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Interplay Between Reactive Oxygen/Reactive Nitrogen Species and Metabolism in Vascular Biology and Disease

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Abstract

Reactive oxygen species (ROS; *e.g.*, superoxide [$O_2^{\bullet-}$] and hydrogen peroxide [H_2O_2]) and reactive nitrogen species (RNS; *e.g.*, nitric oxide [$NO^{\bullet}$]) at the physiological level function as signaling molecules that mediate many biological responses, including cell proliferation, migration, differentiation, and gene expression. By contrast, excess ROS/RNS, a consequence of dysregulated redox homeostasis, is a hallmark of cardiovascular disease. Accumulating evidence suggests that both ROS and RNS regulate various metabolic pathways and enzymes. Recent studies indicate that cells have mechanisms that fine-tune ROS/RNS levels by tight regulation of metabolic pathways, such as glycolysis and oxidative phosphorylation. The ROS/RNS-mediated inhibition of glycolytic pathways promotes metabolic reprogramming away from glycolytic flux toward the oxidative pentose phosphate pathway to generate nicotinamide adenine dinucleotide phosphate (NADPH) for antioxidant defense. This review summarizes our current knowledge of the mechanisms by which ROS/RNS regulate metabolic enzymes and cellular metabolism and how cellular metabolism influences redox homeostasis and the pathogenesis of disease. A full understanding of these mechanisms will be important for the development of new therapeutic strategies to treat diseases associated with dysregulated redox homeostasis and metabolism. *Antioxid. Redox Signal*. 34, 1319–1354.

Keywords: metabolism, oxidative stress, redox signaling, reactive oxygen species, reactive nitrogen species

Introduction

DYSREGULATED REDOX HOMEOSTASIS is a hallmark of cardiovascular disease (CVD) (88, 118, 262). Enzymes that produce reactive oxygen species (ROS; *e.g.*, superoxide [$O_2^{\bullet-}$], hydrogen peroxide [H_2O_2]) and reactive nitrogen species (RNS; *e.g.*, nitric oxide [$NO^{\bullet}$]) are regulated by location-dependent changes in metabolic flux (Figs. 2 and 3). Metabolic changes are among the most prominent features of aging and have been identified in numerous disease states (Fig. 6), because metabolism impacts cellular function through various mechanisms (Fig. 1). How these changes serve to influence the redox balance (and vice versa) is poorly understood (Figs. 4–6) (1, 102, 178, 254, 283). The ROS play the role of a double-edged sword in both physiologic and pathologic processes. Ambient levels at any given time reflect the balance between the rate and the magnitude of ROS production *versus* its elimination (185, 371, 386). At the physiological level, ROS are involved in cellular signaling. However, when present in excess, ROS can drive pathologies associated with aging, cancer, and atherosclerosis (102). Reductive stress, a state in which ROS levels are too low, can also promote and exacerbate a wide spectrum of pathologies ranging from cancer to cardiomyopathy (371).

Metabolism is profoundly affected by oxidative stress (Figs. 5 and 6) (1, 102, 178, 254, 283). For example, ROS/RNS can inhibit multiple glycolytic enzymes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and

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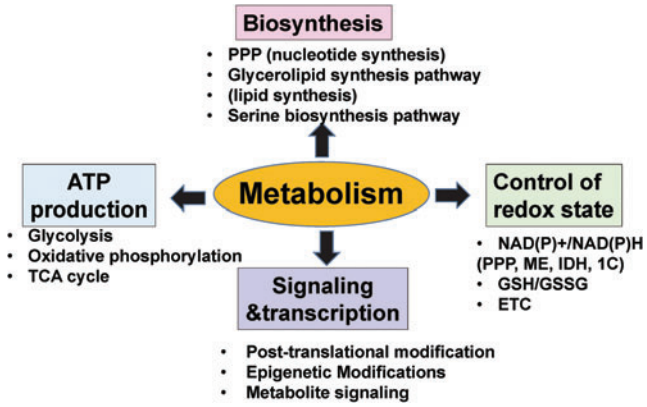

FIG. 1. Role of metabolism in cellular function. Metabolism regulates cellular function by integrating energy production, biosynthesis, control of redox state, cell signaling, and transcription. The main metabolic task is to produce ATP to meet energetic demands for cellular function *via* glycolysis, oxidative phosphorylation and the TCA cycle. Metabolism is also necessary for biosynthetic pathways, including the PPP for nucleotide synthesis, as well as the glycerolipid synthesis pathway (lipid synthesis) and serine biosynthesis pathway. Metabolic pathways also regulate the intracellular redox state by controlling $NAD(P)^+$/NAD(P)H pools and GSH to fuel the TRX/GSH antioxidant defense system. NADPH is derived from PPP, IDHs, MEs, and 1C metabolism, whereas GSH is derived from glutaminolysis. Further, metabolism regulates ETC to produce mitoROS. Finally, metabolism influences signaling and transcription by regulating post-translational modification (*e.g.,* glycosylation *via* the hexosamine biosynthetic pathway), epigenetic modification, and metabolite signaling. 1C, one-carbon; ATP, adenosine triphosphate; ETC, electron transport chain; GSH, glutathione; ME, malic enzyme; mitoROS, mitochondrial ROS; NADPH, nicotinamide adenine dinucleotide phosphate; PPP, pentose phosphate pathway; ROS, reactive oxygen species; TCA, tricarboxylic acid; TRX, thioredoxin. Color images are available online.

pyruvate kinase M2 (PKM2) (4, 259) (Figs. 5 and 6). The inhibition of glycolytic pathways by ROS/RNS promotes metabolic reprogramming away from glycolytic flux toward the oxidative arm of the pentose phosphate pathway (PPP). This shift results in increased production of nicotinamide adenine dinucleotide phosphate (NADPH), which is needed to support antioxidant defense. Cancer cells can promote their own survival *via* antioxidant defense, specifically *via* metabolic reprogramming, to prevent cell death due to excessive ROS accumulation (178, 254, 283). Interestingly, cancer-prone adenomatous polyposis coli-deficient cells exhibit increased mitochondrial- and NADPH oxidase (NOX)-mediated ROS production as well as increases in the TP53-induced glycolysis and apoptosis regulator (TIGAR)-mediated antioxidant defense; however, both pathways contribute to cell proliferation (51) (Fig. 4). In this case, NOX enzymes generate ROS that serve to increase cell proliferation whereas TIGAR limits the damaging effects of ROS (Fig. 4). These findings underscore the apparent importance of both temporal and spatial regulation of redox balance. In this review, we will summarize our current knowledge of this field with a focus on the reciprocal regulation of ROS/RNS and metabolic pathways and their contributions to vascular biology and disease.

ROS Homeostasis

This section summarizes our current understanding of the cellular sources of ROS ($O_2^{\bullet-}$ and H_2O_2) with a focus on the roles played by the NOX enzymes, the mitochondrial electron transport chain (ETC), and uncoupled $NO^{\bullet}$ synthases (NOSs) (81) (Figs. 2 and 3). We will also review the role of coupled NOSs as a source of RNS ($NO^{\bullet}$) (Fig. 4). Other sources of ROS and RNS have been considered extensively in other reviews (321). In this section, we will also consider the roles of antioxidant enzymes, including superoxide dismutases (SODs), catalase, glutathione peroxidases (GPXs), and peroxiredoxins (PRXs), as well as their essential substrates, NADPH and reduced glutathione (GSH) (Figs. 2, 4, and 5). As an example of redox balance, NADPH is essential not only for the functioning of the PRX/thioredoxin (TRX) and GPX/GSH antioxidant systems, but it is also critical for the activities of NOX and NOS, which are the enzymes that generate ROS and RNS, respectively (Fig. 2).

Generation of ROS

NADPH oxidases. The NOXs are flavocytochrome enzymes (32, 294). Both phagocytic and non-phagocytic cells throughout the plant and animal kingdom express functional NOXs, although these enzymes have not been identified in prokaryote species. The NOX proteins produce $O_2^{\bullet-}$ through NADPH electron exchange (Fig. 2). NOX-dependent ROS production has an impact on many metabolic processes and disease states (Fig. 5). There are five NOX isoforms known as NOX1, NOX2, NOX3, NOX4, and NOX5. There are also two isoforms of the related dual oxidases (DUOXes). NOX2 is the prototype NOX enzyme; it is also known as gp91phox (nb: phox is an abbreviation for ''phagocytic oxidase'') because it was first identified in phagocytic cells. The NOX2 complex includes two membrane catalytic subunits, the aforementioned gp91phox and the regulatory subunit, p22phox, and five cytosolic subunits, including p47phox, p67phox, p40phox, p22phox, and Rac1. Structurally, NOX2 shares 20%–50% sequence similarity with the other NOXs (50). These similarities have created difficulties for those designing targeted therapies (184).

NOX1, NOX2, NOX4, and NOX5 are all expressed in vascular tissues (32, 179, 187). NOX1 and NOX2 are $O_2^{\bullet-}$ generating enzymes, whereas NOX4 generates H_2O_2 (32, 179, 187). NOX5 also produces $O_2^{\bullet-}$ in a calcium-dependent manner (107). Results from previous studies suggest that NOX1, NOX2, and NOX5 promote endothelial dysfunction, inflammation, and apoptosis in the vessel wall. By contrast, NOX4 is primarily vasoprotective, as it increases the bioavailability of NO and inhibits apoptotic pathways (32, 102). However, the actions of NOX4 can also be deleterious (15). NOX2 is found in the plasma membrane or in endosomes where it produces $O_2^{\bullet-}$either extracellularly or within the cytosol, respectively. $O_2^{\bullet-}$ is rapidly scavenged to generate H_2O_2 outside the cell by superoxide dismutase 3 (extracellular SOD [ecSOD], SOD3) or within the cytosol by the actions of coper zinc superoxide dismutase (Cu,ZnSOD, SOD1). By contrast, NOX4 is located in focal adhesions, the endoplasmic reticulum, nuclei, and mitochondria and it generates H_2O_2 at these locales (32, 179, 187). NOX1 is found in various subcellular localizations, including the nuclei and caveolae, whereas NOX5 is localized at the plasma

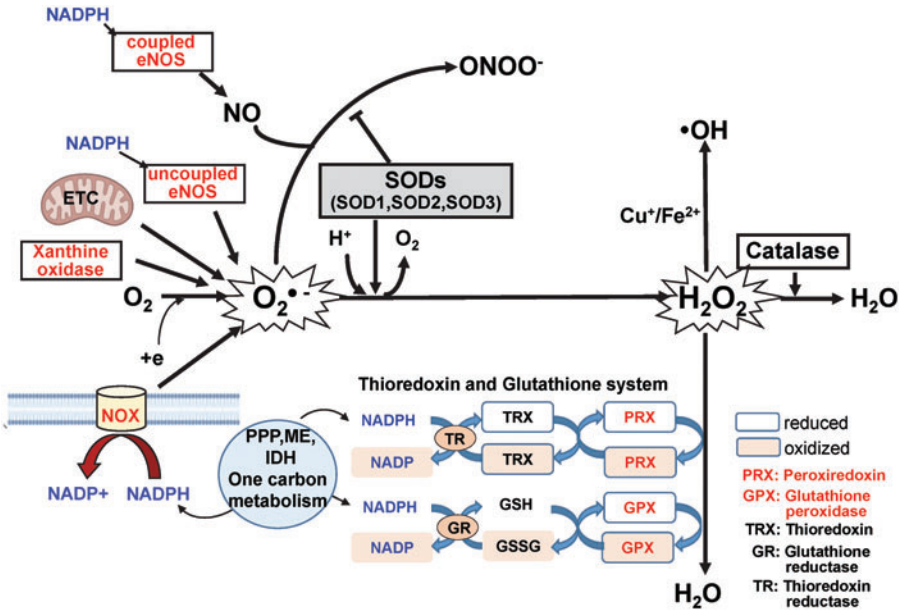

FIG. 2. Generation and metabolism of ROS/RNS. $O_2^{\bullet-}$ is produced by NOXs, the mitochondrial ETC, XO, lipoxygenase, cyclooxygenase, and uncoupled NOS. $O_2^{\bullet-}$ is converted by SODs to H_2O_2, which, in turn, is reduced to water *via* the actions of catalase, GPXs, and PRXs. The PRX/TRX and GPX/GSH systems are fueled by NADPH, which is generated by the PPP, IDHs, MEs, and 1C metabolism. Of note, NADPH is also a substrate for the ROS-generating NOXs and NOS. In the presence of reduced transition metals (Fe^{2+} and Cu^{2+}), H_2O_2 undergoes spontaneous conversion to reactive $OH^{\bullet}$ or related metal-associated reactive species. $NO^{\bullet}$ is produced by coupled NOS. The NOS enzymes utilize NADPH and L-arginine as co-substrates and BH_4 (a product of 1C metabolism) as essential co-factors. Although all NOS isoforms generate $NO^{\bullet}$, they can also generate $O_2^{\bullet-}$ at the expense of $NO^{\bullet}$ *via* a process known as uncoupling. The mechanisms underlying the uncoupling process include the formation of monomers, altered Hsp90 binding, and insufficient levels of BH_4 and L-arginine. Importantly, $NO^{\bullet}$ can be rapidly inactivated *via* a reaction with $O_2^{\bullet-}$, which leads to the formation of the strong oxidant, $ONOO^-$. Thus, SODs are the first line of defense against $O_2^{\bullet-}$-mediated toxicity. The SODs also participate in cell signaling events *via* their capacity to regulate levels of ROS (*e.g.*, $O_2^{\bullet-}$, H_2O_2) while preserving available $NO^{\bullet}$. BH_4, tetrahydrobiopterin; GPX, glutathione peroxidase; H_2O_2, hydrogen peroxide; Hsp, heat-shock protein; $NO^{\bullet}$, nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidase; $O_2^{\bullet-}$, superoxide; $OH^{\bullet}$, hydroxyl radical; $ONOO^-$, peroxynitrite; PRX, peroxiredoxin; RNS, reactive nitrogen species; SOD, superoxide dismutase; XO, xanthine oxidase. Color images are available online.

membrane (32, 107, 179, 187). Other recent reviews include more detailed examples of the roles of NOX enzymes in the pathophysiology of CVD (32, 171, 179, 187, 321, 379).

Mitochondria. The primary site of ROS generation in mitochondria is the ETC (Fig. 2). As shown, Nicotinamide adenine dinucleotide (NADH)–ubiquinone oxidoreductase (complex I) accepts electrons from NADH, which are then transferred to complex II (succinate dehydrogenase [SDH]), which oxidizes succinate to fumarate (12). Electrons continue to travel down the electrochemical gradient to complex III (ubiquinol–cytochrome c oxidoreductase) and then to complex IV (cytochrome c oxidase), where they reduce molecular oxygen (O_2) to water. Approximately 0.2% of the total O_2 undergoes incomplete reduction to become $O_2^{\bullet-}$ (12, 124, 310, 313). Complexes I and III are the major sites of electron leakage involved in the premature reduction of O_2, thereby resulting in the formation of $O_2^{\bullet-}$, whereas complex II can also contribute to $O_2^{\bullet-}$ formation (267). Complexes I and II produce $O_2^{\bullet-}$ that is released into the matrix only (194, 394), whereas complex III can produce $O_2^{\bullet-}$ on both sides of the inner mitochondrial membrane, thereby resulting in its release into the intermembrane space (IMS) (242). $O_2^{\bullet-}$within the IMS is physiologically more important with respect to signaling capacity, as it has easier access to the cytosol from this site; by contrast, matrix $O_2^{\bullet-}$ needs to cross both the inner and the outer mitochondrial membranes to have access to the cytosol. $O_2^{\bullet-}$ is a charged species and, thus, it is not capable of diffusing across mitochondrial membranes. Therefore, $O_2^{\bullet-}$ generated at IMS exits mitochondria through a voltage-dependent mitochondrial anion channel (VDAC) and enters the cytosol, where it is converted to H_2O_2 by cytosolic SOD1 (123). Complex III is the major site of ROS production in human endothelial cells (ECs) during the process of hypoxia reoxygenation and after stimulation with the cytokine, tumor necrosis factor α (TNFα) (63, 327), whereas complex II plays a more important role in lysophosphatidylcholine-induced ROS formation in these cells (355). Complexes I and/or III are responsible for ROS production that elicits dilation in response to shear stress in human coronary arteriolar ECs (207). Thus, the generation of $O_2^{\bullet-}$ by each complex in the ETC appears to be agonist/stimulant-dependent. Several recent reviews provide additional details on the role of mitochondrial ROS (mitoROS) in the pathogenesis of CVD (13, 71, 82, 171, 321, 379).

