Endothelial Cell Metabolism in Normal and Diseased Vasculature

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Abstract: Higher organisms rely on a closed cardiovascular circulatory system with blood vessels supplying vital nutrients and oxygen to distant tissues. Not surprisingly, vascular pathologies rank among the most life-threatening diseases. At the crux of most of these vascular pathologies are (dysfunctional) endothelial cells (ECs), the cells lining the blood vessel lumen. ECs display the remarkable capability to switch rapidly from a quiescent state to a highly migratory and proliferative state during vessel sprouting. This angiogenic switch has long been considered to be dictated by angiogenic growth factors (eg, vascular endothelial growth factor) and other signals (eg, Notch) alone, but recent findings show that it is also driven by a metabolic switch in ECs. Furthermore, these changes in metabolism may even override signals inducing vessel sprouting. Here, we review how EC metabolism differs between the normal and dysfunctional/diseased vasculature and how it relates to or affects the metabolism of other cell types contributing to the pathology. We focus on the biology of ECs in tumor blood vessel and diabetic ECs in atherosclerosis as examples of the role of endothelial metabolism in key pathological processes. Finally, current as well as unexplored EC metabolism-centric therapeutic avenues are discussed. (Circ Res. 2015;116:1231-1244. DOI: 10.1161/CIRCRESAHA.116.302855.)

Key Words: angiogenesis ■ atherosclerosis ■ cancer ■ diabetes mellitus ■ endothelial metabolism

Angiogenesis and Endothelial Cell Biology in a Nutshell

Blood vessels supply tissues with oxygen and nutrients, whereas lymphatic vessels absorb and filter interstitial fluids from these tissues.1,2 Although mostly remaining quiescent throughout adult life, blood vessels maintain the capacity to rapidly form new vasculature in response to injury or in pathological conditions. Key players in this new vessel formation are the blood vessel lining endothelial cells (ECs). Capillary sprouting, an important component of neovascularization, is accomplished by interactions among 3 different EC subtypes, each carrying out highly specific roles during this process2: tip cells are highly migratory, nonproliferative ECs that guide and pull the new sprout in the correct direction, stalk cells elongate the new sprout by their high proliferative capacity, and quiescent phalanx cells mark the more mature part of the vessel by their typical cobblestone shape.3

The specification of ECs into one of these subtypes is mainly driven by the key angiogenic vascular endothelial growth factor (VEGF) and occurs on VEGF production by hypoxic tissues and macrophages trying to regain oxygenation and nutrient supply by attracting new vessel sprouts. These processes have been best studied in retinal angiogenesis where a continuous VEGF gradient will eventually reach the existing vascular front allowing VEGF to bind the VEGF receptor 2 (VEGFR2) in ECs, predestining these ECs to become tip cells. Intriguingly, the newly appointed tip cells themselves instruct their neighboring ECs to adopt a stalk cell phenotype: the Notch ligand delta-like 4 produced by tip cells binds Notch receptors in adjacent ECs, whereby the Notch intracellular domain (better known as NICD) is released and reprograms the cell to express the decoy receptor VEGFR1 at the expense of VEGFR2, causing reduced VEGF sensitivity and enforcing stalk cell behavior.3 Although seemingly rigid, tip/stalk specification is a highly dynamic feature in which, through continuous cell shuffling, the EC with the highest VEGF/VEGFR1 expression ratio (and thus the highest VEGF responsiveness) is at the tip of the new sprout at every given moment.4

When the tip cell encounters another tip cell or a preexisting vessel, both will fuse to form a lumened, perfused vessel, a process referred to as anastomosis. When the new vessel sprout matures, ECs adopt a more quiescent, nonproliferative and nonmigratory, cobblestone-like phenotype called phalanx cells. High VEGFR1 levels and subsequent low VEGF...
responsiveness enable these cells to stay quiescent for years. By virtue of their tight monolayer organization and barrier function, phalanx cells facilitate blood flow within the blood vessel lumen, which further promotes quiescence of phalanx cells.1 In addition, ECs in the maturing vessel secrete platelet-derived growth factor B to attract platelet-derived growth factor receptor β expressing pericytes. Coverage of the nascent vessel with these mural cells contributes to proper vessel functioning and stability.7

**EC Metabolism in Health: Driving Vessel Sprouting**

Although often mistakenly considered as inert lining material with the sole function of guiding and conducting blood, ECs are key players in health, as well as in life-threatening vascular diseases. Before discussing the metabolism of ECs and other cell types involved in vascular pathologies, we will briefly review glucose, fatty acid, and amino acid (AA) metabolism, the 3 major energy and biomass-generating metabolic pathways in healthy ECs (Figure 1), and highlight their importance in normal vessel sprouting. Most of the findings reported below are from in vitro experiments and, although they have tremendously increased our understanding of EC metabolism, await further confirmation in an in vivo setting.

