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The Role of Mitochondria in Angiogenesis

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Abstract

Angiogenesis extends pre-existing blood vessels to improve oxygen and nutrient delivery to inflamed or otherwise hypoxic tissues. Mitochondria are integral in this process, controlling cellular metabolism to regulate the proliferation, migration, and survival of endothelial cells which comprise the inner lining of blood vessels. Mitochondrial Complex III senses hypoxic conditions and generates mitochondrial reactive oxygen species (mROS) which stabilize hypoxia-inducible factor (HIF-1α) protein. HIF-1α induces the transcription of the *vegfa* gene, allowing the translation of vascular endothelial growth factor (VEGF) protein, which interacts with mature and precursor endothelial cells, mobilizing them to form new blood vessels. This cascade can be inhibited at specific points by means of gene knockdown, enzyme treatment, and introduction of naturally occurring small molecules, providing insight into the relationship between mitochondria and angiogenesis. This review focuses on current knowledge of the overall role of mitochondria in controlling angiogenesis and outlines known inhibitors that have been used to elucidate this pathway which may be useful in future research to control angiogenesis *in vivo*.

Keywords

angiogenesis; mitochondria; endothelial; mROS; HIF-1α; VEGF

Angiogenesis, Endothelial Cells, and Hypoxia Sensation by Mitochondria

Angiogenesis is the process of forming new blood vessels as an extension of pre-existing blood vessels to improve oxygen and nutrient delivery to tissues throughout an organism. Ischemia [1], exercise [2] [3], and inflammation [4] [5] create a hypoxic environment in tissue resulting in low levels of oxygen available for use by the cells. Of primary interest in regards to angiogenesis is the role of mitochondria in either promoting or inhibiting the process of blood vessel formation in response to the varying internal state of the organism. Mitochondrial cascades play a major role in controlling angiogenesis by regulating the proliferation, migration, and survival of endothelial cells, which make up the inner lining of

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Declaration of Interest

The authors declare that they have no conflict of interest.

blood vessels [6] [7] [8]. Endothelial cells are highly plastic, and are therefore capable of switching from a resting, quiescent state in established conduit blood vessels, to an active, migratory, proliferative state during the process of angiogenesis [9] [10]. Mitochondria are able to control angiogenesis by sensing fluctuations in oxygen availability in tissue and in turn coordinating changes in energy metabolism and generating reactive oxygen species in order to maintain an internal environment capable of sustaining an organism.

Mitochondrial Complex III and the Generation of Mitochondrial Reactive Oxygen Species

The mitochondrial electron transport chain is composed of four major protein complexes, known as mitochondrial Complex I through IV respectively, which reside on the inner mitochondrial membrane [11]. Mitochondrial Complex III, specifically known as ubiquinolcytochrome *c* oxidoreductase, is made up of eleven distinct proteins encoded by nuclear and mitochondrial genes [12]. Complex III has three major responsibilities in the process of oxidative phosphorylation: electron transfer, ubisemiquinone radical stabilization, and cellular oxygen sensing [13].

Mitochondrial Complex III catalyzes electron transfer from ubiquinol to cytochrome *c*. Ubiquinol (QH_2), which is the reduced form of ubiquinone (Q), and cytochrome *c* serve as small electron carriers which ferry electrons from Complex I and II to Complex III and from Complex III to Complex IV, respectively [11]. The electron transfer across Complex III is carried out by the Q cycle [14]. When electrons are transferred from mitochondrial Complexes I and II to ubiquinone, they do so simultaneously in a paired transfer. This newly reduced ubiquinol can then associate with mitochondrial Complex III at the Q_o site to begin the transfer of electrons onto Complex III. However, the subsequent transfer of electrons from mitochondrial Complex III to mitochondrial Complex IV via cytochrome *c* must be conducted sequentially rather than simultaneously, which is the responsibility of the Q cycle [15]. Mitochondrial Complex III contains both high and low potential redox chains [16]. After one electron is transferred from ubiquinol to the high potential redox chain subunit, the Rieske Iron-Sulfur protein, a radical ubisemiquinone intermediate ($Q^{\bullet-}$) remains until the second electron can be transferred to the low potential redox chain subunit of mitochondrial Complex III, cytochrome *b*, yielding a newly oxidized ubiquinone.