Elimination of ROS

Antioxidant enzymes. The SODs are the primary cellular antioxidant enzymes that can eliminate $O_2^{\bullet-}$. The SODs

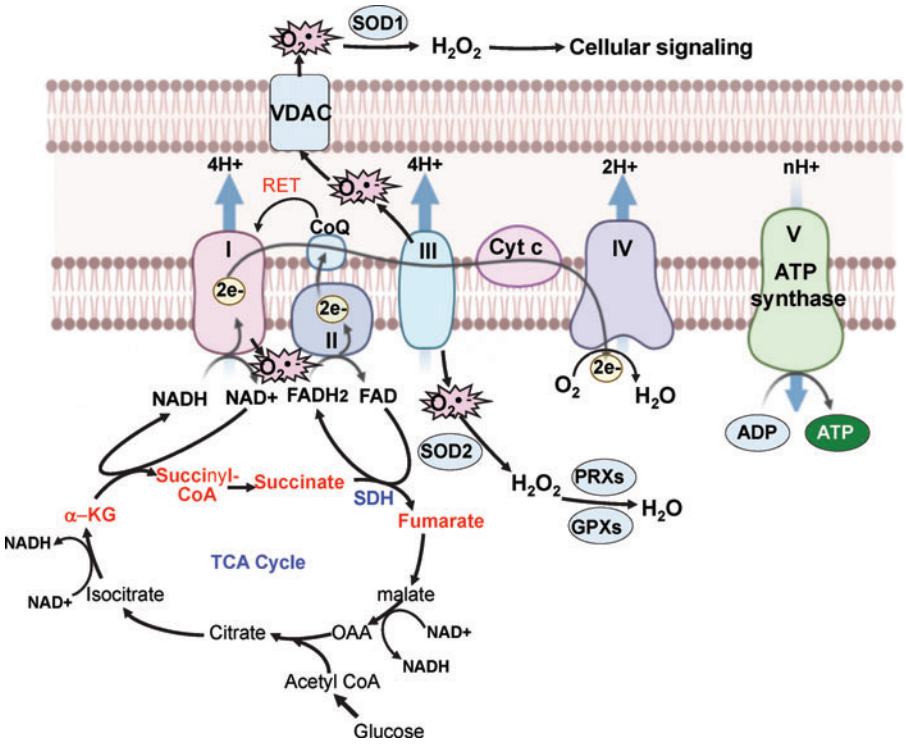

FIG. 3. Interplay between mitoROS production, the ETC, and the TCA cycle. mitoROS are produced by the ETC at complexes I and III during oxidative phosphorylation. The reducing equivalents NADH and $FADH_2$, which are generated by the TCA cycle in a series of enzymatic reactions, transfer electrons to the ETC to produce ATP. Thus, mitoROS, the ETC, and the TCA cycle are closely connected during oxidative phosphorylation. When the pool of CoQ is reduced, mitoROS are produced by complex I *via* RET. Further, VDACs control the release of $O_2^{\bullet-}$ from the mitochondria to the cytosol. NADH is produced during the conversion of α-α-KG to succinyl CoA to provide electrons for complex I in the ETC. NADH is also produced in the conversion of isocitrate to α-KG and the conversion of malate to OAA in the TCA cycle. $FADH_2$ is produced during the conversion of succinate to fumarate *via* the actions of SDH, which is an enzyme that participates in both the TCA cycle and the ETC. α-KG, α-ketoglutarate; CoA, coenzyme A; complex I, NADH–ubiquinone oxidoreductase; complex III, ubiquinol–cytochrome c oxidoreductase; coQ, coenzyme Q; Cyt c: cytochrome c; $FADH_2$, reduced flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; OAA, oxaloacetate; RET, reverse electron transport; SDH, succinate dehydrogenase; VDAC, voltage-dependent anion channel. Color images are available online.

rapidly scavenge $O_2^{\bullet-}$ and use it as a substrate to generate H_2O_2, thereby protecting the cell from the harmful effects of this highly reactive molecule (Fig. 2). The three distinct SODs are found in different cellular locations, including the cytosol (SOD1, or Cu,ZnSOD), the mitochondria (SOD2, or manganese superoxide dismutase [MnSOD]), and the extracellular matrix (ECM; SOD3, or ecSOD) (106). Cellular antioxidant systems capable of scavengingH_2O_2 include catalase, the PRXs/TRX system, and the GPXs/GSH system, which can degrade H_2O_2 to water and molecular O_2 (135) (Fig. 2). These antioxidant scavenging systems also have different cellular localizations, including the cytosol, mitochondria, endoplasmic reticulum, peroxisomes, and extracellular space (22, 36). In the presence of a reduced transition metal (*e.g.*, Fe^{2+} or Cu^{2+}), H_2O_2 can be converted to a hydroxyl radical ($OH^{\bullet}$), which is extremely reactive. In the presence of iron (Fe^{2+}), $OH^{\bullet}$ can generate lipid peroxides that promote ferroptosis; this pathway can be inhibited by GPX4 (314).

Oxidized and inactivated TRX is reactivated and reduced by the enzyme, TRX reductase (TRXR) *via* the oxidation of a reducing equivalent, NADPH (Fig. 2). Similar to PRX and TRX, GPX and GSH cooperate with one another to detoxify H_2O_2 and generate H_2O (Fig. 2). This process yields oxidized GSH (GSSG), which is then reduced by glutathione reductase (GSR) and NADPH (26). Thus, both systems are ultimately dependent on cellular NADPH-reducing equivalents for their regeneration. TRXR and GSR use NADPH to reduce oxidized TRX and GSSG, respectively (Fig. 2). This key reducing equivalent is generated by a complex network of metabolic pathways and enzymes, as discussed later (Fig. 4). Several previous reviews have included a more extensive consideration of the role of SODs, TRX, and GRX in the pathogenesis of CVD (22, 36, 85, 106, 135, 171, 321, 379).

Nicotinamide adenine dinucleotide phosphate. NADPH is an essential electron donor that is found in all eukaryotic cells. NADPH is essential not only for use by the PRX/TRX and GPX/GSH antioxidant defense systems that mitigate ROS-related cellular damage but also as a cofactor for NOS to generate $NO^{\bullet}$ and similarly for NOXs to generate $O_2^{\bullet-}$ or H_2O_2 (Fig. 2). NADPH serves as both a substrate for NOX to generate $O_2^{\bullet-}/H_2O_2$ and a coenzyme for the reductive removal of peroxides (366). NADPH is also required for anabolic biosynthetic reactions that are important for cell growth, such as the synthesis of fatty acids (FAs) and cholesterol, degradation of heme, and metabolism of polyol compounds (160, 372). Approximately 60% of the intracellular

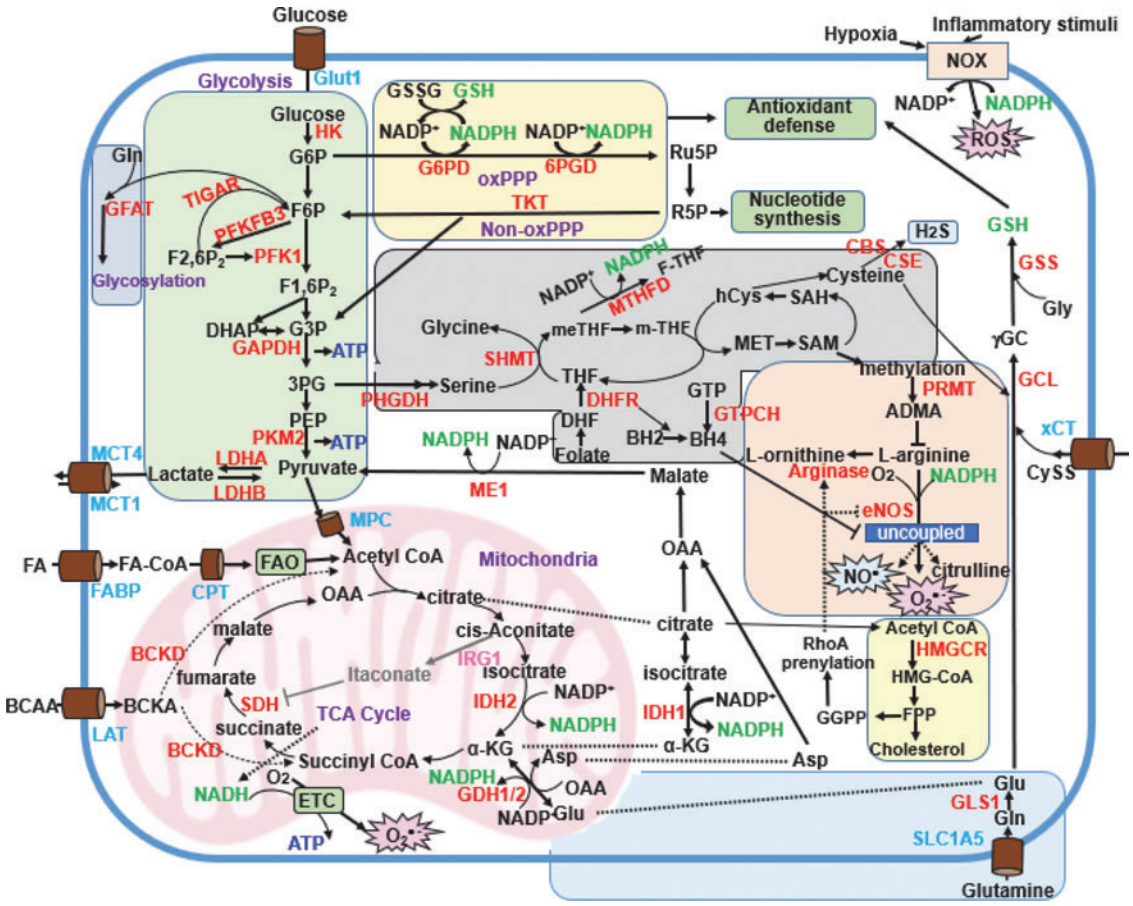

FIG. 4. Cellular metabolic pathway involved in redox homeostasis. The major metabolic pathways that regulate redox homeostasis in ECs are as shown. Parts of metabolic pathways that take place in immune cells (*e.g.,* those involving itaconate) are also included. Metabolic pathways that regulate redox homeostasis are limited to those involved in the production of NADPH and GSH (shown in *green*) and that regulate eNOS activity (shown in *red*). The ECs primarily utilize glycolysis (shaded in *green*) to obtain ATP. During this process, ECs generate pyruvate and lactate from glucose, thereby contributing to four additional pathways. The first of these, known as the PPP (shaded in *yellow*), includes both oxPPP and non-oxPPP pathways that contribute to antioxidant defense and nucleotide synthesis, respectively. Second, 1C metabolism (shaded in *gray*) contributes to protein and nucleotide methylation. Third, the hexosamine pathway (shaded in *blue*) uses F6P to promote protein glycosylation and synthesis of the luminal glycocalyx. Finally, after glycolysis, pyruvate can enter the mitochondria where it is converted to acetyl-CoA and can then enter the TCA cycle (shown in *purple*). NADPH is essential not only for antioxidant defense pathways, including the PRX/TRX and GPX/GSH systems that mitigate ROS-related cellular damage, but it is also necessary for the generation of $NO^{\bullet}$ (a cofactor for NOS) and $O_2^{\bullet-}$ (a cofactor of the NOX enzymes). The major metabolic pathways that generate NADPH include oxPPP, ME1, 1C metabolism, IDH1/2, glutamine metabolism, and CPT1-mediated FAO. *De novo* synthesis of the antioxidant, GSH (shown in *green*) involves CySS import into the cell *via* the CySS/glutamate transporter (xCT), cysteine generated from methionine *via* the trans-sulfuration pathway, and glutamine metabolism. Cysteine is also involved in the synthesis of the gaseous transmitter, H_2S. The primary metabolic pathways contributing to coupled and uncoupled eNOS include the ornithine cycle (shaded in *pink*), the mevalonate pathway (shaded in *pale blue*), and 1C metabolism (*via* BH_4). Lastly, itaconate synthesized from aconitate in activated macrophages *via* the actions of IRG1 inhibits the activity of SDH. This inhibits ROS generation by RET at complex I. CPT1, carnitine palmitoyltransferase-1; CySS, cystine; EC, endothelial cell; eNOS, endothelial NOS; FAO, fatty acid oxidation; H_2S, hydrogen sulfide; IRG1, immune-responsive gene 1; LDH, lactate dehydrogenase; oxPPP, oxidative PPP; xCT, the cystine/glutamate antiporter SLC7A11. Color images are available online.

NADPH is generated *via* the oxidative PPP with the remaining 40% generated by one-carbon (1C) metabolism, isocitrate dehydrogenases (IDHs), and malic enzymes (MEs) (37), as discussed later (Fig. 4). Consequently, cancer cells maintain high levels of NADPH that sustain their rapid growth and protect them from the deleterious effects of excessive ROS (160, 372).

The ROS are closely linked to the systems that generate NADPH, thereby serving to induce antioxidant defense (Fig. 2). Oxidative PPP, one of the major sources of NADPH, is a branch of the metabolic process of glycolysis. In response to ROS, several enzymes that regulate glycolysis, including GAPDH, PKM2, and TIGAR, can redirect glycolytic intermediates to the oxidative PPP (4, 259, 352) (Figs. 4 and 5). The ROS-induced S-glutathionylation and inactivation of specific cysteine residues in both GAPDH and PKM2 contribute to this response (4, 259). Further, inactivation of PKM2 can channel glycolytic precursors into

