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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; VEGF) 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 impacts 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.

Keywords

endothelial cell metabolism; angiogenesis; diabetes; atherosclerosis; cancer

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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]. While 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 three different EC subtypes, each carrying out highly specific roles during this process [3]: *tip cells* are highly migratory, non-proliferative 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 [4].

The specification of ECs into one of these subtypes is mainly driven by the key angiogenic *vascular endothelial growth factor* (VEGF) and occurs upon 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 (Dll4) produced by tip cells binds Notch receptors in adjacent ECs whereby the Notch intracellular domain (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 [5]. Although seemingly rigid, tip/stalk specification is a highly dynamic feature in which, through continuous cell shuffling, the EC with the highest VEGFR2 / VEGFR1 expression ratio (and thus the highest VEGF responsiveness) is at the tip of the new sprout at every given moment [6].

When the tip cell encounters another tip cell or a pre-existing vessel, both will fuse to form a lumenized, perfused vessel, a process referred to as anastomosis. As the new vessel sprout matures, ECs adopt a more quiescent, non-proliferative and non-migratory, 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 [3]. In addition, ECs in the maturing vessel excrete platelet derived growth factor B (PDGF-B) to attract PDGF receptor β (PDGF-Rβ) 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 metabolism, the three major energy and biomass generating metabolic pathways in healthy ECs (Fig. 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 (OXPHOS) 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 [8] 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 flux by over 200-fold [9–11]. Per molecule of glucose, ECs miss out on approximately 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 OXPHOS 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 into which vessels sprout, remains to be determined. Additional advantages of 'aerobic glycolysis' in ECs could be 1) lower OXPHOS-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 pro-angiogenic signaling molecule [12].

Upon 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) [10], which by virtue of its much higher kinase activity (as compared to its phosphatase activity) produces large amounts of fructose-2,6-bisphosphate ($F2,6P_2$) to activate phosphofructokinase-1 (PFK1), a rate-limiting enzyme in glycolysis. Interestingly, both genetic and pharmacologic 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) [10, 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 NICD 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 PFK1 to sustain a metabolically quiescent behavior. Mechanistically, the flow-responsive Krüppel-like factor 2 (KLF2) transcription factor was found to bind to a KLF2 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 two *side branches of glycolysis*: 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 [15]. In ECs, the functionality of key angiogenic proteins like Notch and VEGFR2 depends on their glycosylation status [16, 17]. 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 [18].

Glucose enters the PPP as glucose-6-phosphate (G6P) to fuel 5-carbon sugar (pentose) production for nucleotide and nucleic acid synthesis [19]. Genetic or pharmacologic inhibition of G6P dehydrogenase (G6PDH), the rate-limiting enzyme of the oxidative PPP branch (oxPPP; generating nicotinamide adenine dinucleotide phosphate (NADPH) and ribulose-5-phosphate (Ru5P)), and transketolase, a rate-limiting enzyme in the non-oxidative PPP branch (non-oxPPP, yielding ribose-5-phosphate (R5P)) reduces EC viability and migration [20, 21]. The oxPPP generates NADPH that can be used as reducing equivalent in lipid synthesis and in restoring the antioxidant capacity of glutathione by converting the oxidized form (GSSG) back to the reduced form (GSH) [19].

Besides glucose, *fatty acids* represent another fuel source for ECs. *In vitro* glucose deprivation, for example, causes ECs to increase their *fatty acid* oxidation (FAO) flux in an AMP-activated protein kinase (AMPK)-dependent manner [9]. Interestingly, VEGF enhances the expression of the fatty acid uptake and trafficking protein FABP4, which is required for normal EC proliferation [22]. Given its presumably modest contribution to total ATP levels in ECs [10], 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 [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 [25] (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, though this matter is debated [26, 27]. The required fatty acids are supplied inside the capillary lumen through hydrolysis of triglyceride-rich lipoproteins by lipoprotein lipase (LPL). Intriguingly, LPL 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 [28].

Although gaining increasing attention in cancer cells, *amino acid (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) [29]. The non-essential amino acid glutamine is the most abundant amino acid in the plasma, which makes it a supposedly easily accessible fuel. Glutamine has the added value to contribute both its carbons and nitrogens to ECs' metabolism (though the relative importance of both in vessel sprouting remains to be determined) and can therefore serve different, mostly biosynthetic metabolic fates [30]. ECs express the solute carrier family 1 member 5 (SLC1A5) to take up glutamine and were reported to have high glutaminase (GLS) activity [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) to replenish

