Metabolic Adaptations in Diabetic Endothelial Cells

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In healthy individuals, the endothelium plays a fundamental role in normal health in the maintenance of vascular homeostasis. Endothelial cell (EC) dysfunction results in the development of several pathologies. In diabetes, in particular, sustained hyperglycemia, a characteristic of diabetes, contributes to EC dysfunction and consequently mediates the pathogenesis of diabetes-associated micro- and macrovasculopathies. Hyperglycemia-induced EC dysfunction is triggered by elevated levels of oxidative stress derived from several mechanisms, with the mitochondria as a key source, and is exacerbated by a subsequent hyperglycemia-induced self-perpetuating cycle of oxidative stress and aberrant metabolic memory. Recent reports have highlighted the importance of metabolic pathways in EC and suggested the therapeutic potential of targeting EC metabolism. This review focuses on the current knowledge regarding differences in the metabolism of healthy ECs vs. diabetes-associated dysfunctional ECs, and outlines how EC metabolism may be targeted for therapeutic benefit. (Circ J 2015; 79: 934–941)

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Endothelial Dysfunction and Diabetes

Blood vessels together comprise one of the largest organs in the human body, with a total vascular area estimated to be 800–1,000 m² in adults, an area greater than 3 tennis courts. Once considered a mere selectively permeable interface between the blood and surrounding tissues, blood vessels are now recognized to be crucial for maintaining tissue homeostasis. The endothelium, a single layer of endothelial cells (EC) that lines the blood vessel lumen, controls vessel function and plays a fundamental role in normal health. Indeed, EC dysfunction contributes to the etiology of more diseases than any other tissue in the body. Type II diabetes is a chronic metabolic disorder and has become a public health issue of epidemic proportions, linked to the consumption of vast quantities of energy-dense food coupled with increasingly sedentary lifestyles, globally. Diabetes has a high and increasing prevalence of 8.3% globally (in 2013) according to the International Diabetes Federation, and by 2035, this number is estimated to almost double. Diabetes is predominantly characterized by hyperglycemia (high blood glucose) due to a deficiency of (type I) or resistance to (type II) insulin, which contributes to EC dysfunction. EC dysfunction tilts the physiological balance towards vasoconstrictive, pro-inflammatory and pro-thrombotic effects and has attracted considerable research interests, given that EC dysfunction is an early and key event in the pathogenesis of diabetes-associated micro- and macrovasculopathies, and promotes the accelerated pathogenesis of atherosclerosis, a major complication of diabetes. Microvascular complications include retinopathy, nephropathy, and neuropathy, which eventually develop into blindness, renal failure, and neurological dysfunction of organ systems, respectively. Macrovascular disorders include coronary artery disease, peripheral vascular disease and ischemic stroke. Diabetes is also recognized as an independent risk factor for cardiovascular disease, even when under glycemic control.

While most research has been focused on cellular and molecular mechanisms of endothelial dysfunction in diabetes, this review will focus on the current knowledge regarding the differences in the metabolism of healthy vs. diabetes-associated dysfunctional ECs. EC metabolism is an emerging but understudied therapeutic target, but recent reports have indicated the importance of glycolysis, a key metabolic pathway, in the regulation of EC physiological functions and have highlighted the therapeutic potential of targeting EC metabolism. From the perspective that metabolic aberrations in ECs can mediate the primary complications associated with the pathogenesis of diabetic vascular complications (and other pathologies), this review will also detail how EC metabolism may be targeted for therapeutic benefit.

Endothelial Metabolism in Health

In adults, ECs remain quiescent and function to maintain barrier function and tissue homeostasis, but can respond to angiogenic growth factors induced by stimuli such as hypoxia, nutrient deprivation or tissue damage to re-vascularize tissues to restore oxygen and nutrient delivery, and serve as a conduit for immune cell infiltration in normal wound healing processes. This switch to angiogenesis is accompanied by a “metabolic switch”, which relies on the enhancement of glycolytic flux. Glycolysis involves the breakdown of glucose, producing 2
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of metabolic compensation during insufficiency. Other metabolic pathways have also been implicated in normal EC function. The pentose phosphate pathway (PPP) is a side pathway of glycolysis, which produces NADPH (used in redox homeostasis to convert oxidized glutathione [GSSG] to its reduced form [GSH], a potent antioxidant) and ribose-5-phosphate (used in the synthesis of lipids, nucleotides and histidine). The metabolism of glutamine has been shown to be essential for EC function, given that inhibition of glutaminase, the enzyme that converts glutamine to glutamate, induces senescence in ECs, although it remains to be determined whether this is due to entry into the TCA cycle, or via ornithine synthesis, necessary for the production of polyamines and nitric oxide (NO).