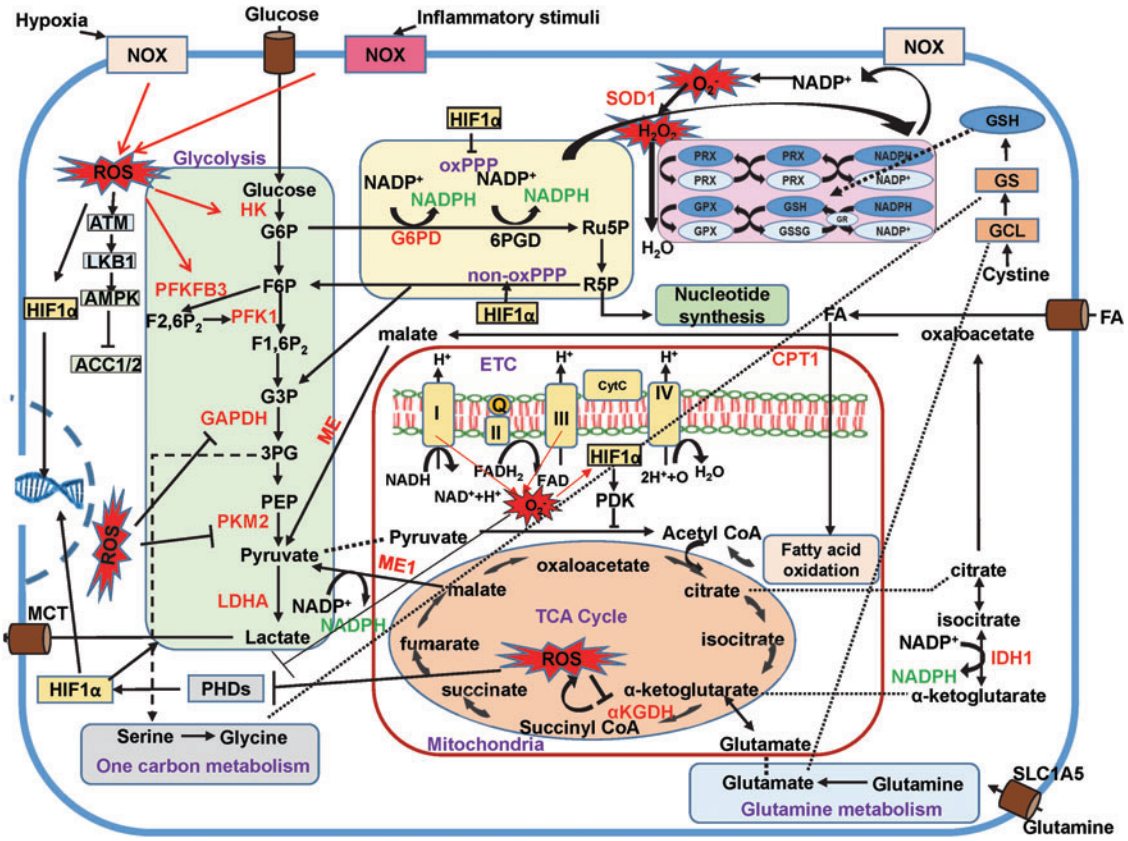

FIG. 5. Interplay between ROS and cellular metabolic pathways. Metabolic pathways contribute to redox homeostasis by regulating ROS generation *via* NOX/NADPH and mitochondrial ETC as well as by their impact on antioxidant systems *via* production of NADPH and GSH, as outlined in Figure 3. Conversely, cytosolic and mitoROS, which are produced by NOX, mitochondrial respiration, as well as metabolic and other enzymes, regulate metabolic pathways by targeting specific enzymes and transcription factors, including AMPK, glycolytic enzymes, mitochondrial enzymes, and HIFs. HIF-1α promotes a shift in metabolism toward glycolysis, while inhibiting mitochondrial O_2 consumption. This leads to decreased production of ATP through oxidative phosphorylation and thus reduced levels of mitoROS. AMPK, 5′ adenosine monophosphate-activated protein kinase; HIF, hypoxia-inducible factor; O_2, oxygen. Color images are available online.

the NADPH-generating 1C metabolism pathway. In this setting, phosphoglycerate dehydrogenase (PHGDH) catalyzes the biosynthesis of serine, and serine hydroxymethyltransferase (SHMT) then incorporates 1C from serine into the folate cycle, which also generates NADPH (28, 381) (Fig. 4). During hypoxia in Myc-transformed cells, hypoxia-inducible factor (HIF) and Myc function cooperatively to increase SHMT levels; this results in the generation of NADPH in the mitochondria and reduces the elevated levels of mitoROS (380). Mitochondrial NADPH can also be generated by the oxidation of malate to pyruvate by MEs, a mechanism that plays an important role in insulin secretion (126) (Fig. 4).

Different cell types most likely rely on different metabolic pathways to generate basal levels of NADPH. In mutant KRAS-driven pancreatic ductal adenocarcinoma cells, glutamine-derived malate was used to generate basal NADPH *via* ME1, rather than *via* oxidative PPP; decreased levels of glucose-6-phosphate dehydrogenase (G6PD) had no impact on the levels of NADPH in these cells (308). Another metabolic enzyme, 5′ adenosine monophosphate-activated protein kinase (AMPK), also regulates NADPH homeostasis. In response to glucose-deprivation stress conditions in which generation of NADPH cannot proceed *via* the PPP, activation of AMPK maintains NADPH levels by inhibiting acetyl-coenzyme A (acetyl-CoA) carboxylases ACC1 and/or ACC2 (154) (Fig. 5).

Reduced GSH. GSH is a highly abundant antioxidant tripeptide (1–10 m*M*) that is produced by most mammalian cells (14, 104, 214) and distributed ubiquitously within the cell, including in the cytosol (90%) as well as in the mitochondria, nucleus, endoplasmic reticulum, and extracellular space (10%) (104, 214). GSH is an antioxidant and detoxifying agent that scavenges ROS/RNS. GSH can be found in the cell in one of three main forms: reduced GSH, oxidized GSSG, and protein-glutathione mixed disulfides (PSSGs) (Figs. 2 and 5). Under physiological conditions, reduced GSH is the predominant form in the cell, where it is 10- to 100 times more abundant than its oxidized form. Thus, together with NADP/NADPH and TRX systems, the relative concentrations of GSH/GSSG determine the redox state at cellular homeostasis (Figs. 2 and 5). Further, GSH is involved in the maintenance of cysteine pools and the detoxification of xenobiotics. In response to oxidative stress, steady-state levels of cellular GSH are regulated by synthesis, recycling of oxidized GSSG, degradation of extracellular

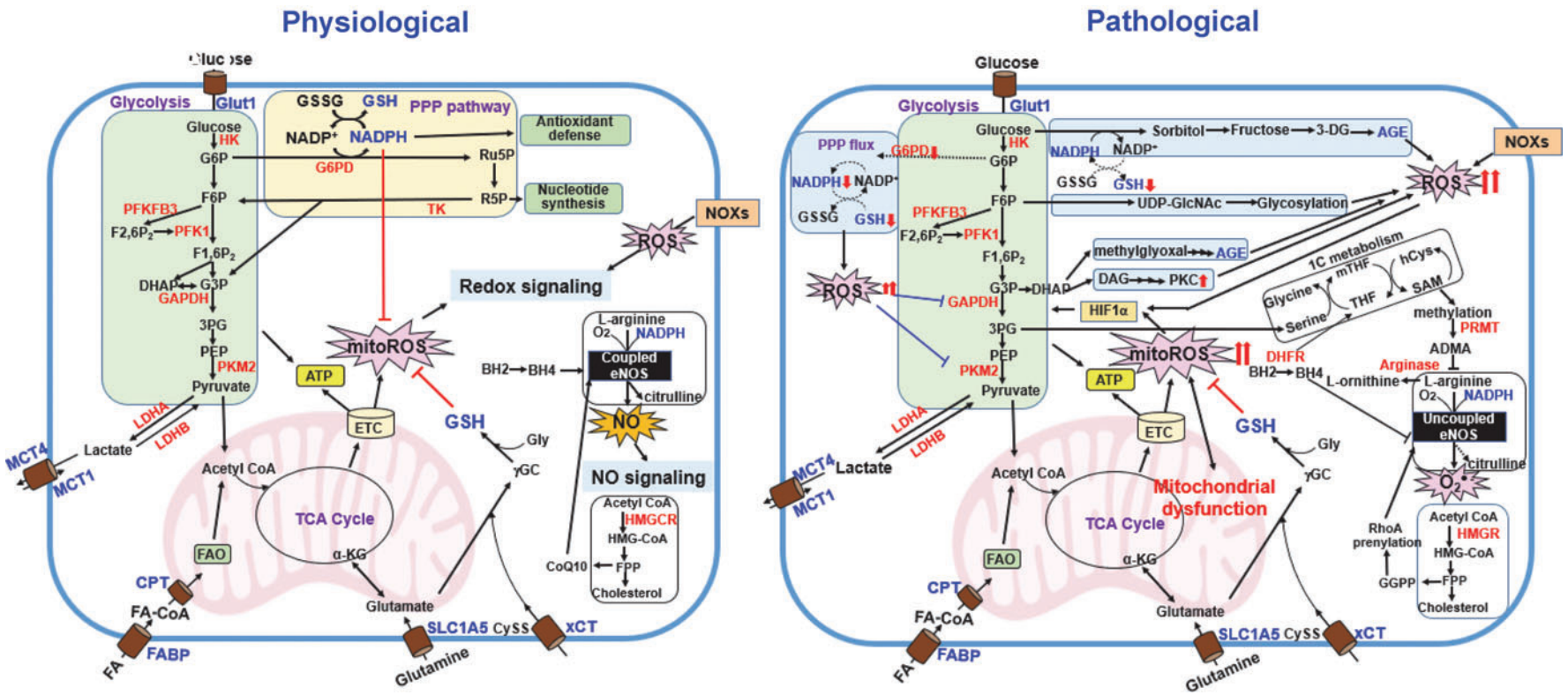

FIG. 6. The interplay between metabolic pathways and ROS (redox homeostasis) in physiological and pathological states. *Left panel*, glycolysis plays an essential role in the control of redox homeostasis by generating PPP-derived NADPH that is involved in the antioxidant system. FAO also generates NADPH through metabolic reactions. Glutaminolysis is involved in redox control by increasing GSH synthesis. During mitochondrial metabolism, ROS will be produced *via* ETC. $NO^{\bullet}$ is produced by NOS by utilizing BH_4, NADPH, and molecular O_2 to convert L-arginine to L-citrulline (coupled eNOS). NOXs- and mitochondria-derived ROS activate redox signaling. *Right panel*, in pathological states such as diabetes and atherosclerosis, excess ROS due to eNOS uncoupling and PPP-GSH impairment inhibit glycolytic flux, which diverts glycolytic intermediates into alternative metabolic pathways such as polyol pathway (AGE production) and PKC activation. This, in turn, further increases mitochondria- or NOX-derived ROS production and mitochondrial dysfunction. mitoROS or Nox-derived ROS stabilize HIF-1α, which induces a metabolic shift toward glycolysis, resulting in reduced oxidative phosphorylation and mitoROS production. The ADMA produced by L-arginine methylation *via* the 1C metabolism as well as arginase inhibit L-arginine binding to NOS, which decreases NO production. The mevalonate pathway also facilitates eNOS uncoupling by RhoAprenylation induced by GGPP, thereby promoting excess ROS production. ADMA, asymmetric dimethylarginine; GGPP, geranylgeranyl pyrophosphate; PKC, protein kinase C. Color images are available online.

GSH, and extrusion of the reduced, oxidized, or conjugated forms (14, 104, 214). GSH is synthesized *de novo* by adenosine triphosphate (ATP)-dependent glutamate-cysteine ligase (GCL) and GSH synthetase (GSS) (Fig. 4). GCL catalyzes the rate-limiting step of this process, which is a glutamate ligation with cysteine to form a dipeptide. GCL levels, together with levels of the cystine/glutamate transporter, are controlled by the critical transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) that activates antioxidant responsive genes (152). This dipeptide is then combined with glycine *via* the actions of GSS to produce GSH.

Cysteine is a rate-limiting substrate for GSH synthesis. Levels of this amino acid are controlled by the cystine/glutamate antiporter SLC7A11 (xCT), which also encodes the cystine/glutamate transporter (65, 304) (Fig. 4). Cysteine can also be generated by the transsulfuration of methionine. In addition to cysteine, glutamine, glutamate, and glycine are also important for GSH synthesis (Fig. 4). The availability of glutamine regulates GSH production in three ways (391) (Fig. 4). First, glutamine is the primary source of glutamate *via* the actions of glutaminases (GLSs) 1 and 2. GLS activity is tightly regulated to maintain appropriate intracellular GSH concentrations. Glutamine can be transported into cells by various amino acid transporter systems, including solute carrier family 1, member 5 (SLC1A5, also known as alanine/serine/cysteine-preferring transporter 2, or ASCT2), which is among the most commonly overexpressed transporters in cancer cells. SLC1A5 and GLSs regulate intracellular GSH levels by controlling glutamine availability and its conversion to glutamate, respectively. Second, glutamine contributes to the maintenance of GSH levels *via* the production of NADPH by ME, as described in the previous section. Third, the cystine/glutamate transporter system also regulates the levels of intracellular glutamine (304). Thus, an overall abundance of glutamine and glutamate is crucial to maintaining appropriate levels of intracellular GSH, which can promote tumor initiation and proliferation (125). In addition, GSH is an essential cofactor of GPX4 and can thus prevent ferroptosis; it also regulates the levels of cysteine, which also can trigger ferroptosis (378). Taken together, these findings highlight the importance of GSH-mediated antioxidant pathways in maintaining cell survival and promoting their growth.

Generation of $NO^{\bullet}$ by NOS:$NO^{\bullet}$/ROS generation

Coupled NOS ($NO^{\bullet}$ generation). $NO^{\bullet}$ is a free radical gas that is synthesized in humans by three distinct NOS isoforms, neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3) (Figs. 2, 4, and 6). $NO^{\bullet}$ has several distinct biological roles that range from mediating antimicrobial immune response, neurotransmission, and endothelium-dependent relaxation; these properties are dictated in an isoform- and cell-specific manner. The nNOS isoform is expressed primarily in the central and peripheral nervous systems, the gastrointestinal tract, and skeletal muscle. At these sites, $NO^{\bullet}$ is synthesized on demand in a calcium-dependent manner to regulate neurotransmission, peristalsis, and penile erection. By contrast, expression of iNOS takes place primarily in activated immune cells, most notably in macrophages and neutrophils that constitutively produce large amounts of $NO^{\bullet}$ and RNS that are used in their microbicidal and immunomodulatory functions. The eNOS isoform is expressed primarily in ECs within the blood vessels where, similar to nNOS, it produces $NO^{\bullet}$ on-demand in a calcium-dependent manner to relax vascular smooth muscle cells (VSMCs), thereby reducing vascular tone and blood pressure. The enzymology underlying $NO^{\bullet}$ biosynthesis by each of the three NOS isoforms is well conserved and involves the NADPH-dependent conversion of L-arginine to $NO^{\bullet}$ with L-citrulline as a byproduct. The three NOS isoforms each contain two domains, including NADPH-binding oxygenase and heme-containing reductase domains that are found in a head-to-tail configuration as parts of a functional homodimer. NADPH binds to a C-terminal binding domain in the reductase domain of one monomer, which delivers electrons *via* a flavin bridge (*i.e.*, flavin adenine dinucleotide [FAD], flavin mononucleotide [FMN]) to an N-terminal heme moiety that binds O_2 at the oxygenase domain of the other monomer. This results in the reduction of molecular O_2 and its insertion into the guanidine nitrogen of L-arginine (103). Electron flow to the heme occurs constitutively in iNOS, but it is controlled by calcium-calmodulin-dependent binding in eNOS and nNOS and is further fine-tuned by post-translational modifications.

