Chapter

Role of Subcellular ROS in Providing Resilience to Vascular Endothelium

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Abstract

For decades, elevated levels of reactive oxygen species (ROS) have been associated with the pathogenesis of cardiovascular diseases (CVD), including myocardial ischemia and infarction (MI). However, several large clinical trials failed to demonstrate beneficial outcomes in response to the global reduction of ROS in patients with underlying CVD. Recent studies from our and other labs showed that it is rather a critical balance between mitochondrial and cytosolic ROS than total ROS levels which determines resilience of coronary endothelial cells (EC). Here, we will discuss published and unpublished work that has helped elucidate the molecular mechanisms by which subcellular ROS levels, duration and localization modulate metabolic pathways, including glycolysis and oxidative phosphorylation, energy production and utilization, and dNTP synthesis in EC. These redox-regulated processes play critical roles in providing resilience to EC which in turn help protect existing coronary vessels and induce coronary angiogenesis to improve post-MI recovery of cardiac function.

Keywords: endothelial cell metabolism, angiogenesis, vascular endothelial growth factor (VEGF), nitric oxide, reactive oxygen species (ROS), glycolysis, dNTP, fatty acid oxidation

1. Introduction

A single layer of endothelial cells (ECs) that covers the vascular lumen and plexus exhibits great plasticity to adapt to environmental cues [1, 2]. It is fascinating how the vascular system, the largest organ system of the body, connects all organs to secure adequate nutrients and blood supply. For that reason, maintaining vascular homeostasis is crucial for the health of the cardiovascular system. In a healthy body, although the ECs are an intricate, dynamic system, they appear to be in a quiescent state [1]. In pathological conditions such as ischemia and infarction, ECs rapidly switch phenotype to form new vessels in a process known as sprouting angiogenesis [3]. Reactive oxygen species (ROS) are believed to play crucial roles in determining the phenotype and fate of EC in both physiological and pathological conditions. Recent work has shown that a critical balance between mitochondrial and cytosolic ROS levels, but not global ROS levels, modulates endothelial function, EC metabolism and angiogenesis, and thus determines resilience of coronary EC [4–8].

In this chapter, we will discuss the molecular mechanisms by which subcellular ROS modulate various metabolic pathways, regulate EC function and angiogenesis.

2. Reactive oxygen species in coronary endothelium

Previously, the pathology of cardiovascular diseases (CVD), including myocardial ischemia and infarction (MI), was believed to be associated with increased levels of ROS [4–8]. Recent studies show that it is rather a critical balance between cytosolic and mitochondrial ROS levels than total ROS levels which determine the resilience of coronary ECs in physiological as well as adverse conditions [6, 7, 9, 10].

ROS are produced in higher levels as a response to injuries by the cellular enzymes and mitochondria [8, 11]. ROS have been reported to contribute to the underlying pathology in almost all organs, and thus the notion that antioxidants would ameliorate pathological effects of ROS came into being. However, clinical trials failed to show beneficial effects of antioxidants in the treatment of CVD [12]. Other studies showed that decreased ROS levels had rather deleterious effects on CVD [6, 7]. Also, decreased ROS levels resulted in the inactivation of endothelial nitric oxide synthase (eNOS) and reduction in NO (nitric oxide) levels [11, 13]. Taken together, global reduction of ROS appeared to reduce endothelial resilience. It is crucial to note that ROS have paradoxical effects on ECs, and thus careful study of the levels, durations and sources of ROS while studying effects of ROS on EC will help advance our understanding of EC resilience during oxidative stress.

2.1 Source of reactive oxygen species in ECs

ROS are produced from different oxidoreductase enzymes and locations including NADPH oxidase, mitochondrial, xanthine oxidase, cytochrome P450 monooxygenase, and uncoupling of NOS [8, 11, 14]. In the vasculature, ECs rely on glycolytic pathways as their source of energy, thus NADPH oxidase enzymes appear to be the major source of ROS in both physiological and pathological conditions [14]. NADPH enzymes have different isoforms, and the major contributors are NOX1, NOX2, NOX4, and NOX5 [15, 16]. Recent studies showed importance of NOX2- and NOX4-derived ROS in endothelial survival or dysfunction, depending on their subcellular location and duration [8].

2.2 Endothelial NADPH oxidase as a major source of ROS in ECs

NADPH oxidase is an intracellular complex enzyme containing membrane-bound and cytosolic regulator subunits [14, 17, 18]. This enzyme produces ROS by transferring electrons from NAD(P)H to an oxygen molecule and is considered the major source of ROS in coronary endothelium. Distinct isoforms of NADPH enzymes have been shown to exhibit different physiological and pathological responses in vascular homeostasis.

NOX1 enzyme is primarily expressed in the vascular smooth muscle cells (VSMC) and it contributes to VSMC proliferation and migration [11, 19–21]. In disease conditions, NOX1 contributes to the impairment of endothelium-dependent vasorelaxation, as well as the augmentation of angiotensin II vasomotor response [11, 22, 23]. A study showed that NOX1-deficient mice attenuated the levels of ROS, neointimal growth, and migration. These findings suggest that the downregulation of NOX1 enzyme can prevent the formation of atherosclerotic plaque [15, 24]. Yet, further studies are warranted to explore the exact role of NOX1 in endothelial signaling.

In contrast, NOX2 enzyme has exhibited positive effects on coronary ECs. NOX2 enzyme stimulates the production of NO by the activation of AMPK-eNOS axis through Ca^{2+}–/calmodulin-dependent protein kinase kinase β (CaMKKβ) [6] resulting in coronary vasodilation, EC proliferation and migration. Although several studies support the beneficial effects of NOX2, they also exert detrimental effects on coronary EC depending on the duration of exposure. Short exposure of elevated ROS levels was associated with the previously mentioned pathway (i.e. CaMKKβ pathway). On the contrary, prolonged exposure of high ROS levels resulted in decrease bioavailability of NO, inactivation of mitochondrial antioxidant MnSOD [7, 8], and decreased EC proliferation and coronary vasodilatation.