Stabilization of the ubisemiquinone intermediate is one facet of a larger mechanism controlled by mitochondrial Complex III; generation of mitochondrial reactive oxygen species. The electron transport chain as a whole is responsible for reducing molecular oxygen (O_2) to water (H_2O), which occurs by means of the final electron transfer from Complex IV to molecular oxygen. The presence of electrophilic molecular oxygen near the Q_o site on mitochondrial Complex III allows the possibility of electron transfer from ubisemiquinone to molecular oxygen rather than the low potential redox chain subunit of Complex III, cytochrome *b* [17]. The probability of this occurring increases in proportion to the amount of time the ubisemiquinone molecule is present [18] [19] [20]. The capture of an electron from ubisemiquinone by molecular oxygen results in the formation of superoxide ($O^{\bullet-}_2$), which, along with other partially reduced oxygen products such as hydrogen

peroxide (H_2O_2) and hydroxyl radicals (•OH), are known as mitochondrial reactive oxygen species (mROS) [21]. Ubisemiquinone stabilization prevents the donation of an electron to molecular oxygen, which inhibits the formation of mROS radicals [18]. These mROS have been shown to contribute to angiogenesis by stabilizing proteins in specific signaling pathways described later [22]. It should be noted that nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) also produces substantial amounts of reactive oxygen species within endothelial cells and other cell types through the reduction of O_2 [23], which can contribute to angiogenesis through similar pathways [22] [24], but this mechanism takes place independently of the mitochondria and is therefore outside the scope of this review. The role of mitochondrial Complex III in cellular oxygen sensing relies on the ubiquinolcytochrome *c* reductase binding protein (UQCRB) subunit, which is a key player in mitochondria's role in angiogenesis, and has therefore been the focus of essential research in this discipline.

Control of mROS Generation by Ubiquinol-cytochrome c Reductase Binding Protein

UQCRB is a 13.4-kDa nuclear-encoded subunit of mitochondrial Complex III which plays a role in the maintenance of mitochondrial Complex III while also assisting in the electron transport function of the complex [25]. The vital nature of this subunit in the overall function of mitochondrial Complex III has been proven over the course of several experiments both *in vivo* and *in vitro* which look to inhibit UQCRB function and subsequently investigate the downstream effects of this inhibition on mitochondrial function and angiogenesis (Table 1). Terpestacin is a naturally occurring bicyclo sesterterpene molecule which has been isolated from multiple organisms, most notably *Embellisia chlamydospora*, a fungus. Early work with this molecule proved its general efficacy in inhibiting angiogenesis [26]. It was then shown that terpestacin contributed its inhibiting effects on angiogenesis by binding specifically to the UQCRB subunit of mitochondrial Complex III [27]. Later work in *Danio rerio* (zebrafish) investigated both terpestacin and gene knockdown of UQCRB with *uqcrb*-MO (Morpholino oligomer) to investigate downstream effects on angiogenesis. Angiogenesis was dose-dependently inhibited by both factors, accompanied by a substantial decrease in *vegfa* gene expression [28]. The introduction of human UQCRB-specific siRNA (siUQCRB) to human umbilical vein endothelial cells (HUVECs) decreased the mobilization and invasiveness of HUVECs dose dependently [29], which helps to strengthen the case for UQCRB's role in the angiogenic cascade as well as the role in angiogenesis of endothelial cell migration and vascular endothelial growth factor (VEGF), which will be described later. mROS generation was also shown to be significantly diminished in cells treated with terpestacin and siUQCRB, implying that the UQCRB subunit also plays a role in mROS production, potentially as a modulator of electron flux through Complex III, which can influence the lifetime of ubisemiquinone, controlling levels of mROS being produced [27]. This inhibition of mROS production decreased the angiogenic proliferation, migration, and survival of endothelial cells [9] [10] [29]. These results indicate that the role of UQCRB in mitochondrial Complex III function and angiogenesis overall involves the production of mROS and VEGF, both of which contribute to downstream factors in the angiogenic pathway of endothelial cells.

