Depolarization of Mitochondria in Endothelial Cells Promotes Cerebral Artery Vasodilation by Activation of Nitric Oxide Synthase

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Objective—Mitochondrial depolarization after ATP-sensitive potassium channel activation has been shown to induce cerebral vasodilation by the generation of calcium sparks in smooth muscle. It is unclear, however, whether mitochondrial depolarization in endothelial cells is capable of promoting vasodilation by releasing vasoactive factors. Therefore, we studied the effect of endothelial mitochondrial depolarization by mitochondrial ATP-sensitive potassium channel activators, BMS-191095 (BMS) and diazoxide, on endothelium-dependent vasodilation.

Approach and Results—Diameter studies in isolated rat cerebral arteries showed BMS- and diazoxide-induced vasodilations that were diminished by endothelial denudation. Mitochondrial depolarization-induced vasodilation was reduced by inhibition of mitochondrial ATP-sensitive potassium channels, phosphoinositide-3 kinase, or nitric oxide synthase. Scavenging of reactive oxygen species, however, diminished vasodilation induced by diazoxide, but not by BMS. Fluorescence studies in cultured rat brain microvascular endothelial cells showed that BMS elicited mitochondrial depolarization and enhanced nitric oxide production; diazoxide exhibited largely similar effects, but unlike BMS, increased mitochondrial reactive oxygen species production. Measurements of intracellular calcium ($[Ca^{2+}]_i$) in cultured rat brain microvascular endothelial cells and arteries showed that both diazoxide and BMS increased endothelial $[Ca^{2+}]_i$. Western blot analyses revealed increased phosphorylation of protein kinase B and endothelial nitric oxide synthase (eNOS) by BMS and diazoxide. Increased phosphorylation of eNOS by diazoxide was abolished by phosphoinositide-3 kinase inhibition. Electron spin resonance spectroscopy confirmed vascular nitric oxide generation in response to diazoxide and BMS.

Conclusions—Pharmacological depolarization of endothelial mitochondria promotes activation of eNOS by dual pathways involving increased $[Ca^{2+}]_i$ as well as by phosphoinositide-3 kinase-protein kinase B–induced eNOS phosphorylation. Both mitochondrial reactive oxygen species–dependent and –independent mechanisms mediate activation of eNOS by endothelial mitochondrial depolarization. **(*Arterioscler Thromb Vasc Biol*. 2013;33:752-759.)**

Key Words: BMS-191095 ■ diazoxide ■ intracellular calcium ■ membrane potential ■ mitochondrial ATP-sensitive potassium channels ■ superoxide

Mitochondria appear to play an important role in the regulation of cerebral vascular tone. Recent, limited evidence shows that mitochondria-derived factors promote relaxation of intact or endothelium-denuded cerebral arteries or isolated vascular smooth muscle (VSM) cells.[1–4] Mitochondrial depolarization in VSM with diazoxide, an activator of mitochondrial ATP-sensitive potassium (mito-K_{ATP}) channels, promotes the generation of reactive oxygen species (ROS) from mitochondria, which sequentially causes the activation of ryanodine-sensitive Ca^{2+} channels on sarcoplasmic reticulum, generation of calcium transients, otherwise known as calcium sparks, and the opening of adjacent large-conductance calcium-activated potassium channels (BK_{Ca}) on the plasma membrane. The efflux of K^+ thus leads to hyperpolarization, decreased global intracellular Ca^{2+} of VSM, and vasodilation.[1,2] However, the contribution of endothelium to the integrated cerebral arterial response to mitochondrial depolarization as well as to other potential mechanisms has never been systematically investigated.

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Our laboratory has provided physical and pharmacological evidence that selective mitochondrial depolarizing agents promote endothelium-dependent vasodilation/vasoconstriction by inducing the release of factors such as nitric oxide and constrictor prostaglandins, which modulate VSM effects in cerebral arteries.[3] However, the precise mechanisms by which endothelial mitochondrial depolarization promotes

Received on: May 21, 2012; final version accepted on: December 17, 2012.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.112.300560/-/DC1.