ATP generation through oxidative phosphorylation could be expected to be the preferred energy-yielding pathway in ECs based on their immediate exposure to blood stream oxygen. However, ECs have a relatively low mitochondrial content and rely primarily on glycolysis with in vitro glycolysis rates comparable to or even higher than in cancer cells and exceeding glucose oxidation and fatty acid oxidation (FAO) flux by >200-fold.8–11 Per molecule of glucose, ECs miss out on ≈34 molecules of ATP by opting for glycolysis instead of oxidative phosphorylation. Notwithstanding the lower ATP per glucose yield, high glycolytic flux can yield more ATP in a shorter time than oxidative phosphorylation when available glucose is unlimited, and has the advantage of shunting glucose into glycolysis side branches (see below) for macromolecule synthesis. Whether large amounts of glucose are indeed available in the metabolically harsh environment in which vessels sprout, remains to be determined. Additional advantages of aerobic glycolysis in ECs could be (1) lower oxidative phosphorylation–generated reactive oxygen species (ROS) levels, (2) preservation of maximal amounts of oxygen for transfer to perivascular cells, (3) adaptation of ECs to the hypoxic surroundings they will grow into, and (4) production of lactate as a proangiogenic signaling molecule.12

On VEGF-stimulation, ECs double their glycolytic flux to meet increased overall energy and biomass demands and to locally supply energy for cytoskeleton remodeling during EC migration. As such, ECs display increased expression of the glycolysis activator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3),13 which by virtue of its much higher kinase activity (as compared with its phosphatase activity) produces large amounts of fructose-2,6-bisphosphate to activate phosphofructokinase-I, a rate-limiting enzyme in glycolysis. Interestingly, both genetic and pharmacological inhibition of PFKFB3 results in decreased sprouting capacity in in vitro spheroid assays and in reduced vessel branching and outgrowth in vivo, even though there is only a partial reduction in glycolytic flux (≈60% of total flux is retained).12,13 Moreover, in vitro knock-down of PFKFB3 diminished tip cell behavior even when Notch was simultaneously knocked-down to create a strong genetic pro-tip cell cue. Conversely, PFKFB3 overexpression was able to push ECs that were genetically instructed to become stalk cells by Notch intracellular domain overexpression, back into a tip cell phenotype, underscoring the pivotal role of glycolysis in ECs.10 As mentioned above, blood flow contributes to phalanx cell quiescence. Remarkably, the laminar shear stress exerted by blood flow reduced glucose uptake, glycolysis, and mitochondrial content in ECs and lowered the expression of PFKFB3 and phosphofructokinase-I to sustain a metabolically quiescent behavior. Mechanistically, the flow-responsive Krüppel-like factor 2 transcription factor was found to bind to a Krüppel-like factor 2–binding site in the PFKFB3 promoter to subsequently repress PFKFB3 transcription.14 Taken together, these findings underscore the pivotal role of glycolysis in EC subtype specification.
Glucose can also be shunted into side branches of glycolysis, such as the hexosamine biosynthesis pathway (HBP) and the pentose phosphate pathway (PPP). The HBP generates N-acetylglucosamine for protein O- and N-glycosylation and uses glucose, glutamine, acetyl-CoA, and uridine for that purpose. In ECs, the functionality of key angiogenic proteins like Notch and VEGFR2 depends on their glycosylation status. Although its in vivo role is less well characterized, the endothelial HBP possibly serves as a nutrient-sensing pathway to guide new vessels to nutrient-rich regions by glycosylating these key angiogenic proteins. Inhibition of the HBP significantly reduces angiogenesis.

Glucose enters the PPP as glucose-6-phosphate to fuel 5-carbon sugar (pentose) production for nucleotide and nucleic acid synthesis. Genetic or pharmacological inhibition of glucose-6-phosphate dehydrogenase, the rate-limiting enzyme of the oxidative PPP branch (oxPPP; generating nicotinamide adenine dinucleotide phosphate [NADPH] and ribulose-5-phosphate), and transketolase, a rate-limiting enzyme in the nonoxidative PPP branch (non-oxPPP, yielding ribose-5-phosphate) reduces EC viability and migration. The oxPPP generates NADPH that can be used as a reducing equivalent in lipid synthesis and in restoring the antioxidant capacity of glutathione by converting the oxidized form back to the reduced form.

Besides glucose, fatty acids represent another fuel source for ECs. In vitro glucose deprivation, for example, causes ECs to increase their FAO flux in an adenosine monophosphate–activated protein kinase (AMPK)–dependent manner. Interestingly, VEGF enhances the expression of the fatty acid uptake and trafficking protein FABP4, which is required for normal EC proliferation. Given its presumably modest contribution to total ATP levels in ECs, the exact role of FAO is uncertain at present. Whether FAO is involved in EC redox
homeostasis (as is the case in stressed cancer cells\textsuperscript{23,24} and biosynthesis of macromolecules, remains to be determined. Interestingly, capillary ECs in fatty acid consuming tissues (such as the heart and skeletal muscle) express FABP4 and FABP5 to transport fatty acids across the endothelium into these tissues\textsuperscript{23} (a process requiring tight control given that excess fatty acid uptake by the tissue can cause insulin resistance). Transendothelial transport of lipids is regulated by VEGF-B, although this matter is debated.\textsuperscript{26,27} The required fatty acids are supplied inside the capillary lumen through hydrolysis of triglyceride-rich lipoproteins by lipoprotein lipase. Intriguingly, lipoprotein lipase is secreted into the interstitium by parenchymal cells and relies on the glycosylphosphatidylinositol-anchored high-density lipoprotein–binding protein 1, expressed by the capillary ECs, to be transported to the capillary lumen.\textsuperscript{28}

