]. Impaired function of the primary cilium, either genetically or due to environmental factors, results in developmental disorders or endothelial dysfunctions, respectively. The main components of the primary cilium are various membrane receptors (specific receptor tyrosine kinases, as platelet-derived growth factor (PDGF) receptor α, fibroblast growth factor receptor; insulin-like growth factor 1 (IGF-1) receptor; G-protein coupled receptors), channel proteins (polycystin-1 and -2), and special arrangement of the microtubules [30, 57]. Both polycystin proteins are importantly involved in intracellular signaling: polycystin-2, in turn, belongs to the TRP channel family which, by interacting with polycystin-1, increases intracellular Ca^{2+} leading to the activation of ryanodine receptors on the endoplasmic reticulum. The released Ca^{2+} subsequently activates a number of signaling pathways, among others the shear-stress induced activation of eNOS and an increase in NO production [57, 58]. Alterations in the patterns and magnitude of biomechanical forces induce disorganization in cilia structure: prolonged exposure to increased shear stress induces disassembly of cilia, rearrangement of cytoskeleton, and increased acetylation of microtubules [59]. Dysfunctional cilia have been implicated in kidney disease, hypertension, and the development of atherosclerosis [60].

In addition to being part of endothelial cilium, microtubules polymerized with heterodimers of alpha- and beta-tubulins are important cytoskeletal proteins in endothelial cells. They regulate numerous cellular functions, including cell shape, adhesion, intracellular transport, mitosis, and migration and thus contribute to endothelial integrity [35, 61]. Increased level of tubulin acetylation has been shown to increase cell migration [62]. On the other hand, cyclic stretch and angiotensin II (AngII) have been shown to increase tubulin deacetylation and tubular reorganization, predisposing to the development of cardiovascular diseases [63]; potential role on the AngII type 1 receptor antagonist in positively affecting endothelial microtubular organization has been implicated [63]. The longevity regulator sirtuin 1 (SIRT1), a potential therapeutic target [64], has been implicated in tubulin deacetylation and the regulation of microtubule function [63].

Disturbed flow patterns and increase of shear-stress have been acknowledged to affect the above targets, thus contributing to endothelial dysfunction in many ways, including increased generation of ROS, promoting cytoskeletal disassembly, increasing cellular permeability, expression of adhesion molecules and inflammation, as well as inducing mitogenic signaling pathways through extracellular signal-regulated kinase (ERK) and Jun kinase (JNK) involved in vascular remodeling which all favor atheroma formation [65]. Moreover, there is ample evidence that nonlaminar flow can result in gene expression of pro-inflammatory molecules in the vascular wall [49, 65].

2.3. On endothelium and inter-cellular communication

As mentioned, endothelial homeostasis involves mutual communication with other cells.

Gap junctions in turn enable a direct transmission of electrical and chemical signals and thus exchange of ions and other small molecules between endothelial cells, VSMC, and pericytes [8, 30, 66]. The main components of endothelial gap junctions are connexins (Cx) which have been shown to be defective in diseased states and thus represent a potential therapeutical target. A total of 21 connexins have been identified in humans displaying cellular specificity [67]. Healthy endothelium mainly expresses Cx-37 and Cx-40 [67, 68]. Alterations in connexin expression might be associated with endothelial dysfunction and increased susceptibility to atherosclerosis: altered expression of Cx-37 was shown to decrease NO bioavailability by decreasing eNOS activity [69], whereas endothelial-specific deletion of Cx-40 was reported to increase CD73-dependent leukocyte adhesion to endothelium [70].

Pericytes are contractile cells that wrap around the endothelial cells of capillaries and venules by sharing their basement membrane [71, 72]. As there is a considerable paracrine signaling between both cell lineages (via the release of transforming growth factor, angiopoietins, PDGF, sphingosine-1-phosphate), pericytes have been implicated in the regulation of capillary blood flow, and in the maturation and survival of endothelial cells by modulating apoptosis, and promoting angiogenesis [71–73]. Disturbance in pericyte-endothelial communication induces various pathological processes, associated with increased proneness to hemorrhage, apoptosis, impaired (tumor) angiogenesis, and endothelial hyperplasia [73].