NOX4 enzyme is abundant in human ECs [8] and produces H_2O_2 molecules rather than O_2^- [9, 11, 25–28]. NOX4 enzyme stimulates vascular angiogenesis through the activation of transforming growth factor β1 (TGF β1), and increases hemoglobin content [29]. NOX4-derived ROS cause vasodilation through endothelium hyperpolarization [30–32]. This occurs via the stimulation of endothelium Ca^{2+}-activated K^+ channel that causes the release of Ca^{2+} from the endoplasmic reticulum [29]. Additionally, NOX4 enzyme activates heme oxygenase-1 (HO-1), which confers a vascular protective response via different mechanisms [29]. Thus, therapeutic modalities that advocate for antioxidants in CVD needs careful consideration of the source and location of ROS.

Calcium-dependent NADPH oxidase, NOX5, is implicated in angiogenic response [33, 34]. It gets its name from its structure because it has an additional N-terminal region that binds to calcium [33]. This unique structure allows the enzyme activation through increased intracellular calcium. Similar to NOX4, NOX5 enzyme seems to produce predominantly H_2O_2 in ECs [24]. H_2O_2 has been implicated in the development of atherosclerotic plaque plausibly by increasing Ca^{2+} levels to promote eNOS-mediated NO synthesis and increasing nitroxide radicals [24]. One mechanism may include increased consumption of NO by ROS. Thus, it has been hypothesized that inhibition of NOX5 enzyme may show beneficial results by precluding oxidant injury to vascular EC.

NADPH enzyme isoforms have distinct locations and EC phenotypes. They have been shown to employ different physiological and pathological responses in vascular homeostasis. As discussed above, NOX1, NOX2, NOX4, and NOX5 are found in the vascular system and they contribute to endothelial resilience through several mechanisms. The roles of NADPH enzymes in physiological and pathological conditions have undergone a considerable evolution in recent years. However, further studies are necessary to deepen our understanding of their roles and contributions to EC resilience.

2.3 Endothelial mitochondrial ROS

Although oxidative phosphorylation in mitochondria play a major role in synthesizing energy in most tissues, EC primarily depends on anaerobic glycolysis for 85% of its ATP generation. ECs have fewer mitochondria and consume lower amounts of O2 than other cell types, and thus mitochondrial ROS are believed to be a minor source of ROS in EC in physiological conditions. However, recent studies demonstrated that sustained increase in NADPH oxidase-derived cytosolic ROS may affect the levels of mitochondrial ROS and thus mitochondrial function in EC [6–8].

3. Metabolic pathways in ECs

EC metabolism plays an important role in facilitating cellular proliferation and migration during the process of angiogenesis. Alterations in metabolic pathways

are necessary to provide energy supplies in the most efficient way under certain circumstances that induces blood vessel sprouting such as hypoxia. In addition, these alterations mediate the formation of important molecules that are essential for cytoskeletal remodeling during the process. This section highlights some of these metabolic pathways and their role in angiogenesis.

3.1 Glycolysis

Glycolysis is a major metabolic pathway that is utilized for energy production through the anaerobic oxidation of glucose molecules [35, 36]. It is the major source of ATP in ECs. Glycolysis involves consumption of 2 ATP molecules, and the end products include 4 ATP, 2 NADH and 2 pyruvate molecules (**Figures 1** and **2**). Subsequently, pyruvate can be shifted to the mitochondria and metabolized into acetyl-CoA to be used in the tricarboxylic acid cycle (TCA). The substrates and products of this process are as follows:

$$\text{Glucose} + 2\,\text{NAD}^+ + 2\,\text{ADP} + 2\,\text{Pi} \rightarrow 2\,\text{Pyruvate} + 2\,\text{NADH} + 2\,\text{H}^+ + 2\,\text{ATP} + 2\,\text{H}_2\text{O}.$$

Glycolysis occurs in the cytosol, and the process does not require oxygen (anaerobic), therefore it constitutes the primary source of energy in cells that lack mitochondria (e.g. red blood cells). In addition, glycolysis is the main source of pyruvate, which is converted to acetyl-CoA to be utilized in the TCA cycle in cells