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endothelium-dependent vasodilation cannot be elucidated from previous studies. The ability of endothelial mitochondrial depolarization to affect cerebrovascular tone is a novel and potentially important area of investigation, as many physiological, pharmacological, pathological, and physical factors impacting endothelium has been shown in other regional circulations,[5–11] but not yet in the cerebral circulation.

Two relatively selective openers of mitoK$_{ATP}$ channels, diazoxide and BMS-191095 (BMS), are available, and we have studied their properties extensively in isolated mitochondria, cultured neurons, and astrocytes.[12–16] Although both depolarize mitochondria, diazoxide, but not BMS, causes increased production of ROS from mitochondria presumably by inhibiting succinate dehydrogenase.[17] We[12,13,16] and others[18–21] are unaware of any effects of BMS other than as an opener of mitoK$_{ATP}$ channels. No previous studies have examined the effects of BMS on cerebral arterial tone, and the endothelium-specific effects of diazoxide have not been specifically studied. In this study, we examined the effect of mitochondrial depolarization in endothelium on integrated cerebral vascular tone and explored the mechanisms involved. Our results have allowed us to document the key elements of signaling steps that link mitochondrial depolarization with the generation of nitric oxide (NO) by cerebral vascular endothelium, including activation of endothelial nitric oxide synthase (eNOS) by dual pathways involving increased $[Ca^{2+}]_i$ as well as by phoshpoinositide 3-kinase (PI3K)-protein kinase B (Akt)–induced phosphorylation.

Results

Intraluminal Diameter Measurements

The resting diameters of the cerebral arteries were similar for each group of experiments (169±4, n=91, in endothelium-intact and 163±4, n=19, in endothelium-denuded; *P*=NS), and they were preconstricted to a similar degree (52±1, n=34, in endothelium-intact and 51±2, n=14, in endothelium-denuded; *P*=NS). The BMS elicited a dose-dependent vasodilation in cerebral arteries with 16.3±1.4%, 49.7±4.7%, and 62.6±4.4% relaxation in response to 10, 50, and 100 μmol/L, respectively (n=6–12). Similarly, diazoxide elicited a dose-dependent vasodilation with 8.9±1.2%, 21.9±2.9%, and 39.5±2.8% relaxation in response to 10, 50, and 100 μmol/L, respectively (n=10; Figure 1). Denudation of endothelium diminished vasodilation to BMS with 5.9±0.9%, 31.6±2.1%, and 38.1±3.8% relaxation in response to 10, 50, and 100 μmol/L, respectively (n=10; *P*<0.05). Similarly, denudation of endothelium diminished vasodilation to diazoxide with 4.8±1.3%, 11.7±3.5%, and 28.5±2.3% relaxation in response to 10, 50, and 100 μmol/L, respectively (n=9; *P*<0.05; Figure 1). Thus, endothelium contributes to vasodilation in response to both BMS and diazoxide.

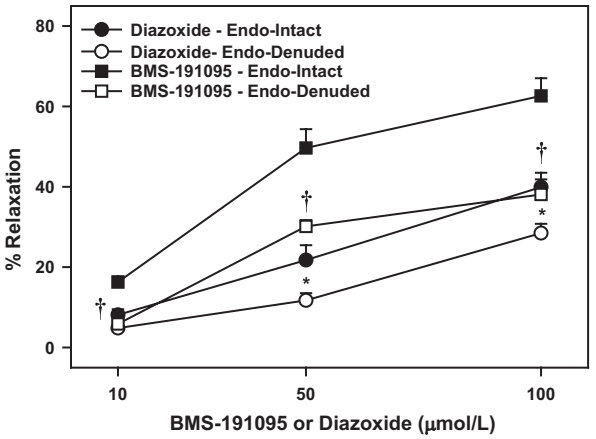

Figure 1. Responses to BMS-191095 (BMS) and diazoxide (10, 50, and 100 μmol/L) in endothelium-intact and -denuded cerebral arteries from Sprague Dawley (SD) rats after development of myogenic tone are shown. Data are mean±SEM of 6 to 14 experiments. * and † indicate significant difference in corresponding response in endothelium-intact arteries with respect to BMS and diazoxide, respectively (*P*<0.05).

