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## Mitochondrial Mechanisms in Cerebral Vascular Control: Shared Signaling Pathways with Preconditioning

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### Abstract

Mitochondrial initiated events protect the neurovascular unit against lethal stresses via a process called preconditioning which independently promotes changes in cerebrovascular tone through shared signaling pathways. Activation of the adenosine triphosphate (ATP)-dependent potassium channels on the inner mitochondrial membrane (mitoK<sub>ATP</sub> channels) is a specific and dependable way to induce protection of neurons, astroglia, and cerebral vascular endothelium. Through the opening of mitoK<sub>ATP</sub> channels, mitochondrial depolarization leads to activation of protein kinases and transient increases in cytosolic calcium (Ca<sup>2+</sup>) levels that activate terminal mechanisms that protect the neurovascular unit against lethal stress. Release of reactive oxygen species (ROS) from mitochondria have similar protective effects. Signaling elements of the preconditioning pathways also are involved in the regulation of vascular tone. Activation of mitoK<sub>ATP</sub> channels in cerebral arteries causes vasodilation, with cell-specific contributions from endothelium, vascular smooth muscle (VSM), and nerves. Pre-existing chronic conditions, such as insulin resistance (IR) and/or diabetes, prevent preconditioning and impair relaxation to mitochondrial centered responses in cerebral arteries. Surprisingly, mitochondrial activation after anoxic or ischemic stress appears to protect cerebral vascular endothelium and promotes the restoration of blood flow; therefore, mitochondria may represent an important, but underutilized target in attenuating vascular dysfunction and brain injury in stroke patients.

### Keywords

cerebral arteries; ischemia; anoxia; ATP-sensitive potassium channels; calcium sparks; endothelium; vascular smooth muscle; neurons; nitric oxide synthase; postconditioning

### INTRODUCTION

The neurovascular unit is an integrated system of vascular and parenchymal cells and their environment working together to match blood flow to metabolic demand and to maintain brain homeostasis. Mitochondria are double membrane organelles which generate chemical energy in the form of ATP which is distributed within cells and promotes the varied activities of the diverse cell types comprising the neurovascular unit (endothelium, vascular smooth muscle [VSM], astroglia, perivascular neurons, parenchymal neurons, pericytes, and

microglia). Although traditionally understood solely as energy producers and relegated to that role, we now appreciate that mitochondria are involved in diverse adaptive activities such as promotion of basal cellular functions, cellular protection, and regulation of vascular tone. Evidence also shows that mitochondrial morphology and function show plasticity and adaptability and can be affected by many factors [12,37,72,107,137]; on the other hand, mitochondrial-centered mechanisms can promote apoptotic and necrotic cell death [56,89]. The purpose of this review is to critically examine the role of mitochondrial-based mechanisms on the tone of cerebral resistance vessels. Specifically, we will examine how the selective targeting of mitochondria in the several cell types comprising the neurovascular unit leads to changes in cerebral vascular tone. The studies on cerebral vascular control in our laboratory originated from studies of pre- and post-conditioning mechanisms in the neurovascular unit when we realized that identical pathways are involved and effects may be interactive. This discussion is limited to the systemic circulation; mitochondrial mechanisms in the pulmonary circulation are different [35]. We will show that even relatively mild metabolic stress, such as that which occurs with IR, a pre-diabetic condition that impairs preconditioning, can dramatically affect mitochondrial related events in the cerebral vasculature. Ultimately, we will present evidence suggesting that mitochondrial activation may unexpectedly benefit the cerebral vasculature after ischemia/anoxia and thus decrease morbidity and mortality in stroke patients. Several excellent reviews which focus primarily on peripheral endothelium [53,85,138] or VSM [108] have been published recently and emphasize the importance of this emerging research area. However, this is the first review to show the similarities between the preconditioning and vascular control pathways and to explore the integration of mitochondrial based signals ranging from multiple cell types to the final vascular response in normal and disease states.

## MITOCHONDRIAL ESSENTIALS

Important structural features of mitochondria are an outer, relatively permeable membrane, a relatively impermeable inner membrane, the intermembrane space, extensions of the inner membrane called cristae, and the intracristae spaces referred to as the matrix [34,68]. Each anatomical component has a specific function which, when integrated, is essential for the optimal production and transfer of energy throughout the mitochondria and into the cytosol. The Krebs cycle is located in the matrix and the complexes associated with the electron transport chain are embedded in the inner mitochondrial membrane. The mitochondrial membrane potential ( $\psi_m$ ) across the inner membrane, which is normally maintained at approximately  $-180\text{mV}$ , provides the proton difference used to drive the synthesis of ATP by the electron transport chain. Several diagnostic and therapeutic approaches take advantage of this negative membrane potential to target positively charged agents specifically to the mitochondria [26,52,69,114]. Detailed descriptions of ATP production by non-mitochondrial glycolysis as well as the mitochondrial electron transport chain are widely available [36,136].

Individual mitochondria are not static entities, rather they are dynamic and can move from one location to another within cells, form networks with other mitochondria or cellular structures (Figures 1 & 2), and undergo replication and fission/fusion in response to physiological and pathological stimuli [26,34,38,137,145]. Because the number and length

of cristae are positively related to ATP generating potential, it appears that changes in the location, number, size, and shape of mitochondria under physiological conditions are determined by the energy needs within a cell [61]. In some cell types, such as VSM, mitochondria do not appear to be mobile in the normal state, but this situation changes during migration following vascular damage [17]. In addition to individual mitochondria forming networks within cells (Figures 1 & 2), the mitochondrial outer membranes are often connected or closely juxtapositioned with the membranes of the endoplasmic/sarcoplasmic reticulum (ER/SR) (Figure 2); cellular structures which are involved in calcium storage and release [145]. The complex interplay among mitochondrial, ER/SR, and cytosolic calcium has been described in several recent reviews [16,23,130]. In VSM, mitochondria and SR often appear to be “bundled” together and this structural feature may account for the lack of significant movement of mitochondria under normal conditions in this vascular cell type (Figure 2) as well as the tight functional interactions between mitochondria and SR. However, we are unaware of any systematic studies comparing the mitochondrial morphology and function in the various cell types within the neurovascular unit.

Mitochondria have their own DNA, distinct from nuclear DNA, and also possess protein synthesizing machinery which produces approximately one-half of their proteins while the other half are encoded by nuclear DNA and imported into mitochondria via several different transport mechanisms. The cytochrome c oxidase complex (Complex IV), which is the last major component of the electron transport chain and is localized to the inner membrane, is a good example of the dual origins of the proteins present in mitochondria since ten subunits are nuclear in origin and three are synthesized within the mitochondria [50,95].

Although it has been suggested that mitochondria contain a variant of nitric oxide synthase (NOS), and thus could produce NO via this enzymatic pathway, there is significant evidence against this view [91,92]. Nonetheless, diffusible NO from non-mitochondrial sources in endothelial cells and neurons [74], or NO arising from mitochondria via non-NOS pathways [94], could affect the functioning of the electron transport chain as well as other mitochondrial functions and could combine with superoxide anion to form peroxynitrite [44,138]. On the other hand, we have shown that NO production from cytosolic NOS occurs due to eNOS phosphorylation and increased cytosolic calcium following activation of mitochondria with diazoxide or BMS-191095 [74].

## ROS PRODUCTION AND RELEASE BY MITOCHONDRIA

Mitochondria are constant producers of ROS which have access to locations throughout the cell as well as to adjacent cells. Although generally thought to be an efficient system, a substantial amount of superoxide anion is continuously “lost” or released by the electron transport chain at different sites under normal conditions [36,138]. For example, there is continuous release of superoxide anion in quiescent neurons, astroglia, VSM, and cerebral vascular endothelium [47,74,122]. Mitochondrial ROS can promote preconditioning as well as directly or indirectly affect cerebral vascular tone, as well as influence other cellular functions.