Uncoupled NOS (ROS generation). Although all NOS isoforms generate $NO^{\bullet}$, they can also generate $O_2^{\bullet-}$ at the expense of $NO^{\bullet}$ *via* a process known as uncoupling (Figs. 2, 4, and 6). The mechanisms underlying uncoupling have undergone intensive investigation and various schemes have been proposed ranging from the formation of monomers, NOS phosphorylation at threonine (T)495, altered heat-shock protein (Hsp)90 binding, and insufficient levels of tetrahydrobiopterin (BH_4) and L-arginine. Of these mechanisms, low levels of BH_4 or low BH_4 to dihydrobiopterin (BH_2) ratios are the most reproducible findings associated with NOS uncoupling (111). BH_4 is an essential cofactor for all NOS isoforms; two molecules of BH_4 are bound stoichiometrically to a single NOS dimer. A BH_4 binding site can be found near the dimer interface on NOS isoforms. This site also binds to the heme iron and converts it from a low-spin to a high-spin state (21). Reduced BH_4 facilitates the oxidation of L-arginine; oxidized biopterin (BH_2) can also bind to NOS but it does not facilitate $NO^{\bullet}$ production. Bound BH_4 also facilitates arginine binding and is important for isoform-specific dimerization (338). BH_4 is a redox-sensitive molecule and is particularly susceptible to degradation by peroxynitrite ($ONOO^-$), which is more potent at oxidizing BH_4 than is either $O_2^{\bullet-}$ or H_2O_2 (188). Indeed, high intracellular levels of $O_2^{\bullet-}$ are not sufficient to block the eNOS-mediated synthesis of $NO^{\bullet}$; however, this condition does reduce its bioavailability (392). Under physiological conditions, there is typically a high ratio of BH_4 to BH_2. By contrast, in disease settings, particularly those associated with increased levels of ROS, BH_2 levels are elevated to the point at which they can become dominant. However, the BH_4:BH_2 ratio is believed to be more important for NOS uncoupling than are the absolute levels of BH_4. BH_2 and BH_4 bind to NOS enzymes with equal affinity, although BH_2 does not facilitate the insertion of activated O_2 into L-arginine; this leads to $O_2^{\bullet-}$escape (337). In addition to the direct oxidation of BH_4, conditions including hyperglycemia and availability of $ONOO^-$ can also prevent BH_4 synthesis by promoting the

ubiquitin-dependent degradation of GTP cyclohydrolase (GTPCH) 1 (373) (Fig. 4). Patients with diabetes, atherosclerosis, and/or hypertension typically have lower levels of BH_4 and thus the potential for dysregulated eNOS activity. Supplementation with BH_4 or folate and increased expression of GTPCH can all serve to improve endothelial function (340, 373). BH_4-deficient macrophages have improved microbicidal activity compared with those that are iNOS-deficient. These findings suggest a functional role for uncoupled NOS enzymes (234). Unexpectedly, reduced levels of BH_4 can promote increased production of mitoROS along with the accumulation of the tricarboxylic acid (TCA) cycle metabolites succinate and fumarate (7). The mitoROS have been shown to have an important role in killing bacteria (359). Low levels of BH_4 in human macrophages have been proposed as a mechanism to explain their comparatively weak ability to generate $NO^{\bullet}$ when compared with rodent macrophages, although they maintain potent microbicidal activity (193). Loss of BH_4 in human macrophages was also linked to impaired activation of Nrf2 (233). To counteract BH_4 deficiency, ascorbic acid, folic acid, and overexpression of the rate-limiting enzyme in BH_4 synthesis, GTP cyclohydrolase (GCH1, the gene encoding GTPCH), have all been shown to promote $NO^{\bullet}$ production and thus to protect against the development of atherosclerosis (79, 182, 312). In another proof-of-concept study, novel analogs of BH_4 have been developed that are resistant to oxidation; administration of these analogs results in improvements in eNOS expression endothelial function and eNOS expression (114). Uncoupled eNOS has been identified as a source of ROS in multiple CVD states. Additional information on this subject can be found in previous reviews (2, 165, 171, 321, 379).

Metabolic Pathways and ROS

Cellular metabolism maintains redox homeostasis by generating ROS *via* the mitochondrial ETC as well as by the actions of the antioxidant systems *via* NADPH and GSH (Figs. 2–6). Further, metabolic reprogramming and increased flux through specific pathways play important roles in shaping both inflammatory and immune responses, to which ROS/RNS are also important contributors (10, 102, 108, 177). In this section, we will highlight how specific metabolic pathways regulate ROS production and inflammation.

Glycolysis and ROS

The ROS and glycolysis are closely linked to one another (Figs. 4–6, shaded in green). For example, to minimize the potential damage to DNA that can occur in cells proliferating under oxidative stress, tumor cells can increase their uptake of glucose and shift the metabolism toward glycolysis to release lactate even in the presence of molecular O_2 (*i.e.*, aerobic glycolysis, also known as the Warburg effect) (354). This results in reduced ROS levels compared with cells undergoing mitochondrial oxidative phosphorylation (200). Pharmacological inhibition or knockdown of glycolytic enzymes will result in the suppression of tumor growth in a variety of cancers (284, 368), whereas induction of mitochondrial respiration will result in slower growth rates both *in vitro* and *in vivo* (48, 290). Similarly, cells with mutant mitochondria with a reduced capacity for oxidative phosphorylation will promote growth through induction of glycolysis (399). The necessity of glycolytic flux in angiogenic ECs (both tip and stalk cells) is supported by observations that include impaired spheroid sprouting, postnatal outgrowth, and branching of murine retinal vasculature in cultured ECs devoid of the glycolytic enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) as well as *in vivo* (74, 375). PFKFB3 overexpression in zebrafish stimulates tip cells (74). Increased glycolysis in ECs provides energy not only for cell proliferation but also for cell migration; this was clear from the results of experiments that colocalized PFKFB3 or other glycolytic enzymes with F-actin in filopodia and lamellipodia, which are sites at which ATP is produced to support rapid remodeling. A recent report showed that loss of PFKFB3 from ECs impairs ischemic muscle revascularization and regeneration by reducing the extent of lactate-mediated macrophage polarization (390).

The role played by glycolysis in promoting oxidative stress associated with atherosclerosis is complex and not fully understood. Disturbed blood flow at aortic bifurcations promotes atherosclerosis as well as induces glycolysis and reduces mitochondrial respiratory capacity in ECs *via* the activation of HIF-1α by NOX4-derived ROS (369) (Figs. 5 and 6). Activated HIF-1α induces the expression of glycolytic enzymes and pyruvate dehydrogenase kinase 1 (PDHK1). This results in a reduction in the mitochondrial respiratory capacity, vascular inflammation, and atherosclerosis (369). The metabolite signatures identified in high-risk atherosclerotic plaques showed increased levels of glycolysis, elevated amino acid utilization, and decreased fatty acid oxidation (FAO), compared with those from low-risk atherosclerotic plaques (329). By contrast, another study revealed that adaptive increases in AMPKα1 induced by disturbed blood flow stimulated EC glycolysis and regeneration and was atheroprotective (376). Further, proinflammatory signaling enhances glycolysis in ECs, which can promote nuclear factor-kappa B (NF-κB)-driven vascular inflammation *via* lactate signaling (393); this will promote a cycle that results in sustained proinflammatory signaling (326). Activated neutrophils responding to oxidative stress shift toward hyperglycolysis *via* the phosphorylation of phosphofructokinase 2 (PFK2) by NOX2-derived ROS (9). Other reports have highlighted the metabolic dependency of astroglial cells on glucose availability for regeneration of the NADPH (181). Finally, hyperproliferative ECs due to pulmonary hypertension also rely on increased glycolytic flux and reduced O_2 consumption, both of which are associated with HIF-1α overexpression (334).

Similar to tumor cells, ECs and brain tissue obtain ATP mainly from glycolysis rather than from oxidative phosphorylation, even in O_2-rich environments (16, 74, 200). Eighty percent of the ATP in ECs is generated by glycolysis, even in the presence of O_2 (74). As glycolysis produces much less energy than does oxidative phosphorylation, the physiologic mechanism underlying this observation remains unclear, although several potential advantages have been proposed (1, 16, 200). For example, one of the benefits of the metabolic shift toward glycolysis in ECs is to reduce the production of ROS by decreasing oxidative phosphorylation, which is the main source of ROS production (199), as well as by generating NADPH *via* PPP to counteract ROS that are produced. Glycolysis is also O_2-sparing and can generate

ATP more rapidly than can be achieved with oxidative phosphorylation. This may be an important adaptive mechanism to promote rapid vascularization of hypoxic tissue.

Therapeutic implications. Given that ECs are both highly glycolytic and angiogenic, therapeutic modulation of glucose metabolism and glucose transporters (GLUTs) are of great interest from a therapeutic perspective (73, 74). However, complete and permanent inhibition of glycolysis with 2-deoxy-D-glucose was associated with unacceptable levels of toxicity and yielded minimal success as monotherapy (324). Interestingly, partial and transient reduction of glycolysis in response to low doses, but not high doses of the PFKFB3 inhibitor, 3PO, reversed excessive vascular growth observed in response to genetic ablation of Notch or vascular endothelial growth factor receptor (VEGFR) 1 in mice with no effect on EC maintenance (287). Other PFKFB3 inhibitors that improve the pharmacokinetic properties and toxicological parameters include PFK-158, an agent that recently entered Phase I clinical trials (57), as well as a phenoxyindole derivative with higher selectivity for PFKFB3 over the other PFKFB isoforms (31). Because glycolysis plays an essential role in controlling redox homeostasis *via* regulation of PPP-derived NADPH, inhibition of glycolysis by the lactate dehydrogenase A inhibitor, FX11, impaired cancer cell growth by decreasing the intracellular ATP levels and inducing oxidative stress (189). Further, inhibition of glycolysis and the PPP combined with the disruption of the TRX system selectively increased its cytotoxicity in several cancers, but not in normal counterparts (285). Thus, one might speculate that a combined approach that included inhibition of glycolysis and the antioxidant system may prove to be an important therapeutic strategy for the treatment of various vascular diseases that depend on glycolysis.

PPP and ROS

Once glucose enters the cell *via* a GLUT, it undergoes phosphorylation by hexokinase (HK) to generate glucose-6-phosphate (G6P) and thus becomes a substrate for glycolysis, glycogen formation, and the PPP. The PPP includes both oxidative and non-oxidative pathways (Figs. 4–6 shaded in yellow). The oxidative PPP produces cellular NADPH that is required for antioxidant defense and FA synthesis, whereas the non-oxidative PPP produces pentose (5-carbon) sugars. Both of these pathways produce ribose 5-phosphate, which is a precursor for nucleotide synthesis. Glycolytic flux can supply the oxidative PPP pathway *via* the actions of G6PD, which is the first committed and rate-limiting step.

The functional significance of oxidative PPP and ROS is revealed by a common human enzyme defect known as X-linked G6PD deficiency, which is an enzyme that protects against oxidative stress. Erythrocytes are very sensitive to oxidative stress and highly dependent on oxidative PPP to maintain adequate levels of NADPH and GSH. One phenotype commonly associated with G6D deficiency is hemolytic anemia after ingestion of agents that can induce oxidative stress (*e.g.*, sulfonamides and fava beans, among others). Under conditions of oxidative stress, glucose utilization is shifted from glycolysis to PPP to produce more NADPH and to generate GSH from GSSG (276). The glycolytic enzyme, GAPDH, has critical cysteine residues in its active site that can be oxidized and thus inactivated by H_2O_2 (259). PKM2 is another key glycolytic enzyme that can be S-glutathionylated at C358 by H_2O_2; this compromises its enzymatic activity, thereby reducing the rate of glycolysis and increasing flux into the PPP pathway to increase levels of NADPH (4).

Although NADPH functions to maintain the GSH and TRX levels (Figs. 2 and 5), in some tissues, NADPH may take on a pro-oxidative role *via* its actions as a cofactor for the enzyme, NOX (32) (Figs. 2 and 4). The enzyme, G6PD, which is a rate-limiting enzyme for PPP (a key source of NADPH in oxidative stress), can play distinct roles depending on the specific cell types and conditions. Under conditions of pathologic oxidative stress, for example, vascular tissue challenged with angiotensin II (228) or macrophages responding to lipopolysaccharide (LPS) (122), G6PD promotes ROS production in VSMCs (228), while also protecting against ROS in ECs (195). G6PD deficiency inhibits oxidant-mediated angiotensin II-induced signaling pathways by limiting the production of NADPH, which, in these situations, serves as a substrate for NOX (228). In the context of atherosclerosis, G6PD-mutant/apolipoprotein E $(ApoE)^{-/-}$ mice display reduced G6PD activity (20%) together with decreased levels of vascular $O_2^{\bullet-}$, inflammatory responses, and atherosclerotic lesions (229). By contrast, G6PD overexpression in ECs reduces TNFα-induced ROS production and increases eNOS activity because NADPH is also a substrate for eNOS (195). Thus, whether or not NADPH-derived from G6PD can function as an antioxidant seems to be dependent on the levels of NOX and peroxidases that are upregulated in disease states associated with oxidative stress.

Glutaminolysis and ROS

Glutamine is the most abundant non-essential amino acid in the human body. Circulating concentrations of glutamine are typically between 400 and 600 μM. Glutamine is a key source of carbon and nitrogen for biosynthetic processes. The enzyme, GLS, converts glutamine to glutamate, which then undergoes decarboxylation to produce α-ketoglutarate (α-KG) (Figs. 4 and 5, shaded in blue). The Krebs cycle intermediate, α-KG, is ultimately used to produce ATP. The importance of glutamine as a carbon source to supply the TCA cycle in ECs was also demonstrated by the enhanced levels of glutamine metabolism observed in association with pulmonary artery hypertension (89). Further, the ongoing metabolism of glutamine is essential to meet the metabolic needs of hyperproliferative vascular cells and proceeds *via* a mechanism that relies on both mechanotransducer Yes-associated protein (YAP) and the transcriptional coactivator with PDZ binding motif (TAZ) (23). The ECM stiffening results in mechanoactivation of YAP/TAZ, which then stimulates GLS expression. The GLS is critical for proliferation and migration *via* its role in replenishing the amino acid, aspartate (23). Glutamine metabolism is also essential for EC proliferation. Inhibition of glutamine metabolism in ECs by inhibiting GLS-1 or glutamine deprivation prevents EC proliferation by impairing lipid biosynthesis *via* reductive carboxylation and complete loss of TCA intermediates. This inhibition of glutamine metabolism also increases oxidative stress by decreasing the rate of GSH synthesis (141, 174). The importance of glutamine in angiogenesis has been demonstrated by observations on the proliferation of aggressive cancer cells

and its dependence on glutamine availability (45, 347). As one example, glutamine deprivation induces apoptosis in human breast cancer cells (47). However, the role of glutamine metabolism in EC migration remains controversial (141, 174, 260). Glutamine metabolism also plays a role in injury-induced neointimal formation in arteries by regulating VSMC proliferation (250). The TEA domain transcription factor 1 (TEAD1) promotes VSMC proliferation *via* transcriptional induction of the glutamine uptake transporter, SLC1A5. This results in the activation of the mammalian target of rapamycin complex 1 (mTORC1) signaling and promotion of neointima formation (250).

Glutamine is involved in ROS homeostasis as a precursor for GSH. To be available to cells, glutamine must be transported into cells by specific transporters, including SLC1A5/ASCT2, and converted to glutamate by GLS. Glutamate directly contributes to GSH synthesis by promoting the uptake of cystine through the cystine/glutamate exchanger, Slc7a11. Thus, the availability of glutamine, glutamate, and cysteine regulates the biosynthesis of cellular GSH. Glutamine can also produce NADPH *via* the malate system, and it also serves as a precursor for the GSH system. Similarly, TCA cycle intermediates, for example, citrate, can be exported into the cytosol, where ME or IDH1 uses them to generate NADPH (39). Since NADP/NADPH levels control the oxidative state of GSH, tumor cells maintain the GSH pool in a reduced state, thereby supporting the TRX system. Further, the mitochondrial enzyme, glutamate dehydrogenase 1, positively regulates the enzymatic activity of the antioxidant enzyme GPX by controlling intracellular levels of fumarate (157). Thus, glutamine promotes ROS homeostasis by regulating the synthesis of GSH, NADPH, and the mitochondrial antioxidant enzyme, GPX.

Therapeutic implications. Given the importance of glutamine in promoting angiogenesis (141, 174), GLS1 inhibitors have been used in clinical trials for solid tumor and leukemia cells and might ultimately be repurposed for the treatment of pathologic angiogenesis in vascular disease (168). The GLS inhibitors include compound 968, BPTES, and CB-839 (149, 168). Because glutamine also plays an important role in maintaining redox balance, inhibition of glutaminolysis can result in the depletion of the intracellular GSH and subsequent generation of ROS, both of which contribute to impaired cell proliferation (117, 147).

FAO and ROS

The FAO is important for NADPH homeostasis and redox balance (Fig. 4). The FAs are an excellent source of energy, as they can produce twice as much ATP as can be obtained from carbohydrates. They are also a source of NADPH and thus serve as an alternative to the PPP. When NADPH generation by the PPP is impaired under conditions of energy stress, for example, glucose deprivation, the actions of AMPK result in an increase in NADPH *via* FAO, which will ultimately inhibit cell death (154). Inhibition of FAO results in decreased NADPH and GSH levels and elevated levels of intracellular ROS (261). In macrophages, inhibition of FAO results in the generation of mitoROS; this promotes the recruitment of NOX to the phagosomal membrane to limit the growth of the pathogen, *Mycobacterium tuberculosis* (Mtb) (41).