Scavenging of the ROS with MnTBAP did not affect vasodilation to BMS (51.1±4.3, n=5; *P*=NS) confirming that BMS-induced vasodilation was independent of ROS generation (Figure 2A). In contrast, MnTBAP diminished vasodilation to diazoxide (8.6±1.3, n=5; *P*<0.05) confirming that diazoxide-induced vasodilation was ROS-dependent (Figure 2B). Inhibition of mitoK$_{ATP}$ channels alone with 5-hydroxydeconoic acid (11.7±2%, n=14; *P*<0.05) and fluoxetine (19.5±4%, n=5; *P*<0.05), or both mitochondrial and plasma membrane K$_{ATP}$ with glibenclamide (22.3±4%, n=6; *P*<0.05) decreased vasodilation to BMS in endothelium-intact arteries (Figure 2A). Wortmannin pretreatment

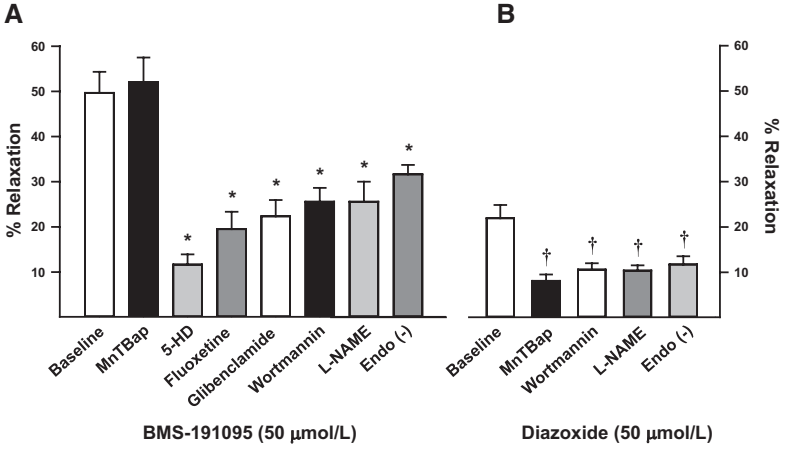

Figure 2. Vascular responses to 50 μmol/L BMS-191095 (**A**) and diazoxide (**B**) in endothelium-intact rat cerebral arteries in the presence and absence of 100 μmol/L manganese(III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), 1 mmol/L 5-hydroxydeconoic acid (5-HD), 5 μmol/L fluoxetine, 10 μmol/L glibenclamide, 100 nmol/L wortmannin, and 100 μmol/L N$^{\omega}$-nitro L-arginine methyl ester (L-NAME) are shown as bar graphs. In addition, vascular responses to 50 μmol/L BMS-191095 and diazoxide in endothelium-denuded rat cerebral arteries are also displayed in the bar graphs. Data are mean±SEM of 4 to 14 experiments. * and † indicate significant difference in corresponding response in endothelium-intact arteries with respect to BMS-191095 and diazoxide respectively (*P*<0.05).

reduced the vasodilation to 50 µmol/L BMS (25.6±2.4%, n=5; $P<0.05$) and 50 µmol/L diazoxide (10.6±1.4%, n=5; $P<0.05$), implicating PI3K in the vasodilation. Finally, inhibition of NOS with L-NAME resulted in diminished vasodilation to 50 µmol/L BMS (25.6±4%, n=8; $P<0.05$) and 50 µmol/L diazoxide (10.4±1.2%, n=6; $P<0.05$), suggesting that the major part of the vasodilation to BMS and diazoxide was mediated by NO (Figure 2A and 2B).