Superoxide anion from mitochondrial and other cellular locations appears to be an important, if not essential, signaling agent involved in the maintenance of basal cell functions such that prolonged suppression of ROS availability is detrimental to cell viability [141]. Superoxide anion acting at sites within the mitochondria or following transport across the inner mitochondrial membrane can influence events throughout the cell. It has been suggested that superoxide anion can leave the mitochondria via voltage dependent anion channels (VDACs) [57,101]. However, VDACs are located in the outer, more porous mitochondrial membrane and it is not clear how superoxide anion would transverse the inner mitochondrial membrane. Alternatively, superoxide anion formed at Complex III may be released into either the matrix or the intermembrane space during certain conditions and then may be transported across the outer mitochondrial membrane by VDACs [60,118]. Superoxide anion also can leave the matrix [66] following enzymatic conversion to hydrogen peroxide through aquaporin-like channels in the inner mitochondrial membrane [8,15,103]. Hydrogen peroxide is considered to be less reactive and has a longer half-life than superoxide anion [120] and can be produced directly by mitochondria under certain conditions [120,143]. A number of factors such as substrate availability, status of the electron transport chain complexes, and the composition of the local environment are able to affect mitochondrial production of ROS [120]. In addition, a physical distortion of the relationship between the two mitochondrial membranes caused by increased shear stress in endothelium leads to enhanced ROS release by mitochondria [143]. It has generally been accepted that mitochondrial depolarization is always accompanied by enhanced ROS release. However, as described in detail below, mitochondrial depolarization and enhanced ROS production are not necessarily linked and can occur separately under a variety of conditions.

The primary sites of superoxide anion production and release are Complex I (NADH-ubiquinone oxidoreductase), Complex II (succinate dehydrogenase, SDH), and Complex III (ubiquinolcytochrome c oxidoreductase). Complex I and II accept electrons from NADH + H<sup>+</sup> and FADH<sub>2</sub>, respectively, which are transferred to Complex III and finally to Complex IV (cytochrome c oxidase), where the final electron acceptor is oxygen and the final product is water [121,143]. Superoxide anion from these three Complexes is released into the matrix. A number of metabolic poisons are available which inhibit one or more of the respiratory chain complexes: rotenone: Complex I; 3-NPA: Complex II; Antimycin A: Complex III; cyanide: Complex IV; and oligomycin: Complex V. The result of inhibition, especially of Complexes I-III, is enhanced ROS release.

Although “normal” continuous release of ROS from mitochondria appears to play a positive role in the maintenance of basal cellular function, transiently elevated ROS levels can promote selective protein synthesis, preconditioning, and changes in vascular tone. However, chronically but modestly elevated ROS production by mitochondria can lead to cellular dysfunction. For example, we have shown that a mutation of the inner mitochondrial membrane peptidase 2-like (Imp2l) gene leads to chronically enhanced ROS release by mitochondria and subsequently causes reduced vascular dilation to carbachol in mesenteric arteries [100]. Similarly, chronically elevated mitochondrial ROS production due to the metabolic syndrome impairs cerebral vascular function through multiple mechanisms [71]. It also has been reported that a genetic deficiency of MnSOD in mice increases basal

superoxide levels in cerebral arteries and aorta [10]. Additionally, Chilian et al. have shown that chronic inhibition of Complex I by rotenone blocks ischemia induced collateral artery growth in the coronary circulation via activation of adenosine monophosphate activated kinase, and the subsequent inhibition of mTOR and p70 ribosomal S6 kinase [119]. Higher levels of cellular ROS, such as occurs during injury, can lead to cell death via both mitochondrial- and non-mitochondrial-pathways, especially in metabolically compromised conditions [57,89].

Production of ROS by mitochondria appears to be an organelle-independent process under most basal conditions, but recent reports indicate that ROS production by mitochondria can either promote ROS production by the extra-mitochondrial NADPH oxidase system, or *vice versa*, via a positive feedback system [23,29,146]. Thus, the interaction between the mitochondria and cytosolic NADPH oxidase axis leads to cellular damage due to excessive production of ROS by a ROS induced ROS mechanism. The best example of this interaction occurs with the exposure of vascular and non-vascular cells to angiotensin II [29,113]. Deficiency of MnSOD leads to cerebral vascular endothelial dysfunction, especially in aged mice to angiotensin II [21,110].

## MITOCHONDRIAL DEPOLARIZATION AND RELATED EVENTS

The most reproducible and robust approach to depolarizing mitochondria experimentally can be achieved by targeting the mitochondrial ATP-sensitive potassium (mitoK<sub>ATP</sub>) channel on the inner mitochondrial membrane with drugs such as BMS-191095 or diazoxide. The physical structure of mitoK<sub>ATP</sub> channels is not yet known with certainty but appears to differ substantially from the previously described plasmalemmal K<sub>ATP</sub> channels [1,43,125,127]. Nonetheless, the pharmacological identification and selectivity to agonists of the mitoK<sub>ATP</sub> channels is convincing to the majority of investigators. Recently, Foster et al. [4] provided evidence that the renal outer medullary potassium channel (ROMK) is a component of the K<sup>+</sup> channel of the cardiac mitoK<sub>ATP</sub>. They also showed that overexpression and suppression of ROMK promoted or reduced preconditioning, respectively. Succinate dehydrogenase might also be involved in either the assembly or function of the mitoK<sub>ATP</sub> channel [124,139]. Nonetheless, additional studies are needed to define completely the structure of the mitoK<sub>ATP</sub> channels.

Isolated mitochondria and mitochondria *in situ* in cultured cells, tissue slices, and in isolated pressurized cerebral arteries depolarize in a dose-dependent manner to selective mitoK<sub>ATP</sub> channel openers such as diazoxide and BMS-191095 [13,47] and mitoK<sub>ATP</sub> channel activity is affected by endogenous factors such as the ADP/ATP ratio [1], peroxynitrite [90,91], superoxide anion [89,91], and cytosolic protein kinase C epsilon (PKCε) [118]. Nonetheless, we expect that other, yet unidentified, physiological and pathological factors will be able to directly or indirectly activate mitochondria including the mitoK<sub>ATP</sub> channel. The classical K<sub>ATP</sub> channel antagonist glibenclamide as well as 5-hydroxydecanoic acid (5-HD), which needs to be metabolized before becoming active [59], block the actions of diazoxide, BMS-191095, and/or PKCε [13,47,118]. Diazoxide, a drug previously used against acute hypertension or hypoglycemia in people, is the most commonly used mitoK<sub>ATP</sub> channel opener [42], but it has the additional effect of inhibiting succinate dehydrogenase (SDH;

complex II), especially at high doses [22,82]. Diazoxide also readily crosses the BBB and thus is effective in the brain when given intravenously [96]. Although applications of diazoxide or BMS-191095 depolarize mitochondria, diazoxide, but not BMS-191095, also causes the liberation of ROS [14], which our findings indicate is secondary to SDH inhibition. This view is supported by examination of the effects of the specific inhibitor of SDH, 3-nitropropionic acid (3-NPA), which increases ROS production by mitochondria [13] and also induces preconditioning [63] and changes in vascular tone [71]. Nonetheless, the primary actions of diazoxide on the cells of the neurovascular unit are still specific to mitochondria [14,82], and the associated ROS increase appears to enhance the degree of depolarization [90,91]. In contrast, BMS-191095 is very selective for mitoK<sub>ATP</sub> channels and has no known non-specific effects to complicate the interpretation of the results [14,54,55].

A potential role for mitochondrial calcium activated potassium (mitoK<sub>Ca</sub>) channels in depolarizing mitochondria has been suggested based primarily on the use of the multiple target drug NS1619 [6]. Although NS1619 results in mitochondrial depolarization, it seems likely that, at least in neurons, effects are due to other factors such as inhibition of Complex I and subsequent increased release of ROS [48]. Given the multiple potential sites of action of NS1619 within various cell types, it is also possible that mitochondrial effects to this drug are secondary to non-mitochondrial events. Nonetheless, more research in this area is warranted and the development of more specific agonist would aid these efforts.