intermediates that were used for biosynthesis purposes (anaplerosis)) (Fig. 1). Inhibition of glutaminase causes ECs to senesce prematurely and to stop proliferating [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 [34, 35], whereas GLS2 is downstream of p53 and preferably shunts glutamine carbons and nitrogen into glutathione for antioxidant purposes [36]. Remarkably, transcriptomic analysis of laser capture micro-dissected tip cells from the microvasculature in mouse postnatal retinas, showed increased expression of GLS2 [37]; the exact biological significance hereof is unknown at present. Glutamine-derived glutamate can be used for the production of other non-essential amino acids. Serine is of particular interest here since its synthesis requires the α-nitrogen from glutamate and 3-phosphoglycerate (3PG) from the glycolytic pathway and thus exemplifies the functional interconnection between glucose and glutamine metabolism [38] (Fig. 1). ECs express D-3-phosphoglycerate-dehydrogenase, the rate-limiting enzyme in serine synthesis. Serine seems to predominantly affect blood pressure by promoting vasodilation through activation of K_{Ca} channels present in the endothelium [39, 40]. Furthermore, by virtue of its interconversion with glycine, serine can feed one-carbon metabolism, which is crucial for redox balance and for nucleotide, protein and lipid synthesis [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 adenosine monophosphate (AMP)-activated protein kinase (AMPK). Unmet energy demands, reflected by increased intracellular AMP 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 nitric oxide (NO) and statins (see below) the reader is referred to the following reviews [42, 43]. For additional information on how signaling proteins drive cellular metabolism (not restricted to ECs), the reader is referred to the following reviews [44–46].

The endothelium is one of the largest organs in the body and probably also one of the most heterogeneous. The endothelium comprises of 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 in order 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 [47]. Brain microvascular ECs have significantly more mitochondria than peripheral ECs [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 [49] (tumor endothelium heterogeneity is reviewed in ref [50]). Whether the role of muscle ECs in trans-endothelial 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 disease 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 (Fig. 2A) and atherosclerosis (a frequent complication in diabetes) (Fig. 2B) and on excessively growing ECs in tumor blood vessels (Fig. 3A,B). 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 G6PDH-mediated entry of glucose into the PPP, thereby lowering production of the main intracellular reductant NADPH and increasing oxidative stress levels [21]. Contributing to this is the high glucose-induced activation of NADPH oxidases generating ROS [51]. 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 (PARP1). The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH; converting glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphoglycerate (1,3BPG)) is ADP-ribosylated by PARP1 (ref [52]), causing glycolytic flux to stall and glycolytic intermediates to pile up and to be diverted into the following ‘pathological’ pathways (Fig. 2A): 1) Excess glucose is shunted into the polyol pathway where it is converted to sorbitol by the rate-limiting enzyme aldose reductase (AR) [53], a reaction that further depletes NADPH and increases ROS. Sorbitol in turn is converted to fructose by sorbitol dehydrogenase (SDH), which leads to the production of 3-deoxyglucosone (3DG), a highly reactive α-oxo-aldehyde that contributes to the non-enzymatic generation of toxic advanced glycation endproducts (AGE) [54]. 2) The fructose-6-phosphate (F6P) 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 [55]. 3) G3P and dihydroxyacetone phosphate (DHAP) are diverted towards methylglyoxal production, further contributing to ROS and AGE production. Also, G3P and DHAP are used for *de novo* synthesis of diacylglycerol (DAG) and subsequent protein kinase C (PKC) activation that has been shown to cause vascular abnormalities [56]. Remarkably, on top of stalling glycolysis and subsequently impacting 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 (reviewed in ref [48]). Of note, GAPDH inactivation and glucose-shunting into glycolytic side-branches were remedied by normalizing mitochondrial ROS levels [57, 58].

As end products of dysfunctional EC metabolism, AGEs have far reaching effects on the ECs' immediate extracellular surroundings but also on other cells types. AGEs can crosslink key-molecules (*eg*, laminin, elastin, collagens) in the extracellular matrix (ECM) basement membrane causing increased vessel stiffness, which further contributes to the diabetes-related vascular complications (for more details the reader is referred to the following review [59]). 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 (SMC) and ECs themselves [60]. Contributing to vascular dysfunction, AGEs cause hyperpermeability and induce tissue factor expression leading to a more procoagulant endothelium [59]. RAGE ligation in monocyte-derived macrophages increases the expression of macrophage scavenger receptor class A and CD36, favoring uptake of oxidized low-density lipoproteins (LDL) [61]. Conversely, efflux of cholesterol to high-density lipoprotein (HDL) 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 [62]. 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 [59, 60, 63]). In VSMCs, AGE-RAGE also induces autophagy via ERK/Akt signaling to metabolically sustain the increased proliferation [64], and induces inducible NOS (iNOS) activity through NADPH-oxidase derived ROS in an NF-κB-dependent manner [65]. In addition, AGEs in SMCs have been reported to cause increased fibronectin/ECM production and (vascular) calcification [66–68].