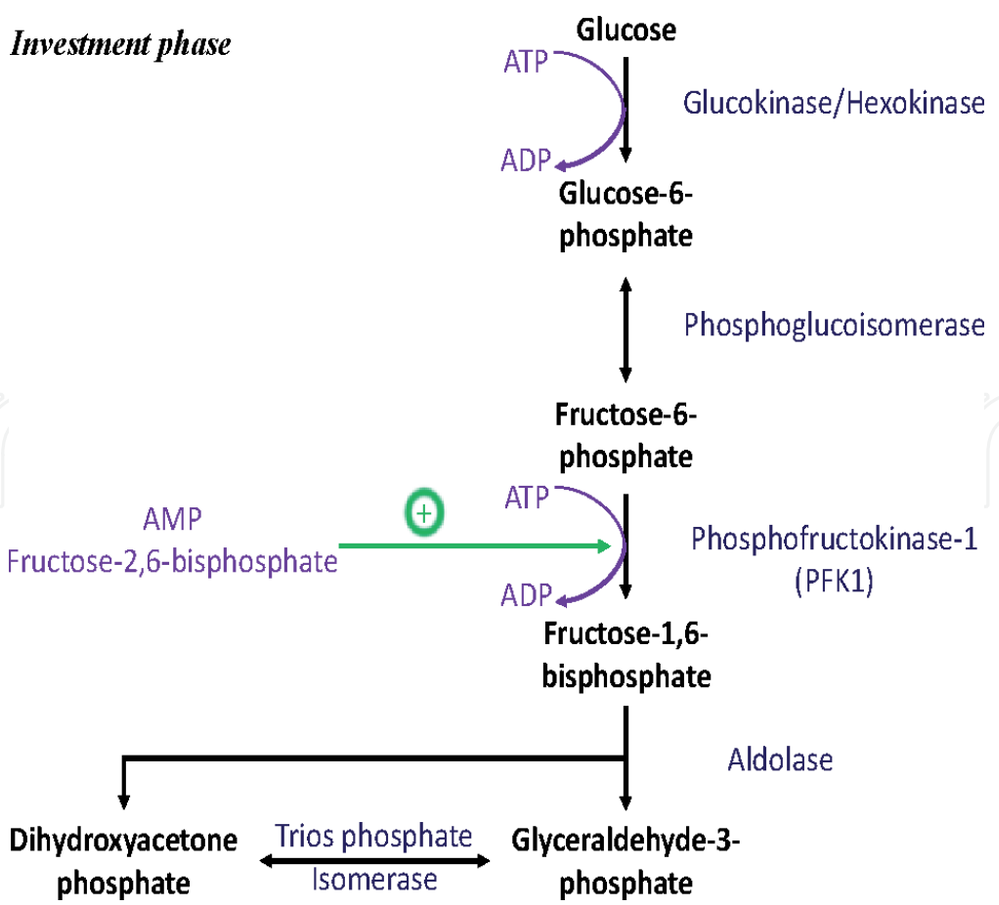

Figure 1.
The investment phase of glycolysis and regulation of the rate limiting PFK1 enzyme by fructose-2, 6-bisphosphate and AMP.

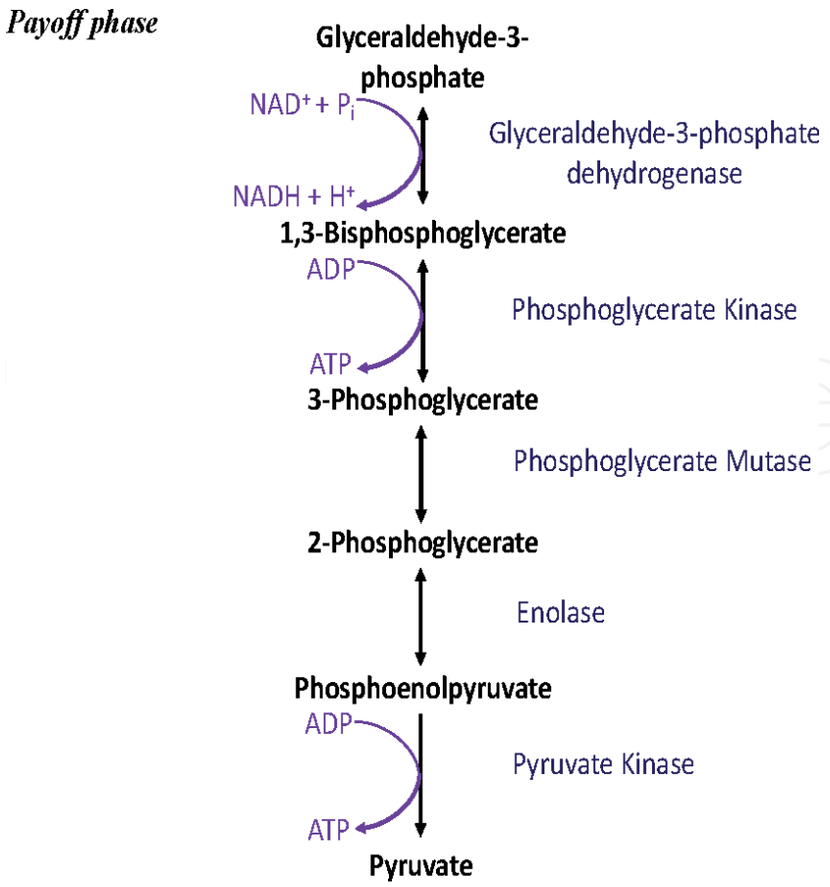

Figure 2.
The payoff phase of glycolysis.

that use oxidative phosphorylation (aerobic respiration) as a primary source of ATP. Also, glycolysis is a more efficient source of energy in periods of hypoxia and ischemia when oxygen supply becomes scarce.

3.1.1 Mechanisms of glycolysis

The first step in glycolysis constitutes the *investment* phase of glycolysis, in which 2 ATP molecules are consumed as shown in **Figure 1**. It involves trapping of the glucose molecule inside the cell via phosphorylation into glucose-6-phosphate [35, 36]. This reaction is catalyzed by glucokinase in the liver and pancreatic β cells, or a hexokinase enzyme in the rest of body cells. It also involves the transfer of a phosphate group from an ATP molecule. Next, glucose-6-phosphate is converted to fructose-6-phosphate by an isomerase. This is followed by the rate-limiting step of glycolysis, which involves the phosphorylation of fructose-6-phosphate into fructose-1-6-bisphosphate by phosphofructokinase 1 (PFK1). This step is critical in the glycolytic pathway and the PFK1 enzyme is highly regulated by multiple factors that determine the direction of the reaction. Fructose-1-6-bisphosphate is subsequently converted by an aldolase into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P).

The following reactions constitute what can be referred to as the *payoff* phase of glycolysis. It is also important to remember that at this stage, we have two 3-carbon molecules per 1 glucose molecule as shown in **Figure 2**. G3P is converted to 1-3-diphosphoglycerate, generating NADH in the process. 1-3-diphosphoglycerate then loses a phosphate group to 3-phosphoglycerate via phosphoglycerate kinase, which generates an ATP molecule. 3-phosphoglycerate is subsequently converted

in a two-step reaction into phosphoenolpyruvate (PEP). Finally, pyruvate kinase converts PEP into pyruvate, generating ATP in the process. Thus, the end product of glycolysis includes 4 ATP molecules, but because of the initial consumption of 2 ATP, the return on investment includes 2 ATP molecules per glucose [35, 36].

3.1.2 Regulation of glycolysis

The availability of glucose regulates the rate of glycolysis and is determined by two main mechanisms: glucose uptake from the blood, and breakdown of glycogen [35, 37]. In addition, the amount of oxygen can also regulate glycolysis through what is called the "Pasteur Effect", which describes how increased oxygen levels inhibit glycolysis, and decreased availability results in acceleration of glycolysis [35]. Within the glycolytic pathway, PFK1, which catalyzes the rate limiting step is considered the main player in terms of glycolysis regulation, and its activity can be affected in a number of ways.