## **MITOCHONDRIAL MEMBRANE POTENTIAL AND ROS PRODUCTION INDEPENDENCE**

The use of BMS-191095 has led to findings which challenge accepted views concerning the linkage between mitochondrial depolarization and enhanced mitochondrial ROS release. The selectivity of BMS-191095 for mitoK<sub>ATP</sub> channels and the failure to detect non-specific effects [14,54,55], which complicate the interpretation of results, has shown that it is possible to depolarize mitochondria without eliciting the enhanced release of ROS in a variety of cells including neurons, cerebral vascular endothelium, and cerebral VSM. The previous concept linking mitochondrial membrane potential and ROS release was developed using inhibitors of the electron transport chain which, in addition to increasing ROS release from Complexes I-III, probably depolarized mitochondria via reduced ATP availability necessary for maintaining the negative transmembrane potential. The more recent finding with BMS-191095, which allows dissociation between mitochondrial depolarization and mitochondrial ROS production, is supported by other laboratories [133,137] as well as other experimental approaches in our laboratory [33]. The dissociation between mitochondrial membrane potential and ROS release is not absolute, however, but probably occurs within a limited range of depolarization. The relative independence of mitochondrial membrane potential from ROS production and release, with the subsequent induction of different signaling pathways, underscores the versatility of mitochondria to initiate appropriate and selective cellular responses to varied stimuli.

## ROLE OF MITOCHONDRIA IN PRECONDITIONING

Preconditioning represents the condition in which transient exposure of cells to an initiating event leads to protection against subsequent, potentially lethal stimuli (Figure 3) by limiting ROS availability, suppressing large increases in cytosolic levels of free calcium, and/or by other mechanisms which promote cell survivability (Figure 4). The acute signaling mechanisms promoting preconditioning also affect cerebral vascular tone, and the changes in cells following preconditioning resulting from the altered cellular calcium and ROS dynamics probably affect subsequent cerebral artery responses to changing conditions (Figure 5). However, this possibility has not been tested.

Beginning with our original observation [31], a number of independent studies by other laboratories have established that mitochondrial-centered mechanisms are important initiators of the pre- and post-conditioning response in neurons, astroglia, and cerebral endothelial cells [12,14,30,31,42,46,64,65,96,99,104,109,121,125,128]. Mitochondrial-centered preconditioning also occurs in tissues such as myocardium [70] and skeletal muscle [55] and peripheral endothelium [9,99]. Important mitochondrial specific targets for inducing preconditioning by pharmacological approaches include: (1) potassium channels located on the inner mitochondrial membrane; (2) respiratory chain enzymes; and (3) metabolic substrate restriction.

Although the mitochondrial-initiated mechanisms involved in preconditioning are not fully understood, the activation of protein kinases, transient but modest calcium fluxes, and/or production of ROS appear to be essential signaling events leading to preconditioning (Figure 4). There are two general types of preconditioning: immediate and delayed. Immediate preconditioning occurs within minutes of the initiating stimulus and lasts for several hours before disappearing, whereas delayed preconditioning takes several hours to develop and persists for several days [51,81]. Given these temporal aspects, it seems probable that immediate preconditioning primarily involves changes in the activity or function of enzymes, second messengers, and ion channels already present whereas delayed preconditioning principally is due to *de novo* protein synthesis such as increased catalase levels [14]. Many subsequent signaling events not only induce preconditioning but also promote changes in vascular tone. Postconditioning, in which the initiating event occurs following the onset of the potentially lethal event [125,144] has also been reported and the mechanisms, especially those involving mitochondria and the PI3-kinase/Akt signaling pathway, appear to be similar to those that promote preconditioning. In cultures of primary rat brain microvascular endothelium, we found that postconditioning was as effective as preconditioning against oxygen-glucose deprivation (OGD) (Figure 3), and that activation of the PI3Kinase/Akt signaling pathway was essential in both types of cellular protection (Figures 3 & 4). The ability of mitochondria from cerebral vascular endothelium to function at the end of OGD, where almost 50% of cells would eventually die without treatment, indicates that mitochondria remain a potentially, useful target in stroke patients.

Although some authors have presented a case for a role of mitochondrial calcium activated potassium (mitoK<sub>Ca</sub>) channels in promoting preconditioning based primarily on the use of the multiple target drug NS1619 [18,27], the experimental evidence is not convincing due to

the lack of a specific mitoK<sub>Ca</sub> channel opener and the numerous non-specific effects of NS1619. We were the first to show that NS1619 is both neuroprotective [134] and vasoactive in cerebral [5] arteries, and that preconditioning of neurons by this agent was likely due to inhibition of Complex I or other mechanisms rather than a mitoK<sub>Ca</sub> channel-specific effect [48,49,80,87]. Administration of NS1619 dilates other peripheral arteries [60].

Removal or reductions in key metabolic substrates, such as glucose and oxygen, can induce preconditioning and changes in cerebral vascular tone. For example, transient withdrawal of glucose, the major energy substrate for neurons, as well as removal of amino acids results in mitochondrial depolarization and reduced ATP production and in delayed tolerance against various insults such as OGD, glutamate excitotoxicity, and exogenous hydrogen peroxide toxicity [45]. Adenosine, AMP, ADP, and ATP are vasoactive stimuli in cerebral arteries [116,142].

## MITOCHONDRIA AND CEREBRAL VASCULAR TONE

Our studies of preconditioning indicated that many of the signaling events associated with the induction of mitochondrial-targeted cellular protection also affect cerebrovascular tone (Figures 4 & 5). Although it is known that extracellular ATP, from either glycolysis or oxidative phosphorylation, as well as the metabolites adenosine, AMP, and ADP (also preconditioning stimuli) [2,109] can alter cerebrovascular tone via plasmalemmal purinergic receptors [28], only a few studies have examined direct mitochondrial influences on the diameter of cerebral resistance vessels [19,37,71,75,140]. Thus, the study of mitochondrial effects on the cerebral vasculature is a relatively new field. Only a few studies have examined mitochondrial influences on the cerebral vasculature during disease states, but increasing amounts of information are rapidly becoming available in other systemic circulations [54,85,138] and VSM [108]. Although the majority of studies involving mitochondrial influences on the cerebral vasculature have used pharmacological agents, a few studies have used genetically altered mice in which a deficiency of MnSOD has resulted in increased basal levels of superoxide anion and endothelial dysfunction in cerebral arteries especially with exposure to angiotensin II and aging in male rats [21].

### Vascular smooth muscle

Jaggar and colleagues [19,140] were the first to show that activation of mitochondria by diazoxide promoted relaxation of VSM cells in endothelium-denuded cerebral arteries or freshly dissociated VSM via a mechanism primarily involving ROS. Thus, diazoxide application enhanced the generation of ROS from mitochondria, which sequentially caused the activation of ryanodine-sensitive Ca<sup>2+</sup> channels on the SR, the generation of Ca<sup>2+</sup> transients called “Ca<sup>2+</sup> sparks”, and the opening of adjacent large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (BK<sub>Ca</sub>) channels on the plasma membrane. The resulting K<sup>+</sup> efflux led to VSM hyperpolarization, decreased global intracellular Ca<sup>2+</sup>, and vasodilation. We have reported similar findings in endothelium denuded arteries with diazoxide [71,74]. The role of mitochondrial ROS resulting from inhibition of SDH in promoting VSM relaxation in cerebral arteries is also shown by application of 3-NPA [71]. However, BMS-191095 has a similar effect in VSM without the involvement of ROS [72,73] (Figure 5). Thus,



BMS-191095 does not increase vascular cytosolic or mitochondrial levels of ROS in any of the cell types that we have examined and dilation to BMS-191095 is not affected by ROS scavengers; nonetheless, calcium spark activity increases with BMS-191095 application. The reasons for these differences in findings are unclear but taken together underscore the importance and robustness of mitochondrial mechanisms in promoting relaxation of VSM. We speculate that there is direct electrophysiological coupling between mitochondria and SR in VSM and/or that microdomains involving the outer mitochondrial and SR membranes link functional interactions between these two organelles. The close physical association and thus the possibility of direct coupling of mitochondria and SR are clearly seen with electron microscopy in cerebral VSM (Figure 2).