The exact role for glutamine (metabolism) in diabetic endothelial cell 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 (F6P) to yield glucosamine-6-phosphate (GlucN6P) in the glutamine fructose-6-phosphate aminotransferase (GFAT)-mediated first and rate-limiting step of the HBP [32, 69]. However, disease-mimicking high glucose treatment (25 mM) of the human umbilical vein endothelial cell line EA.hy926 revealed a small reduction in glutamine being oxidized [70]. Interestingly, a genome-wide association study identified a single nucleotide polymorphism (SNP) on chromosome 1q25, which causes a 32% reduction in the expression of glutamine synthetase (GS or GLUL: the enzyme responsible for *de novo* glutamine synthesis) in ECs. Only in diabetic patients, and not in the non-diabetic participants, this SNP (occurring at an approximate allelic frequency of 0.7 in diabetics) leads to increased risk for coronary heart disease (CHD), with each risk allele carrying a 36% higher risk for CHD. Plasma pyroglutamic acid (an intermediate of the γ-glutamyl cycle and direct precursor for glutamic acid) to glutamic acid ratios were altered in diabetics homozygous for the SNP, but the exact causative mechanism remains to be determined [71].

The available data on FAO in diabetic ECs appear highly contextual and somewhat contradictory. High glucose treatment of EA.hy926 ECs caused increased palmitate oxidation [70], 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-1 (CPT-1), the rate-limiting enzyme for FAO) and substantial decrease in FAO [72]. Of note, leptin, of which the circulating levels are increased in diabetes, induces FAO in ECs by increasing CPT-1 activity and by lowering acetyl-CoA carboxylase (ACC; rate-limiting enzyme for fatty acid synthesis) activity [73]. Insulin resistance itself (under normal glucose levels/tolerance) increases FAO in aortic ECs [74].

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 towards the muscle interstitium (for more details and possibly contradictory findings on insulin-induced capillary blood flow, the reader is referred to the following review [75]). Muscle (micro)-vascular dysfunction and reduced eNOS activity are among the earliest signs of insulin resistance and type 2 diabetes [76].

Atherosclerosis

ECs in atherosclerosis display perturbations in metabolic pathways pertaining to NO generation. Atherosclerosis is characterized early on by uncoupled and reduced eNOS, resulting in an imbalance between the anti-atherogenic molecule NO and pro-atherogenic superoxides [77]. In the endothelium, eNOS normally generates NO through enzymatic oxidation of arginine to citrulline, requiring several cofactors including NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), Ca^{2+}/calmodulin and tetrahydrobiopterin (BH_4) [78–80]. eNOS-derived 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 [81, 82]. When the availability of the NO-precursor arginine and cofactor BH_4 is reduced, eNOS fails to produce NO and citrulline and instead produces ROS [77, 83], a process called eNOS uncoupling (Fig. 2B).

The amino acid arginine is the main source for the generation of NO with approximately 1% of the daily arginine intake being metabolized through this pathway [84]. Even though endothelial and plasma levels of arginine are sufficiently high to support eNOS-dependent NO synthesis, arginine did appear to be rate-limiting in atherosclerotic patients with impaired endothelium-dependent vasodilation [85]. A possible explanation for this is the existence of endogenous arginine analogues such as asymmetric dimethyl arginine (ADMA) which antagonizes endothelium-dependent vasodilation and NO synthesis, by competing with arginine for binding to the catalytic center of eNOS [86]. ADMA plasma concentrations have been shown to be elevated up to 10-fold in atherosclerotic patients compared to healthy subjects and is now considered a major cardiovascular risk factor [87]. Approximately 80% of ADMA is eliminated through metabolization into citrulline and dimethylamine via dimethyl-arginine dimethyl-aminohydrolase (DDAH) [88]. DDAH is impaired by oxidative stress, allowing the accumulation of ADMA and subsequent inhibition of NO production [89].

The remaining 20% of ADMA can also be metabolized by alanine-glyoxylate aminotransferase (AGXT2), 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 endothelium-dependent vasodilation, but was also shown to reverse the hyperadhesive phenotype of monocytes and T-lymphocytes in atherosclerotic patients [90].

Next to eNOS uncoupling, NADPH oxidases are important sources of ROS in the vasculature [91]. In early atherosclerotic vessels, endothelial-derived NADPH oxidases produce superoxides, whereas further along the disease, mainly VSMC-derived NADPH oxidases produce superoxides [92, 93]. NADPH oxidases are induced in ECs and macrophages by plaque components such as oxidized LDL. 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 (see above), atherosclerosis-inducing triglycerides and oxidized low-density lipoprotein (oxLDL) can cause endothelial mitochondria to become dysfunctional by damaging mitochondrial DNA and other vital components, eventually leading to increased ROS [48].