Arteries took ≈20 to 30 minutes to attain a stable diameter both before and after the administration of pharmacological agents. In general, addition of pharmacological blockers had minimal effects on the diameter of preconstricted arteries. When compared with vehicle-treated arteries (0.5±0.4%, n=27), pretreatment with L-NAME (8.9±1.2%, n=14; $P<0.05$) caused slight constriction of arteries, whereas pretreatment with wortmannin (8.2±5%, n=5; $P<0.05$) caused only slight vasodilation. However, pretreatment with MnTBAP (0.45±0.7%, n=10), 5-hydroxydeconoic acid (0.6±1%, n=14), fluoxetine (–0.7±4%, n=5), and glibenclamide (–0.1±1%, n=6) had no significant effect on vascular diameter.

Mitochondrial Membrane Potential, [Ca2+]i, NO, and Mitochondrial ROS Measurements

The BMS and diazoxide depolarized the mitochondria of CMVECs indicated by reduction of rhodamine 123 fluorescence compared with vehicle-treated cells (Figure 3A and 3E). Background subtracted rhodamine 123 fluorescence

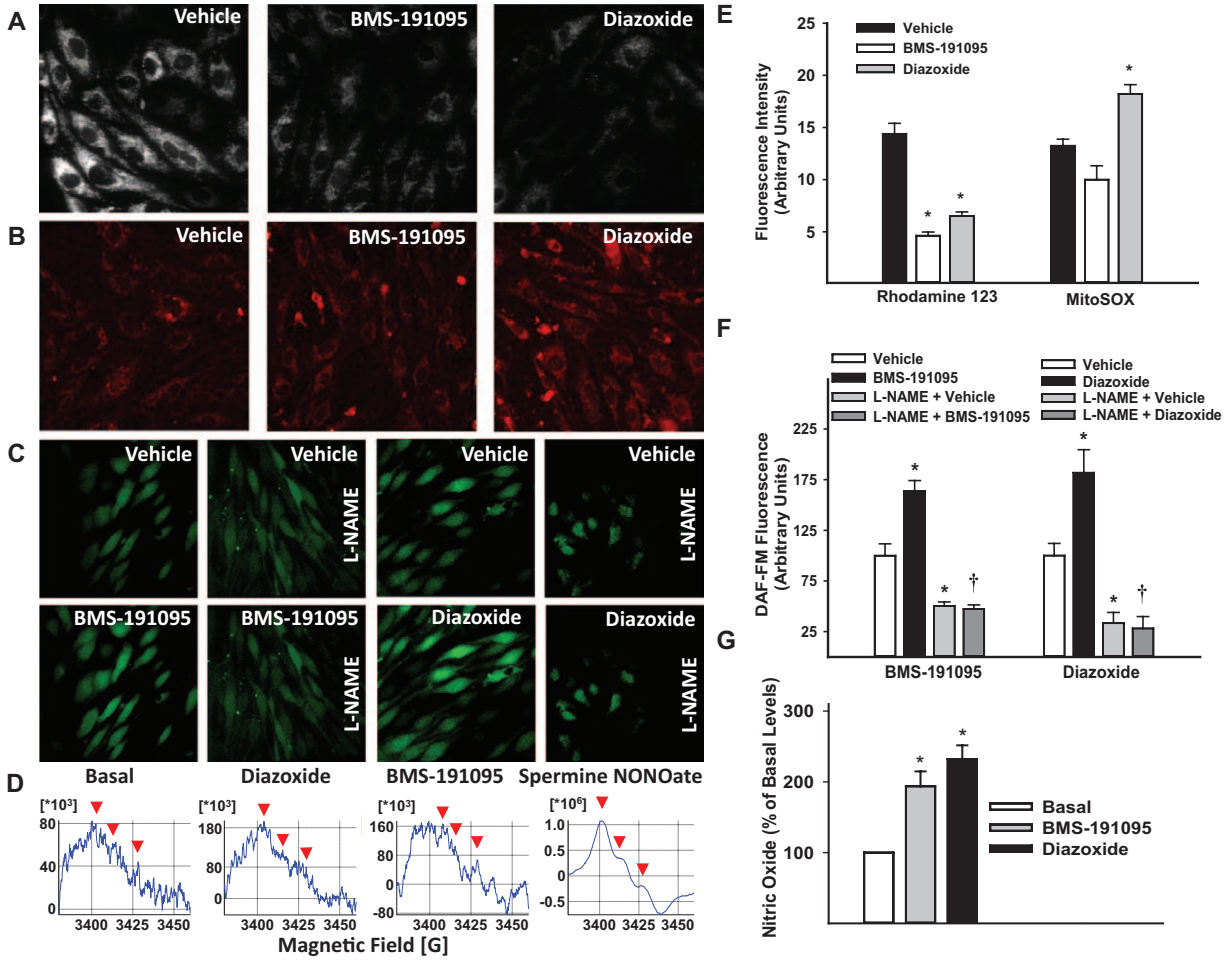

Figure 3. Fluorescence images of cultured primary rat brain microvascular endothelial cells (CMVECs) loaded with various fluorescent probes treated with vehicle (DMSO) or 50 µmol/L BMS-191095 (BMS) or 100 µmol/L diazoxide are shown. Fluorescence images of CMVECs loaded with rhodamine 123, a mitochondrial membrane potential marker, are shown in **A**. Mitochondrial depolarization is marked by the decrease in fluorescence. Fluorescence images of MitoSOX, a mitochondrial ROS-sensitive dye, are shown in **B**. **C**, Images of CMVECs loaded with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM), a nitric oxide (NO)-sensitive dye, and treated with or without N^{ω}-nitro L-arginine methyl ester (L-NAME). Electron spin resonance (ESR) spectra of rat aortas incubated for 90 minutes at 37°C with 0.4 mmol/L colloid iron diethyldithiocarbamate ,$Fe^{2+}(DETC)_2$, are shown in **D**. The vertical axis represents signal intensity in