## Endothelium

Mitochondrial content in the cerebral vascular endothelium is higher than in endothelium in other peripheral circulations, probably due to the transport requirements of the BBB [107]. We investigated the contribution of mitochondrial factors arising within the endothelium on the integrated response of intact cerebral arteries using several approaches. First, removal of the endothelium altered the vasodilation to diazoxide and BMS-191095, implying that traditional endothelium-derived factors such as NO and prostaglandins contribute to changes in vascular tone [71]. However, the endothelial effects are complicated. In intact blood vessels, inhibition of NOS with L-NAME administration reduced relaxation to diazoxide, indicating a dilator role of NO. In contrast, inhibition of cyclooxygenase (COX) with indomethacin enhanced dilation in diazoxide treated arteries, implying a role for constrictor prostanoids. Indomethacin did not affect vasodilation in endothelium-denuded arteries. Similar results were obtained with BMS-191095 with respect to L-NAME administration [74]. However, unlike diazoxide, indomethacin administration did not enhance dilation to BMS-191095, suggesting that a ROS-mediated mechanism leading to COX activation may account for these differences. Fluorescence and electron spin resonance measurements of NO in intact arteries or cultured cerebral microvascular endothelial cells confirmed the production of NO in response to diazoxide and BMS-191095. In addition, fluorescence measurements showed that a global increase in free cytosolic calcium rather than calcium sparks was temporally associated with increased NO production. We have demonstrated that NO arises from cytosolic rather than mitochondrial sites during normal conditions [92-94]. Thus, BMS-191095 and diazoxide application led to mitochondrial depolarization, activation of eNOS by calcium- and phosphorylation-specific mechanisms, production of NO, and relaxation of VSM (Figure 5). Gutterman and colleagues [143] showed that a physiological stimulus such as increased shear stress is able to directly activate mitochondria in coronary endothelial cells via filamentous connections and dilate coronary arteries via effects of hydrogen peroxide. Whether this endothelium-specific mechanism operates in response to increases in flow or shear stress in cerebral arteries is unclear, since alternative mechanisms promoting either dilation or constriction in cerebral resistance vessels of different sizes and locations have been proposed by Koller and Toth [86]. Mitochondrial depolarization by the protonophore, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), in endothelial tubes from mesenteric arteries, has also been shown to activate calcium-activated  $K^+$  channels presumably by elevation of endothelial  $Ca^{2+}$  [7]. Even though precise mechanisms may be different in cerebral and coronary arteries, the common

feature is that stimulation of mitochondria is able to liberate substances by endothelium which can affect VSM tone.

Mitochondrial numbers and composition in the cerebral vascular endothelium are affected by factors such as ischemic stress (unpublished observations) and hormones such as estrogen [79,123]. Administration of 17beta-estradiol profoundly affects mitochondrial function in cerebral blood vessels via estrogen receptor alpha, thereby enhancing efficiency of energy production and suppressing mitochondrial oxidative stress. These results indicate that ovarian hormones normally act through a distinctive regulatory pathway involving peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 to support cerebral endothelial mitochondrial content and guide mitochondrial function to favor ATP coupling and ROS protection. An interesting observation is that female mice with MnSOD deficiency are largely protected compared with male mice against cerebral vascular dysfunction [21]. The basis for this gender difference is unclear at this time but may reflect yet unknown compensatory mechanisms in the vascular mitochondria of female mice.

### Perivascular and parenchymal nerves

The potential for mitochondrial influences from perivascular nerves or parenchymal neurons on cerebral vascular responses has never been specifically investigated. However, there are a number of factors suggesting an important neuronal contribution from both sources. Mitochondrial density is relatively high in perivascular nerves observed under confocal imaging (unpublished observations) and as seen in our preconditioning studies; mitochondria in neurons are responsive to both diazoxide and BMS-191095 [47]. Cerebral arteries receive innervation from sympathetic, parasympathetic, and nitroxidergic nerves [11,25,131]. These speculations are supported by our preliminary studies. Specific inhibition of neuronal NOS with 7-NI in perivascular nerves, or blockade of perivascular nerves with tetrodotoxin (TTX), inhibits dilation in both intact and denuded cerebral arteries to BMS-191095 [81]. The effects of TTX may be due to interactions between different types of perivascular nerves or between astroglia and perivascular nerves. Electrical activation of perivascular nerves, which might mimic superfusion with diazoxide and BMS-191095, elicits dilation of cerebral arteries which is blocked by TTX [3]. Additionally, BMS-191095 depolarizes mitochondria in cultured cortical neurons and enhances NO production by these cells. Thus, in addition to the endothelium, perivascular nerves, parenchymal neurons, and possibly astroglia as well [67,122], can provide mitochondrially-initiated vasoactive signals to VSM [82-84] for the final determination of integrated changes in cerebral vascular diameter (Figure 6).

## MITOCHONDRIA IN INSULIN RESISTANCE

Insulin resistance often precedes the development of type II diabetes by years or decades, and is considered a relatively silent phase of the metabolic syndrome despite developing vascular dysfunction and moderate arterial hypertension. We and others have characterized many of the general cardiovascular and brain effects of this disease [39-41,60] due to tissue and vascular inflammation and increased basal levels of ROS from both mitochondrial and non-mitochondrial sources [41,42].

We were among the first investigators to demonstrate that several pathological stressors are able to disrupt normal functioning of plasmalemmal  $K_{ATP}$  channels in cerebral arteries [4,39,40] and therefore it is not surprising that mito $K_{ATP}$  channels would be affected in a similar manner. For example, ischemia/reperfusion as well as nutritional and genetic models of IR reduced function of several types of potassium channels in cerebral VSM, including  $K_{ATP}$  channels, through mechanisms involving enhanced baseline vascular levels of ROS [39,40]. However, effects of ischemia on  $K_{ATP}$  channels are transient, so that normal responses return by 4 hours of reperfusion. Thus, ROS scavengers [40], PKC inhibition [39], or anti-inflammation therapies such as statin administration [41] are able to restore normal dilation to potassium channel activators despite continued IR. Even though enhanced ROS from mitochondria is associated with IR, reductions in ROS production from NADPH oxidase results in restored  $K_{ATP}$  channel function in cerebral arteries. We speculate that ROS induced ROS production, involving the mitochondrial electron transport chain and NADPH oxidase, might be necessary for the full dysfunction of  $K_{ATP}$  channels in IR. Similarly, a more severe, acute event, namely ischemia/reperfusion is able to reduce  $K_{ATP}$  channel-dependent dilation in cerebral arteries [4]. Additionally, we have shown that immediate preconditioning which is dependent upon activation of mito $K_{ATP}$  channels is abolished in hearts of IR rats [70]. Thus, ischemic- as well as diazoxide-induced preconditioning fails to protect hearts from ischemia/reperfusion in IR rats whereas both of these approaches limited infarct size in hearts from non-IR rats. In these IR animals, prior to ischemia/reperfusion, a substantial number of the mitochondria in the heart were swollen or showed disruption of the normal pattern for cristae. Moreover, the isolated mitochondria from the hearts of IR animals had reduced responses to diazoxide. An additional finding from this study was that infarct size was enhanced in the hearts from Zucker obese compared with lean rats, which is similar to that which we [106] and others [10] have found in brains of IR, obese mice following MCAO. These results may indicate that normal protective mechanisms initiated at the level of the mitochondria are impaired in many common disease states and thus the brain and other organs are more at risk during ischemic episodes.