In addition, while fatty acids (FA) can serve as fuels for energy production in oxidative cell types such as skeletal muscle or cardiomyocytes, the reliance of ECs on glycolysis for ATP generation suggests they may metabolize fatty acids for other purposes. FA can be metabolized in the mitochondria by a process called fatty acid β-oxidation (FAO), which produces acetyl-CoA that can enter the tricarboxylic acid (TCA) cycle. Whether this may be for energy generation, redox homeostasis, or other functions remains to be determined, but ECs increase their fatty acid β-oxidation (FAO) flux in vivo upon glucose deprivation, suggesting there may be a degree of metabolic compensation during insufficiency.

Other metabolic pathways have also been implicated in normal EC function. The pentose phosphate pathway (PPP) is a side pathway of glycolysis, which produces NADPH (used in redox homeostasis to convert oxidized glutathione [GSSG] to its reduced form [GSH], a potent antioxidant) and ribose-5-phosphate (used in the synthesis of lipids, nucleotides and histidine). The metabolism of glutamine has been shown to be essential for EC function, given that inhibition of glutaminase, the enzyme that converts glutamine to glutamate, induces senescence in ECs, although it remains to be determined whether this is due to entry into the TCA cycle, or via ornithine synthesis, necessary for the production of polyamines and nitric oxide (NO).

The hexosamine biosynthetic pathway (HBP) is another side branch of glycolysis, which produces N-acetylglucosamine necessary for N-linked and O-linked glycosylation. Flux through this pathway facilitates the glycosylation of key angiogenic molecules such as vascular endothelial growth factor receptor 2 and Notch, among others, and perturbation of glycosylation impairs angiogenesis, although the effects are context dependent and precise mechanisms remain to be elucidated.
Overall, numerous metabolic pathways play key roles in physiological angiogenesis (Figure 1). We further discuss the primary perturbations to redox homeostasis and generation of reactive oxygen species (ROS) in the context of these metabolic pathways in diabetes-associated EC dysfunction.

Metabolism in Diabetes-Associated EC
The progression of diabetes-associated vasculopathies is highly dependent on the severity and duration of hyperglycemia, a hallmark of diabetes (both types I and II). Consequently, hyperglycemia is postulated to mediate these diabetes-associated vasculopathies through increased oxidative stress originating from both the cytosol and mitochondria in ECs. ECs are capable of producing ROS from a variety of enzymatic sources. Mechanisms contributing to elevated oxidative stress in diabetic ECs are overviewed in Figure 2, and include: (1) hyperglycemia-induced elevated ROS (cytosolic or mitochondria-derived); (2) hyperglycemia-induced diversion of glycolytic flux into alternative metabolic pathways; (3) hyperglycemia-induced block of PPP; (4) hyperglycemia-associated formation of advanced glycation end products (AGEs); and (5) the protein kinase C (PKC) pathway.

While a considerable body of evidence in humans indicates that endothelial dysfunction is closely associated with the vasculopathies reported in diabetes, many of the findings discussed here are observations from in vitro experiments and, although they have increased our understanding of the alterations to EC metabolism in response to hyperglycemia, definitive in vivo verification is required before the possibility of clinical translation.

### Hyperglycemia Increases ROS

**Cytosolic ROS**
Hyperglycemia-induced increases in ROS can be derived from auto-oxidation of glucose or hyperglycemia-activated NADPH oxidases. Increased ROS, derived from xanthine or NADPH oxidases, can result in endothelial NO synthase (eNOS) uncoupling (a process whereby eNOS fails to produce NO and citrulline and instead produces ROS), given that superoxide anions can react directly with NO yielding peroxynitrite (ONOO−), ultimately reducing the levels of NO. eNOS uncoupling results in increased oxidative stress due to the generation of superoxide instead of NO, creating a vicious cycle. Under normal conditions, eNOS-derived NO mediates endothelium-dependent vasodilation, required for normal vascular homeostasis, and inhibits events promoting atherosclerosis. Not surprisingly, diabetic patients show an impairment of endothelium-dependent vasodilation. eNOS uncoupling was also recently shown to participate in EC dysfunction in diabetic mouse models, and to mediate peripheral neuropathy in Zucker diabetic rats. Other mechanisms leading to eNOS uncoupling include reduced availability of the NO-precursor l-arginine and co-factor tetrahydrobiopterin (BH4), aberrant