The cofactor BH_4 is *de novo* produced from guanosine triphosphate (GTP) in the pathway where the highly regulated GTP cyclohydrolase I (GTPCH) is the first and rate-limiting enzyme. The endothelial cell-autonomous need for *de novo* BH_4 production was only recently confirmed in mice with EC-specific knock-out of GTPCH showing loss of EC NO activity and increased $O_2^{\bullet -}$ production [94]. 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 [95]. BH_4 is recovered through a salvage pathway in which dihydrofolate reductase (DHFR) reduces BH_2 back to BH_4 (ref [96]). The involvement of DHFR links BH_4 levels (and eNOS activity by extension) to one-carbon metabolism through which single carbon units from folates are transferred to methyl-acceptors (folate cycle) and in which methyltetrahydrofolate (mTHF) donates one-carbon to recycle methionine (and subsequently the methyldonor S-adenosylmethionine (SAM)) from homocysteine (methionine cycle) (for more details the reader is referred to the following review [97]) (Fig. 2B). Both homocysteine by itself (in *in vitro* assays) as well as inactivating mutations in methylenetetrahydrofolate reductase (MTHFR; the enzyme generating mTHF) leading to hyperhomocysteinemia lower BH_4 availability, possibly by affecting GTPCH and DHFR activity. Finally, recycling of the methyldonor SAM provides a link between one-carbon metabolism and ADMA (see above), which is generated through methylation of arginine residues by protein arginine N-methyltransferase (PRMT).

The EC mevalonate or isoprenoid pathways, which use acetyl-CoA to generate cholesterol, have a peculiar effect on eNOS transcript levels. Active RhoA/ROCK signaling, involved in regulating cell shape, polarity, contractility and locomotion by controlling cytoskeletal dynamics (see reviews [98, 99]), reduces eNOS mRNA stability [100]. However, in order 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 (see under translational opportunities below) inhibit the rate-limiting 3-hydroxyl-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) in the mevalonate pathway and subsequently reduce cholesterol levels [101]. A beneficial non-lipid 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 [102]. 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 [103, 104] (see also under Translational Opportunities). 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 (*eg* ref [105]). Noteworthy, regular-dose statins primarily affect Rac1 signaling by increasing the expression of the small GTP-binding protein GDP dissociation stimulator (SmgGDS), which causes nuclear translocation and subsequent degradation of Rac1 leading to reduced ROS levels [106]. For further reading on the pleiotropic effects of statins on ECs the reader is referred to the following reviews [107, 108].

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 [109]. Subsequently, the endoplasmic reticulum (ER) enzyme acyl-CoA:cholesterol acyltransferase (ACAT) converts free (unesterified) cholesterol into cholesteryl fatty acid esters through re-esterification; these cholesteryl fatty acid esters are the main component of foam cells [110]. 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 (ABCA1) transporter-mediated cholesterol efflux to lipid-deficient apolipoprotein (Apo) A-I. Macrophages in ABCA1-deficient mice indeed have reduced cholesterol efflux [111], while overexpression of ABCA1 in macrophages was associated with a substantial reduction in atherosclerosis [112]. A similar cholesterol efflux pathway to mature HDL exists via the scavenger receptor class B type I (SR-BI) or the ATP-binding cassette protein G1 (ABCG1) transporter [113, 114]; the expression of ABCG1 is reduced by AGEs produced by diabetic ECs (see above). Finally, apoA-I binding protein (AIBP)-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 [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 likely resembles the metabolism of highly activated ECs, since the tumor-induced switch from quiescence to proliferation and migration during sprouting is metabolically taxing. Normal, quiescent ECs display higher than expected glycolysis rates in order to generate sufficient energy to

maintain crucial functions (*eg* tight barrier function in certain vascular beds) [3, 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 [117, 118] and the capability of (tumor-derived) VEGF to induce PFKFB3 expression [10] (Fig. 3A). In this respect, ECs and cancer cells are highly alike [10] given that most cancer cells are highly glycolytic (Warburg effect [119]). Furthermore, both cell types co-compartmentalize glycolytic enzymes with actin-rich regions in invading structures such as filopodia (ECs) and invadopodia [120] (cancer cells) to ensure efficient energy production required for motility and invasion [10].

Although the exact role of EC glutamine metabolism and the relative contribution of the two GLS isoforms (GLS1 *vs* GLS2) is undetermined, blocking GLS causes reduced EC proliferation and increased senescence [33], which suggests a crucial role for glutamine in tumor vessel sprouting, further supported by the notion that glutamine is the most abundant amino acid and a readily available carbon and nitrogen source. Cancer cells from their side have indeed rewired their glutamine metabolism to maximize glutamine carbon flux towards replenishment of the TCA cycle and nitrogen usage for nucleotide, amino acid 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' [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 (α-KG) into citrate for *de novo* lipid synthesis [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 non-essential amino acid 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 [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 [124, 125].