Fructose 2–6 bisphosphate is an allosteric regulator of PFK1, which increases the enzyme activity [35, 37]. It is produced by phosphofructokinase 2 (PFK2), an enzyme that has both kinase and phosphorylase activity and can transform fructose 6 phosphates to fructose 2,6 bisphosphate and vice versa. Insulin dephosphorylates PFK2 activating its kinase activity, and increasing fructose 2,6 bisphosphate production, which subsequently activates PFK1 (**Figure 1**). Moreover, Glucagon phosphorylates PFK2, activating its phosphatase, which transforms fructose 2,6 bisphosphate back to fructose 6 phosphate. This decreases fructose 2,6 bisphosphate levels and decreases PFK1 activity. Low energy levels within the cell which result in increased AMP and low ATP/AMP ratio, induce allosteric activation of PFK1.

3.1.3 Glycolysis and EC angiogenesis

The endothelium is one of the most diverse tissues in the human body, which displays significant organ-specific heterogeneity. This diversity determines the function of the endothelium according to the organ being supplied [38]. Since ECs lining blood vessels are responsible for supplying oxygen and nutrient to body tissues, the ability to expand this network of blood vessels via angiogenesis is critical for organ growth and function in health and disease [39]. Low oxygen levels serve as a primary stimulus for angiogenesis, which in its classic meaning refers to the sprouting of branches from the existing vessels.

3.1.3.1 Angiogenesis

ECs are essential for the normal functioning of the vascular system. They drive the vascular system expansion during physiologic organ growth to supply sufficient nutrients, as well as under pathologic conditions through a process known as angiogenesis (**Figure 3**). Angiogenesis depends highly on the coordinated orchestra of several regulatory steps [1]. Briefly, this process is guided by the migratory non-proliferative "tip" cells at the forefront from an existing vessel, while the "stalk" cells trail the proliferative and elongation part of the sprout. "Tip" and "stalk" cells continuously switch their phenotype between being either tip or stalk cells. For example, the "tip" cell becomes a "stalk" cell when it loses its migratory behavior, and the "stalk" cell will compete for the position [1]. Several studies found that the vascular endothelial growth factor (VEGF) controls the "tip" cells induction, filopodia formation, and expression of the Notch ligand Delta–like 4 (NLD4) [40, 41]. NLD4, subsequently, suppresses VEGF receptor 2

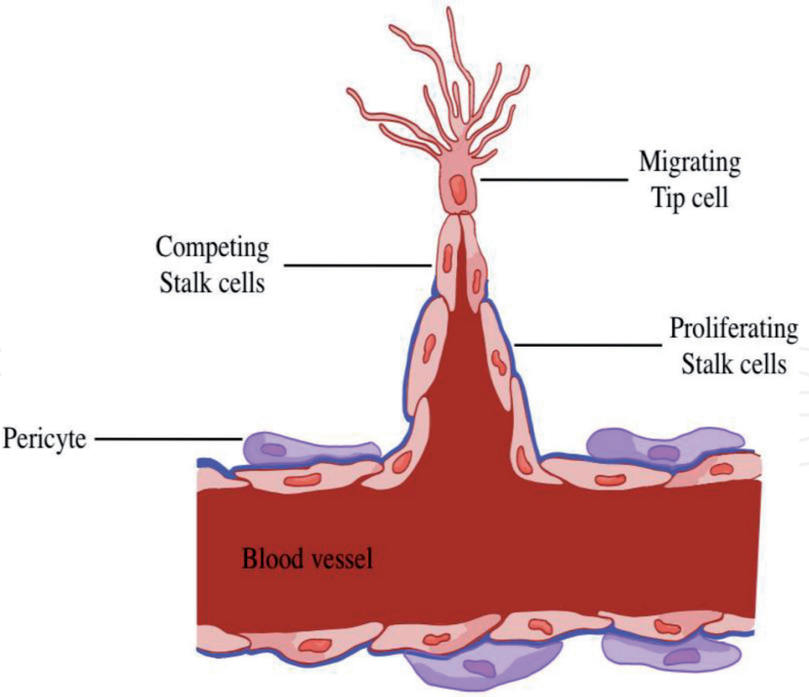

Figure 3.
Angiogenesis is mainly regulated via VEGF. Tip cells require increasing amounts of ATP necessary for migration into hypoxic tissues while proliferating stalk cells generate building blocks (dNTP, protein) to maintain their growth and cellular division.

(VEGFR2/kdr/Flk1) and thus modulates the tip cell behavior. While many genetic and molecular signaling pathways were recognized to be part of this process, the role of ECs metabolism has not been studied and explored until recently.

Switching on the angiogenic machinery of ECs has significant consequences on EC metabolism. This is because angiogenic ECs require nutrients and energy not only for motility but also for the synthesis of building blocks (proteins, nucleotides, and lipids) for cellular proliferation. Hence, during angiogenesis, ECs must increase their metabolic activity to generate energy quickly, while at the same time meeting the challenge of scarce resources as they proliferate in harsh hypoxic environments. Therefore, EC metabolism has to be flexible to support vessel formation under different conditions [39, 42].

Upon switching from quiescence state to vessel branching, the rate of glycolysis is increased in order to fuel subcellular processes required for migration such as cytoskeleton remodeling. Notably, the pro-angiogenic VEGF increases expression of the glycolysis activator phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3) [43]. PFKFB3 generates higher levels of fructose-2,6-bisphosphate, which activates phosphofructokinase 1, the rate limiting enzyme in glycolysis [43, 44]. In fact, studies have shown that genetic and pharmacologic inhibition of the phosphofructokinase 2 reduced EC sprouting and branching capacity [44, 45]. Another regulator enzyme is the hexokinase-2 (HK2) which phosphorylate glucose to glucose-6-phosphate [44, 46]. Several transcription factors such as KLF2 and forkhead box 1 (FOXO1) were found to suppress these key glycolytic enzymes in the quiescent phalanx cells [47, 48]. However, the rate of glycolysis increases in the actively sprouting tip and stalk cells due to VEGF-mediated activation of PFKFB3 and the decreased levels of KLF2 and FOXO1. Interestingly, PFKFB3 and other glycolytic enzymes are highly concentrated in filopodia to generate ATP at the so-called 'ATP hot-spots'. And several studies showed that pharmacologic or genetic inhibition PFKFB3 impairs new vessel formation [43, 44].