The FAO includes a series of cyclic oxidation reactions *via* which long- and short-chain FAs are degraded, resulting in the generation of NADH, $FADH_2$, and acetyl-CoA. The FAO-derived acetyl-CoA can be introduced into the TCA cycle to generate ATP and aspartate for the synthesis of deoxynucleotide triphosphates (dNTPs) that are required for DNA replication in proliferating ECs. In cancer cells, however, only a fraction of the acetyl-CoA produced completes the TCA cycle to produce ATP; the acetyl-CoA that remains is used to generate citrate. Citrate is then exported into the cytosol, where it ultimately supports the production of large amounts of NADPH with the help of ME and IDH1 (39) (Fig. 4). In ECs, stalk cells depend on FAO for vessel sprout elongation, specifically *via* its capacity to sustain the synthesis of dNTPs (80, 286, 367). By contrast, tip cells depend on PFKFB3-driven glycolysis for rapid production of ATP for vessel sprouting (74, 375). Interestingly, Notch signaling serves as a molecular switch and promotes the transition from FAO from nucleotide synthesis pathways in proliferating ECs to NADPH regeneration in quiescent ECs. This promotes the protection of the vasculature against oxidative stress-induced cell damage (162). Mice with an EC-selective deletion of the FAO rate-limiting enzyme, carnitine palmitoyltransferase-1 (CPT1) showed endothelial dysfunction, including inflammatory cell recruitment and barrier disruption typically associated with increased oxidative stress (162).

In the heart, FAs are the main source of energy. In normal states, FAO, followed by carbohydrate (glucose and lactate) oxidation from mitochondrial oxidative phosphorylation, are the major sources of ATP production (98, 153). Interestingly, there is a reciprocal relationship between FAs and glucose oxidative metabolism; this is known as the Randle Cycle or the glucose/FA cycle (271). In various heart diseases, including ischemic heart disease and heart failure, the relationship between FAO and glucose oxidation is disrupted; this results in impaired cardiac efficiency and function (167, 209). During ischemia/reperfusion, circulating FAs and cardiac FAO levels are elevated. This results in decreased glucose oxidation through the inhibition of pyruvate dehydrogenase (PDH) activity, which is the rate-limiting enzyme that catalyzes the conversion of pyruvate to acetyl-CoA and NADH in mitochondria (167, 209). It has been proposed that reduced cardiac efficiency in disease states associated with elevated FAO is due to the use of a less efficient energy source than glucose oxidation based on the amount of ATP produced per O_2 molecules consumed (97, 209). Consistent with this concept, inhibiting FAO and/or increasing glucose oxidation can result in improved cardiac function in ischemic heart disease, heart failure, and diabetic cardiomyopathy (87, 98, 153, 166).

Therapeutic implications. The introduction of FAO inhibitors has provided clear therapeutic benefits to patients with type II diabetes or myocardial ischemia (39), because inhibition of FAO alleviates both O_2 shortage and insulin resistance. The CPT1 inhibitor, perhexiline, has been evaluated as a potential treatment for heart disease. Many other CPT1 inhibitors are undergoing preclinical evaluation (*e.g.*, oxfenicine) or exhibited toxic side effects in clinical trials (*e.g.*, etomoxir) (137). Both trimetazidine (164) and ranolazine (245) inhibit 3-ketoacylthiolase (3-KAT), the enzyme

that catalyzes the final step in FAO, and they are in use for the treatment of angina. In macrophages, inhibition of FAO promotes key antimicrobial functions and overcomes the immune evasion mechanisms associated with infection with Mtb (41), as mentioned earlier. The FAO also plays a critical role in tumor growth *via* its capacity to regulate NADPH homeostasis and oxidative stress (261). In human glioblastoma cells, pharmacological inhibition of CPT1 by etomoxir enhances cell death by promoting decreased levels of NADPH and GSH and by elevating the levels of intracellular ROS (261). Overexpression of CPT1A has been associated with a high tumor grade, unfavorable clinical outcomes in acute myeloid leukemia and ovarian cancer (218). Thus, targeting FAO may be a promising approach toward reducing pathologic angiogenesis and heart failure (80, 166).

1C metabolism and ROS

In proliferating cells, 1C metabolism (in particular, serine-glycine 1C metabolism, or SGOC) is one of the major sources of NADPH (Fig. 4, shaded in gray) other than the oxidative PPP (94, 196, 226). SGOC is mediated by a folate cofactor and is a universal metabolic process that serves to activate and transfer 1C units to support the biosynthesis of purines and thymidine, amino acid homeostasis (glycine, serine, and methionine), epigenetic maintenance, and redox balance (3). Serine and glycine 1C metabolism involves three pathways (Fig. 4, shaded in gray), including the folate cycle, the methionine cycle, and the transsulfuration pathway. PHGDH catalyzes the biosynthesis of serine; SHMT subsequently introduces 1C unit from serine into the folate cycle. Carbon units then enter the methionine cycle, resulting in the generation of S-adenosyl-methionine (SAM), which undergoes further conversion to homocysteine and ultimately to cysteine that can be diverted toward the synthesis of GSH. A tracing study of NAPDH compartmentalization revealed that serine was used predominantly in the mitochondria of mammalian cells to generate NADPH (196) (Fig. 4). During hypoxia, HIF-1α and MYC work cooperatively to increase the expression of SHMT2 (the mitochondrial isoform of SHMT) to promote the production of mitochondrial NADPH and to counteract the elevated levels of mitoROS (380). Upregulation of 1C metabolism is another metabolic shift used to evade ROS-induced cell death.

In addition to reducing ROS production by supplying NADPH, 1C metabolism plays an important role in supporting endothelial function and preventing CVD (Fig. 4, shaded in gray). These activities are mediated *via* the modulation of eNOS activity and the regulation of its cofactor, BH_4 (20), and the methylation of arginine residues in proteins, which will be discussed in the section focused on RNS to follow (Figs. 4 and 6, shaded in orange). Further, 1C metabolism is also involved in the generation of homocysteine. For example, inactivating mutations in the 1C folate metabolism gene encoding methylenetetrahydrofolate reductase (MTHFR) result in hyperhomocysteinemia, which is a prominent risk factor associated with CVD (110) (Fig. 4, shaded in gray).

Therapeutic implications. Anti-folates that target 1C metabolism have been explored as treatments for cancer (84). Further studies will be needed to develop therapeutic strategies that selectively inhibit SHMT2 for the treatment of CVD. Since 1C metabolism is involved in supporting antioxidant defense *via* the actions of GSH and NADPH in a cell-type and context-dependent manner, it may be possible to achieve greater selectivity than the simple inhibition of DNA synthesis alone. Therapeutic approaches that modulate NOS activity by targeting SGOC in BH_4 and arginine metabolism will be discussed in the section focused on RNS.

Branch-chain amino acids and ROS

Branch-chained amino acids (BCAAs), including valine (V), leucine (L), and isoleucine (I), function as critical nitrogen donors in processes involving intracellular nitrogen shuttling. BCAA uptake is facilitated by the large neutral aminoacid transporter (LAT/SLC7a5); once inside the cells, BCAAs are converted to branched-chain ketoacids (BCKAs) by the actions of the enzyme, branched-chain aminotransferase (BCAT). BCKAs undergo further conversion to acetyl-CoA and succinyl-CoA *via* the actions of the BCKA dehydrogenase (BCKD) complex, which is linked to the TCA cycle (Fig. 3). Although most amino acids are metabolized in the liver, catabolism of BCAAs takes place in several non-hepatic tissues, including cardiac muscle, adipose tissue, brain, and kidney (49, 143, 151, 358). Treatment with BCAAs can be beneficial, but, paradoxically, increased circulating levels of BCAA have also been associated with obesity and diabetes. For example, Tanada *et al.* (319) showed that supplementation with BCAAs resulted in clinical improvement in a rat model of heart failure. Further, Zhao *et al.* (395) found that leucine (L) supplementation reduced the size of atherosclerotic lesions in $ApoE^{-/-}$ mice; this finding was associated with an improved plasma lipid profile and reduced levels of systemic inflammation. By contrast, 3-hydroxy-isobutyrate (3HIB), a catabolic intermediate of the BCAA valine (V), activates trans-endothelial FA transport and thus stimulates FA uptake in muscle tissue *in vivo* and promotes lipid accumulation and insulin resistance (151). Thus, BCAAs may provide a novel therapeutic strategy for atherosclerosis and cardiometabolic disease (387).

Oxidative stress has been closely associated with the pathophysiology of an inherited metabolic genetic disorder of BCAA metabolism known as maple syrup urine disease (MSUD). The MSUD results from a BCKD dehydrogenase deficiency and results in the accumulation of all BCAAs (151, 274). The patients with MSUD show high levels of lipid and protein oxidation in plasma (17) and an inflammatory profile that results from unbalanced ROS production (235). The importance of oxidative stress in the pathophysiology of other inherited metabolic disorders of BCAA metabolism, including methylmalonic acidurias (MMAs) and homocystinuria, has been well characterized. Fibroblasts derived from these patients showed elevated ROS, apoptosis, and phosphorylation of the stress kinases p38MAPK and JNK (274). Interestingly, BCATs have redox-active CXXC motifs. When these enzymes are S-glutathionylated, they support the chaperone role of BCAT and promote appropriate protein folding (60, 90). Therefore, BCAA metabolism also plays an important role in regulating the redox balance (274).

Alpha-ketoglutarate dehydrogenase and ROS

The primary sources of ROS in mitochondria are complex I and III of the ETC. Also, metabolic enzymes, including

alpha-ketoglutarate dehydrogenase (α-KGDH) and PDH complexes, produce mitoROS (266, 332) (Fig. 5). The enzyme, α-KGDH catalyzes the conversion of α-KG to succinyl-CoA *via* the actions of IDH2 or IDH3 and produces NADH that provides electrons for the ETC. Importantly, α-KGDH together with PDH complexes are believed to produce more ROS than complex I, which is regulated by NADH/NAD ratio (266, 332). In addition to generating ROS, KGDH can also be inactivated by oxidative stress (332), which, in turn, limits the supply of NADH to the ETC. In tumors grown under hypoxic conditions or in the presence of a defective ETC, α-KGDH plays an important role in maintaining cell proliferation and lipogenesis (236, 364). During hypoxia, citrate is generated from glutamine-derived α-KG *via* reductive carboxylation by cytosolic and mitochondrial NADPH-dependent IDH1 and IDH2. The generation of isocitrate from α-KG implies a reduced level of α-KGDH activity and an unbalanced α-KG/citrate ratio. This will lead to a reverse TCA cycle that ultimately promotes FA synthesis and favors tumor growth. α-KGDH can also be inhibited *via* the degradation of its E3 subunit by HIF-1 (96).

Inflammation, metabolic shifts, and ROS

Chronic low-grade inflammation plays a key role in promoting CVD *via* the regulation of energy metabolism (10). The master transcription factor, NF-κB, is one of the critical regulators of metabolic reprogramming that promotes aerobic glycolysis in innate immune defense and during acute inflammation (10, 230, 331). By contrast, activation of sirtuin 1 (SIRT1) inhibits NF-κB signaling, enhances oxidative metabolism, and promotes the resolution of inflammation (140). Thus, both innate immunity and energy metabolism can be regulated by antagonistic crosstalk between NF-κB-and SIRT1-mediated signaling pathways (169). Given that ROS regulate the actions of NF-κB in response to inflammatory agonists (227) and that SOD2 overexpression inhibits ROS-induced NF-κB (43), ROS, inflammation, and metabolism appear to be closely linked.

To meet their bioenergetic, biomass, and redox demands, T cell activation and differentiation require coordinated programming of cellular metabolism. However, several studies have revealed that the T cell metabolic program differs depending on the specific cell type (99, 216, 256). For example, activated effector T (Teff) cells generate energy by augmenting aerobic glycolysis, whereas memory T cells (Tm) engage FAO. Moreover, regulatory T cells (Tregs) activate AMPK and depend on lipid oxidation for their energy requirements (237, 257, 303, 351). The results of several studies suggest that mitochondrial dynamics control T cell fate through metabolic reprogramming and by altering the morphology of their cristae (35). Tm cell fusion configures the ETC complex associations to favor oxidative phosphorylation and FAO, whereas fission in Teff cells leads to expansion of the cristae, reduced ETC efficiency, and augmented glycolysis (35). Cell growth, clonal expansion, and the effector functions of Teff cells require enhanced aerobic glycolysis, the PPP, and glutaminolysis (5, 100, 105, 112, 148).

During T cell activation, increased mitochondrial biogenesis results in more mitochondria, an expanded mitochondrial-dependent metabolic flux, and the production of ROS (68). Also, mitoROS-induced by fission contributes to NF-κB-mediated activation in T cells (278). T cell activation, proliferation, differentiation, and immune responses all require ROS-mediated signaling and activation of transcription factors such as NF-κB and activator protein 1 (AP-1) (76, 163, 243). However, excessive ROS production induces apoptosis in T cells *via* mechanisms that depend on B cell lymphoma 2 (Bcl-2), FAS ligand (FasL), and the mitochondrial membrane potential (130, 131). Thus, a fine-tuned balance between glycolysis, the PPP, and glutaminolysis ensures appropriate levels of intracellular ROS that drive T cell activation, differentiation, and immune responses (296).

Activation of proinflammatory macrophages during inflammation is caused by metabolic reprogramming from oxidative phosphorylation to glycolysis. The mitoROS stabilize and activate hypoxia-inducible factor HIF-1α, which, in turn, increases both glycolytic capacity and expression of the proinflammatory cytokine, interleukin (IL)-1β (66, 238); this pathway also regulates the formation and activation of inflammasomes. Of note, succinate can drive mitoROS production at complex I *via* reverse electron transport (RET) as part of the pathogenesis of ischemia–reperfusion injury (53, 258, 292) (Fig. 3). The SDH deficiency directs macrophages toward an anti-inflammatory phenotype (*i.e.*, production of IL-10), thereby resulting in RET-induced generation of mitoROS. This, in turn, enhances ATP production *via* oxidative phosphorylation and reduced mitochondrial membrane potential (158, 238, 398). Interestingly, the endogenous metabolite, itaconate, which is highly induced in activated macrophages, inhibits SDH-mediated succinate oxidation, thereby promoting anti-inflammatory effects (186) (Fig. 4). Thus, mitoROS produced in response to metabolic reprogramming and itaconate-induced succinate oxidation plays a key role in macrophage phenotype switching from M1 (inflammatory) to M2 (anti-inflammatory).

The NLR family pyrin domain containing 3 (NLRP3) inflammasome functions as a sensor of metabolic stress and regulates inflammation *via* interactions with thioredoxin-interacting protein (TxNIP) (289, 299, 333, 397, 398); TxNIP binds to TRX, thereby reducing its activity (382). In response to glucose stimulation, TxNIP dissociates from TRX and interacts with NLRP3 *via* an ROS-sensitive mechanism to activate inflammasome and inflammatory cytokine signaling (289, 299, 333, 397, 398). In addition to inflammasome activation, ROS also promote critical efferocytotic activities (*i.e.*, removal of apoptotic cells) of macrophages, which is a critical aspect of the resolution of inflammation (101). Lysophosphatidylcholine released from apoptotic cells reduces mitochondrial membrane potential and ATP production; this results in the generation of mitoROS and activation of AMPK (156). The AMPK activation facilitates metabolic reprogramming toward glycolysis and induces the synthesis of tubulin that is needed to promote macrophage chemokinesis and efferocytosis (156). Thus, ROS play an important role in inflammasome activation by regulating the actions of TXNIP and macrophage-mediated efferocytosis by metabolic reprogramming *via* AMPK.

Therapeutic implications. The results of the Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) trial support the inflammatory hypothesis of atherosclerosis and cancer in humans by demonstrating the beneficial

effects of canakinumab, which is an anti-IL-1β neutralizing monoclonal antibody (204, 357). However, limited effects on cardiovascular mortality and the prominence of side effects, including the higher incidence of fatal infections, warrant further investigations directed at new therapeutic strategies. Although promising clinical outcomes have resulted from immunotherapy, including immune checkpoint blockade, these therapies have proven to be ineffective for a significant number of patients (119). Given that metabolic reprogramming of immune cells influences the responses to immunotherapy and that metabolic programs differ among immune cell subsets, inhibitors designed to target specific metabolic pathways may be a promising therapeutic approach for inflammatory CVD. This topic is covered in detail in previous reviews (24, 119, 370).