![Figure 2](image-url). Elevated oxidative stress and metabolic perturbations in diabetic endothelial cells (ECs). (A) Diabetic ECs have hyperglycemia-induced elevated reactive oxygen species (ROS) derived from a variety of intracellular sources. (B) Hyperglycemia-induced elevated ROS in ECs activates and perturbs several metabolic pathways resulting in a self-perpetuating cycle of oxidative stress. Abbreviations as in Figure 1. AGEs, advanced glycation end products; AMPK, AMP-activated protein kinase; BH4, tetrahydrobiopterin; eNOS, endothelial NO synthase; GAPDH, glyceraldehyde-3-phosphate; PARP1, polyADP-ribose polymerase-1; PGC1α, peroxisome proliferator activated receptor gamma coactivator 1 α; PKC, protein kinase C.
O-glycosylation via the HBP,30 and increased AGEs.31,32

Mitochondrial-Derived ROS
Recent evidence also suggests that hyperglycemia causes endothelial mitochondriopathy, highlighted by aberrations in mitochondrial biogenesis and autophagy (resulting in the accumulation of damaged mitochondria), impaired mitochondrial function and increased mitochondrial fragmentation.33,34 Given that ECs produce their energy primarily through anaerobic glycolysis (and not through oxidative phosphorylation [OxPhos] in the mitochondria), endothelial mitochondria are essential for Ca2+ homeostasis and the generation of ROS, and are considered as the sensors and initiators of EC death. Due to dysfunctional mitochondria, ROS production is further increased and there is a Ca2+ overload, resulting in the exacerbation of oxidative stress in mitochondria and subsequent increased EC dysfunction or apoptosis.35 Vascular damage is linked with hyperglycemia-induced increase in ROS production by the mitochondrial electron transport chain in ECs.35 Streptozotocin-induced diabetic mouse models have shown a major role for oxidative stress-mediated damage in mitochondrial DNA in the pathogenesis of diabetic nephropathy.36 In other cells, increased OxPhos-linked ROS production in mitochondria is also believed to be a key mediator of hyperglycemic tissue injury, such as nephropathy.37

Mechanistically, the metabolic regulators AMP-activated protein kinase (AMPK) and peroxisome proliferator activated receptor gamma coactivator 1α (PGC1α) have been closely implicated in hyperglycemia-induced ROS effects. For instance, NO has been shown to mediate protection against ROS via upregulation of PGC1α.38,39 and PGC1α has been shown to regulate mitochondrial antioxidant defense in ECs.40 High glucose, however, induces PGC1α expression, resulting in impaired EC migration and vasculogenesis, and the antioxidant N-acylcysteine blocks the induction of PGC1α.41 Whether these divergent activities of PGC1α are due to different effects on quiescent vessels vs. growing vessels, or due to other reasons remains to be determined.

Interestingly, activation of AMPK in mature ECs and endothelial progenitor cells reduced hyperglycemia-induced ROS generation by reducing NADPH oxide activity,42 increasing the expression of mitochondrial-specific manganese dismutase, a mitochondrial antioxidant,43 and promoting mitochondrial biogenesis,44 despite the aforementioned role of dysfunctional mitochondria in ROS production. Endothelial-specific activation of AMPK also prevents diabetes-induced EC dysfunction.45 Incidentally, AMPK activators (such as the drug metformin) have the potential to improve mitochondrial oxidative stress-induced EC dysfunction.

Hyperglycemia Diverts Glycolytic Flux to Alternative Metabolic Pathways
Elevated ROS (through the aforementioned mechanisms), together with elevated reactive nitrogen species, lead to the activation of polyADP-ribose polymerase-1 (PARP1), a DNA repair mechanism enzyme triggered upon oxidative DNA damage (DNA single-strand breaks), and the subsequent inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) through ribosylation.46–48 GAPDH is a glycolytic enzyme whose activity is essential for maintenance of glycolytic flux, and hence inhibition of GAPDH results in the accumulation of glycolysis pathway intermediates and their subsequent diversion into 3 alternative metabolic pathways known to be altered in ECs by hyperglycemia: (1) the polyl pathway; (2) the HBP; and (3) the glycation pathway.