Similar to preconditioning, mitochondrial-dependent responses in cerebral arteries are impaired in IR rats [71]. This attenuation of dilation appears to be due to reduced mitochondrial depolarization of VSM as well as reduced ROS generation in response to diazoxide. In addition, decreased NO production/bioavailability, despite increased endothelial NOS expression in IR arteries, appears to contribute to diminished relaxation of VSM. We have found similar impairment of cerebral vascular dilation to BMS-191095 in IR animals [74]. An unexpected result was that, in contrast to mitochondrial activation with diazoxide or BMS-19095, dilator responses to 3-NPA were modestly enhanced in arteries from IR rats [71]. Additionally, co-application of 3-NPA with diazoxide led to more than just an additive dilator effect which was not attenuated in arteries from IR animals. The reason for this effect is unclear, but it seems to indicate that IR interferes with some, but not all vasoactive influences from mitochondria. The potential for the uncoupling of the tight relationship between metabolic need and blood flow in the brain due to cerebral vascular dysfunction associated with IR, as well as the elimination of normal, protective mechanisms involving mitochondria such as preconditioning, may account for the increased risk and

severity of neurological diseases and strokes in patients suffering from the metabolic syndrome. Similar to our findings with IR, anti-ROS treatment restores morphological structure in coronary endothelial cells in a mouse model of type 1 diabetes [102]. In addition, Kizhakekuttu et al. [84] provided evidence that mitochondrial derangements lead to reduced endothelium-induced dilation in human arteries. A possible therapeutic approach might involve the early detection and treatment of IR individuals with the aim toward reducing vascular inflammation and mitochondrial dysfunction prior to the further progression of this chronic disease. We have shown that even very short term treatments with a statin, which reduced vascular ROS levels and restored normal  $K_{ATP}$  channel function without lowering blood cholesterol levels, was effective in IR rats [41].

## MITOCHONDRIA AND CEREBRAL ISCHEMIA

Although mitochondrial failure is a major cause of cell death due to ischemia, our recent findings indicate that mitochondria in endothelium may represent a useful target to prevent cell death and restore cerebral blood flow after stroke. Preconditioning before ischemia preserves endothelium-dependent dilation [32] and reduces permeability of the BBB *in vivo* [96], and post conditioning protects endothelium *in vitro* against prior OGD (Figure 3). In recent preliminary studies, we examined the effects of experimental stroke on middle cerebral artery responses two days following reperfusion, and found that dilation was greater on the side ipsilateral to the middle cerebral artery occlusion compared with the contralateral, non-stroke side (Figure 7). At this time, dilator and constrictor responses to other, non-mitochondrial stimuli were reduced in middle cerebral arteries on the stroke side. Thus, not only are endothelial cells responsive to mitochondrial-induced postconditioning after anoxic stress, but vascular responsiveness to mitochondrial activators is also enhanced [134]. Therefore, because strokes occur unpredictably, therapeutic targeting of mitochondrial *after* the onset of ischemic stroke may be a clinically useful approach. Cerebral ischemia, resultant cerebral vascular dysfunction, and parenchymal damage occur unexpectedly following occlusive and hemorrhagic strokes as well as from severe hypotension due to heart attacks and trauma. The only proven therapeutic approach for occlusive strokes is surgical or enzymatic removal of the clot from a cerebral artery, but even after clot removal, patients often suffer from cerebral hypoperfusion, damage to the BBB, and continued parenchymal damage. We believe that activation of mitochondrial mechanisms *after* the insult may benefit the brain by protecting cerebral vascular cells by preventing further injury to the endothelium and VSM, correcting cerebral hypoperfusion, restoring normal cerebral vascular responsiveness and the BBB, and restricting further neuronal and glia cell death.

## CONCLUSIONS, SIGNIFICANCE, AND PERSPECTIVE

We can draw four major conclusions. *First*, mitochondrial influences are important initiators of preconditioning and postconditioning in the most vulnerable cell types within the neurovascular unit. Thus, targeting mitochondria may be a useful therapeutic approach to protect the cerebral vasculature and brain of people prior to elective surgeries and also following the onset of strokes. *Second*, individual mitochondrial influences from endothelium, perivascular nerves, and perhaps parenchymal neurons and astroglia are able to

contribute to the direct mitochondrial-initiated dilator effects of VSM into a final, integrated change in cerebral vascular tone. Thus, production of vasoactive factors following activation of mitochondria in response to physiological stimuli in one or several of the cells comprising the neurovascular unit may represent the elusive signaling link between metabolic rate and blood flow. *Third*, important mitochondrial-derived mechanisms such as those that induce preconditioning or cause changes in cerebral vascular tone are disrupted by chronic disease processes such as IR and diabetes, even when relatively mild. Thus, direct or indirect targeting of mitochondria in the early stages of the metabolic syndrome might prove to be an effective treatment to reduce morbidity and mortality in these patients related to the increased risk of cardiovascular disease, strokes, and dementias such as Alzheimer's disease. *Fourth*, preliminary evidence indicates that mitochondrial mechanisms promoting vasodilation are usually present to some extent following experimental strokes. Thus, targeting mitochondria following a patient's ischemic injury may help correct cerebral hypoperfusion and restore the BBB, and thereby restrict further neuronal and glia cell death.

The study of mitochondrial influences on the vasculature is a rapidly evolving field of investigation; however, there is much research to do in order to understand the intricacies of mitochondrial biology and the mitochondrial mechanisms involved in cerebral vascular control during health and disease. Nonetheless, it seems likely that the manipulation of mitochondrial numbers or function will represent an important therapeutic approach for the future treatment of many chronic and acute disease states in people.