Ammonia, produced during the first two steps of glutaminolysis (deamination of glutamine to glutamate and glutamate to α-KG), is an auto- and paracrine inducer of autophagy [126]. In co-culture 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 [127]. *In vitro* glutamine deprivation causes osteosarcoma and lung cancer cells to excrete the pro-inflammatory chemokine interleukin-8 (IL-8), which has pro-angiogenic activity [128]. An apparent ER stress and depletion of TCA intermediates underlies this phenomenon; treatment with dimethyl α-KG replenished the TCA and abrogated IL-8 excretion[129]. Although the *in vivo* relevance of these findings remains to be determined, they exemplify how cancer cell metabolism instructs the stromal component (*cq* 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 upon proliferation [130]. CAFs have increased glycolysis rates to sustain a very peculiar relationship with cancer cells. The activity of oxygen-sensing prolyl-hydroxylase domain (PHD) proteins in CAFs is inhibited by high ROS levels coming from neighboring cancer cells. Subsequent hypoxia-inducible factor (HIF)-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 [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 [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 (Fig. 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 upon 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 [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 non-responsiveness and reduced effector function normally caused by constant antigen exposure (*cfr* chronic inflammation), and increase the expression of the immune-inhibitory programmed death receptor 1 (PD1) and the cytotoxic T-lymphocyte antigen-4 (CTLA4), ligation of which has been shown to reduce glycolysis [135, 136]. Lactate excreted by cancer cells promotes IL-17A production, which negatively regulates T cell-mediated anti-tumor mechanisms [137]. Although the metabolism of tumor associated macrophages (TAMs) 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 [12, 138] 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 [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 [10, 13]. The current approach in tumor angiogenesis relies on blocking VEGF or its receptors; a 'growth factor-centric' treatment that suffers from tumor-based escape mechanisms (*ie* use of alternative additional growth factors to induce excess angiogenesis), leading to resistance [140]. The recent data on how the glycolysis regulator PFKFB3 in ECs controls vessel sprouting in parallel to genetic signaling [10] have generated seminal follow-up studies showing the advantage of (chemical) inhibition of PFKFB3 in treating pathological angiogenesis [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-propen-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 [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 KLF2 represses the expression of PFKFB3 to ensure the exact glycolytic flux required to maintain the ECs (phalanx cells) quiescent [14]. Given the high prevalence of atherosclerotic plaque formation at sites where disturbed flow impacts the ECs (*eg* bifurcations, the aortic lesser curvature) and possibly causes reduced KLF2 expression (reviewed in ref [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 HMG-CoA reductase blocking statins, for example, are among the most often prescribed drugs world-wide [101]. Interestingly, statins have additional beneficial effects such as inhibiting inflammatory processes but also a direct protective effect on the endothelium by increasing NO production (see above) [143]. Even though they do mostly not outweigh the benefits, side effects such as myopathy, (possibly) increased diabetes incidence and statin intolerance, are linked to statin treatment [101]. Furthermore, by blocking HMG-CoA reductase in the mevalonate pathway, statins reduce the availability of farnesyl pyrophosphate, a precursor for Coenzyme Q10, an important antioxidant and co-factor for eNOS in ECs [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 nano-carriers which use EC-expressed inflammation markers as landing platform are a promising strategy to deliver possible drugs to the atherosclerotic lesion [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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Non-standard abbreviations and acronyms

α-KG	α-ketoglutarate
(ox)LDL	(oxidized) low-density lipoprotein
(non-)oxPPP	(non-)oxidative pentose phosphate pathway
(V)SMC	(vascular) smooth muscle cell
1,3BPG	1,3-bisphosphoglycerate
3DG	3-deoxyglucosone
3PG	3-phosphoglycerate
3PO	3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one
AA	amino acid
ABCA1	ATP-binding cassette protein A1
ABCG1	ATP-binding cassette protein G1
ACAT	acyl-CoA:cholesterol acyltransferase
ACC	acetyl-CoA carboxylase
acetyl-CoA	acetyl-coenzyme A
ADMA	asymmetric dimethyl arginine
AGE	advanced glycation end product
AGXT2	alanine-glyoxylate aminotransferase 2
AIBP	ApoA-I binding protein
AMPK	adenosine monophosphate activated protein kinase
Apo	apolipoprotein
AR	aldose reductase
ATP	adenosine triphosphate
BH_2	7,8-dihydrobiopterin
BH_4	tetrahydrobiopterin
CAF	cancer associated fibroblast
CD36	cluster of differentiation 36
CHD	coronary heart disease
CPT-1	carnitine palmitoyltransferase-1