Although gaining increasing attention in cancer cells, AA metabolism is largely understudied in ECs. Arginine is the exception to this rule and has been broadly studied for its conversion to citrulline and nitric oxide (NO), an important regulator of EC function, by endothelial nitric oxide synthase (eNOS).\textsuperscript{29} The nonessential AA glutamine is the most abundant AA in the plasma, which makes it a supposedly easily accessible fuel. Glutamine has the added value to contribute both its carbons and nitrogen to ECs’ metabolism (although the relative importance of both in vessel sprouting remains to be determined) and can, therefore, serve different, mostly biosynthetic metabolic fates.\textsuperscript{30} ECs express the solute carrier family 1 member 5 to take up glutamine and were reported to have high glutaminase (GLS) activity\textsuperscript{31,32}; this enzyme converts glutamine to glutamate in the first and rate-limiting step of glutaminolysis (the series of reactions that serves to supply glutamine carbons to the tricarboxylic acid [TCA] cycle to replenish intermediates that were used for biosynthesis purposes [anaplerosis]; Figure 1). Inhibition of glutaminase causes ECs to senesce prematurely and to stop proliferating.\textsuperscript{33} Two different GLS isozymes have been identified in mammalian cells; kidney-type GLS1 and liver-type GLS2. In cancer cells at least, these isozymes serve different functions: GLS1 is a c-MYC target and mainly drives glutamine carbons into the TCA,\textsuperscript{34,35} whereas GLS2 is downstream of p53 and preferably shunts glutamine carbons and nitrogen into glutathione for antioxidant purposes.\textsuperscript{36} Remarkably, transcriptomic analysis of laser capture microdissected tip cells from the microvasculature in mouse postnatal retinas, showed increased expression of GLS2\textsuperscript{37}; the exact biological significance hereof is unknown at present. Glutamine-derived glutamate can be used for the production of other nonessential AAs. Serine is of particular interest here because its synthesis requires the α-nitrogen from glutamate and 3-phosphoglycerate from the glycolytic pathway and thus exemplifies the functional interconnection between glucose and glutamine metabolism\textsuperscript{38} (Figure 1). ECs express α-3-phosphoglycerate-dehydrogenase, the rate-limiting enzyme in serine synthesis. Serine seems to predominantly affect blood pressure by promoting vasodilation through activation of KC\textsubscript{a} channels present in the endothelium.\textsuperscript{39,40} Furthermore, by virtue of its interconversion with glycine, serine can feed 1-carbon metabolism, which is crucial for redox balance and for nucleotide, protein, and lipid synthesis.\textsuperscript{41}

The above metabolic pathways in ECs (and the rate-limiting enzymes therein) are not only controlled by substrate and end product availability but also by key metabolic sensors like the highly conserved serine threonine kinase AMPK. Unmet energy demands, reflected by increased intracellular adenosine monophosphate levels cause AMPK to increase flux through energy-generating metabolic pathways (eg, FAO) to maintain cellular energy levels. Specifically in ECs, AMPK can also be activated by EC-specific stimuli, such as hypoxia/ischemia and (blood flow) shear stress. For more details on the role of endothelial AMPK in angiogenesis and ischemia, as well as on the link with NO and statins (see below) the reader is referred to the following reviews.\textsuperscript{42,43} For additional information on how signaling proteins drive cellular metabolism (not restricted to ECs), the reader is referred to the following reviews.\textsuperscript{44–46}

The endothelium is one of the largest organs in the body and probably also one of the most heterogeneous. The endothelium comprises not just one stereotype EC but rather a large collection of EC subtypes differing in phenotype, function, and location. Exactly how this heterogeneity translates to EC metabolism—or vice versa how EC metabolism drives this heterogeneity—remains largely unknown. Probably if not certainly, the different EC types adapt the flux through the metabolic pathways generalized above to meet their highly specific energy, redox, and biosynthesis demands. Arterial, venous, microvascular, and lymphatic ECs each have different functions and face different oxygen levels, most probably reflected by differences in their core metabolism. As such, pulmonary microvascular ECs differ from pulmonary arterial ECs in glucose and oxygen consumption and in total intracellular ATP levels.\textsuperscript{47} Brain microvascular ECs have significantly more mitochondria than peripheral ECs\textsuperscript{48}; whether this implies increased oxidative metabolism in these cells remains unknown. EC heterogeneity and possible metabolic consequences also apply to the disease state (see below). Tumor ECs, for example, differ significantly when isolated from high-versus low-metastatic tumors.\textsuperscript{49} Tumor endothelium heterogeneity is further discussed in the following review.\textsuperscript{50} Whether the role of muscle ECs in transendothelial insulin transport (described further below) also reflects a tissue-specific EC characteristic remains to be determined. As such, EC heterogeneity is a given; its translation to differences in metabolic wiring, however, remains to be explored.

Metabolic Features of ECs and Accomplices in Diseased Vessels

Given that ECs take the lead part but are not soloists in vascular disorders, the following section highlights the main metabolic changes in ECs in the disease state and looks for parallels and differences with or effects on the metabolism of other cell types involved, for as far as they have been studied. We focus on dysfunctional ECs in diabetes mellitus (Figure 2A) and atherosclerosis (a frequent complication in diabetes mellitus) (Figure 2B) and on excessively growing ECs in tumor blood vessels (Figure 3A and 3B). Much like in healthy ECs, the
data on EC metabolism in disease are mostly from in vitro/ex vivo experiments.