Itaconate and ROS

Itaconate is one of the most abundant metabolites in activated macrophages (186). Itaconate is synthesized from aconitate, a molecule that would otherwise contribute to the TCA cycle *via* the actions of aconitate decarboxylase 1 (ACOD1, also known as immune-responsive gene 1 [IRG1]) (Fig. 4). Itaconate inhibits the activity of SDH; inhibition or knockout of SDH suppresses succinate-mediated inflammatory processes (including responses mediated by IL-1β and HIF-1α) (238) and induces the expression of anti-inflammatory Nrf2 factor 3 (ATF3) (248). Interestingly, itaconate formation contributes to the decreased mitochondrial O_2 consumption observed in response to LPS (186). Succinate oxidation leads to an elevated mitochondrial membrane potential and ROS production likely *via* RET at complex I of the ETC (Fig. 3). Elevated levels of mitoROS are responsible for driving the increased inflammatory response (238). Alternatively, elevated levels of succinate can promote the succinylation of susceptible lysine residues. Numerous succinylated substrates have been identified in the cytosol, nucleus, and mitochondria that play major roles in modulating metabolic processes (309). The enzyme, PKM2, plays an important role in promoting glycolysis; elevated levels of succinate can induce succinylation of PKM2 on K498, thereby inhibiting its activity (344). The SDH converts succinate into fumarate, and endogenous fumarate can succinylate and covalently modify cysteine residues of numerous substrates (Fig. 4). One of the best-characterized examples of this process is the succinylation of C152 found within the active site of GAPDH, thereby resulting in reduced levels of glycolysis and inflammation (180). Kelch-like ECH-associated protein 1 (KEAP1) can also undergo succinylation. Both dimethyl fumarate and monomethyl fumarate promote succinylation of KEAP1, which results in the activation of Nrf2 (291). Itaconate can also form covalent attachments *via* post-translational modification. A cell-permeable form of itaconate can form a covalent linkage with C22 of GAPDH, thereby inhibiting its enzyme activity and glycolytic flux (203).

Metabolism and RNS

Since all NOS activity (*i.e.*, the actions of nNOS, iNOS, and eNOS) depends on the availability of L-arginine and BH_4, the metabolic pathways leading to the synthesis of these factors (*i.e.*, 1C metabolism) may have an impact on the availability of $NO^{\bullet}$ (Figs. 2 and 4). In the sections that follow, we review the pathways *via* which various metabolic processes regulate RNS homeostasis.

L-*arginine metabolism and RNS*

Cellular metabolism plays an important role in the generation of RNS. As described in the earlier sections, NOS enzymes utilize NADPH and L-arginine as co-substrates, whereas BH_4 is an essential co-factor that is not consumed (Figs. 2 and 4). L-arginine levels are typically high in the circulation ($\sim 100\,\mu M$) and are even higher within ECs ($>400\,\mu M$). Depletion of cellular L-arginine has been identified as an important mechanism to prevent full activation of NOS isoforms and eNOS-mediated uncoupling and endothelial dysfunction in CVD. Elevated expression arginase I and arginase II, which are enzymes that consume L-arginine, has been observed in association with CVD and has been proposed as a mechanism that might be used to decrease L-arginine levels to a point at which the NOS enzymes no longer have sufficient fuel to promote catalysis. However, despite many beneficial effects that have been attributed to L-arginine in animal models (62) and humans (365), long-term L-arginine consumption has not been associated with the reduced incidence of myocardial infarction nor has it been implicated in reducing the rates of post-infarction mortality (343). Mechanistically, the Michaelis constant (K_m) for L-arginine with respect to the NOS enzymes is $\sim 2\,\mu M$; it is unlikely (and potentially threatening to cell viability) that arginine levels can be reduced to an extent that will severely compromise $NO^{\bullet}$ synthesis. Indeed, it has been shown that high levels of arginase expression can result in decreased eNOS activity, but only by $\sim 10\%$. The specific intracellular location of arginase has no impact on this ability (91). High-dose L-arginine can also induce compensatory changes over time, including the upregulation of arginase enzymes. This response may result in the synthesis of deleterious quantities of metabolites such as ornithine and thus contribute to maladaptive vascular remodeling. The metabolite, L-citrulline, is recycled to L-arginine by the actions of argininosuccinate synthase and argininosuccinate lyase (121). This has been proposed as an alternative approach, as supplementation with L-citrulline has been associated with cardiovascular benefits, including reducing systemic blood pressure (170).

Asymmetric dimethylarginine (ADMA) and N-monomethyl L-arginine (L-NMMA) are naturally occurring metabolites that regulate NOS activity. Concentrations of ADMA and L-NMMA are increased in renal failure (336), atherosclerosis (240), and homocysteinemia (316). Although L-NMMA is a potent inhibitor of NOS activity, ADMA is a weak inhibitor. However, ADMA can promote uncoupling of eNOS more effectively than L-NMMA (83) (Fig. 4). High levels of methylated arginines promote endothelial dysfunction (61), which is believed to involve both reduced NOS activity and uncoupled NOS and ROS production (318). ADMA and L-NMMA are metabolized by the enzyme, dimethylarginine dimethylaminohydrolase 1 (DDAH1), to form L-citrulline. The activity of DDAH1 can be compromised by ROS and RNS (191), a response that leads to increased levels of ADMA. Homocysteinemia has been associated with impaired endothelium-dependent relaxation responses (335). Elevated levels of homocysteine and methionine can promote

increased protein methylation and thus increased levels of ADMA, thereby compromising endothelial function (27). Administration of folate can restore endothelial function and eNOS uncoupling (340). Similarly, $NO^{\bullet}$ may be capable of regulating homocysteine levels *via* direct inhibition of methionine synthase (MTR), an enzyme that uses 5-methyltetrahydrofolate to convert homocysteine to methionine (70). This mechanism could limit the effectiveness of high levels of $NO^{\bullet}$ donors on cardiovascular health.

BH_4 metabolism and RNS

It is not clear whether NADPH is rate-limiting for eNOS activity, as it also has many important roles in regulating other enzyme systems, including those contributing to both prooxidant and antioxidant pathways. The affinity of eNOS for NADPH (*i.e.*, calmodulin-bound eNOS) is $\sim 1\ \mu M$ (231). Cellular levels of NADPH levels have been estimated at $\sim 100\ \mu M$ (202) and are tightly regulated (372). NADPH in the cytosol is primarily generated by the PPP, including the actions of G6PD, which converts $NADP^+$ to NADPH. Overexpression of G6PD results in increased eNOS activity (195) although it is not clear whether or not this response is due to increased levels of NADPH. NADPH is also important for the synthesis of BH_4 (Fig. 4). Dihydrofolate reductase (DHFR) and dihydropteridine reductase (DHPR, also known as quinoid dihydropteridine reductase [QDPR]) can catalyze the reduction of BH_2 to BH_4 in an NADPH-dependent manner. Human DHFR has a reduced affinity for BH_2. Likewise, conversion to BH_4 can be inhibited by folate, which may limit the effectiveness of folic acid for the treatment of endothelial dysfunction (362). Mitochondrial function is also critical for BH_4 synthesis. Depletion of CR6-interacting factor 1 (CRIF1), a factor that is important for the assembly of mitochondrial subunits and complexes, leads to loss of GTPCH expression, diminished BH_4 levels, and uncoupled eNOS (190). The expression of NOS enzymes can also be regulated by cellular metabolism, most notably by glycolysis. For example, LPS-dependent expression of iNOS can be inhibited by glycolysis (301). C-terminal-binding protein (CtBP) is an NADH-sensitive transcription factor that can regulate transcription of genes *via* mechanisms that are dependent on cellular metabolism. Suppression of CtBP activity impairs LPS-mediated induction of iNOS, thereby connecting changes in metabolism with gene transcription (301). The expression of eNOS can also be regulated by glycolysis in ECs. Lactate promotes increases and 2-deoxyglucose results in inhibition of the expression of eNOS messenger RNA (mRNA) (134).

1C metabolism and RNS

1C metabolism plays an important role in modulating eNOS activity *via* the regulation of its cofactor BH_4 (20) and methylation of arginine residues in proteins. These actions contribute to endothelial function and CVD (Fig. 4). DHFR, which is a key enzyme in both folate and 1C metabolism, plays an important role in regulating eNOS activity *via* a salvage pathway in which BH_2 is consumed to maintain endothelial BH_4 levels, together with *de novo* biosynthesis *via* the rate-limiting enzyme, GTPCH (20). Reduced BH_4 availability contributes to eNOS uncoupling and results in the production of $O_2^{\bullet -}$ instead of $NO^{\bullet}$, thereby inducing endothelial dysfunction. Similarly, ADMA inhibits the activity of eNOS *via* competition with its cofactor, L-arginine; this results in increased ROS production and reduced bioavailability of $NO^{\bullet}$ (26, 192). The ADMA is generated by protein arginine methyltransferase (PRMT) in the presence of SAM as part of the methionine cycle of 1C metabolism. The SAM also plays a major role in promoting epigenetic modifications in its role as a universal methyl group donor for methyl transfer reactions (Fig. 3).

Therapeutic implications. Although impaired metabolism can lead to the production of RNS, therapeutic strategies, there are currently few to no metabolic strategies available to counter the resulting endothelial dysfunction. Supplementation with BH_4 limits atherosclerosis (127) and sepiapterin, which can result in increased levels of BH_4 *in vivo*, and it improves endothelial function in numerous models (64, 77). However, the impact of folate or BH_4 supplementation as a means to prevent CVD in clinical and preclinical settings remains controversial (55, 67, 339). There is a considerable body of evidence in support of the notion that preserving endothelial function has positive effects on metabolism. The loss of endothelial $NO^{\bullet}$ observed in eNOS-deficient mice results in insulin resistance and hyperlipidemia (86, 142) secondary to lower energy expenditures, reduced O_2 consumption, and mitochondrial dysfunction with lower rates of beta-oxidation.

Metabolic Enzymes and ROS

The ROS induce a variety of post-translational protein modifications, including cysteine oxidation in the form of sulfenylation (SOH) and S-glutathionylation (136). These modifications can have a direct influence on the activity of susceptible metabolic pathways. In this section, we will highlight how ROS can affect metabolic enzymes, including mitochondrial protein dynamics.

AMPK and ROS

AMPK is a critical metabolic redox sensor in glucose metabolism that promotes angiogenesis in ECs and postnatal neovascularization. AMPK also protects against atherosclerosis (201, 244, 311, 374, 376). Interestingly, ROS can stimulate AMPK activity to regulate metabolic requirements even in the presence of appropriate levels of ATP (38) (Fig. 4). For example, H_2O_2 can induce GLUT4 translocation *via* the activation of AMPK in cardiac myocytes. (139). H_2O_2 directly activates AMPK *via* oxidative S-glutathionylation of the AMPKα and AMPKβ subunits at C299 and C304 (401). The mitoROS can regulate autophagy (307), efferocytosis (156), and HIF-1α-dependent longevity (132, 144) *via* activation of AMPK. In response to hypoxic conditions, mitoROS activate AMPK *via* a mechanism that functions independently of the adenosine monophosphate (AMP)/ATP ratio (92). As an adaptive response, mitoROS-induced AMPK activation induces upregulation of peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α-dependent expression of antioxidant enzymes, which, in turn, limits excess mitoROS production (268). Taken together, AMPK functions not only as a metabolic sensor but also as a redox sensor to regulate cellular metabolism and redox state (38, 401).

AMPK also contributes to vascular health *via* its capacity to confer potent antioxidant defense. For example, under metabolic stress, AMPK increases NADPH levels and thus decreases levels of H_2O_2 by induction of FAO (154). AMPK activation also improves endothelial function by reducing oxidative stress associated with atherosclerosis *via* increasing the expression of uncoupling protein 2 (UCP2) in ECs (349). UCP2 deficiency in mice increases oxidative stress, which amplifies the progression of atherosclerotic plaques (25). Additional evidence supporting AMPK-mediated protection against oxidative stress has emerged from studies that demonstrate its capacity for negative regulation of NOX *via* reduced phosphorylation of the p47phox NOX subunit. This prevents p47phox translocation from cytosol to membrane, which is required for the activation of NOX (232).

Therapeutic implications. AMPK activators (*e.g.*, metformin) have been used to treat type 2 diabetes and CVD (72, 116, 388). Metformin activates the Reperfusion Injury Salvage Kinase (RISK) pathway (388) as part of its cardioprotective mechanism. Recently, a small molecule activator of AMPK (*e.g.*, A-769662, MIF20) showed efficacy at protecting the heart tissue from the negative sequelae of ischemia–reperfusion injury (172, 346).

PKM2 and ROS

Pyruvate kinase (PK) catalyzes the final rate-limiting step in glycolysis by transferring the phosphate from phosphoenolpyruvate (PEP) to ADP to generate pyruvate and ATP. There are four isoforms of PK, including PKM1, PKM2 (encoded by PKM), PKL, and PKR (encoded by PKLR) (377). PKM1 and PKM2 are alternatively spliced products of the PKM gene (247). Interestingly, PKM2 (but not PKM1) is expressed exclusively in growing and confluent ECs (173). The PKM1 homo-tetramer has high constitutive PK activity, whereas the activity of PKM2 is regulated by post-translational modifications that promote the formation of the less active dimer or the more active tetrameric form (4, 54, 133, 215).

Oxidative stress due to H_2O_2, diamide, or hypoxia inactivates PKM2 and prevents the formation of the active tetramer *via* oxidation of Cys358; oxidation can be inhibited in the presence of the reducing agent, dithiothreitol (DTT) (4) (Fig. 4). Inhibition of PKM2 increases the levels of G6P and redirects the glycolytic flux toward the oxidative PPP pathway to generate additional NADPH. Since NADPH is one of the factors required to maintain appropriate levels of reduced GSH, inactivation of PKM2 leads to the reduction of oxidative stress (4). Also, PKM2 has been shown to promote increased expression of genes encoding glycolytic proteins (*e.g.*, SLC2A1 [solute carrier family 2 member 1, Glut1], LDHA, PDHK1) and VEGFA *via* its interactions with the transcription factors HIF-1α and HIF-2α (212, 213). Therefore, PKM2 not only reduces oxidative stress by generating NADPH but also alleviates ischemia by increasing the expression of vascular endothelial growth factor (VEGF). Further, in the setting of pulmonary arterial hypertension, Guo *et al.* (120) reported that ROS-induced phosphorylation and inhibition of PKM2 stimulates cell proliferation and survival *via* increased flux through the PPP pathway. In proliferating human T cells (*e.g.*, acute lymphoblastic leukemia), phosphorylation of PKM2 reduces glycolytic flux and activates the PPP (345). In proliferating ECs, PKM2 maintains cell cycle progression by suppressing p53-mediated signaling (173). By contrast, in quiescent ECs, PKM2 maintains vascular barrier function by suppressing NF-κB/angiopoietin 2 signaling (173, 315) independent of its canonical protein kinase activity. PKM2 activation also promotes angiogenic differentiation that maintains ROS at low levels; this has been associated with enhanced glycolysis and mitochondrial fission (273). The mitoROS promote dimerization of the PKM2 in monocytes and macrophages from atherosclerotic patients. PKM2 dimerization stimulates its nuclear translocation and thereby promotes signal transducer and activator of transcription 3 (STAT3) phosphorylation and production of IL-6 and IL-1β (305). Moreover, activation of PKM2 promotes angiogenesis *via* its actions on vascular resident endothelial progenitor cells *via* modulation of glycolysis as well as mitochondrial fission and fusion, which are required for the treatment of diseases and injuries requiring strategies that promote or inhibit angiogenesis (273).