CTLA4	cytotoxic T-lymphocyte antigen-4
DAG	diacylglycerol
DDAH	dimethyl-arginine dimethyl-aminohydrolase
DHAP	dihydroxyacetone phosphate
DHFR	dihydrofolate reductase
Dll4	Delta like 4
EC	endothelial cell
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
ETC	electron transport chain
F1,6P$_2$	fructose-1,6-bisphosphate
F2,6P$_2$	fructose-2,6-bisphosphate
F6P	fructose-6-phosphate
FA(BP)	fatty acid (binding protein)
FAD	flavin adenine dinucleotide
FAO	fatty acid oxidation
FMN	flavin mononucleotide
G3P	glyceraldehyde-3-phosphate
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAT	glutamine fructose-6-phosphate amidotransferase
GGPP	geranylgeranyl pyrophosphate
GLS	glutaminase
GlucN6P	glucosamine-6-phosphate
GLUT	glucose transporter
GS/GLUL	glutamine synthetase
GSH	reduced glutathione
GSSG	oxidized glutathione
GTP	guanosine triphosphate

GTPCH	GTP cyclohydrolase
HBP	hexosamine biosynthesis pathway
hCYS	homocysteine
HDL	high-density lipoprotein
HIF	hypoxia-inducible factor
HMG-CoA	hydroxymethylglutaryl coenzyme A
IL	interleukin
iNOS	inducible nitric oxide synthase
KLF2	Krüppel-like factor 2
LDH	lactate dehydrogenase
LPL	lipoprotein lipase
MET	methionine
mTHF	5-methyltetrahydrofolate
MTHFR	methylenetetrahydrofolate reductase
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor κB
NICD	Notch intracellular domain
nNOS	neuronal nitric oxide synthetase
NO	nitric oxide
OAA	oxaloacetate
OXPHOS	oxidative phosphorylation
PARP1	polyADP-ribose polymerase 1
PD1	programmed death receptor 1
PDGF-B	platelet derived growth factor B
PDGF-Rβ	platelet derived growth factor receptor β
PFK1	phosphofructokinase-1
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3
PHD	prolyl hydroxylase domain
PKC	protein kinase C
PRMT	protein arginine N-methyltransferase
R5P	ribose-5-phosphate
RAGE	receptor for advanced glycation end products

ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
Ru5P	ribulose-5-phosphate
SAM	S-adenosylmethionine
SDH	sorbitol dehydrogenase
SLC1A5	solute carrier family 1 member 5
SmgGDS	small GTP-binding protein GDP dissociation stimulator
SNP	single nucleotide polymorphism
SR-B1	scavenger receptor class B type I
TAM	tumor associated macrophage
TCA	tricarboxylic acid
THF	tetrahydrofolate
UDP-glucNAc	uridine diphosphate N-acetylglucosamine
VEGF(R)	vascular endothelial growth factor (receptor)

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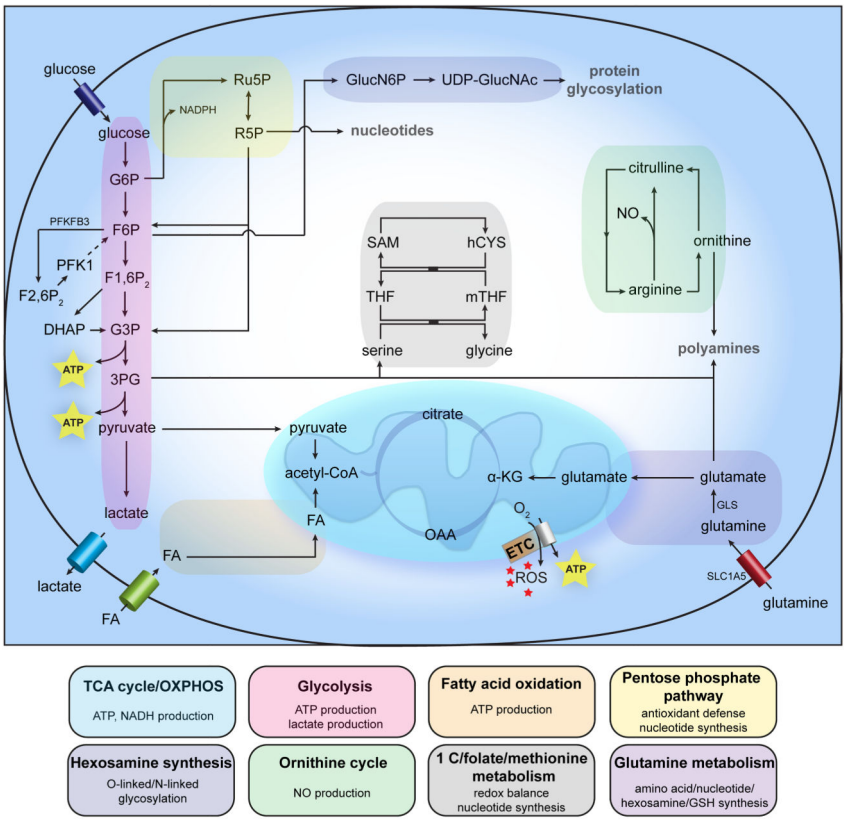