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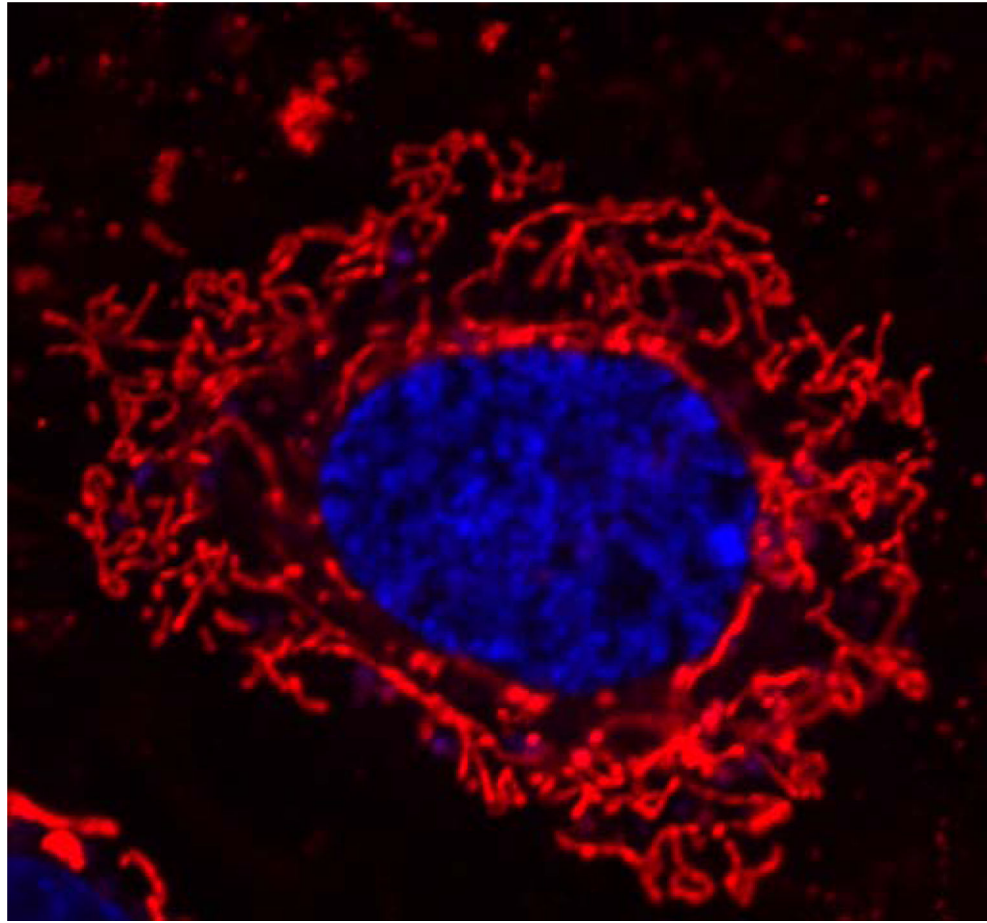
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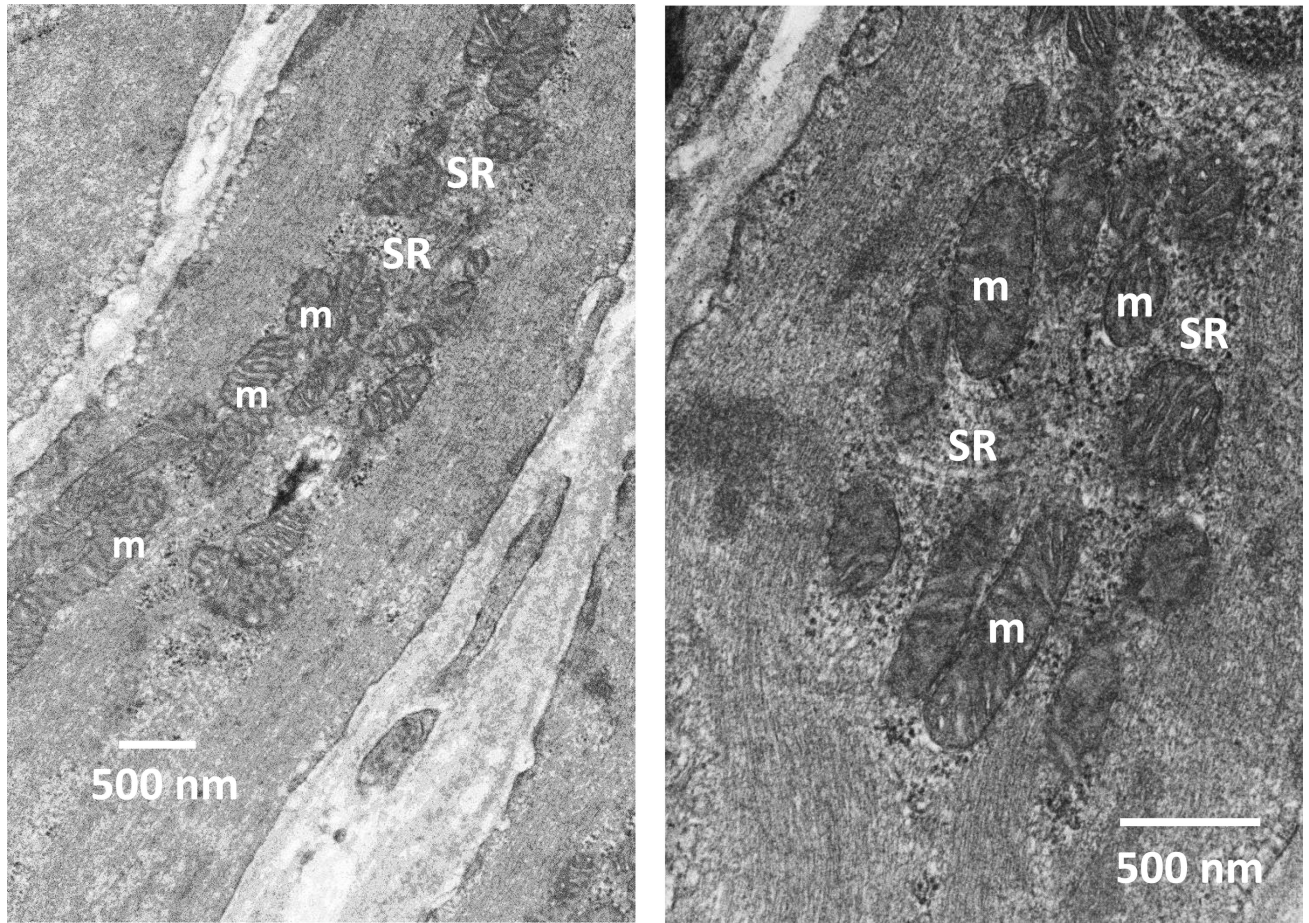
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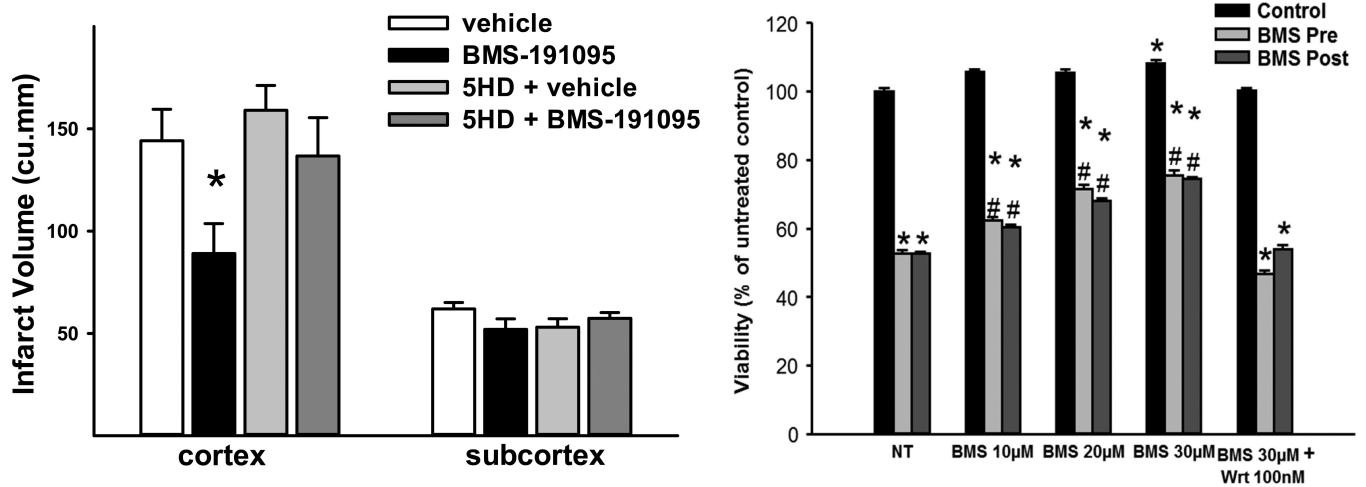


**Figure 1.** Mitochondrial morphology within a cerebral vascular endothelial cell. The mitochondria (red) and the nuclei (blue) of live rat brain microvascular endothelial cells were stained using mitochondrial stain (1:10000;  $I_{ex}$ : 514 nm and  $I_{em}$ : 538-681 nm) and Hoechst nuclear stain (1: 1000;  $I_{ex}$ : 405 nm and  $I_{em}$ : 410-585 nm), respectively (Chromeo™ Live Cell Mitochondrial Staining kit, Active Motif, Carlsbad, California). Fluorescence images were obtained using LSM 710 AxioObserver (Carl Zeiss, Hamburg, Germany) with plan-Apochromat 63×/Numerical Aperture 1.4 oil immersion objective.



**Figure 2.**

Lower and higher magnifications of VSM cells from cerebral arteries from normal rats showing clustering of mitochondria (m) and sarcoplasmic reticulum (SR) in distinct locations. Arteries were fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide and embedded in Epon812. Ultrathin sections (80-90 nm) were mounted on formvar-coated nickel grids (200 mesh), air dried, and stained with 4.7% uranyl acetate and lead citrate (at 10 min and 2 min, respectively). The sections were put on grids and viewed using a FEI Tecnai BioTwin 120 keV TEM with a digital imaging setup (Wake Forest University Health Sciences, Winston-Salem, NC).