**Diabetic ECs**

Diabetics have increased blood glucose levels that drastically change EC metabolism and cause EC dysfunction. Hyperglycemia reduces glucose-6-phosphate dehydrogenase–mediated entry of glucose into the PPP, thereby lowering production of the main intracellular reductant NADPH and increasing oxidative stress levels. Contributing to this is the high glucose-induced activation of NADPH oxidases generating ROS. Excess glucose induces arginase activity, which consumes the NO-precursor arginine and as such uncouples the NO generating eNOS activity; instead superoxide anions are being produced. ROS together with reactive nitrogen species cause DNA strand breaks, which activate the enzyme polyADP-ribose polymerase. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (converting glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate) is ADP-ribosylated by polyADP-ribose polymerase, causing glycolytic flux to stall and glycolytic intermediates to pile up and to be diverted into the following pathological pathways (Figure 2A). (1) Excess glucose is shunted into the polyol pathway where it is converted to sorbitol by the rate-limiting enzyme aldose reductase, a reaction that further depletes NADPH and increases ROS. Sorbitol, in turn, is converted to fructose by sorbitol dehydrogenase, which leads to the production of 3-deoxyglucosone, a highly reactive α-oxo-aldehyde that contributes to the nonenzymatic generation of toxic advanced glycation endproducts (AGE). (2) The fructose-6-phosphate overload causes increased flux through the HBP, which, as discussed above, is crucial for protein glycosylation, but under hyperglycemic conditions impedes normal angiogenic behavior. eNOS activity, for example, is reduced by increased O-glycosylation. (3) Glyceraldehyde-3-phosphate and dihydroxyacetone phosphate are diverted to yield fructose-6-phosphate overload causes increased flux through the HBP, which, as discussed above, is crucial for protein glycosylation, but under hyperglycemic conditions impedes normal angiogenic behavior. eNOS activity, for example, is reduced by increased O-glycosylation. (3) Glyceraldehyde-3-phosphate and dihydroxyacetone phosphate are diverted to yield fructose-6-phosphate, a rate-limiting enzyme aldose reductase, a reaction that further depletes NADPH and increases ROS. Sorbitol, in turn, is converted to fructose by sorbitol dehydrogenase, which leads to the production of 3-deoxyglucosone, a highly reactive α-oxo-aldehyde that contributes to the nonenzymatic generation of toxic advanced glycation endproducts (AGE). (2) The fructose-6-phosphate overload causes increased flux through the HBP, which, as discussed above, is crucial for protein glycosylation, but under hyperglycemic conditions impedes normal angiogenic behavior. eNOS activity, for example, is reduced by increased O-glycosylation. (3) Glyceraldehyde-3-phosphate and dihydroxyacetone phosphate are diverted to yield fructose-6-phosphate, a rate-limiting enzyme aldose reductase, a reaction that further depletes NADPH and increases ROS. Sorbitol, in turn, is converted to fructose by sorbitol dehydrogenase, which leads to the production of 3-deoxyglucosone, a highly reactive α-oxo-aldehyde that contributes to the nonenzymatic generation of toxic advanced glycation endproducts (AGE).

Remarkably, on top of stalling glycolysis and subsequently affecting glycolytic side branches, hyperglycemia causes endothelial mitochondriopathy featuring defects in mitochondrial biogenesis and mitophagy (the latter causing damaged mitochondria to pile up), mitochondrial fragmentation and impaired functionality and increased ROS production. Of note, glyceraldehyde-3-phosphate dehydrogenase inactivation and glucose-shunting into glycolytic side branches were remedied by normalizing mitochondrial ROS levels. As end products of dysfunctional EC metabolism, AGEs have far-reaching effects not only on the ECs’ immediate extracellular surroundings but also on other cell types. AGEs can cross-link key molecules (eg, laminin, elastin, and collagen) in the extracellular matrix basement membrane causing increased vessel stiffness, which further contributes to the diabetes mellitus-related vascular complications. The broad intracellular effects of circulating AGEs are mediated through ligating the receptor for advanced glycation end products (RAGE), which is expressed in monocytes, smooth muscle cells, and ECs themselves. Contributing to vascular dysfunction, AGEs cause hyperpermeability and induce tissue factor expression leading to a more procoagulant endothelium. AGE ligation in monocyte-derived macrophages increases the expression of macrophage scavenger receptor class A and CD36, favoring uptake of oxidized low-density lipoproteins. Conversely, efflux of cholesterol to high-density lipoprotein is hampered by reduced expression of the ATP-binding cassette transporter G1 in human macrophages exposed to AGE-bovine serum albumin; a finding that mainly depended on RAGE. Both phenomena contribute to foam cell transformation, a key process in atherosclerosis (see below). In vascular smooth muscle cells (VSMCs), AGE–RAGE ligation increases proliferation and chemotactic migration, which contributes to VSMC accumulation in atherosclerotic plaques and is mediated by different signaling cascades (for more details the reader is referred to other reviews). In VSMCs, AGE–RAGE also induces autophagy via extracellular signal regulated kinase/Akt signaling to metabolically sustain the increased proliferation, and induces inducible NOS (iNOS) activity through NADPH-oxidase–derived ROS in an nuclear factor xB–dependent manner. In addition, AGEs in smooth muscle cells have been reported to cause increased fibronectin/extracellular matrix production and (vascular) calcification.

The exact role for glutamine (metabolism) in diabetic EC dysfunction is not fully understood, although the effect of hyperglycemia on the HBP and subsequent eNOS inhibition (see above) proves the involvement of glutamine given that its γ-nitrogen is coupled to fructose-6-phosphate to yield glucosamine-6-phosphate in the glutamine fructose-6-phosphate aminotransferase–mediated first and rate-limiting step of the HBP. However, disease-mimicking high glucose treatment (25 mmol/L) of the human umbilical vein EC line EA.hy926 revealed a small reduction in glutamine being oxidized. Interestingly, a genome-wide association study identified a single nucleotide polymorphism on chromosome 1q25, which causes a 32% reduction in the expression of glutamine synthetase (the enzyme responsible for de novo glutamine synthesis) in ECs. Only in diabetic patients, and not in the nondiabetic participants, this single nucleotide polymorphism (occurring at an approximate allelic frequency of 0.7 in diabetics) leads to increased risk for coronary heart disease, with each risk allele carrying a 36% higher risk for coronary heart disease. Plasma pyroglutamic acid (an intermediate of the glutamine cycle and direct precursor for glutamic acid) to glutamic acid ratios were altered in diabetics homozygous for the single nucleotide polymorphism, but the exact causative mechanism remains to be determined.