3.1.3.2 Endothelial cell metabolism

Despite the fact that oxygen is readily available for EC consumption, the glycolytic pathway remains primary source of energy for EC [38, 44, 49]. In fact, 85% of EC energy production in the form of ATP is generated through glycolysis, even though oxidative phosphorylation (OXPHOS) can generate significantly larger amounts of ATP molecules at much faster rate [43, 49, 50]. However, ECs have fewer mitochondria and consume lower amounts of O2 than other cell types, especially in the presence of abundant supplies of glucose, where only a small fraction of pyruvate is shifted to the TCA cycle [39, 43, 50]. Nonetheless, ECs retain their capacity for oxidative metabolism when glycolysis is compromised or in conditions of stress. Surprisingly, even though the amount of energy generated per glucose molecule via oxidative phosphorylation is significantly greater, higher rates of glycolysis can provide more ATP in a shorter period of time when glucose supply is unlimited. In fact, the rate of glycolysis is high in EC compared to other normal cells, and their glucose consumption is comparable with that of some cancer cells [43].

A logical question that can be asked here is, why do ECs depend on glycolysis for their energy production when they have direct supply of oxygen from blood? There are several explanations for this observation. First, despite the fact that the energy yield via glycolysis is significantly low compared to aerobic respiration, glycolysis can generate ATP molecules at a much faster rate [39, 49]. This is especially important when considering the energy requirements of ECs during angiogenesis. In addition, anaerobic glycolysis facilitates ECs sprouting and proliferation in hypoxic tissues and makes them resistant to hypoxic insults [39]. Also, it limits ROS generation and produces larger amounts of lactic acid, which acts as a pro-angiogenic factor [38, 39, 44]. Moreover, oxygen can be spared to be utilized by the underlying tissue cells. The low oxygen dependence allows sprouting cells to explore and reach distant hypoxic tissues [44]. Also, low oxygen consumption by ECs facilitates oxygen delivery to vital organs. Furthermore, glycolysis provides essential metabolites that are used in multiple cellular pathways such as pentose phosphate pathway (PPP), hexosamine biosynthesis pathway (HBP) and 3-phosphoglycerate (G3P) which generate important molecules and compounds that are used in different cellular processes [39, 49]. Thus, glycolysis provides a metabolic platform that allows ECs to perform diverse roles in the growing and resting vasculature with minimal ROS generation.

3.1.3.3 Alternative metabolism of glucose

ECs engage in several other pathways that can potentially affect angiogenesis, but their exact roles are understudied. Once phosphorylated by hexokinase (HK), glucose-6-phosphate (G6P) can be used to form glycogen, which could serve as an endogenous source of glucose when ECs sprout into glucose-deprived milieu. In fact, inhibition of glycogen phosphorylase (PYG), was found to impair EC migration [51].

G6P can also enter the pentose phosphate pathway (PPP) to generate NADPH [44]. NADPH is essential for restoring the reduced form of glutathione (GSH) from its oxidized form (GSSG), which serves as an antioxidant [38, 52]. PPP provides two intermediates of glycolysis, fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P). Interestingly, inhibition of G6P dehydrogenase (G6PD) or Transketolase (TKT) in the PPP was found to impair EC viability and migration [44].

3.1.3.4 Pathways regulation

ECs react to environmental conditions and energy requirements through several mechanisms that involve cellular molecules sensing changes in energy levels. One

of these molecules is the AMP-kinase (AMPK), which gets activated by the rising levels of AMP as energy levels dwindle. Activation of AMPK-mediated phosphorylation of metabolic targets promotes catabolic pathways and ATP production, while inhibiting anabolic pathways that consume ATP [39, 53]. This allows ECs to balance their energy level according to environmental changes. For instance, AMPK increases energy production via fatty acid oxidation (FAO) in EC mitochondria and help maintain ATP levels when glucose supplies are low [39, 54]. In addition, AMPK is activated by EC-specific stimuli such as hypoxia and shear stress generated by blood flow [38]. Interestingly, inhibition of AMPK was found to hinder EC angiogenesis in response to hypoxia [55].

3.2 Oxidative phosphorylation

The mechanism by which ATP is produced in the mitochondria via oxidative phosphorylation (OxPhos) was first discovered in the second half of the twentieth century [56, 57]. OxPhos is a process that involves the use of high-energy intermediates for energy transduction between the electron transport chain of the mitochondria and the chemical synthesis of ATP from ADP and phosphate. OxPhos generates 15 times the amount of ATP produced by glycolysis during anaerobic conditions. The reaction involves oxygen consumption, and energy is released from the high energy molecules (NADH, $FADH_2$) and stored in the form of an electrochemical proton gradient across the inner mitochondrial membrane. This energy extraction occurs in three steps each catalyzed by a specific membrane complex including Complex I (NADH dehydrogenase), Complex III (Cytochrome bc_1) and Complex IV (Cytochrome oxidase/COX). Complex II (Succinate dehydrogenase) converts succinate to fumarate, a TCA cycle intermediate, and in the process H^+ is produced from $FADH_2$, which is then shunted by Complex III across the inner mitochondrial membrane. COX is also considered the rate-limiting step of this aerobic respiration. Eventually, the electrochemical proton gradient is utilized by Complex V (ATP Synthase) to produce ATP, or it can be dissipated in the form of heat by passive proton leakage [56, 58, 59].

The electron transport chain is regulated through different mechanisms. Allosteric effectors such as ADP and ATP regulate the process by binding to their specific binding sites on the different mitochondrial complexes. Regulation of the enzyme activity by ATP or ADP binding to the same site on the complex subunit depends on the ATP/ADP ratio. For instance, the exchange of bound ADP by ATP on COX results in an allosteric ATP synthesis inhibition at an ATP/ADP ratio of 28 [60]. In addition, phosphorylation and dephosphorylation of the enzyme complexes is considered another mean of regulating the electron transport chain. For example, phosphorylation of COX was found to inhibit the enzyme activity [56].