Mitochondrial Metabolism and Intracellular Redox Reactions

Under normoxic conditions, oxidative phosphorylation is the primary metabolic pathway used to recycle ADP to ATP as an energy source for the cell [11]. The electron transport chain on the inner mitochondrial membrane transfers electrons from NADH and $FADH_2$ to a series of specific protein complexes, which then deposit four total electrons onto molecular oxygen, forming water as a final byproduct. This electron transfer powers the transport of protons across the inner mitochondrial membrane into the intermembrane space, creating a mitochondrial membrane potential that contributes to the proton motive force driving the phosphorylation of ADP to ATP [30] [31] [32] [11]. mROS are a natural byproduct of oxygen metabolism, but under normal metabolic conditions, only 0.1% of the total oxygen consumed in the mitochondria is converted to mROS [33].

Hypoxic conditions, or low levels of available oxygen in a tissue, can be caused by ischemia [1], exercise [2] [3], or inflammation [4] [5]. Mitochondria, more specifically the UQCRB subunit of mitochondrial Complex III, play an important role as oxygen sensors in the cell [27]. Sensation of hypoxic conditions increases the generation of mROS which can then be used to regulate downstream hypoxic response pathways [34] [35] [36]. Although it appears to be counterintuitive, due to the decreased availability of oxygen in the cell, studies have shown hypoxic conditions increase mROS production at mitochondrial Complex III [37]. One experiment contributing to the discovery of this phenomenon involved low concentrations of the probe dichlorofluorescein diacetate which is oxidized during the formation of mROS to yield fluorescent dichlorofluorescein (DCF). Under control conditions, the rate of probe oxidation matched the rate of leakage of oxidized probe from the cell, resulting in a stable fluorescence intensity. Hypoxic conditions lead to an increased fluorescence intensity, indicating an overall increase in oxidation within the cell, resulting from the amplified production of mROS [34] [38] [39]. This follows the common redox reaction blueprint, in which the O_2 within the mitochondria are being reduced to reactive oxygen species by gaining electrons, and the other entities within the mitochondria, DCF in the case of this experiment, are being oxidized by losing electrons as a result of this reaction. The extent of hypoxia was shown to be proportional with fluorescence intensity, with 1% O_2 showing greater fluorescence intensity than 3% and 5% O_2 [40], indicating higher levels of oxidation in the mitochondria under increasingly hypoxic conditions, a result of increased mROS production. A review of additional experimentation further confirmed this notion of increased oxidant production under physiological hypoxia in a large range of cell types [37]. Likewise, blocking the ability of mitochondrial Complex I and II to generate ubiquinol, a key step in the electron transport chain during metabolism, was carried out using specific inhibitors [40], and led to attenuation of oxidation in the cell. This confirmed the role of mitochondrial Complex III in mROS production under hypoxic conditions [40], oxidizing ubiquinol to ubisemiquinone, which allows for the reduction of O_2 to mROS. mROS can then be used by the mitochondria in hypoxic response signaling, namely, stabilizing the major downstream factor in the angiogenesis pathway, the HIF-1α protein.