Experimentally, hyperglycemia-induced activation of each of these pathways was abolished by blocking PARP1 activity in aortic ECs using competitive PARP1 antagonists.47 Therefore, oxidative stress is considered to be the initial event in EC dysfunction. In addition, pharmacological inhibition of PARP1 emerges as a potential approach for the experimental therapy of diabetic vascular dysfunction.

Polypeptide Pathway
Excess glucose is diverted to the polyl pathway, in which aldose reductase (ALR2) reduces glucose to sorbitol in an NADPH-dependent reaction.48 Such consumption of NADPH results in the oxidation of GSH to GSSG, resulting in decreased superoxide scavenging and subsequently enhancing oxidative stress. In addition, sorbitol dehydrogenase can convert sorbitol into fructose, generating 3-deoxyglucosone (3-DG), a highly reactive dicarboxyl compound, contributing to the non-enzymatic generation of noxious AGEs.49 ALR2 was shown to be responsible for the early events in the pathogenesis of diabetic retinopathy (leading to a cascade of retinal lesions, including blood-retinal barrier breakdown, loss of pericytes, neovascularization, neuro-retinal apoptosis, etc) in diabetic db/db mice.51

Hexosamine Biosynthetic Pathway
Excess glucose enters the HBP via fructose-6-phosphate (F6P), where it is converted into glucosamine-6-phosphate and subsequent uridine 5’-diphosphate N-acetylglucosamine (UDP-GlcNAc) via enzymatic activity of glutamine:fructose-6-phosphate amidotransferase. Under normal conditions, UDP-GlcNAc is crucial for protein glycosylation, but under hyperglycemic conditions, protein glycosylation is dysregulated and results in altered protein expression. For example, under hyperglycemic conditions, O-glycosylation deregulates eNOS activity.50

Glycation Pathway
The glycolytic intermediates glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) are pushed towards the production of highly reactive dicarboxyl compounds such as methylglyoxal, glyoxal (also generated from glucose autoxidation) and 3-DG in the glyoxalase system. These reactive aldehydes can react with and modify (through glycation) both DNA and (cytosolic and mitochondrial) proteins, leading to the non-enzymatic formation of toxic AGEs.

In ECs, methylglyoxal is most likely the main reactive aldehyde formed.52,53 Several reports have implicated elevated methylglyoxal in diabetic patients in diabetic-associated vasculopathies.54–56 Methylglyoxal also triggers eNOS uncoupling, in vitro, associated with superoxide formation and BH4 depletion, but the exact mechanism remains poorly defined.54 Recently, a novel mechanism was proposed via which methylglyoxal exposure results in alterations in EC redox status, via the inhibition of NADPH-generating enzymes (G6P and 2-deoxyglucose-6-phosphate), thereby reducing thiol and GSH.57 Depleted GSH results in decreased rates of reactive aldehyde detoxification and superoxide scavenging and thus intensified damaging oxidative stress. Rats that systemically and moderately overexpressed glyoxalase I, a key enzyme detoxifying methylglyoxal, showed reduced endothelial glycative and oxidative stress and attenuated EC dysfunction.58 Remarkably, normalization of glucose level in diabetic patients did not entirely prevent elevation of methylglyoxal and subsequent diabetic complications, and progressive ROS.
production and AGE accumulation persisted after glucose normalization.59,60 Such observations lead to the concept of “metabolic memory” (discussed in the next section).

Given that inhibition of hyperglycemia-induced oxidative stress prevents the hyperglycemia-associated activation of the 3 aforementioned pathways, oxidative stress is considered to be the initial event in EC dysfunction. But, because these pathways result in increased levels of damaging end products (ROS and AGEs) this creates a hyperglycemia-induced self-perpetuating cycle of oxidative stress.

Hyperglycemia-Induced PPP Block
G6P, F6P and G3P are glycolytic intermediates (Figure 1) that either feed into or are generated out of the PPP-associated reactions depending on substrate availability. Thus, under hyperglycemic conditions, it was postulated that the PPP could be protective by diverting the aforementioned excess glycolytic metabolites away from the 3 alternative metabolic pathways, thereby decreasing the levels of damaging end products. Additionally, increased flux through the PPP could result in increased levels of NADPH and GSH redox cycle, thereby increasing the antioxidant capacity of ECs. Hyperglycemia, however, was shown to reduce glucose-6-phosphate dehydrogenase (G6PD)-mediated entry of glucose into the PPP, resulting in increased oxidative stress and decreased NO bioavailability in ECs.18 Restoring PPP flux, possibly through activating G6PD, might be an attractive target to reduce oxidative stress in the context of hyperglycemia.