An important communication exists between the *perivascular adipose tissue* (PVAT), endothelial cells, and VSMCs: PVAT secretes a number of adipokines (tumor necrosis factor, TNFα, interleukin-6, IL-6, resistin, irisin) with various pro- and antiatherogenic properties [8, 11]. The level of adipocytokines has been suggested as an independent predictor of endothelial dysfunction in healthy subjects [17]. In a healthy individual, PVAT importantly influences

metabolism [74] and inflammatory response and modifies vascular tone regulation by secreting protective adipokines which exert paracrine actions directly and indirectly by stimulating the release of endothelial vasodilators on VSMC [75, 76]. Moreover, healthy PVAT has been shown to enhance insulin-induced vasodilation by releasing adipokines [77], as well as prostacyclin (PGI_2) [74]. Adiponectin, the most abundant adipokine, has been shown to decrease tissue inflammation [78]. However, these anti-contractile and anti-inflammatory properties of PVAT are blunted in disease, such as obesity, and hypertension where an imbalance of secreted adipokines from PVAT and alterations in metabolism predispose to inflammation and the development of endothelial dysfunction [79]. Hypoxia is one of the main mechanisms increasing the release of inflammatory cytokines (IL-6 and TNFα) from PVAT [80], and inducing macrophage activation which all favors a pro-inflammatory and pro-contractile state [81]. Moreover, in response to vascular injury [82] or high fat diet [83], PVAT has been shown to express pro-inflammatory phenotype and a significant reduction of adiponectin levels leading to atherosclerosis. Interestingly, the inflammatory profile of PVAT has been shown to differ between distinct fat depots [84]. Independent human studies have proven beneficial effects of exercise and diet on PVAT, and consequently also on endothelial dysfunction [85, 86].

Endothelial and VSMC are abundantly innervated with the fibers of *the sympathetic nervous system*, and there is a considerable and complex cross talk between various neurotransmitters released from these fibers and endothelial autacoids [87–89]. Besides noradrenaline, that binds on various adrenoceptors on endothelial cells and VSMC, other transmitters (such as adenosine triphosphate (ATP), calcitonin gene-related peptide (CGRP) acetylcholine (ACh), and neuropeptide Y) are released from nerve varicosities, thereby profoundly modulating endothelial function. Noradrenaline was shown to activate β_3-adrenergic receptors on endothelium, thereby increasing NO production [89, 90], while the co-transmitted ATP mediates endothelial hyperpolarization by acting on endothelial purinoceptors (P2Y) [88]; both effects finally induce vasorelaxation and, respectively, modulate the "classical" sympathetic vasoconstrictor effects on VSMC. Moreover, NO affects neurotransmission at the level of blood vessels, acting on presynaptic α_2-adrenergic receptors, and it also interferes with the sympathetic neurotransmission in the central nervous system [87].

Endothelial dysfunction and autonomic nervous system imbalance often coexist in the development of cardiovascular diseases [88]. Indeed, an inverse relationship between markers of endothelial function and the sympathetic activity in healthy conditions is well known: in young adults, acute increase of the sympathetic activity, as assessed by measuring the plasma noradrenaline concentration, has been shown to impair endothelium-dependent dilation [91, 92]. Sympathetic nervous system activity is also proposed to be an important factor contributing to endothelial dysfunction with age [92].

2.4. Other endothelial functions: involvement in hemostasis, inflammation, and angiogenesis

Endothelium maintains blood fluidity, preventing intravascular coagulation and thrombus formation, respectively; endothelial cells express a variety of intraluminal surface proteins (such as thrombomodulin) and secrete molecules with anticoagulant and antithrombotic properties: ectonucleotidases and protein C and S as well as substances which inhibit platelet adhesion and aggregation (PGI_2 and NO).