Therapeutic implications. Many studies have reported the clinical relevance and therapeutic potential of agents that target PKM2 in CVD. The results of one study revealed significant increases in PKM2 expression in failing compared with non-failing hearts. Thus, induction of PKM2 is a signature of not only cardiotoxicity but also cardiac stress in general (272). Tetrameric PKM2–stabilizing drugs (*e.g.*, TEPP-46) suppress p53-mediated transcriptional activity and cardiomyocyte apoptosis, thereby preventing anthracycline-induced cardiotoxicity (280). The therapeutic potential of PKM2 was further demonstrated in cardiomyocytes in which PKM2 participates in cell cycle regulatory mechanisms that enhance myocardial regeneration after myocardial infarction (220).

PFKFB3 and ROS

PFKFB is a bifunctional and rate-limiting enzyme in glycolysis that controls the cellular levels of fructose-2, 6-bisphosphate (F-2, 6-BP), which is an allosteric activator of PFK-1 (249, 275). Mammalian PFKFB (also known as PFK2) consists of four isoenzymes, including PFKFB 1, 2, 3, and 4. Among them, PFKFB3 plays the most crucial role in promoting the production of fructose-2, 6-bisphosphate (F-2,6-BP) and glycolysis, as it has the highest kinase to bisphosphatase activity ratio (740:1). PFKFB3 is expressed mainly in leukocytes, vascular cells, and cancer cells. Oxidative stress regulates PFKFB3 activity and protein stability mainly *via* post-translational modification (217, 277, 297) (Fig. 4). In cancer cells, oxidative stress inactivates PFKFB3, but not other PFKFB isoenzymes, by S-glutathionylation of C206. This results in a shift from glycolysis to the PPP, and thus NADPH production, regeneration of GSH, and ROS detoxification (297). The ROS generated by NOX2 in cells from patients with acute myeloid leukemia promotes glycolysis by increasing the expression of PFKFB3 (277). Further, activation of cSrc promotes cell proliferation and migration *via* PFKFB3 phosphorylation at Y194; this results in increased flux through the glycolytic and

TCA cycle pathways and increasing ROS levels *via* associated reductions in the oxidative PPP, NADPH, and antioxidant systems (217). Given that ROS are known to activate cSrc, the cSrc-pY-PFKFB3-ROS axis may represent a positive feedback loop that can induce sustained ROS production. Taken together, these findings suggest that persistent glycolysis and redox regulation are closely linked to one another. The therapeutic implications of these observations are discussed in the section focused on glycolysis and ROS.

HIF-1 and ROS

HIF-1 is a key regulator of the developmental and physiological states required for the maintenance of O_2 homeostasis. HIF-1 also promotes the adaptive responses to reduced O_2 availability by regulating gene expression (161, 222, 295). HIF contributes to the metabolic shift from glucose oxidation to aerobic glycolysis in various CVDs and cancer (222, 295). HIF-1 is a heterodimeric transcription factor that consists of an $O_2^{\bullet-}$-labile α subunit and a stable β subunit. Under conditions of normoxia, two proline residues on HIF-1α are hydroxylated by prolyl hydroxylase domain-containing proteins (PHDs) to become substrates for ubiquitination; they are then degraded by the von Hippel-Lindau (VHL) complex. By contrast, in response to hypoxia, mitoROS derived from respiratory complex III or cytosolic ROS can stabilize both HIF-1$\alpha$and HIF-2α by inhibiting PHDs (19, 222) (Figs. 5 and 6). The actions of HIF-1α and HIF-2α serve to increase the expression of angiogenic genes such as VEGF and thereby promote angiogenesis associated with embryonic vascular development (270), hindlimb ischemia (29), hypertrophic cardiomyopathy (281), myocardial infarction (129), skin wound healing (219), and retinal neovascularization (155).

HIF-1-mediated regulation of the glycolytic pathway is closely linked to ROS homeostasis (Figs. 5 and 6). Overexpression of HIF-1 increases the uptake of glucose through GLUTs (*i.e.*, GLUT1 and sodium-glucose transporters [SGLTs]) (75). Hypoxia-induced HIF-1α increases the extent of metabolic pathway reprogramming from oxidative phosphorylation to glycolysis to maintain ATP production. This metabolic reprogramming results from the upregulation of GLUTs and glycolytic enzymes (222), downregulation of PDH complexes *via* activation of PDHK1 (175, 211), or through suppression of mitochondrial respiration by inducing the expression of NADH dehydrogenase 1 alpha subcomplex, 4-like 2 (NDUFA4L2) (322). Loss of SIRT3, another regulator of HIF, results in impaired glycolysis *via* the reduction of signaling *via* the HIF-2α-PFKFB3 axis. This will ultimately result in impaired myocardial angiogenesis and cardiac dysfunction (128). Macrophage proinflammatory signaling is also induced in part *via* HIF-1α, which preferentially promotes ATP production *via* glycolysis (238). In this case, RET-derived mitoROS generated by mitochondrial hyperpolarization and succinate oxidation serve to stabilize HIF-1α; this leads to increases in glycolytic capacity and IL-1β mRNA and protein expression (320).

PDHK1 activation by HIF-1α shunts pyruvate away from the mitochondria, resulting in decreased flux through the TCA cycle and the ETC and potential attenuation of mitoROS production. The functional significance of this adaptive response has been demonstrated as follows: (i) Chronic hypoxia in HIF-1α-deficient fibroblasts results in cell death due to excessive accumulation of ROS accumulation (175, 295); (ii) hypoxia-induced expression of HIF-1α induces mitophagy *via* the actions of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), which results in a reduction in mitochondrial mass, O_2 consumption, and ROS generation (389); and (iii) hypoxia-induced HIF-1 increases the expression of the mitochondrial isoforms of SHMT1 and SHMT2, leading to increased mitochondrial NADPH and decreased mitoROS levels in Myc-transformed cells (380). Thus, the critical adaptive response involving the hypoxia-HIF1 axis reduces the production of mitoROS *via* decreases in mitochondrial mass, O_2 consumption, and increased NADPH, in addition to the impact of metabolic reprogramming.

Therapeutic implications. Given the importance of HIF for both physiological and pathological angiogenesis, several strategies have been developed to target HIF-related pathways for the treatment of ischemic diseases and cancer (222). PHD inhibitors (*e.g.*, FG-2216, FG-4592), HIF activators (HIF-1α adenoviral-based therapy), and HIF inhibitors (EZN-2968, digoxin, anthracyclines) are all under consideration for clinical use (222). Additional details on these issues can be found in several excellent reviews (222).

Mitochondrial dynamics, ROS, and metabolism: mitochondrial dynamics and ROS

Mitochondrial dynamics refers to a multifaceted process that includes both the fission and fusion of the mitochondrial outer and inner membranes. Mitochondrial fission and fusion proteins coordinate this dynamic process. These proteins are closely associated with mitochondrial function and can influence and be regulated by mitoROS production (Figs. 3 and 5). The ROS can regulate mitochondria dynamics and vice versa. The ROS induce post-translational modifications of proteins that regulate mitochondrial dynamics, thereby influencing their expression and/or their activity. These actions serve to regulate mitochondrial dynamics, morphology, and function (221, 282, 353, 363). Increases in ROS generation during conditions of oxidative stress, high glucose concentrations, or elevations in FAs can contribute to mitochondrial fragmentation whereas sublethal amounts of H_2O_2 can induce hyperfusion.

Mitochondrial fission is mainly mediated by small GTPase, dynamin-related protein 1 (Drp1), and its receptor proteins mitochondrial fission factor (Mff), mitochondrial fission 1 protein (Fis1), mitochondrial dynamics protein of 49 kDa (MiD49), and MiD50. Drp1 is localized in the cytosol during resting states. When activated, Drp1 is recruited to the mitochondria outer membranes where it induces constriction and scission of the mitochondria in a GTP-dependent manner (360). The ROS can induce phosphorylation of S616 of Drp1 and activation of Drp1 GTPase activity. For example, ROS (including mitoROS) induced by ischemia/reperfusion injury promote Drp1 oligomerization and phosphorylation at S616. This leads to excessive mitochondrial fission in target ECs (115). In the setting of neurodegenerative diseases, $NO^{\bullet}$ has been found to induce S-nitrosylation of Drp1, which increases its GTPase activity and mitochondrial fragmentation (52); however, this finding remains controversial (30). Sulfenylation (Cys-OH formation) is a reversible initial step in

ROS-mediated oxidation of reactive cysteine residues of proteins that participate in redox signaling (255, 263). We reported that Drp1 sulfenylation at C644 as a result of the loss of protein disulfide isomerase A1 augments Drp1 GTPase activity and mitochondrial fragmentation. These events drive the production of mitoROS, which leads to EC senescence (176). Thus, targeting cysteine oxidation or other modifications of Drp1 may be a potential therapeutic strategy for diseases associated with mitochondrial dysregulation and dysfunction associated with oxidative stress. Several reports document a link between Drp1-mediated mitochondrial fission and ROS. First, hyperglycemia induces mitochondrial fragmentation *via* Ca^{2+} and extracellular signal-regulated kinase (ERK)1/2-dependent phosphorylation of Drp1 S616 or increases in Drp1 expression. These responses induce the overproduction of mitoROS (223, 279, 302, 341, 350, 383, 384). Second, H_2O_2 stimulation of cardiac myocytes promotes increased mitochondrial fragmentation. This response results in mitochondrial membrane depolarization and increased resistance to insulin *via* the upregulation of Drp1 (356). Thus, mitochondrial fission and ROS are interconnected.

Mitochondrial fusion is mediated by three GTPases, including the dynamin proteins mitofusin 1 (Mfn1), Mfn2, and optic atrophy 1 (Opa1). Mfn1 and Mfn2 are outer membrane proteins that facilitate the fusion of this membrane, whereas Opa1 is associated with the inner membrane and facilitates inner membrane fusion. Increased expression of Mfn2 induced by H_2O_2 is both necessary and sufficient to induce apoptosis of heart muscle cells in response to oxidative stress (300). In ECs, knockdown of the Mfns disrupts mitochondrial networks and decreases mitochondrial membrane potential, VEGF-induced migration, and capillary network formation (210). Depletion of Mfn2 limits ROS generation and blunts expression of components of the ETC and transcription factors associated with oxidative metabolism, whereas ablation of Mfn1 inhibits VEGF-induced Akt-eNOS signaling (210). GSSG stimulates mitochondrial fusion by inducing disulfide bond-mediated oligomerization of Mfn1 and *via* the C684 of Mfn2 (306). Taken together, these findings point to the regulation of redox-regulated mitochondrial hyperfusion of the mitochondrial IMS. Importantly, mutation of C684, which is found within a disulfide bridge when cells are in an oxidative state, renders Mfn2 more susceptible to alterations in the redox environment. Thus, the thiol switching of C684 in Mfn2 plays an important role in mediating redox-induced alternations of mitochondrial shape and activity (325). Thus, an understanding of how the mechanisms that define the relationships between redox homeostasis, mitochondrial structure, and mitochondrial dynamics are disrupted in pathological conditions may lead to the development of new therapeutic strategies.

Mitochondrial dynamics and metabolism

Mitochondrial dynamics mediated by fission and fusion and bioenergetics have a reciprocal influence on one another. Dynamic properties of the mitochondria are regulated by cellular signaling events and have a discernible impact on cellular metabolism (239, 342). Changes in mitochondrial morphology are frequently observed in response to alterations in the surrounding cellular milieu (*e.g.*, metabolic flux), which influence cellular bioenergetics. Thus, an understanding of the mechanisms that govern mitochondrial morphology and their emerging role in mitochondrial bioenergetics will be of critical importance (109). Deletion of any of the components that support mitochondrial dynamics will perturb oxidative phosphorylation and glycolysis even at baseline (205). Several reports suggest roles for Mfn and Drp1 in cell metabolism and the pathogenesis of metabolic disorders (239, 288, 342). Mitochondrial fusion is particularly important in cells undergoing cellular respiration, as it facilitates the dissemination of metabolites, enzymes, and mitochondrial gene products throughout the entire mitochondrial compartment. This serves to optimize mitochondrial function and counteracts the effects of mitochondrial mutations that accumulate during the aging process. Muscle-specific gene-deletion of Mfn2 disrupts glucose homeostasis (293). Similarly, Mfn deficiency in ECs inhibits migration, network formation, viability, and mitoROS production *via* reduced expression of coenzyme Q and transcription factors associated with oxidative metabolism (210). Although Mfn gene-deleted embryos show developmental delays, expression of Mfn2 is increased in tissues under conditions of nutrient deprivation in the nervous system, skeletal muscle, and heart (6, 93). Loss of Mfn2 reduces mitochondrial membrane potential, O_2 consumption, and activity of the ETC and the TCA cycle whereas anaerobic respiration increases as a compensatory mechanism (44, 241). Depletion of Mfn2 also results in the decreased expression of complexes that support oxidative phosphorylation. By contrast, Mfn2 overexpression increases both glycolysis and mitochondrial membrane potential (95) as well as the expression of ETC proteins, including subunits that contribute to complexes I, IV, and V (208). Recently, Buck *et al.* (35) reported that, by remodeling mitochondrial cristae, fusion in Tm configures associations of the ETC complex that favor oxidative phosphorylation and FAO. By contrast, fission in Teff cells leads to expansion of the mitochondrial cristae, thereby reducing ETC efficiency and promoting aerobic glycolysis. Thus, remodeling of the mitochondrial cristae *via* fusion/fission is a signaling mechanism that directs T cell fate *via* metabolic programing.

Fragmented mitochondria are frequently found in resting cells. Mitochondrial fission plays an important role in removing damaged organelles by autophagy. Thus, both mitochondrial fusion and fission contribute to the maintenance of mitochondrial function and the optimization of bioenergetic capacity. Multiple signaling pathways regulate the machinery of mitochondrial dynamics so that the shape of the mitochondrial compartment will adapt to specific metabolic conditions within the cell (361). Ablation of Drp1 in liver cells results in reduced adiposity and elevated whole-body energy expenditure, thereby protecting mice from diet-induced obesity (348). Drp1-mediated fission also regulates glycolysis during cell transformation (298). Activation of protein kinase A/A-kinase anchoring protein 1 (AKAP1) results in the phosphorylation of Drp1 at S637. This modification inhibits Drp1 activity and mitochondrial fission, thereby resulting in enhanced mitochondrial tubulation that promotes ATP production (42).

Mitochondrial dynamics as a therapeutic target

Alterations in the proteins that support mitochondrial dynamics can lead to CVD. As an example, reduced expression

of Mfn2 led to hyperproliferation of VSMCs and accelerated cardiac hypertrophy and cardiomyopathy in mouse and rat models (46). Another report suggested that lactate accelerated vascular calcification that resulted from excessive Drp-mediated mitochondrial fission and a deficiency in BNIP3-related mitophagy (400). Also, increased mitochondrial fission contributes to impaired endothelial function in patients diagnosed with diabetes mellitus (302) as well as to the hyperproliferation of pulmonary artery smooth muscle cells in patients with pulmonary arterial hypertension (225). Drp1 is also crucial for the O_2-induced constriction and closure of the ductus arteriosus at birth in healthy humans and rabbits (138) as well as for arterial constriction (206). Finally, Drp1 is upregulated in response to pathological conditions of the heart; excessive mitochondrial fission appears to be detrimental in this setting (145, 330).