3.3 Fatty acid oxidation contribute to dNTP synthesis

Deoxyribonucleoside Triphosphate (dNTP) is a molecule consisting of a deoxyribose sugar attached to three phosphate groups and one of the nucleotide bases, adenine, guanine, cytosine, or thymine as shown in **Figure 4** [61]. Apart from DNA replication, dNTPs may also function as a source of energy for different cellular reactions and signaling pathways [62].

3.3.1 dNTP formation

There are two biosynthetic pathways for nucleotides formation: *de novo* and salvage [62]. The *de novo* pathways require high energy and the use of raw material

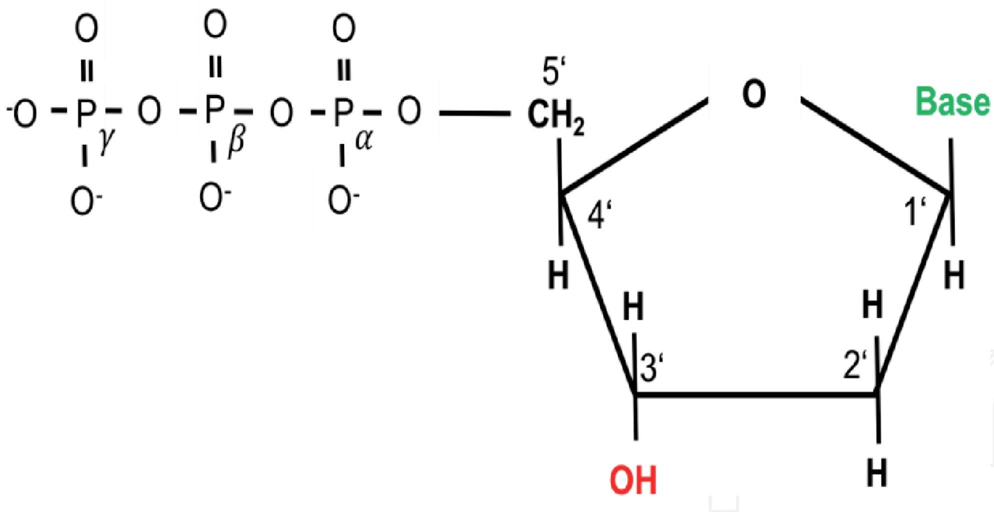

Figure 4.
Structure of Deoxyribonucleoside Triphosphate (dNTP).

like glucose, glutamine, aspartate, and HCO_3 to form nucleotides [62, 63]. However, salvage pathways exist as an alternative energy-efficient route to form nucleotides [63].

The enzyme ribonucleotide reductase (RR), which is NADPH-dependent, is responsible for catalyzing the rate-limiting reaction in which ribonucleotides are converted to their respective deoxyribonucleotides [62, 63]. This reaction is regulated by the number of RR enzymes and allosteric control mechanism [62, 63]. RR consists of two nonidentical subunits, α and β. α subunit has the catalytic site, substrate-specificity site, and activity site; whereas the β subunit contains a stable tyrosyl free radical [63]. The activity of RR enzymes is tightly controlled by allosteric mechanism [63, 64]. The reduction of ribonucleotides requires a specific positive effector, however, the product dNTP can also serve as a negative effector on the enzyme (**Table 1**) [61, 63].

dNTPs levels and RR enzyme activity are important to control the fidelity of nuclear and mitochondrial DNA replication and repair. It has been reported that increased levels of dNTP, *in vitro*, decreased the length of 'S phase' of the cell cycle during DNA replication, which implies that under physiological conditions, nucleotides are used mainly for DNA synthesis [65, 66]. Interestingly, whereas elevated levels of dNTP resulted in delay in the S phase entry through unclear mechanisms [67, 68], depletion of dNTP pool also resulted in inhibition of DNA replication, and fork stalling [69]. In fact, when the enzyme RR was blocked, DNA synthesis was arrested, preserving the dNTP for DNA damage repair under suboptimal conditions [69, 70].

3.3.2 Mitochondrial dNTP

Mitochondria are one of the major endomembrane organelles in eukaryotic cells [14, 71, 72] owing to their ability to produce ATP through oxidative phosphorylation as discussed in Section 3.2. Yet they participate in cellular function and dysfunction, including calcium regulation, activation of cellular death, ROS formation, and cellular building block synthesis [73, 74]. In ECs, the mitochondria compromise only 6% of cell volume, implicating that EC rely on anaerobic glycolysis rather than mitochondria-derived energy [7, 71]. However, mitochondria act primarily as major signaling organelles in the ECs and maintain mitochondrial dNTP pools for proper EC functions. Additionally, alternation in the levels of mitochondrial ROS has been

Substrate	ADP	GDP	CDP	UDP
Positive Effector	dGTP	dTTP	ATP	ATP
Negative Effector	dATP	dATP	dATP dGTP dTTP	dATP dGTP dTTP

Table 1.
Ribonucleotide reductase enzyme regulators.

shown to be associated with impaired one-carbon metabolism, which is essential for purines and pyrimidines nucleotides [75, 76].

In response to mild oxidative stress, the mitochondria attempt to re-establish homeostasis by ROS-buffering capacity of mitochondria. For example, the activity of adenine nucleotide translocase is impaired under mitochondrial oxidation, leading to shortage of adenine diphosphate (ADP) [77]. On the other hand, up-regulation of mitochondrial anti-oxidant systems and other molecules counteract ROS-induced protein unfolding [78, 79]. If oxidative stress is persistent, mitochondria may translate the adaptive response into activation of cellular death [74]. These responses and deregulation of ROS levels contribute to the pathogenesis of cardiovascular system, including coronary artery diseases.

3.3.3 Fatty acid oxidation (FAO)

Long chain fatty acids are a major source of energy productions, primarily in mitochondria [61, 80, 81]. Fatty acids are broken up into acetyl CoA, NADH and $FADH_2$ in the mitochondria [80]. These three products are used by the mitochondrial matrix for energy production through TCA and oxidative phosphorylation [80].