On the other hand, endothelium enables *hemostasis* when needed through the expression of specific membrane glycoproteins which enable adhesion of platelets to the damaged vascular wall (i.e., subendothelial matrix); and secretion of diverse substances, such as vWF, thrombin, tissue factor, and many others. Additionally, endothelium plays a crucial role in the fibrinolytic system. Nevertheless, prothrombotic, procoagulation, and antifibrinolitic states are associated with thrombi formation and cardiovascular events.

Endothelium is importantly involved in *inflammatory processes* and tissue repair as it adjusts changes in vascular reactivity and permeability in response to various cytokines and autacoids, increasing plasma extravasation and larger molecules trafficking as well as the adhesion and recruitment of leucocytes across the vessel walls, mainly of the postcapillary venules to the site of injury/inflammation. It itself produces many substances involved in inflammation and subsequent tissue repair, i.e., VEGF and various cytokines (e.g., IL-1, TNF-α). Moreover, cytokines released from endothelium in an autocrine manner augment the inflammatory response by affecting intracellular signaling pathways which activate various transcription factors, especially the transcription factor NF-kB, to finally increase the expression of proadhesive and procoagulant genes. In addition, cytokines and VEGF also promote endothelial and VSMC proliferation [93]. In response to inflammation, endothelial cells increase the expression of a variety of adhesion molecules, belonging to three gene families, namely selectins, integrins, and the immunoglobulin (Ig) superfamily (comprising VCAM, intercellular adhesion molecules (ICAM), and PECAM-1) on their surface which enable leucocytes to recognize the affected sites, adhere to endothelium and cross the vessel wall. As the level of circulating adhesion molecules is increased in diseases, they have been used as a marker of endothelial dysfunction. Inappropriate regulation of inflammatory processes has been acknowledged as an early step in the development of atherosclerosis as well as other pathological processes [94].

Endothelium is crucial for vasculo- and *angiogenesis*. Postnatally, endothelial cells are relatively quiescent and the growth of new vessels (neoangiogenesis) in healthy adults only occurs in uterine cycle, reproduction (i.e., placenta formation), and wound healing, as well as in response to repeated exercise and endurance training in myocardium and skeletal muscles. Neoangiogenesis requires a fine orchestrated interplay between endothelial cells, VSMC, pericytes, and a variety of signaling molecules, including growth factors [95], chemokines, angiopoietins, semaphorins, angiogenic enzymes, adhesion molecules, ephrins, and MMPs [93]. Neoangiogenesis involves many interdependent processes; in brief, upon stimulation by various angiogenic growth factors, endothelial cells get activated and release protease to degrade and invade basal lamina and the underlaying extracellular matrix. Endothelial cells then migrate into the interstitial space, where they proliferate and differentiate to form solid sprouts connecting neighboring vessels [38, 93, 96]. The role of VEGF and angiopoietins and the involvement of caveolae have shortly been mentioned above. In addition, circulating EPCs have also been shown to play a role in angiogenesis as they can differentiate to mature endothelial cells and replace injured or senescent endothelial cells [41, 97]. The number of circulating EPCs has been shown to be increased in cancer [98]. One of the most important (patho)physiological stimuli for neoangiogenesis in adult period is hypoxia which modifies gene expression dependent on the activation of hypoxia-inducible factor-1 (HIF-1) and, among others, it triggers endothelial cells to get activated [99]. Excessive angiogenesis in adult

period also occurs in many pathological conditions, such as cancerogenesis, tumor growth, and metastasis. To this end, the processes connected to angiogenesis represent an important therapeutic niche: on one side, antiangiogenic drugs are targeted against tumor growth [100], and on the other, proangiogenic drugs have to be designed to augment the angiogenic potential [101].