mROS Mediated Stabilization of HIF-1α and the VEGF Pathway

Hypoxia inducible factor (HIF-1) is a heterodimer transcription factor protein made up of HIF-1α and HIF-1β domains [41]. Under normoxic conditions, the HIF-1β subunit is stable, while the HIF-1α subunit is easily degradable through hydroxylation by prolyl hydroxylase and subsequent ubiquitination by the von-Hippel-Lindau protein (pVHL)/E3 ubiquitin ligase, allowing for degradation by proteasomes [42] [43] [44] [45] [46]. Under hypoxic conditions, HIF-1α remains viable, accumulating and moving into the nucleus to enable the transcription of genes necessary for a cell and an organism as a whole to respond to hypoxia [11]. One major protein transcriptionally activated by the HIF-1 protein complex is vascular endothelial growth factor (VEGF) [47] [48] [49] [50]. When HIF-1α is in its active state under hypoxic conditions it is able to bind to the 5' flanking region of the *vegfa* gene, inducing transcription and leading to translation of the VEGF protein [48]. VEGF, an endothelial cell-specific mitogen, is the major factor affecting angiogenesis [51] [52] [53]. VEGF possesses paracrine function and is released by cells in nearby tissue to bind to vascular endothelial growth factor receptor-2 (VEGFR2), also known as KDR/Flk-1 [54] [55] [56], which has been implicated as the primary mitogenic receptor for VEGF in the angiogenesis pathway [57] [58]. Activation of VEGFR2 leads to phosphorylation of specific downstream signal transduction effectors, including extracellular signal transduction kinase (ERK) [59] [60] and Akt [61]. ERK, a subtype of mitogen-activated protein kinase (MAPK), contributes to angiogenesis by promoting endothelial cell growth and differentiation [62]. Akt inhibits apoptosis to promote endothelial cell survival [63] [64] [65], while also contributing to angiogenesis through the transcription and translation of additional VEGF [66] [67] and promoting the migration of endothelial cells [68] [69]. Increased levels of VEGF in circulation stimulates the mobilization of mature endothelial cells in existing blood vessels as well as the maturation of circulating endothelial precursor cells (CEPs) [70], which both express the VEGFR2 receptor [71] [72], and coordinate to form the inner lining of new blood vessels.

The transcription factor subunit HIF-1α, however, must be stabilized and activated in order to carry out its function. We have discussed how hypoxic conditions lead to increased levels of mROS, which in turn stabilizes the HIF-1α protein [73] and allows transcription of the *vegfa* gene. Several experiments have implicated the role of hypoxia-induced mROS in the stabilization of HIF-1α by manipulating this pathway through treatment with specific inhibitors (Table 1). For example, treating cells with matairesinol, a natural small molecule originating in *Cedrus deodara* plants, suppresses hypoxia-induced generation of mROS both *in vitro* and *in vivo*, which in turn suppresses HIF-1α and VEGF levels as well as HUVEC tube formation [74]. mROS, as oxidative stressors, carry out this stabilization by contributing to the deactivation of prolyl hydroxylases in the cell, which prevents the hydroxylation and degradation of HIF-1α [75]. To determine which mROS are required for HIF-1α stabilization, copper zinc superoxide dismutase (SOD1) and manganese superoxide dismutase (SOD2), which breakdown superoxide to hydrogen peroxide, or glutathione peroxidase 1 (GPX1) and catalase, which breakdown hydrogen peroxide to water, were introduced to cells *in vitro*. HIF-1α stabilization was decreased in cells overexpressing GPX1 and catalase, indicating a role for hydrogen peroxide in prolyl hydroxylase

deactivation and the accompanying stabilization of HIF-1α [76]. The breakdown of superoxide into hydrogen peroxide by SOD1 and SOD2 contributes to this stabilizing effect of the mROS, but is not sufficient to trigger HIF-1α protein stabilization in the absence of high levels of hydrogen peroxide [76].

Other experiments confirming the role of mROS in HIF-1α stabilization *in vitro* have employed the knockout of specific mitochondrial components in mutant cell or mitochondrial strains. One study confirmed both the role of the cytochrome *b* subunit as the second, low potential electron acceptor on mitochondrial Complex III and the role of mROS in HIF-1α stabilization. Cells that were deficient of cytochrome *b*, while not completing final electron transfer to convert molecular oxygen to water due to this missing link in the electron transport chain, were still able to produce mROS, as evidenced by the stabilization of HIF-1α protein [17]. This supports the ubisemiquinone mechanism of mROS production described earlier, and provides further evidence for the role of mROS in stabilizing HIF-1α. Inhibiting Complex III with a specific inhibitor, stigmatellin, inhibits hypoxic stabilization of HIF-1α *in vitro*, again confirming the role of this complex in the production of mROS [17]. The binding of terpestacin to the UQCRB subunit of mitochondrial Complex III was also shown to inhibit HIF-1α stabilization by inhibiting mROS production, as discussed. This led to the inhibition of angiogenesis which coincided with decreased expression of VEGF [27], as would be expected without the activated transcription factor for the *vegfa* gene, the HIF-1 protein complex.