The available data on FAO in diabetic ECs seem highly contextual and somewhat contradictory. High glucose treatment of EA.hy926 ECs caused increased palmitate oxidation, whereas a similar high glucose treatment on primary human umbilical vein ECs caused an increase in malonyl-coenzyme A levels (known to inhibit carnitine palmitoyltransferase-I, the rate-limiting enzyme for FAO) and substantial decrease in FAO. Of note, leptin, of which the circulating levels are increased in diabetes mellitus, induces FAO in ECs by increasing carnitine...
Figure 2. Metabolic pathways involved in disease characterized by endothelial cell dysfunction. A, In diabetes mellitus, hyperglycemia triggers mainly increased reactive oxygen species (ROS) production through endothelial nitric oxide synthase (eNOS) uncoupling and pentose phosphate pathway (PPP) impairment resulting in stalled glycolytic flux with glycolytic intermediates being diverted into alternative metabolic pathways leading to additional excess ROS and advanced glycation end products (AGEs) production.

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B. Atherosclerosis is characterized at a metabolic level mainly by eNOS uncoupling resulting in excess ROS production and loss of nitric oxide (NO)–dependent vasodilation. 1,3BPG indicates 1,3-bisphosphoglycerate; 3DG, 3-deoxyglucosone; ADMA, asymmetrical dimethyl arginine; AGXT2, alanine-glyoxylate aminotransferase; AR, aldose reductase; BH₄, 7,8-dihydrobiopterin; BH₂, tetrahydrobiopterin; DAG, diacylglycerol; DDAH, dimethyl-arginine dimethyl-aminohydrolase; DHAP, dihydroxyacetone phosphate; DHFR, dihydrofolate reductase; eNOS, endothelial nitric oxide synthase; F1,6P₂, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; FA, fatty acid; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAT, glutamine fructose-6-phosphate amidotransferase; GA3P, geranylgeranyl pyrophosphate; GlucN6P, glucosamine-6-phosphate; HCS, homocysteine; GTP, guanosine triphosphate; GTPCH, GTP cyclohydrolase; HMG-CoA, hydroxymethylglutaryl coenzyme A; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; MET, methionine; mTHF, 5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PARP1, polyADP-ribose polymerase 1; PKC, protein kinase C; SAM, S-adenosylmethionine; and SDH, sorbitol dehydrogenase.
palmitoyltransferase-1 activity and by lowering acetyl-CoA carboxylase (rate-limiting enzyme for fatty acid synthesis) activity. Insulin resistance itself (under normal glucose levels/tolerance) increases FAO in aortic ECs.

On a more systemic level, capillary blood flow in muscle is increased by insulin through increased eNOS expression and NO levels and subsequent vasodilation (capillary recruitment). This drives nutritive flow and ensures transport across capillary ECs of glucose and insulin itself toward the muscle interstitium (for more details and possibly contradictory findings on insulin-induced capillary blood flow, the reader is referred to the following review). Muscle (micro)-vascular dysfunction and reduced eNOS activity are among the earliest signs of insulin resistance and type 2 diabetes mellitus. Atherosclerosis

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NO mediates endothelium-dependent vasodilation, required for normal vascular homeostasis, and inhibits important events promoting atherosclerosis, such as platelet aggregation, smooth muscle cell proliferation and migration, leukocyte adhesion and oxidative stress. When the availability of the NO-precursor arginine and cofactor BH4 is reduced, eNOS fails to produce NO and citrulline and instead produces ROS, a process called eNOS uncoupling (Figure 2B).

The AA arginine is the main source for the generation of NO with ≈1% of the daily arginine intake being metabolized through this pathway. Even though endothelial and plasma levels of arginine are sufficiently high to support eNOS-dependent NO synthesis, arginine did seem to be rate-limiting in atherosclerotic patients with impaired endothelium-dependent vasodilation. A possible explanation for this is the existence of endogenous arginine analogs such as asymmetrical dimethyl arginine (ADMA), which antagonizes endothelium-dependent vasodilation and NO synthesis, by competing with arginine for binding to the catalytic center of eNOS. ADMA plasma concentrations have been shown to be elevated ≥10-fold in atherosclerotic patients compared with healthy subjects and is now considered a major cardiovascular risk factor. Approximately 80% of ADMA is eliminated through metabolism into citruline and dimethylamine via dimethyl-arginine dimethyl-aminohydrolase.

Dimethyl-arginine
dimethyl-aminohydrolase is impaired by oxidative stress, allowing the accumulation of ADMA and subsequent inhibition of NO production. The remaining 20% of ADMA can also be metabolized by alanine-glyoxylate aminotransferase, a mitochondrial aminotransferase primarily expressed in the kidney. The competition between arginine and ADMA may explain why arginine supplementation is only beneficial in atherosclerotic patients with high ADMA plasma levels, and not in healthy patients with low ADMA plasma levels. Finally, oral supplementation of arginine not only benefits endothelial cells in atherosclerotic patients.