Endothelial cells also express enzymes for degradation of other autacoids, and enzymes which convert humoral factors to attend their full activity, as, e.g., ACE.

Endothelium, therefore, is a highly metabolic organ. Energy supply for its pleiotropic functions is derived mainly from glucose by anaerobic metabolism although endothelium is directly exposed to blood with high oxygen partial pressure (pO_2); therefore, enough O_2 to be transported to other cells may be conserved [102]. Fatty acids represent another potential fuel for endothelial cells; yet, as they have relatively few mitochondria, fatty acids have been proposed to only modestly contribute to total ATP generation [102]. Glucose is transported into endothelial cells via insulin-independent GLUT-1 transporter [103]; respectively, endothelial cells, particularly the microvascular ones [104], are susceptible to adverse effects of hyperglycemia which in multiple ways increases the level of oxydative stress. The mechanism involves mitochondrial hyperpolarization, which affects ROS production [105, 106]. However, ROS should not only be regarded as a foe: in recent years, they have been acknowledged as important players in endothelial homeostasis, modulating endothelium-dependent vasodilation, permeability, and angiogenesis [105, 106]. On the other hand, dysfunctional mitochondria have been implicated in endothelial dysfunction and vascular aging [107–109] and as such represent a potential therapeutic target [109]. Moreover, mitochondria might be regarded as oxygen sensors since in hypoxic conditions, the generation of ROS is increased and connected to hypoxia-mediated responses, such as increased permeability, changes in cell surface adhesion molecules, cell proliferation, and angiogenesis [110]. Exercise and diet have been shown to beneficially impact mitochondria dysfunction [108, 111]. Caloric restriction has also been connected with SIRT1: decreased ATP level activates AMP-activated protein kinase (AMPK), the main cellular energy sensor which in turn activates SIRT1 [112].

3. Endothelium and vascular tone regulation: endothelial vasodilators

One of the main functions of endothelium is its involvement in vascular tone regulation. In response to mechanical and pharmacological (ACh, histamine, bradykinin, VEGF, various hormones as estrogen, CGRP, substance P, insulin, and platelets products (serotonin, ADP)) stimuli, endothelium releases a number of vasoactive mediators which, by affecting VSMC, regulate vascular tone and thus help to adjust blood flow to tissue demands. There is a considerable and complex interplay between endothelial and other humoral vasoactive substances, as well as the sympathetic nervous system. Mostly investigated endothelial vasodilators are NO, PGI_2, and endothelium-derived hyperpolarizing factor(s) (EDHF), whereas the main constrictors include endothelin, thromboxane, AngII, the cytochrome P450

(CYP)-hydroxylase-derived 20-hydroxyeicosatetraenoic acids (20-HETE) [113, 114], and constrictor prostanoids [115]. In a healthy state, vasodilators predominate whereas in endothelial dysfunction, reduced vasodilator bioavailability, in particular NO and an excessive release of vasoconstrictors result in an increased vascular tone. Moreover, in the settings of endothelial dysfunction, other vasodilators may compensate for the reduced NO bioavailability [116–118].

Endothelial vasodilators mediate the endothelium-dependent vasodilation. Yet, it must be noted that the contribution of each to the regulation of vascular tone differs between the vascular beds: while NO has mainly been implicated in the regulation of larger vessels, other vasodilators seem to play the prominent role in microcirculation [116, 117, 119].

FMD denotes endothelium-dependent decrease of vascular tone in response to increased blood flow, which is noted down—as well as upstream of the vessel tree, and has been used as a surrogate marker of endothelial function in the clinics. It is mostly tested by performing a transient occlusion of a distal (or proximal) artery to induce local ischemia and assessing blood flow increase after reperfusion. The phenomenon of postocclusive hyperemia (PORH) is a good example to explain the mechanism of FMD: endothelium-dependent vasodilation elicited by increased shear stress due to increased flow as a consequence of the vasorelaxant effect of locally accumulated metabolites additionally increases the flow to meet tissue metabolic demands and simultaneously oppose the pressure-induced myogenic vasoconstrictory response [116].