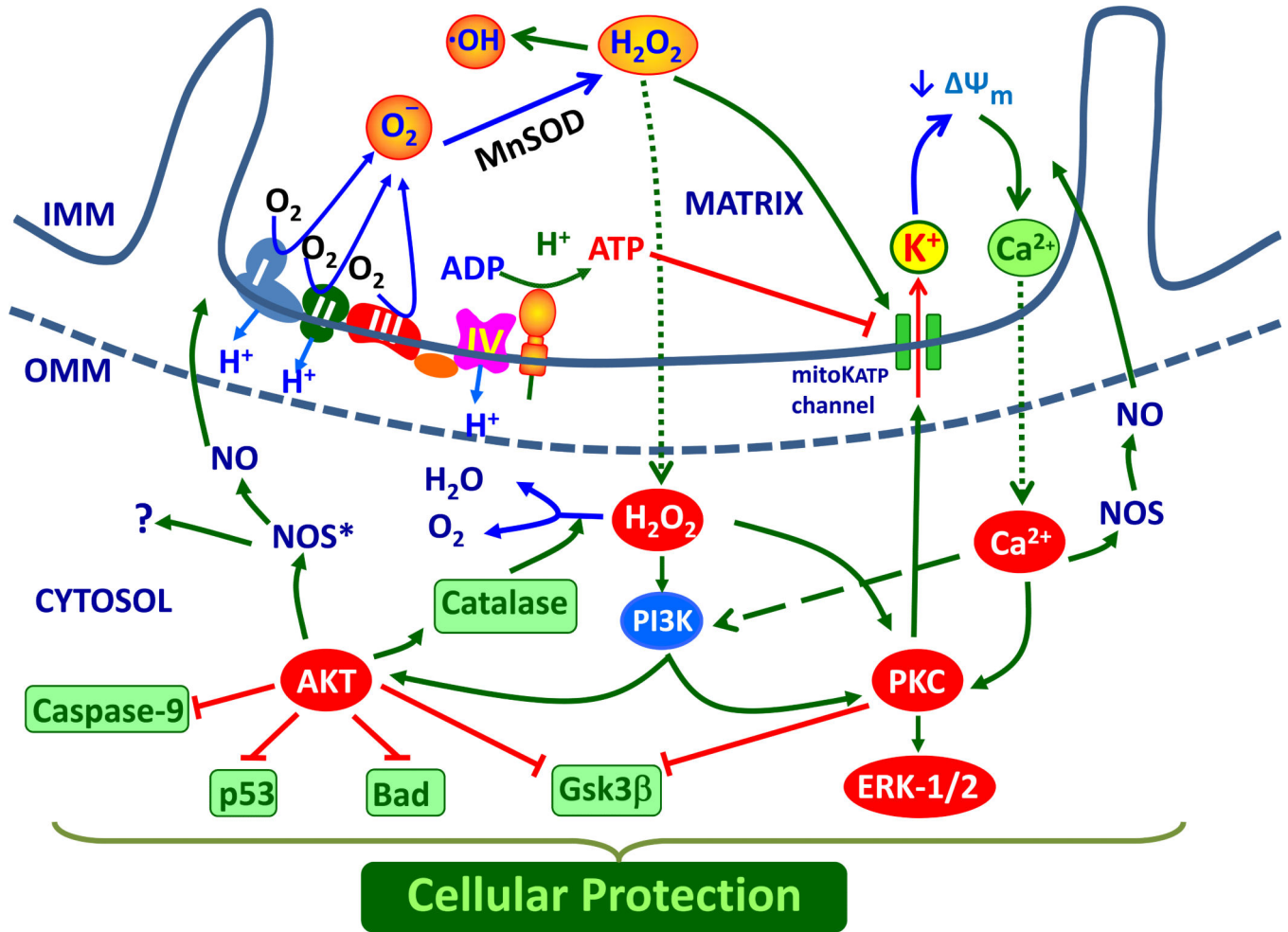
**Figure 3.** Examples of preconditioning and postconditioning with mitoK<sub>ATP</sub> channel opener BMS-191095. **Left.** BMS-191095 protected the rat brain *in vivo* against 90 min of middle cerebral artery occlusion when given before ischemia [128]. **Right.** Original data showing that BMS-191095, when given prior to or following OGD, improved survival of cultured rat cerebral endothelial cells. Inhibition of PI3Kinase with wortmannin (Wrt) counteracted beneficial effects of BMS-191095 (unpublished observations). \*P<0.05.

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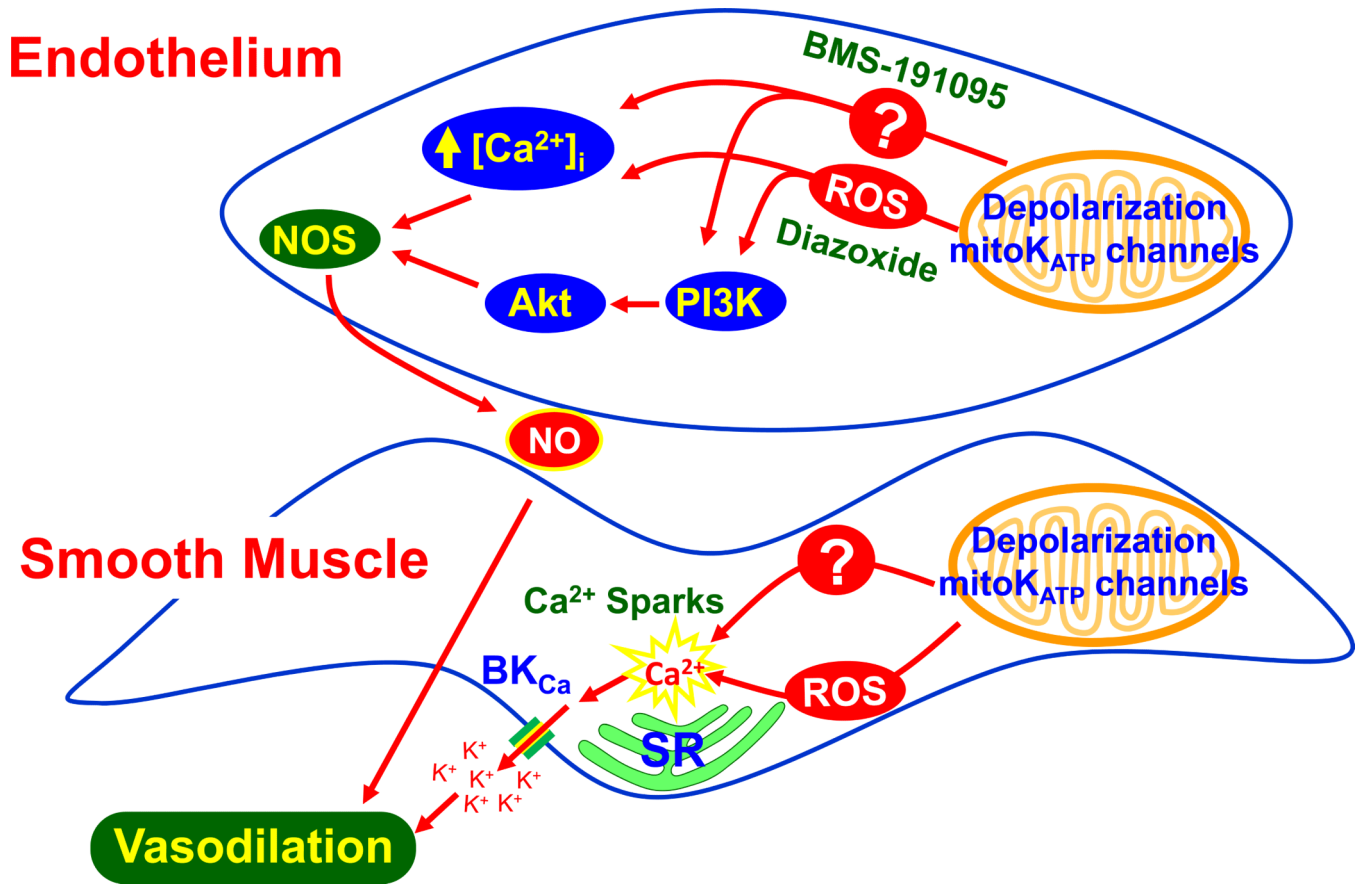
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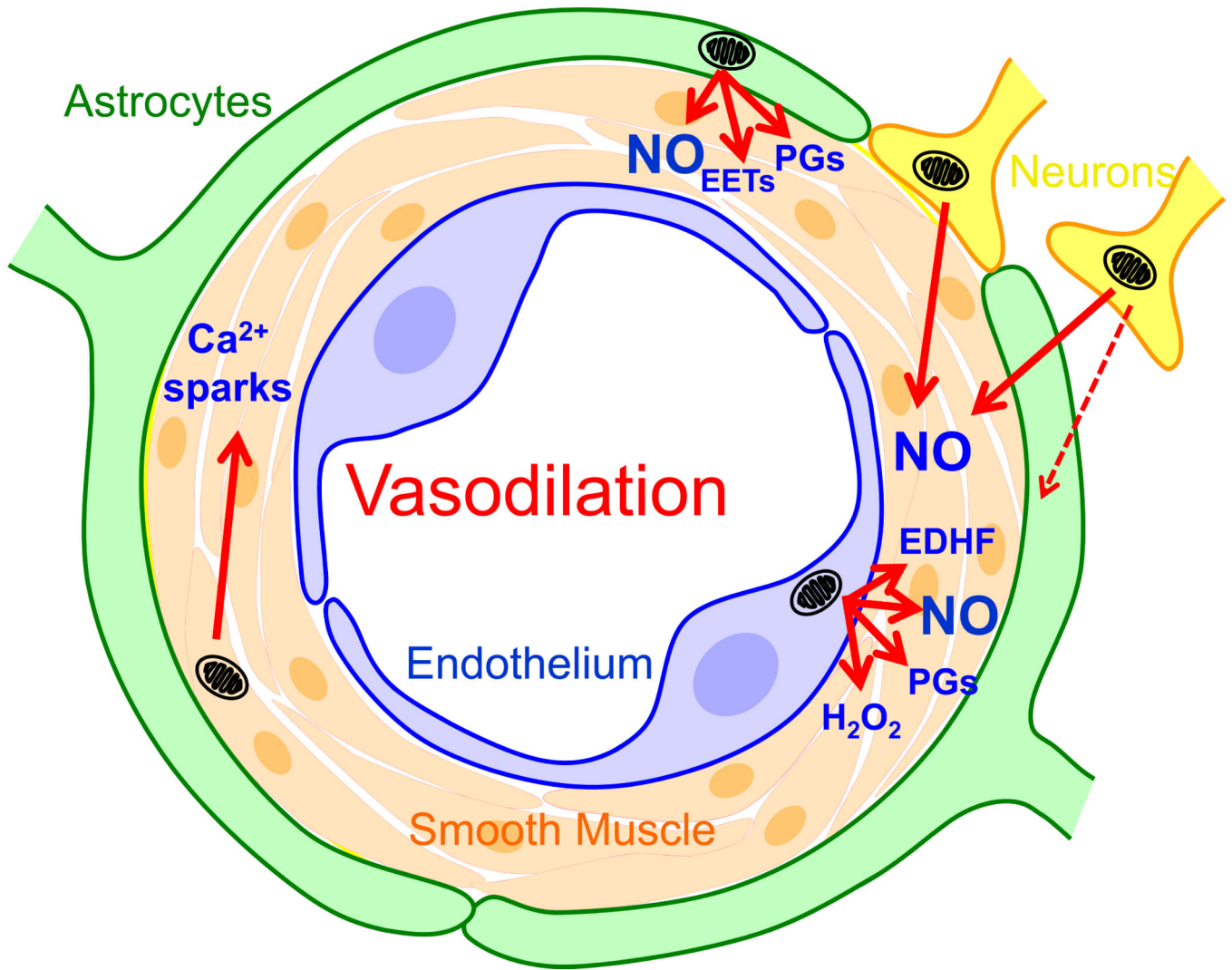