Figure 1. General metabolism in healthy ECs

Schematic and simplified overview of general EC metabolism. Abbreviations used: 3PG: 3-phosphogylcerate; α-KG: α-ketoglutarate; ATP: adenosine triphosphate; DHAP: dihydroxyacetone phosphate; ETC: electron transport chain; $Fu1,6P_2$: fructose-1,6-bisphosphate; $F2,6P_2$: fructose-2,6-bisphosphate; F6P: fructose-6-phosphate; FA: fatty acid; G3P: glyceraldehyde-3-phosphate; G6P: glucose-6-phosphate; GLS: glutaminase; GlucN6P: glucosamine-6-phosphate; hCYS: homocysteine; mTHF: 5-methyltetrahydrofolate; NADPH: nicotinamide adenine dinucleotide phosphate; NO: nitric oxide; OAA: oxaloacetate; PFK1: phosphofructokinase-1; PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; R5P: ribose-5-phosphate; ROS: reactive oxygen species; Ru5P: ribulose-5-phosphate; SAM: S-adenosylmethionine; SLC1A5: solute carrier family 1 member 5; THF: tetrahydrofolate; UDP-GlucNAc: uridine diphosphate N-acetylglucosamine.

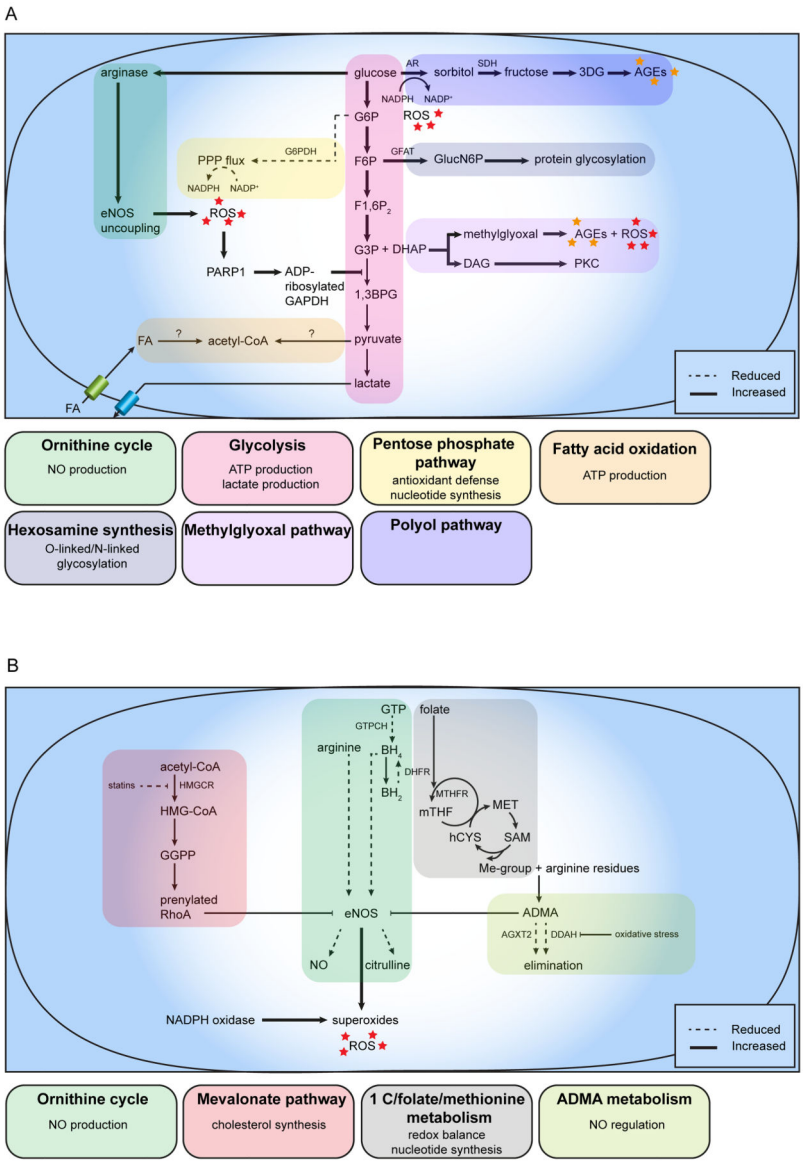

Figure 2. Metabolic pathways involved in disease characterized by EC dysfunction

A, In diabetes, hyperglycemia triggers mainly increased ROS production through eNOS uncoupling and PPP impairment resulting in stalled glycolytic flux with glycolytic intermediates being diverted into alternative metabolic pathways leading to additional excess ROS and AGEs production. **B,** Atherosclerosis is characterized at a metabolic level mainly by eNOS uncoupling resulting in excess ROS production and loss of NO-dependent vasodilation. Abbreviations used: as in Figure 1. 