3.3.3.1 Fatty acid oxidation as a major energy-producing pathway

Fatty acid oxidation (FAO) is an important catabolic and anabolic process. On the outer membrane of mitochondria, FAO transfers the acyl group from CoA to carnitine by carnitine palmitoyltransferase I (CPT1). Acyl-carnitine is then exchanged across the inner membrane of mitochondria. The acyl group is transferred back again to CoA by carnitine palmitoyltransferase II (CPT II) as shown in **Figure 5** [82]. CPT1 is an important enzyme for FAO and is a rate limiting factor for FAO in the mitochondria. Malonyl CoA, an intermediate product of fatty acid synthesis, is an inhibitor of CPT1.

β-oxidation is a four steps process carried by enzymatic oxidation, hydration, and oxidation that act on acyl CoA to yield a shorter acyl CoA and acetyl CoA [83]. The four-step process is shown in the schematics of **Figure 6**.

3.3.3.2 Role of fatty acid oxidation in vessel sprouting

Recent studies have shown the critical role of FAO for vessel sprouting [42, 84]. In a study, the levels of FAO and dNTP synthesis were reduced when mitochondrial CPT1A was silenced. This resulted in impaired vascular sprouting due to reduction ECs proliferation but not migration. Additionally, silencing long-chain acyl-CoA dehydrogenase (ACADVL) has yielded similar results, supporting the role of FAO in vessel sprouting. Overexpression of CPT1A obtained opposite results, further supporting a crucial role of CPT1A in angiogenesis.

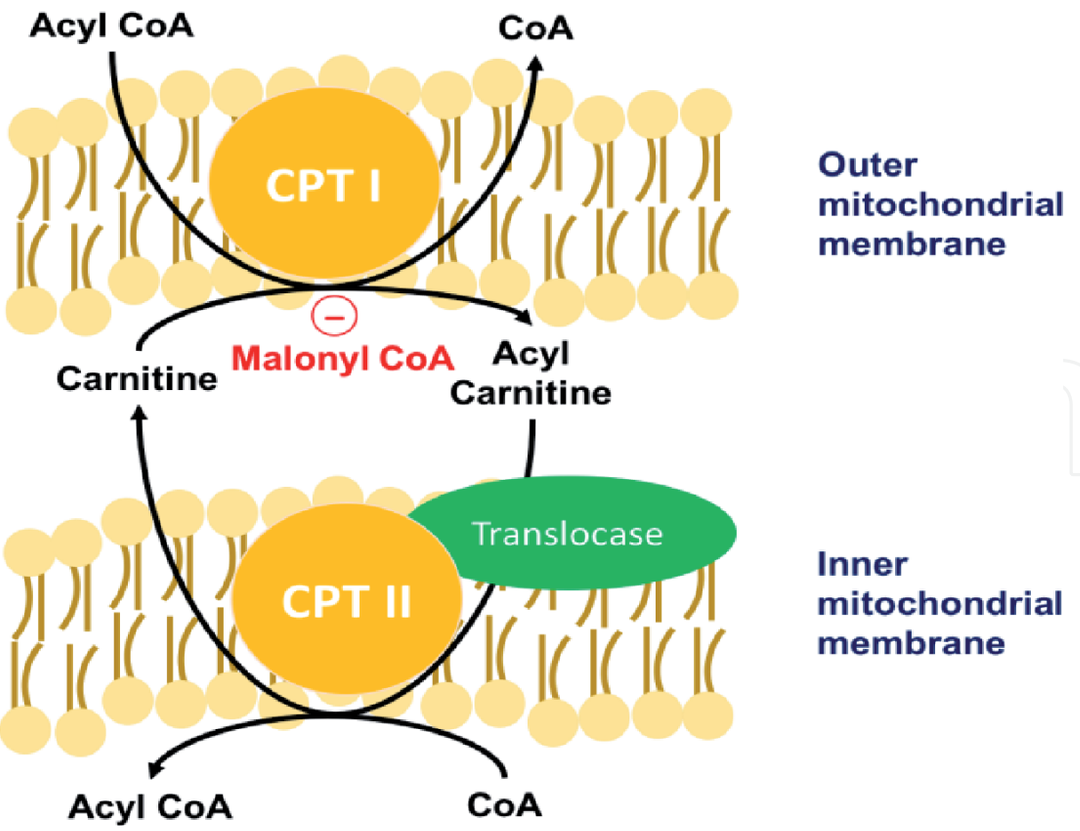

Figure 5.
Long-chain fatty acid transportation in the mitochondra. Fatty acids are transported through the mitochondrial membrane as acyl CoA for subsequent oxidation. Malonyl CoA acts as a key inhibitor molecule for CPT I, and thus regulating the rate of fatty acid oxidation (FAO).

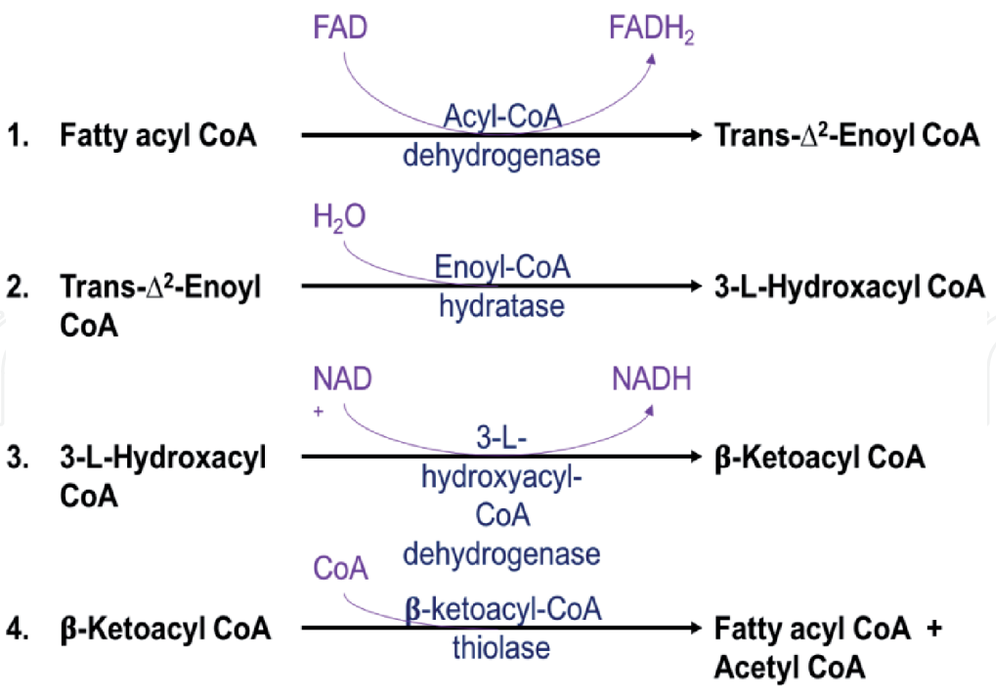

Figure 6.
Fatty acid β-oxidation pathway.