**Figure 4.** Schematic illustration showing signaling events (red circles) and ultimate mechanisms (green boxes) resulting in cellular protection following opening of mitoK<sub>ATP</sub> channels or liberation of ROS from the protein complexes which form the electron transport chain. Abbreviations: OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane;  $\Psi_m$ , mitochondrial membrane potential; O<sub>2</sub><sup>-</sup>, superoxide anion; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; Ca<sup>2+</sup>, calcium; ADP, adenosine di-phosphate; ATP, adenosine tri-phosphate; PKC, protein kinase C; Gsk3β, phosphoglycogen synthase kinase 3 beta; PI3K, phosphoinositide 3-kinase; Bad, Bcl-2 associated death promoter, Akt; protein kinase B, eNOS\*; phosphorylated endothelial NOS.

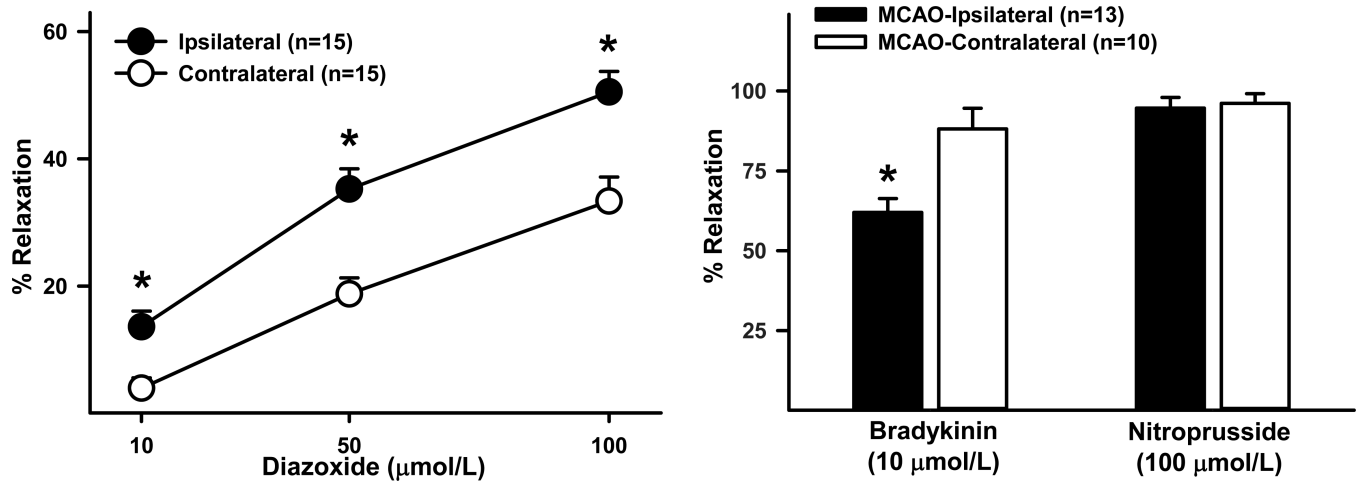




**Figure 5.** Schematic illustration showing signaling events (red circles) and ultimate mechanisms (green boxes) resulting in changes in vascular tone following opening of mitoK<sub>ATP</sub> channels or liberation of ROS from the protein complexes which form the electron transport chain. Abbreviations:  $\Psi_m$ , mitochondrial membrane potential;  $O_2^-$ , superoxide anion;  $H_2O_2$ , hydrogen peroxide;  $Ca^{2+}$ , calcium; ADP, adenosine di-phosphate; ATP, adenosine tri-phosphate; PKC, protein kinase C; Gsk3 $\beta$ , phospho-glycogen synthase kinase 3 beta; PI3K, phosphoinositide 3-kinase; Bad, Bcl-2 associated death promoter, Akt; protein kinase B.



**Figure 6.** Schematic illustration showing interaction among mitochondrial influences originating from VSM, endothelium, perivascular nerves, and parenchymal astroglia and neurons. Factors produced following activation of mitochondria by physiological, pharmacological, and pathological stimuli in any of these cell types can affect VSM and the interaction of these factors will determine the final, integrated arterial tone. Abbreviations: NO, nitric oxide; PGs, prostaglandins; EDHF, endothelial derived relaxing factor; EETs, epoxyeicosatrienoic acids, K<sup>+</sup>, potassium ion.



**Figure 7.**

Original data from Wistar rats showing that dilator responses of isolated, pressurized middle cerebral arteries to diazoxide are substantially enhanced compared to the non-ischemic, contralateral artery two days following 90 minutes of middle cerebral artery occlusion via the filament method. At the same time dilator responses to other endothelial-dependent responses such as bradykinin were reduced. \* $P < 0.05$ .