Next to eNOS uncoupling, NADPH oxidases are important sources of ROS in the vasculature. In early atherosclerotic vessels, endothelial-derived NADPH oxidases produce superoxides, whereas further along the disease, mainly VSMC-derived NADPH oxidases produce superoxides. NADPH oxidases are induced in ECs and macrophages by plaque components, such as oxidized low-density lipoprotein. Subsequent NADPH oxidase-derived ROS production has a detrimental effect in atherosclerosis by triggering increased expression of adhesion molecules, induction of VSMC proliferation and migration, apoptosis of ECs and oxidation of lipids. Much like hyperglycemia in diabetes mellitus (see above), atherosclerosis-inducing triglycerides and oxidized low-density lipoprotein can cause endothelial mitochondria to become dysfunctional by damaging mitochondrial DNA and other vital components, eventually leading to increased ROS.

The cofactor BH$_4$ is de novo produced from guanosine triphosphate (GTP) in the pathway where the highly regulated GTP cyclohydrolase I is the first and rate-limiting enzyme. The EC–autonomous need for de novo BH$_4$ production was only recently confirmed in mice with EC-specific knock-out of GTP cyclohydrolase showing loss of EC NO activity and increased O$_2$. production. Cellular BH$_4$ is oxidatively degraded to inactive 7,8-dihydrobiopterin (BH$_2$) and it is assumed that the intracellular BH$_4$/BH$_2$ ratio, rather than absolute BH$_4$ levels, is the key factor for eNOS uncoupling. BH$_4$ is recovered through a salvage pathway, in which dihydrofolate reductase reduces BH$_2$ back to BH$_4$. The involvement of dihydrofolate reductase links BH$_4$ levels (and eNOS activity by extension) to 1-carbon metabolism through which single carbon units from folates are transferred to methylacceptors (folate cycle) and, in which methyltetrahydrofolate donates 1-carbon to recycle methionine (and subsequently the methyl donor S-adenosylmethionine) from homocysteine (methionine cycle; for more details the reader is referred to the following review). Both homocysteine by itself (in vitro assays), as well as inactivating mutations in methylenetetrahydrofolate reductase (the enzyme generating methylenetetrahydrofolate) leading to hyperhomocysteinemia lower BH$_4$ availability, possibly by affecting GTP cyclohydrolase and dihydrofolate reductase activity. Finally, recycling of the methyl donor S-adenosylmethionine provides a link between 1-carbon metabolism and ADMA (see above), which is generated through methylation of arginine residues by protein arginine N-methyltransferase.

The EC mevalonate or isoprenoid pathways, which use acetyl-CoA to generate cholesterol, have a peculiar effect on eNOS transcript levels. Active RhoA/Rho-associated protein kinase signaling, involved in regulating cell shape, polarity, contractility, and locomotion by controlling cytoskeletal dynamics, reduces eNOS mRNA stability. However, to be active and acquire its correct membranous localization, RhoA needs to be prenylated with geranylgeranyl pyrophosphate (GGPP), an intermediate of the mevalonate pathway. The serum lipid-lowering statins (Translational Opportunities section of this article) inhibit the rate-limiting 3-hydroxyl-3-methylglutaryl coenzyme A reductase in the mevalonate pathway and subsequently reduce cholesterol levels. A beneficial nonlipid-lowering effect of statins, originating from the same inhibitory action, is to inhibit Rho activation in ECs by reducing the availability of geranylgeranyl and as such to restore eNOS expression levels. In contrast, by blocking the mevalonate pathway, statins also reduce the availability of farnesyl pyrophosphate, required for the synthesis of Coenzyme Q10, an important cofactor for eNOS (Translational Opportunities section of this article). However, the effects on Rho prenylation are observed at statin doses far exceeding the clinically relevant dose, an important remark given the reported dose-dependency of statin effects on ECs. Noteworthy, regular-dose statins primarily affect Rac1 signaling by increasing the expression of the small GTP-binding protein GDP dissociation stimulator, which causes nuclear translocation and subsequent degradation of Rac1 leading to reduced ROS levels. For further reading on the pleiotropic effects of statins on ECs, the reader is referred to the following reviews.

Furthermore, cholesterol metabolism drives the mischievous behavior of monocyte-derived macrophages in atherosclerosis. After ingestion by macrophages, lipoproteins’ cholesteryl esters are converted into free cholesterol and fatty acids through hydrolysis in late endosomes. Subsequently, the endoplasmic reticulum enzyme acyl-CoA:cholesterol acyltransferase converts free (unesterified) cholesterol into cholesteryl fatty acid esters through re-esterification; these cholesteryl fatty acid esters are the main component of foam cells. Incessant accumulation of free cholesterol may result in free cholesterol-induced cytotoxicity and subsequent inflammation and atherosclerotic lesion development. Cholesterol accumulation can be counteracted by ATP-binding cassette protein A1 transporter-mediated cholesterol efflux to lipid-deficient apolipoprotein A-1. Macrophages in ATP-binding cassette protein A1–deficient mice indeed have reduced cholesterol efflux, whereas overexpression of ATP-binding cassette protein A1 in macrophages was associated with a substantial reduction in atherosclerosis. A similar cholesterol efflux pathway to mature high-density lipoprotein exists via the scavenger receptor class B type 1 or the ATP-binding cassette protein G1 transporter, the expression of ATP-binding cassette protein G1 is reduced by AGEs produced by diabetic ECs (see above). Finally, apolipoprotein A-I–binding protein–mediated cholesterol efflux from ECs regulates proper EC function and proper VEGFR2-mediated angiogenesis, exemplified by the findings
that knock-down or overexpression of aibp in zebrafish caused dysregulated and reduced angiogenesis, respectively.\textsuperscript{115}