3.1. Nitric oxide exerts many vasoprotective functions

NO is constantly being formed from the amino acid L-arginine by constitutively expressed eNOS. It can also be produced by other isoforms of NOS, namely inducible (iNOS) and neuronal (nNOS), present in various cell types (endothelial cells, platelets, neurons and VSMC, macrophages, polymorphonucleated leukocytes) which are important in the settings of endothelial activation and/or dysfunction [43]. eNOS is a calmodulin-dependent enzyme requiring cofactors as Ca^{2+}, tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide phosphate (NADP), etc. Its activity is regulated by complex interactions of the endothelial microdomain proteins whereby its association with the heat-shock protein increases and with the caveolin-1 decreases its activity [44]. Moreover, it is modulated posttranslationally in a Ca^{2+}-dependent way through the activation of various Ca^{2+}-channels on the cell membrane and by Ca^{2+}-independent way which is the main mechanism of shear-stress-induced eNOS activation. The Ca^{2+}-independent pathway mainly affects the phosphorylation of eNOS by Akt kinase, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA), or AMPK, depending on the stimulus; a variety of stimuli, including hormones, growth factors, purines, histamine, bradykinin, serotonin, noradrenaline, etc., affect G-protein-coupled receptors and finally activate the corresponding target to subsequently phosphorylate eNOS and increase its activity [43]. On the other hand, hyperglycemia has been suggested to adversely affect eNOS phosphorylation thus attenuating NO production [120]. Another common posttranslational modification of eNOS activity is acetylation/deacetylation: for example, SIRT-1, a class III histone deacetylase enhances the ACh- and shear-stress-induced-NO production by deacetylating eNOS, thereby enhancing its binding to calmodulin [121]. Moreover, aspirin has been shown to enhance eNOS activation by acetylating eNOS, independently of its effect on cyclooxygenase inhibition [122].

Of note is the dual role of eNOS: when the bioavailability of its substrates and/or cofactors is reduced and in conditions of increased oxidative stress, eNOS can get uncoupled and produces superoxide anion (O^{2-}) instead of NO; O^{2-} is a highly reactive radical which forms peroxynitrite ($ONOO^-$) with NO, and in this way, it additionally decreases the bioavailability of NO and contributes to endothelial dysfunction [43, 44, 90].

Besides directly inducing vascular relaxations by activating the soluble guanylate cyclase (sGC) in VSMC, NO importantly modulates other endothelial autacoids (e.g., PGI_2, EDHF, endothelin) affecting vascular tone [123, 124], and interferes with the sympathetic neurotransmission pre- and postsynaptically [89].

NO exerts other effects: it inhibits platelet aggregation (interestingly, activated-platelet-derived substances increase the activity of eNOS, thus producing more NO) and the adhesion of leucocytes to the vascular wall by decreasing the expression of adhesion molecules on endothelial surface [90]. Moreover, it interferes with cellular metabolism [125] by modulating mitochondrial function, and oxygen metabolism [106, 126]. As stated, NO forms ROS ($ONOO^-$) with increased levels of O^{2-} which, among others, impairs the mitochondrial respiratory chain [127]. On the other hand, the depolarization of mitochondrial membrane induced by mitochondrial K_{ATP}-channel activators has been reported to increase the activity of eNOS in rat cerebral arteries [128]. Other gaseous mediators involved in vascular tone regulation have also been proposed to interfere with the mitochondrial function [127].

In the settings of hypoxia, NO could alternatively be derived from the reduction of $ONOO^-$ which may partly compensate for reduced eNOS activity [129].

A number of factors have been known to exert beneficial vasoprotective effects by interfering with eNOS activity and consequently increasing NO bioavailability, including female sex hormones (the protecting effects of estrogens have long been appreciated), insulin, glucagon-like peptide, thyroid hormones, erythropoietin, high density lipoproteins, etc., as well as endothelial exposure to repetitive increases in shear stress as occurs in exercise [25, 130].