1,3BPG: 1,3-bisphosphoglycerate; 3DG: 3-deoxyglucosone; ADMA: asymmetric dimethyl arginine; AGE: advanced glycation end product; AGXT2: alanine-glyoxylate aminotransferase; AR: aldose reductase; BH_2: 7,8-dihydrobiopterin; BH_4: tetrahydrobiopterin; DAG: diacylglycerol; DDAH: dimethyl-arginine dimethyl-aminohydrolase; DHFR: dihydrofolate reductase; eNOS: endothelial nitric oxide synthase; G6PDH: glucose-6-phosphate dehydrogenase; GADPH: glyceraldehyde-3-phosphate dehydrogenase; GFAT: glutamine fructose-6-phosphate

amidotransferase; GGPP: geranylgeranyl pyrophosphate; GTP: guanosine triphosphate; GTPCH: GTP cyclohydrolase; HMG-CoA: hydroxymethylglutaryl coenzyme A; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; MET: methionine; MTHFR: methylenetetrahydrofolate reductase; PARP1: polyADP-ribose polymerase 1; PKC: protein kinase C; PPP: pentose phosphate pathway; SDH: sorbitol dehydrogenase.

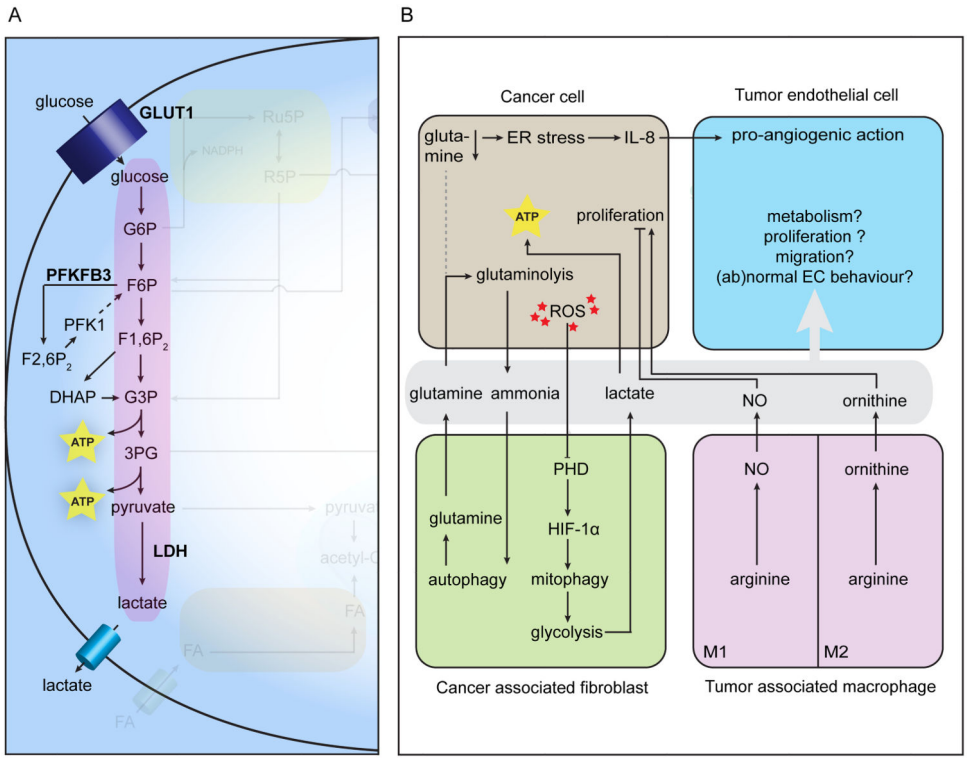

Figure 3. Metabolic pathways in tumor vessels and metabolic interactions between cancer and stromal cells

A, ECs in tumor vasculature are presumably characterized by increased glycolysis. **B,** Metabolic interactions between cancer and stromal cells. Abbreviations used: as in Figures 1 and 2. ER: endoplasmic reticulum; GLUT: glucose transporter; HIF: hypoxia-inducible factor; IL-8: interleukin-8; LDH: lactate dehydrogenase; PHD: prolyl hydroxylase domain.