Conclusion

The major link between mitochondria and angiogenesis centers on the production of VEGF in response to oxygen sensation and mROS production by the mitochondria. As the centers of oxidative metabolism, the mitochondria play an integral role in sensing an inflamed or otherwise hypoxic tissue environment, and allowing the transcription of the genes necessary to ensure adequate delivery of nutrients and oxygen to these tissues through the extension of blood vessels by angiogenesis. This angiogenic response ensures the health and survival of both specific tissues and whole organisms. It has been clearly established that mROS are a major signaling molecule generated by the mitochondria to stabilize HIF-1α, leading to the production of VEGF which is released from cells and acts in a paracrine function to activate VEGFR2, inducing downstream pathways. Inhibitors of various steps in this pathway have revealed the overall link between mitochondria and angiogenesis. Future research should be directed toward discovering efficient methods of controlling angiogenesis *in vivo* by employing these inhibitors, and better understanding mROS signaling, possibly beyond the scope of prolyl hydroxylase deactivation, investigating the possibility of a greater role for these oxidative stressors in the angiogenesis pathway.

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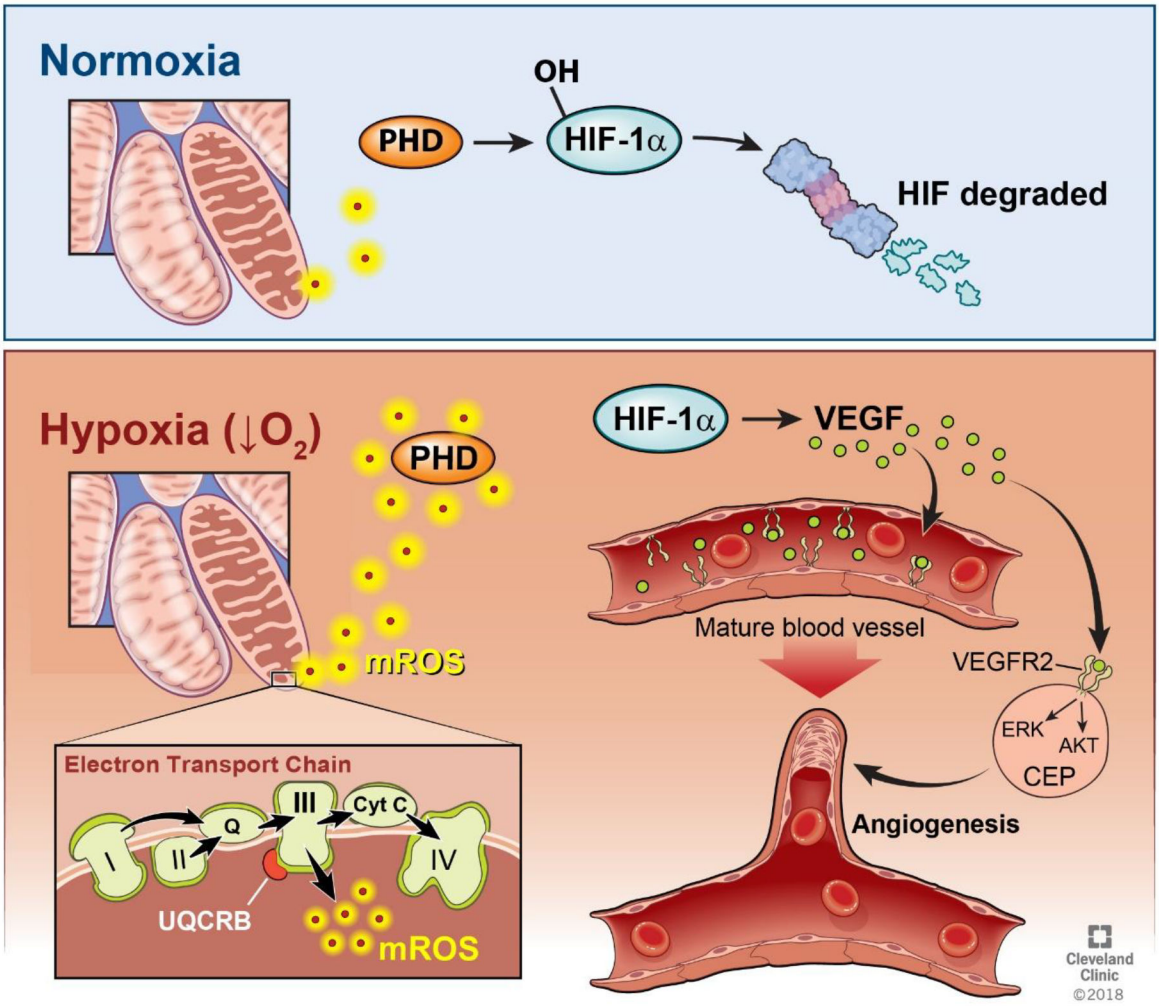