3.3.3.3 Fatty acid oxidation for de novo *synthesis of nucleotides*

As noted above, silencing CPT1A in ECs showed impaired *de novo* synthesis of dNTPs. This impaired *de novo* dNTP synthesis contributed to reduced vessel

sprouting [82]. Nonetheless, the levels of glucose oxidation were increased to compensate for the FAO loss, yet it was not sufficient to help in the proliferative defects of ECs with knockdown CPT1A. This reflects the irreplaceable role of FAO for *de novo* dNTP synthesis in ECs [82].

3.3.3.4 Fatty acid β-oxidation in quiescent vs. proliferating endothelial cells

Depending on the cellular status, the FAO are directed either toward DNA synthesis or redox homeostasis. FAO are involved in regenerating $NADP^+$ to NADPH, they also upregulate the expression of $NADP^+$ producing genes, which are critical for redox homeostasis [43, 82]. Quiescent ECs upregulate FAO, but do not rely on them for ATP production or nucleotide synthesis, rather utilize it for redox homeostasis [85]. Unlike quiescent ECs, proliferating ECs utilize FAO for DNA synthesis, as previously discussed [82].

CPT1A, the rate limiting enzyme for FAO in mitochondria, has been shown to be critical for redox homeostasis in EC. In quiescent ECs, CPT1A inhibition caused the levels of ROS to elevate, leading to decreased anti-fibrinolytic gene expression, endothelial leakage, and increased leukocytes adhesion and/or infiltration [80, 85]. Thus, it is believed that quiescent ECs require more redox buffering capacity compared to proliferating ECs due to higher levels of ROS.

Besides the involvement of FAO in redox balance in quiescent ECs, they are also involved in other vasculo-protective NADPH-regenerating pathways such as oxidative PPP and nicotine nucleotide transhydrogenase [85].

4. Endothelial metabolism in atherosclerosis

The generation of increased amounts of NO in atherosclerosis is critical for its anti-atherogenic effects, including vasodilation, inhibition of platelet aggregation, smooth muscle proliferation as well as leukocyte migration and oxidative stress [38]. Endothelial cells produce NO through enzymatic oxidation of arginine to citrulline via eNOS enzyme. eNOS requires several co-factors including NADPH, flavin adenine dineucleotide (FAD), flavin mononeucleotide (FMN), Calcium/Calmodulin and tetrahydrobiopterin (BH4). Decreased availability of Arginine or deficiency of BH4 results in the paradoxical generation of ROS instead of NO by eNOS, a process known as eNOS uncoupling [38, 86]. Arginine in particular, has been found to be rate-limiting for NO synthesis in patients with atherosclerosis [87]. It was demonstrated that an arginine analog asymmetric dimethyl arginine (ADMA), that acts as a competitor for eNOS, impaired NO production. ADMA levels are markedly increased in atherosclerosis and therefore it is recognized as a major cardiovascular risk factor [88]. Moreover, Dimethyl arginine dimethyl aminohydrolase (DDAH), an enzyme that metabolizes ADMA into citrulline and dimethylamine is impaired by the oxidative stress in atherosclerosis [38]. Interestingly, because of this competition, Arginine supplements have been found to be of great benefit in atherosclerotic patients with high ADMA levels, by enhancing endothelial-dependent vasodilation and inhibition of leukocyte adhesion and migration to the atherosclerotic plaque [89].

Furthermore, endothelial NADPH oxidase is induced by certain atherosclerotic plaque components such as the oxidized LDL (oxLDL). The NADPH oxidase-derived ROS were found to have detrimental effects in promoting plaque progression. These include oxidation of LDLs, inducing vascular smooth muscle proliferation and migration and EC apoptosis as well as promoting the expression of vascular adhesion adhesion molecules [38].

5. Conclusions

The endothelium is one of the most diverse tissues in the human body. It maintains the integrity of the vascular system and provides nutrition to underlying tissues. In addition, EC drives the growth and proliferation of blood vessels under physiologic and pathologic conditions. ECs exhibit significant flexibility in response to various environmental changes such as hypoxia and ischemia. Careful analysis of the process of sprouting angiogenesis explains how ECs function in such an orchestrated way to reach their end goal of providing nutrients and oxygen supply to the affected tissues. ECs display phenomenal resilience in the process through various mechanisms, one of which is their metabolic adaptation and the other is critical balance between subcellular levels of ROS (cytosolic versus mitochondrial). ECs limit their oxygen consumption in order to preserve it for the tissues that they supply to and also to maintain a balanced intracellular redox state. Although ECs do not utilize mitochondrial OxPhos for ATP synthesis and thus generate very little mitochondrial ROS, NADPH oxidase-derived ROS appear to regulate many critical EC functions in health and disease. However, EC has intricated intracellular mechanisms by which subcellular oxidants may communicate at the subcellular levels [7]. Unlike most cells in the body (except tumor cells), ECs upregulate and accelerate their glycolytic pathways in order to generate energy (ATP production) and certain molecules that act as building blocks (dNTPs) and are essential for supporting EC proliferation and migration. CPT1A-mediated FAO appears to play a significant role in synthesizing dNTPs and $NADP^+$, NADPH in EC mitochondria. Depending on the metabolic states of ECs (quiescent versus proliferative), FAO-generated NADPH is utilized for quiescent EC's redox homeostasis or dNTPS for cell proliferation in angiogenic endothelium. Further studies aimed at understanding the molecular mechanisms by which subcellular ROS modulate EC metabolism in health and disease will help develop therapeutics modalities for CVD.

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Conflict of interest

The authors declare no conflict of interest.

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