**Tumor Vasculature**

Within the tumor microenvironment, metabolic features of the cancer cells are mostly hardwired (driven by genetic alterations), whereas stromal cells adapt their normal metabolism to their environment and to meet the demands of the tumor. Even though full characterization of tumor EC metabolism is in its infancy, it most probably resembles the metabolism of highly activated ECs because the tumor-induced switch from quiescence to proliferation and migration during sprouting is metabolically taxing. Normal, quiescent ECs display higher than expected glycolysis rates to generate sufficient energy to maintain crucial functions (eg, tight barrier function in certain vascular beds).\textsuperscript{10,116}; tumor ECs have increased lactate dehydrogenase B expression and probably further increase glycolysis as indicated by the induction of the glucose transporter GLUT1 in the tumor endothelium\textsuperscript{117,118} and the capability of (tumor derived) VEGF to induce PFKFB3 expression\textsuperscript{10} (Figure 3A). In this respect, ECs and cancer cells are highly alike\textsuperscript{10} given that most cancer cells are highly glycolytic (Warburg effect\textsuperscript{119}). Furthermore, both the cell types compartmentalize glycolytic enzymes with actin-rich regions in invading structures, such as filopodia (ECs) and invadopodia\textsuperscript{120} (cancer cells) to ensure efficient energy production required for motility and invasion.\textsuperscript{10}

Although the exact role of EC glutamine metabolism and the relative contribution of the 2 GLS isoforms (GLS1 versus GLS2) is undetermined, blocking GLS causes reduced EC proliferation and increased senescence,\textsuperscript{33} which suggests a crucial role for glutamine in tumor vessel sprouting, further supported by the notion that glutamine is the most abundant AA and a readily available carbon and nitrogen source. Cancer cells from their side have indeed rewired their glutamine metabolism to maximize glutamine carbon flux toward replenishment of the TCA cycle and nitrogen usage for nucleotide, AA, and glutathione production. The c-MYC oncogene has been reported to underlie this rewiring by inducing glutaminolysis-related enzymes and rendering cancer cells glutamine-addicted.\textsuperscript{34,35} This dependency on glutamine is further exemplified by the recent findings that, under hypoxic conditions or impaired mitochondrial respiration, cancer cells can reductively carboxylate glutamine-derived α-ketoglutarate into citrate for de novo lipid synthesis.\textsuperscript{121–123} If and to what extent reductive carboxylation contributes to lipid synthesis in the predominantly glycolytic/hypoxic (see above) tumor ECs and total stromal compartment is currently an open question.

The glutamine-derived nonessential AA arginine is, as mentioned above, pivotal in EC behavior because of its precursor role in eNOS-mediated NO synthesis. Generation of the correct perivascular NO gradient by eNOS promotes vessel maturation.\textsuperscript{124} Cancer cells from their side express neuronal NOS (nNOS) or iNOS to generate NO, which perturbs the optimal perivascular NO gradient and renders tumor vessels abnormal.\textsuperscript{124,125}

Ammonia, produced during the first 2 steps of glutaminolysis (deamination of glutamine to glutamate and glutamate to α-ketoglutarate), is an auto- and paracrine inducer of autophagy.\textsuperscript{126} In coculture systems, ammonia produced by high glutaminolysis rates in breast cancer cells, induced autophagy in cancer-associated fibroblasts (CAFs) leading to increased proteolysis and increased glutamine levels, which in turn feeds the high glutaminolysis rate in the cancer cell compartment and as such closes the loop.\textsuperscript{127} In vitro glutamine deprivation causes osteosarcoma and lung cancer cells to excrete the proinflammatory chemokine interleukin (IL)-8, which has proangiogenic activity.\textsuperscript{128} An apparent endoplasmic reticulum stress and depletion of TCA intermediates underlies this phenomenon; treatment with dimethyl α-ketoglutarate replenished the TCA and abrogated IL-8 excretion.\textsuperscript{129} Although the in vivo relevance of these findings remains to be determined, they exemplify how cancer cell metabolism instructs the stromal component (in this case tumor ECs).

Much like ECs, fibroblasts maintain a relatively high glycolytic flux in quiescence to sustain basal cell functions and they approximately double this flux on proliferation.\textsuperscript{120} CAFs have increased glycolysis rates to sustain a peculiar relationship with cancer cells. The activity of oxygen-sensing prolyl hydroxylase domain proteins in CAFs is inhibited by high ROS levels coming from neighboring cancer cells. Subsequent hypoxia-inducible factor 1α stabilization causes excess NO production through autophagic degradation of caveolin-1, a repressor of NO production. These high NO levels cause mitochondria in CAFs to become dysfunctional and to be cleared through mitophagy; consequently, CAFs need to turn to glycolysis for energy production and as such supply lactate (and pyruvate), which cancer cells use to generate ATP in the TCA cycle.\textsuperscript{131,132} This phenomenon contradicts the predominant view on cancer cells as the absolute Warburgian cells and has been coined the Reverse Warburg effect; the stromal compartment is suspected to be glycolytic (through mitochondrial dysfunction) and to feed energy-rich lactate into the TCA cycle of the cancer cell for highly efficient aerobic ATP production for anabolism and growth.\textsuperscript{132,133} It remains to be determined if highly glycolytic tumor ECs engage in a similar host–parasite–like relationship with cancer cells as CAFs do (Figure 3B).