On the other hand, many factors negatively impact eNOS or scavenge NO, thereby reducing the beneficial effects of NO: ROS, reduced availability of BH_4 or arginine, due to increased levels of arginase, asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS, cortisol, aldosterone, and numerous others. Moreover, environmental factors, such as smoking, radiation, increased salt intake or hyperglycemia, as well as other metabolic disturbances, such as insulin resistance and metabolic syndrome, hypercholesterolemia and obesity, homocysteinemia, uric acid, diminish the NO-dependent vasodilation and other NO-associated effects [130]. The latter settings predispose to endothelial dysfunction and the development of diseases, such as hypertension and atherosclerosis, and increase the risk for cardiovascular events.

Therefore, the biosynthetic pathways of NO and its downstream targets might all represent potential therapeutic targets [90, 131].

Increased levels of ROS could be regarded as a common denominator of reduced NO bioavailability resulting in endothelial dysfunction. Fortunately, the endogenous ROS-scavenging enzymes convert O^{2-} to hydrogen peroxide (H_2O_2) which is proposed as one of important endothelial factors inducing VSMC hyperpolarization and vasodilation, respectively [75, 132, 133].

The bad ROS are turned into the good ones thus ameliorating their damaging effects [133]. Indeed, increased levels of H_2O_2 have also been suggested to overtake the role of NO in the settings of reduced NO bioavailability also in humans [134].

3.2. Endothelium-dependent relaxations beyond NO: hyperpolarization

Shear stress and various agonists also stimulate the production of other vasodilators, among which the derivatives of arachidonic acid (AA) play an important role.

AA is liberated from membrane phospholipids by the action of Ca^{2+}-stimulated phospholipase A_2, and subsequently metabolized into biologically active eicosanoids with a variety of functions. Three main complex enzymatic pathways of AA metabolism are the cyclooxygenase pathway, the cytochrome (CYP)-P450 pathway, and the lipoxygenase pathway; however, AA can be transformed into isoprostanes nonenzymatically by ROS [135]. Regarding vascular tone regulation, AA metabolites include a number of vasodilators and vasoconstrictors [113–115, 135–138].

One of the most investigated AA metabolites is PGI_2, formed by prostacyclin synthase, which belongs to the CYP-450 family and is highly expressed in endothelial cells and associated with cyclooxygenase (COX)-2 [115, 135–137]. Its vasodilator effects mainly involve binding to prostacyclin (IP) receptors and activation of adenylate cyclase increasing cAMP level in VSMC and subsequent relaxation. Yet, the role of PGI_2 as endogenous mediator of endothelium-dependent vasodilation *in vivo* has often been questioned and its other effects, such as inhibition of platelet adhesion and aggregation, and reduction of oxidative stress [135, 139] might rather account for its vasculoprotective effects. Moreover, COX-2-derived PGI_2 might play a compensatory role for the decreased NO bioavailability [117, 118]. To this end, it must be noted that other prostanoids from the COX metabolic pathways also affect vascular tone: due to various metabolic pathways as well as various prostaglandin receptors coupled to different signaling pathways, they might either induce vasoconstriction or vasodilation [115, 135, 137–139]. It is the delicate balance between vasoconstrictors and vasodilators which enables normal functioning of healthy endothelium; in endothelial dysfunction, the effect of vasoconstrictive prostanoids predominates, predisposing to the development of hypertension, atherosclerosis, and various other diseases.

Besides NO and PGI_2, endothelial hyperpolarization (EDH) accounts for endothelium-dependent, flow-mediated vasodilation; by blocking eNOS (by L-NMMA) and COX (by more or less specific COX inhibitors), the role of non-NO-non-PGE-dependent vasodilation has unequivocally been confirmed not only in *in vitro* assays and in animal models but also *in vivo* in various human vessels during resting [140–143] and exercise [144].