Hyperglycemia-Associated Formation of AGEs
The accumulation of AGEs associated with hyperglycemia results in elevated oxidative stress (elevated superoxide, hydroperoxide and ONOO-) and contributes to impaired angiogenesis, apoptosis and upregulation of inflammatory and tissue-injury-provoking molecules, mediated at least in part through the interaction of AGEs with the receptor for AGEs and subsequent NFκB activation.65 AGEs also modify collagen-type proteins through protein cross-linking to decrease vessel elasticity, cell adhesion and matrix-matrix interaction, contributing to the progression of diabetic nephropathy.

PKC Pathway
Under conditions of hyperglycemia and high levels of circulating free fatty acids (FFA), excess DHAP (a glycolytic intermediate) fuel de novo diacylglycerol (DAG) synthesis, which subsequently activates PKC.66 DAG and PKC were found to be significantly elevated in diabetic animal models, and PKC activation contributes to diabetic-related macrovascularopathies, because excess DAG negatively affects blood flow, vasodilation, vascular permeability, and fibrosis.66 In addition, PKC results in the activation of vascular NADPH oxidases and alterations in eNOS expression.67 Besides hyperglycemia and circulating FFA, the accumulation of AGEs can also result in the activation of the PKC and MAPK pathways, leading to the dephosphorylation of platelet-derived growth factor receptor β and subsequent apoptosis of perivascular mural pericytes.67 Importantly, loss of pericyte coverage is one of the earliest signs of diabetic retinopathy.68

Metabolic Memory
From the information given here, it is clear that hyperglycemia plays a key role in the induction of oxidative stress and subsequent endothelial dysfunction. Therefore, normalization of glucose level was thought to counteract these effects. In many cases, however, vascular dysfunction persisted even after restoration of glycemic control in diabetic patients.69,70 Metabolic (hyperglycemic) memory (a hypothesis suggesting that aberrant mechanisms associated with hyperglycemia induce stable modifications resulting in persistent EC dysfunction, even despite normalization of glucose) may explain why intensive glucose control regimens have failed to improve cardiovascular outcome in patients with diabetes type I.70 This poor reversibility of hyperglycemia-induced modifications has also been shown in EC-specific in vitro and in vivo studies.71,72

Implications and Perspectives
Overall, recent work has defined a role for EC metabolism in the regulation of physiological angiogenesis.13-15 While the role of other metabolic pathways remain to be investigated in normal endothelial function to provide a holistic roadmap, we can now begin to divert our attention to studying the metabolic alterations that occur in diabetes. Utilizing state-of-the-art 1C metabolic flux tracer assays,84 definitive evidence of the metabolic perturbations that may arise in the ECs in patients with diabetes is needed to further our understanding of the underlying complications that result in excess ROS, oxidative stress and EC dysfunction leading to vascular complications. The existence of metabolic memory could facilitate such metabolic studies in ECs in vivo and ex vivo. The production and involvement of ROS sources, however, vary across ECs from different parts of the vasculature.85-89 There is also a need to understand the underlying mechanisms mediating the variations in hyperglycemia-induced EC dysfunction across different vascular beds, given the increasing evidence that ECs in different tissues greatly differ,90 and that diabetes preferentially affects ECs in some but not in all vascular beds.10,19 Lastly, considering the disappointing outcomes of clinical trials using antioxidant therapies,92,93 a "prophylaxis rather than cure" approach or combination therapy may be required in the management of hyperglycemia-induced oxidative stress in diabetic ECs. A promising metabolic target may be aldose reductase, which has several inhibitors currently undergoing clinical trials, but
there is currently no specific treatment for vascular complications in diabetes. Another emerging target is anti-VEGF-B therapy, which has been shown to decrease plasma triglycerides and non-esterified FA, and normalize high-density lipoprotein:low-density lipoprotein ratios, along with lowering ins and non-esterified FA, and normalize high-density metabolism of ECs in the context of the micro- and macrovas-
to be determined. Finally, whether limiting aberrant pathways transporter for FA, or metabolize it for their own needs remains to be determined. Finally, whether limiting aberrant pathways generating ROS or enhancing metabolic pathways involved in redox homeostasis and ROS scavenging, targeting the cellular metabolism of ECs in the context of the micro- and macrovas-
culopathies associated with diabetes is an attractive target for future focus.

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