Fig. 1. Summary of the Angiogenesis Pathway

Ischemia, exercise, and inflammation create a hypoxic tissue environment, resulting in decreased oxygen availability in cells. Under these hypoxic conditions, the electron transport chain produces mROS at mitochondrial Complex III. These mROS exit the mitochondria and deactivate prolyl hydroxylase (PHD). Under normoxic conditions, the levels of mROS being produced are insufficient to deactivate PHD, and PHD therefore hydroxylates the HIF-1α protein, marking it for eventual degradation by proteasomes. When high levels of mROS are produced under hypoxia, HIF-1α is stabilized, allowing the production of the VEGF protein. This VEGF protein attaches to the VEGFR2 receptor on both mature endothelial cells lining blood vessels and circulating endothelial precursors (CEP). This activates the ERK and Akt pathways, causing the maturation and mobilization of endothelial cells, allowing angiogenesis to occur.

Table 1

Inhibitors of the Angiogenesis Pathway

Inhibitor	Cell Type	Process Inhibited	Pathway	Reference
Stigmatellin	WT cybrids; *in vitro*	Inhibits HIF-1α stabilization and accumulation	Binding to Q_o site inhibits mROS production	[17]
Terpestacin *in vivo*	Murine FM3A breast cancer cells; *in vivo*	Inhibits tumor angiogenesis	Inhibition of HIF-1α Mediated VEGF expression	[27]
Terpestacin *in vitro*	Human HT1080 fibrosarcoma cells, HUVECs; *in vitro*	Inhibits VEGF production; inhibits tube formation and angiogenesis by HUVECs	Decreases mROS levels by inhibiting UQCRB which inhibits HIF-1α stabilization and accumulation, inhibiting VEGF production	[27]
Uqcrb-MO	Zebrafish embryos	Inhibits VEGF production	Gene knockdown of *uqcrb* gene leads to decreased expression of *vegfa* gene	[28]
siUQCRB	HUVECs; *in vitro*	Inhibits endothelial cell invasiveness through inhibition of ERK and Akt pathways	Gene knockdown of *uqcrb* leads to decreased mROS levels, decreased activation of VEGFR2	[29]
Rotenone and thenoyltrifluoroacetone (TTFA)	Cardiomyocytes; *in vitro*	Inhibits mROS production	Inhibits formation of ubiquinol by preventing transfer of electrons from Complex I and Complex II	[40]
Matairesinol	HUVECs; *in vitro* and *in vivo*	Inhibits HUVEC tube formation	Inhibits mROS production which decreases HIF-1α stabilization and VEGF production	[74]
GPX1 and catalase	A549 adenocarcinomic human alveolar basal epithelial cells; *in vitro*	Inhibits HIF-1α stabilization and survival	Breaks down hydrogen peroxide to water which prevents breakdown of prolyl hydroxylase	[76]