Many of endothelial mediators and signals are known to induce the hyperpolarization of VSMC [8, 130, 135, 136, 145, 146]: epoxyeicosatrienoic acids (EETs) produced in the CYP-450-dependent metabolism of AA [135, 136]; the above mentioned H_2O_2 [133, 140], potassium ions released from the endothelial cells via K_{ca} channels, and direct transmission of endothelial cell hyperpolarization by myoendothelial gap junctions. Thus, EDHF comprises a variety of factors which activate various potassium channels: small (SK_{Ca}), intermediate, and

large-conductance K_{ca} channels; inward rectifier K^+ channels (K_{IR}) [147], and TRPV4 channels [148] have been implicated in finally inducing EDH. To this end, the corresponding channels have been investigated as potential targets to affect EDH [147, 149]. Endothelial hyperpolarization can spread via myoendothelial gap junctions to directly induce VSMC hyperpolarization: the role of gap junctions has been implicated also in humans *in vitro* and *in vivo* as carbenoxolone, a nonspecific gap junction blocker, diminished conducted vasodilation in isolated human coronary arterioles [150], and, moreover, reduced FMD in the brachial artery of healthy volunteers [151]. The term conducted dilation has been used to denote electrotonic transmission of local hyperpolarization, and may spread several mm upstream independent of alterations in shear stress [116, 119, 152]. It reflects the involvement of gap junctions and enables a coordinated adjustment of vascular resistance in larger and smaller arterioles and capillaries [153].

Age and gender might affect the EDHF-mediated response as male animals were reported to exhibit smaller EDHF-mediated endothelium-dependent relaxation compared to females; similarly, aging affected EDH. A reduced SK_{Ca} activity and a reduced expression of gap junction proteins, Cx-40 and Cx-43, have been suggested to account for these differences [154]. Moreover, 17b-estradiol has been proposed to activate AMPK and/or SIRT1, both implicated to be involved in increasing the EDHF-mediated signaling [154]. Alteration of the EDH contributes to endothelial dysfunction observed in various pathologies [145, 154].

Finally, other endothelium-derived vasodilators should be listed: adenosine and purines, various peptides, including CGRP, C-type natriuretic peptide (CNP), adrenomedullin, endocannabinoids, and gaseous transmitters other than NO, namely hydrogen sulfide (H_2S), and carbon monoxide (CO) which have been suggested to compensate for decreased NO bioavailability [127, 155, 156]. Adenosine, one of the most potent vasodilators (exerting also other vasculoprotective effects) is mainly formed from extracellular nucleotides (ATP, AMP) by the action of ectonucleotidases, expressed in endothelial cells and also investigated as potential therapeutic targets [157]. Interestingly, circulating ATP itself has been proposed to mediate vasodilation in humans *in vivo* by inducing vascular hyperpolarization via activation of K_{IR} channels [158].

4. Conclusion

By spreading throughout the vascular system and exerting pleiotropic functions, the endothelium could be regarded as one of the main players in cardiovascular physiology. The integrity of endothelium is crucial for vascular homeostasis and health. On the other hand, endothelial cells are susceptible to changes in blood composition and hemodynamic forces and as such vulnerable to developing endothelial dysfunction. Endothelial dysfunction nowadays is acknowledged a key initiating event in atherosclerosis, and connected to several pathological conditions and cardiovascular events. Accordingly, understanding endothelial function and dysfunction is crucial for recognition and treatment, or, optimally, for prevention of the development of cardiovascular diseases, the leading cause of death worldwide. To this end,

it should be emphasized that the mechanistic studies on isolated vessels or on animal models cannot always be extrapolated directly to humans. Therefore, in spite of intensive investigations, additional studies to elucidate mechanisms of endothelial function and dysfunction are necessary to accomplish endothelium-targeted interventions.

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