Given that mitochondrial dynamics include responses and adaptations to metabolic demands and are involved in the regulation of mitophagy, compounds that target mitochondrial fission and fusion are currently of great interest. For example, mitochondrial division inhibitor (Mdivi)-1, which can inhibit the GTPase activity of Drp1, protects the heart from ischemia–reperfusion injury *via* the inhibition of mitochondria outer membrane permeabilization, which will result in mitochondrial-mediated cell death (146). In addition to Mdivi-1, the Drp1 inhibitor P110 can also protect the heart against ischemia–reperfusion injury (330). The antihypertensive drug, cilnidipine, is a small molecule that inhibits the interaction between filamin and Drp1. Administration of cilnidipine to mice after the induction of myocardial infarction limited the extent of mitochondrial fission, cardiomyocyte senescence, and myocardial dysfunction independent of its capacity to block Ca^{2+} channels (246). P110 also blocks the interaction between Drp1 and Fis1. This drug was shown to be neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) animal model of Parkinson's disease *via* its capacity to inhibit hyperactivation of Drp1 (265, 317). Similarly, 15-oxospiramilacone (S3) is an anticancer agent that inhibits Wnt/beta-catenin signaling that can also enhance Mfn1/2 activity and induce mitochondrial fusion by targeting the deubiquitinase ubiquitin carboxyl-terminal hydrolase 30 (USP30) in mitochondria. USP30 enhances the irreversible ubiquitination of Mfn1/2 (385). However, administration of S3 can restore mitochondrial fusion and function in cells that are deficient in either Mfn1 or Mfn2, suggesting its potential therapeutic potential as a means to target mitochondrial dynamics in patients with CVD.

Metabolic Enzymes and RNS

$NO^{\bullet}$ and its metabolites have long been associated with maintaining metabolic homeostasis within cells. $NO^{\bullet}$ can inhibit and activate numerous metabolic pathways as described in the sections to follow.

Glycolytic enzymes and $NO^{\bullet}$

$NO^{\bullet}$ is a regulator of glycolysis. Endothelial $NO^{\bullet}$ promotes glycolysis *via* activation of HIF-1α (33) and the AMPK-dependent activation of PFK1. $NO^{\bullet}$ also supports glycolysis in dendritic cells (328). $NO^{\bullet}$ derived from eNOS can limit ischemic injury by inhibiting the activity of PKM2, a response that is mediated *via* direct nitrosylation of a specific cysteine residue. Inhibition of PKM2 leads to the accumulation of glycolytic intermediates, which can then be shunted to other pathways including the PPP for the production of NADPH, a molecule that is necessary for antioxidant activity (396). Alternatively, iNOS can promote the nuclear translocation of PKM2, thereby increasing glycolytic flux (198). The relative concentration of $NO^{\bullet}$ may be one mechanism to account for its different effects on metabolism. For example, low concentrations of $NO^{\bullet}$ can promote glycolysis whereas higher concentrations can be inhibitory.

Mitochondrial enzymes and $NO^{\bullet}$

Aside from its role in promoting vasodilation, physiologic levels of $NO^{\bullet}$ can support reversible binding and inhibition of the mitochondrial enzyme, complex IV, an action that serves to suppresses mitochondrial respiration (34, 56). Complex IV is the terminal oxidase (*i.e.*, complex IV) of the mitochondrial ETC (Fig. 3). $NO^{\bullet}$, but not O_2 binding to heme results in the accumulation of O_2. $NO^{\bullet}$-mediated inhibition of complex IV is a competitive process that can be reversed by O_2. Physiological levels of $NO^{\bullet}$ may repress complex IV, thereby beneficially reducing O_2 consumption and ATP formation due to the inhibition of electron flux at this site. This inhibition results in the redistribution of O_2 to other sites, most notably under conditions of increased O_2 demand (323). By contrast, prolonged exposure to $NO^{\bullet}$ can lead to S-nitrosylation and inhibition of complex I (58), which will also inhibit cellular respiration. In ECs, endogenous $NO^{\bullet}$ can suppress mitochondrial respiration (59).

$NO^{\bullet}$ has been shown to stimulate the biogenesis of mitochondria *via* activation of peroxisome proliferator-activated receptor-gamma (PPARγ) and PGC1-α (30). $NO^{\bullet}$ can also stimulate mitochondrial fragmentation that results from activation of mitochondrial fission or suppression of fusion, which can lead to mitochondrial dysfunction. In carbon tracing studies and *via* an analysis of O_2 consumption, $NO^{\bullet}$-mediated inhibition of metabolism was tracked to the TCA cycle, mitochondrial aconitase, and PDH (253). Ultimately, $NO^{\bullet}$ accumulation leads to suppression and loss of mitochondrial ETC complexes. The cytokine IL-10 can alter glycolytic responses by regulating the production of $NO^{\bullet}$ to limit iNOS-mediated suppression of oxidative phosphorylation (18).

The reaction between $NO^{\bullet}$ and $O_2^{\bullet-}$ generates $ONOO^-$ and occurs at or near-diffusion-limited rates and more rapidly than SOD-mediated $O_2^{\bullet-}$ dismutation. $ONOO^-$ reacts avidly with proteins and contributes to the nitration of tyrosine residues. Within cells, nitrotyrosine staining is concentrated in the mitochondria (76); $ONOO^-$ is a potent inhibitor of the mitochondrial ETC (269). $ONOO^-$ generated outside or within the mitochondria can oxidize and inhibit complexes I and II of the ETC, as well as aconitase and manganese (Mn)-dependent SOD, also known as SOD2 (40). Mitochondria are known to emit $O_2^{\bullet-}$ and have a dedicated SOD (*i.e.*, SOD2). In pathological states, the amount of $O_2^{\bullet-}$ produced increases. Therefore, in the presence of $NO^{\bullet}$, mitochondria are the major sites of $ONOO^-$ production. $ONOO^-$ promotes the depolarization and cyclosporine-sensitive calcium efflux from the mitochondria *via* the mitochondria permeability transition pore or mitochondrial permeability transition pore (251). The formation of $ONOO^-$ in mitochondria is largely driven by $O_2^{\bullet-}$ flux. Although there have been reports of a

mitochondrial NOS isoform that is similar to NOS1 that might contribute to $ONOO^-$ formation in the mitochondria (114), this issue remains controversial (183). Forced subcellular targeting of NOS isoforms to the mitochondria revealed that although levels of BH_4 and L-arginine are sufficient to support $NO^•$ synthesis, only calcium-independent enzymes are active in the mitochondrial IMS (150). This result suggests that the mitochondrial NOS isoform involved in this process must either reside outside the mitochondria or be an isoform of NOS2 (as opposed to NOS1).

Mitochondrial enzymes and NO in inflammation

Macrophages that express NOS2 and generate large amounts of $NO^•$ have been found to suppress mitochondrial respiration. $NO^•$ is important for metabolic reprogramming as part of the proinflammatory shift toward glycolysis (8). Of note, these findings have been reported in mouse macrophages, but not in human macrophages, which produce comparatively lower levels of $NO^•$. In addition to its ability to suppress the ETC and respiration, under conditions of relative hypoxia, $NO^•$ can stimulate the production of mitoROS that promotes proinflammatory signaling (59, 252). Suppression of mitochondrial respiration is not an essential feature of inflammatory polarization, compared with the upregulation of glycolysis. $NO^•$ is important for the regulation of TCA metabolism and its intermediates, citrate, and succinate, as well as production of the immunometabolite, itaconate (8). Itaconate production and its ability to shape the immune response are examples of the importance of metabolic reprogramming in support of this phenotypic change.

Therapeutic implications. Pharmacological modification of $NO^•$ signaling can have a direct impact on metabolism. $NO^•$ donors can promote glycolysis in multiple cell types (78, 224). Further, $NO^•$ and elevated levels of cyclic guanosine monophosphate (cGMP) contribute to the browning of adipose tissue in lean animals. However, in obesity, treatment with sildenafil and elevated levels of cGMP does not promote adipose tissue browning and result in compromised glucose disposal (159). In the heart, elevated levels of cGMP can promote glycolysis associated with the accumulation of both malate and α-KG and the increased activity of malate dehydrogenase (113). In pulmonary hypertension, a disorder associated with the upregulation of glycolytic pathways, administration of sildenafil results in the decreased expression of HK 2 (197). Thus, although $NO^•$ donors and modulators of cGMP signaling can have an impact on cellular metabolism *via* the inhibition of phosphodiesterase, these actions can be both dose and context-dependent. As one example of an intriguing connection between glycolysis and $NO^•$ signaling, GAPDH has been shown to function as a heme chaperone for soluble guanylate cyclase, an action that is necessary for binding $NO^•$ and production of cGMP (69). Similarly, H_2O_2 can activate PKG-1α to control vasodilation and blood pressure *via* a mechanism that is independent of the $NO^•$-cGMP pathway (264). The role of crosstalk between ROS and RNS in metabolism warrants future investigation.

Summary and Conclusion

Mitochondrial oxidative phosphorylation, glycolytic metabolism, and redox homeostasis are all closely connected in vascular and inflammatory cells and are important in shaping cell behavior (Figs. 5 and 6). Imbalances in any of these pathways will compromise cellular function and contribute to CVD and cancer. Reciprocal interactions between the pathways that regulate metabolic flux and redox balance are important drivers of both physiological and pathophysiological processes (Figs. 5 and 6). For example, inhibition of glycolysis by ROS/RNS promotes metabolic reprogramming so that the cells shift from glycolytic flux to the oxidative arm of the PPP to generate NADPH. This shift will serve to increase antioxidant defense, which is important for preserving $NO^•$ and endothelial function. In macrophages, $NO^•$ is important for metabolic reprogramming and contributes to the proinflammatory shift away from mitochondrial oxidation and toward the glycolytic pathways. Thus, these are not merely passive metabolic adaptations; these changes play active roles in shaping physiological and pathological processes.

Given the pivotal role of metabolism in the process of angiogenesis, there is substantial interest in the therapeutic possibilities associated with the manipulation of EC metabolism (Fig. 1). However, strategies that target single pathways have been largely unsuccessful. Combined approaches using both metabolic inhibitors and ROS-modulating agents may offer greater promise, as they can function synergistically to stimulate angiogenesis required for cell repair or eradicate cancer cells *via* the regulation of intracellular ROS levels and cellular metabolism to achieve the desired outcome. An improved understanding of these mechanisms and how they are integrated into and contribute to various disease settings may lead to new opportunities for intervention and improved therapeutic strategies for CVD. Drugs targeting metabolic pathways are currently in use for the treatment of cancer; the findings obtained may have critical implications for the development of treatments for CVD. For CVD, drugs targeting ATP citrate synthase (ACLY), which links carbohydrate and lipid metabolism, may serve to reduce cholesterol levels and limit atherosclerosis *via* its actions primarily in the liver. The roles in this process played by other cell types are currently emerging.

Authors' Contributions

T.F., M.U.-F., D.A., and S.N. prepared the figures; T.F., M.U.-F., D.A., S.N., and D.J.R.F. drafted the article; T.F., M.U-F., D.F., and E.J.B.C. edited and revised the article; and T.F., M.U.-F., D.A., S.N., D.J.R.F., and E.J.B.C. approved the final version of the article.

Author Disclosure Statement

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Abbreviations Used

α-KG = α-ketoglutarate
α-KGDH = alpha-ketoglutarate dehydrogenase
1C = one-carbon
ACC = acetyl-CoA carboxylase
ADMA = asymmetric dimethylarginine
AMPK = 5′ adenosine monophosphate-activated protein kinase
ApoE = apolipoprotein E
ATP = adenosine triphosphate
BCAA = branch-chained amino acid
BCAT = branched-chain aminotransferase
BCKA = branched-chain ketoacids
BCKD = BCKA dehydrogenase
BH_2 = dihydrobiopterin
BH_4 = tetrahydrobiopterin
BNIP3 = BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
cGMP = cyclic guanosine monophosphate
CoA = coenzyme A
Complex I = NADH–ubiquinone oxidoreductase
Complex III = ubiquinol–cytochrome c oxidoreductase
Complex IV = cytochrome c oxidase
CoQ = coenzyme Q
CPT1 = carnitine palmitoyltransferase-1
CtBP = C-terminal-binding protein
Cu,ZnSOD, SOD1 = coper zinc superoxide dismutase
CVD = cardiovascular disease
CySS = cystine
Cyt c = cytochrome c
DDAH1 = dimethylarginine dimethylaminohydrolase 1
DHFR = dihydrofolate reductase
dNTPs = deoxynucleotide triphosphates
Drp1 = dynamin-related protein 1
EC = endothelial cell
ECM = extracellular matrix
ecSOD, SOD3 = extracellular SOD
ETC = electron transport chain
F6P = fructose-6-phosphate
FA = fatty acid
FAD = flavin adenine dinucleotide
FAO = fatty acid oxidation
Fis1 = mitochondrial fission 1 protein
G6P = glucose-6-phosphate
G6PD = glucose-6-phosphate dehydrogenase
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GCL = glutamate-cysteine ligase
GGPP = geranylgeranyl pyrophosphate
GLS = glutaminase
GLUT = glucose transporter
GPX = glutathione peroxidase
GSH = glutathione
GSR = glutathione reductase
GSS = GSH synthetase
GSSG = oxidized glutathione
GTPCH = GTP cyclohydrolase
H_2O_2 = hydrogen peroxide
H_2S = hydrogen sulfide
HIF = hypoxia-inducible factor
HK = hexokinase
Hsp = heat-shock protein
IDH = isocitrate dehydrogenase
IL = interleukin
IMS = intermembrane space
IRG1/ACOD1 = immune-responsive gene 1/aconitate decarboxylase 1
KEAP1 = Kelch-like ECH-associated protein 1
LDH = lactate dehydrogenase
L-NMMA = N-monomethyl L-arginine
LPS = lipopolysaccharide
Mdivi = mitochondrial division inhibitor
ME = malic enzyme
Mfn = mitofusin
mitoROS = mitochondrial ROS
MnSOD, SOD2 = manganese superoxide dismutase
mRNA = messenger RNA
MSUD = maple syrup urine disease
Mtb = *Mycobacterium tuberculosis*
NADH = nicotinamide adenine dinucleotide
NADPH = nicotinamide adenine dinucleotide phosphate
NF-κB = nuclear factor-kappa B
NLRP3 = NLR family pyrin domain containing 3
$NO^{\bullet}$ = nitric oxide
NOS = nitric oxide synthase
NOS1, nNOS = neuronal NOS
NOS2, iNOS = inducible NOS
NOS3, eNOS = endothelial NOS
NOX = NADPH oxidase
Nrf2 = nuclear factor erythroid 2-related factor 2
O_2 = oxygen
$O_2^{\bullet-}$ = superoxide
OAA = oxaloacetate
$^{\bullet}OH$ = hydroxyl radical
$ONOO^-$ = peroxynitrite
Opa1 = optic atrophy 1
PDH = pyruvate dehydrogenase
PDHK1 = pyruvate dehydrogenase kinase 1
PDHK = pyruvate dehydrogenase kinase
PFK = phosphofructokinase
PFKFB3 = 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3
PGC = peroxisome proliferator-activated receptor-gamma coactivator
PHD = prolyl hydroxylase domain-containing protein
PHGDH = phosphoglycerate dehydrogenase
PK = pyruvate kinase
PKC = protein kinase C
PKM2 = pyruvate kinase M2
PPP = pentose phosphate pathway
PRX = peroxiredoxin
RET = reverse electron transport
RNS = reactive nitrogen species
ROS = reactive oxygen species
SAM = S-adenosyl-methionine
SDH = succinate dehydrogenase
SGOC = serine-glycine one-carbon metabolism
SHMT = serine hydroxymethyltransferase
SIRT = sirtuin

Abbreviations Used (Cont.)

SLC1A5/ASCT2 = solute carrier family 1, member 5/alanine/serine/cysteine-preferring transporter 2
TAZ = transcriptional coactivator with PDZ binding motif
TCA = tricarboxylic acid
Teff = effector T
TIGAR = TP53-inducible glycolysis and apoptosis regulator
Tm = memory T cells
TNF = tumor necrosis factor
TRX = thioredoxin
TRXR = thioredoxin reductase
TxNIP = thioredoxin-interacting protein
UCP = uncoupling protein
USP30 = ubiquitin carboxyl-terminal hydrolase 30
VDAC = voltage-dependent mitochondrial anion channel
VEGF = vascular endothelial growth factor
VSMC = vascular smooth muscle cells
xCT = the cystine/glutamate antiporter SLC7A11
XO = xanthine oxidase
YAP = yes-associated protein