Although the immune component of the tumor is intensively studied nowadays, insights to the metabolism of tumor-associated immune cells are lacking. Aerobic glycolysis is induced on switching from a naive T cell to an activated T cell to fulfill the energy needs for proliferation, differentiation, and activity (for more detailed information on the metabolic features of different T-cell subtypes, we refer to other reviews\textsuperscript{12,134}). If and how the move from the oxygen and nutrient-rich blood and lymph vessels to the harsh tumor microenvironment changes the metabolism of activated T cells is less well characterized. T cells in the tumor microenvironment display an exhaustion-like phenotype, a state of nonresponsiveness and reduced effector function normally caused by constant antigen exposure (compare with chronic inflammation), and increase the expression of the immune-inhibitory programmed death receptor 1 and the cytotoxic T-lymphocyte antigen-4, ligation of which has been shown to reduce glycolysis.\textsuperscript{135,136} Lactate excreted by cancer cells promotes IL-17A production, which negatively regulates T-cell–mediated antitumor mechanisms.\textsuperscript{137} Although the metabolism of tumor-associated macrophages is not fully known, these cells display a peculiarly
divergent use of arginine within the tumor stroma depending on their polarization status (M1 versus M2 phenotype; see other reviews\textsuperscript{12,13} for more detail). M1 macrophages suppress tumor growth and use arginine and iNOS to produce NO, which is toxic for cancer cells. Tumor growth promoting M2 macrophages use arginase 1 to convert arginine to ornithine, which can feed proliferation of cancer cells.\textsuperscript{139}

**Translational Opportunities**

The need for efficient and specific therapeutics to treat life-threatening vascular disorders is high. As evidenced above, diseased ECs reorient their core metabolism in dysfunction and during imbalanced angiogenesis raising the question if more EC metabolism-centric treatment strategies should be considered. Given that the necessary technical and conceptual advances to fully understand diseased EC metabolism to the tiniest details are starting to emerge only now, EC metabolism as a therapeutic target is still in its infancy. Nevertheless, recent publications provided convincing proof of concept.\textsuperscript{10,13} The current approach in tumor angiogenesis relies on blocking VEGF or its receptors; a growth factor-centric treatment characterized by tumor-based escape mechanisms (ie use of alternative additional growth factors to induce excess angiogenesis), leading to resistance.\textsuperscript{104} The recent data on how the glycolysis regulator PFKFB3 in ECs controls vessel sprouting in parallel to genetic signaling\textsuperscript{10} have generated seminal follow-up studies showing the advantage of (chemical) inhibition of PFKFB3 in treating pathological angiogenesis.\textsuperscript{13,141}

Noteworthy and probably of critical importance for future EC metabolism-centric approaches is the paradigm altering concept of partial and transient glycolysis reduction. The small molecule PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propan-1-one (3PO) blocks EC glycolysis in vivo only transiently and by only 40% (corresponding to the difference in glycolysis rate between quiescent and proliferating/migrating ECs), sufficient to reduce pathological angiogenesis without affecting the healthy, quiescent vasculature.\textsuperscript{13} Contrary to earlier belief, it is thus not necessary to block glycolysis permanently and completely—furthermore, this bears an increased risk for adverse effects.

Whether targeting PFKFB3 in ECs is a valuable strategy in atherosclerosis too is an unanswered question at present. In vitro, in response to the shear stress mimicking normal blood flow in mature vessels, the transcription factor Krüppel-like factor 2 represses the expression of PFKFB3 to ensure the exact glycolytic flux required to maintain the ECs (phalanx cells) quiescent.\textsuperscript{14} Given the high prevalence of atherosclerotic plaque formation at sites where disturbed flow affects the ECs (eg, bifurcations, the aortic lesser curvature) and possibly causes reduced Krüppel-like factor 2 expression,\textsuperscript{142} it is tempting to speculate that ECs in these athero-prone regions have increased PFKFB3 levels and subsequently higher glycolysis rates; if so, partial glycolysis inhibition as described above might also prove valuable in atherosclerosis. In the context of atherosclerosis (or cardiovascular disorders by extension), serum lipid-lowering treatments have proven to be highly beneficial. The hydroxymethylglutaryl coenzyme A reductase blocking statins, for example, are among the most often prescribed drugs worldwide.\textsuperscript{101} Interestingly, statins have not only additional beneficial effects such as inhibiting inflammatory processes but also a direct protective effect on the endothelium by increasing NO production (see above).\textsuperscript{143} Even though they do mostly not outweigh the benefits, side effects such as myopathy, (possibly) increased diabetes mellitus incidence and statin intolerance, are linked to statin treatment.\textsuperscript{100} Furthermore, by blocking hydroxymethylglutaryl coenzyme A reductase in the mevalonate pathway, statins reduce the availability of farnesyl pyrophosphate, a precursor for Coenzyme Q10, an important antioxidant and cofactor for eNOS in ECs.\textsuperscript{103,104} Therefore, in atherosclerosis too, additional/other EC metabolism centric approaches, focused on normalizing eNOS activity, can be of future importance. The use of vascular-targeted nanocarriers which use EC-expressed inflammation markers as landing platform are a promising strategy to deliver possible drugs to the atherosclerotic lesion.\textsuperscript{144} Although challenging to accomplish, normalizing the pathologically increased or decreased flux through a given metabolic pathway will be key to successful novel treatments.

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**References**


competitive effect of ADMA on NO-mediated bioactivity was observed in human umbilical vein endothelial cells (HUVECs) and in human aortic endothelial cells (HAECs), specifically in terms of NO production, protein arginine nitration, and nitrotyrosine expression. These findings underscore the role of ADMA in modulating NO availability and suggest potential therapeutic strategies that target ADMA to improve endothelial function and prevent vascular diseases. The importance of this research lies in its potential to elucidate the mechanisms underlying endothelial dysfunction and to inform the development of novel therapeutic interventions.


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