arbitrary units, and the horizontal axis represents the magnetic field (**G**). A characteristic NO–$Fe(DETC)_2$ signal with 3 peaks (indicated by **arrows**) was detected in rat aortas incubated with $Fe(DETC)_2$ and various drugs. The representative ESR spectra of the basal (vehicle-treated), 100 µmol/L diazoxide, 50 µmol/L BMS, and 10 µmol/L spermine NONOate (10 µmol/L, N-[2-Aminoethyl]-N-[2-hydroxy-2-nitrosohydrazino]-1,2-ethylenediamine)-treated aortas are shown along with the cumulative data expressed as percentage change from basal level as a bar graph (**G**). The cumulative data of fluorescence intensity from the images were determined and plotted as a bar graph for rhodamine 123, MitoSOX, and DAF-FM (**E** and **F**). In **E**, *significant difference in response vs vehicle for corresponding fluorescent probes ($P<0.05$). In **F**, * and † indicate significant difference in response vs vehicle and untreated BMS or diazoxide, respectively ($P<0.05$). In **G**, * indicates significant difference in response vs basal level of normalized NO–$Fe(DETC)_2$ signal ($P<0.05$).

(arbitrary units) was reduced to 4.6±0.3 by BMS (n=6) and 6.5±0.4 by diazoxide (n=8) treatment versus 14.4±1, in vehicle-treated (n=6) cells. Measurements of MitoSOX fluorescence (arbitrary units) showed that BMS-treated CMVECs did not display increased fluorescence (10±1.3, n=5; *P*=NS) compared with the vehicle (13.3±0.6, n=8), indicating ROS production from mitochondria was unchanged (Figure 3B and 3E). In contrast, diazoxide treatment enhanced the fluorescence (18.2±0.9, n=7; *P*<0.05) compared with vehicle-treated cells, indicating generation of ROS (Figure 3B and 3E). In addition, normalized measurements of DAF-FM in CMVECs showed increased fluorescence intensity in response to BMS (164±10%, n=7; *P*<0.05) and diazoxide (182±23%, n=6; *P*<0.05) compared with the vehicle (100±11% and 100.1±12%, respectively; n=6 each), indicating generation of NO. Inhibition of NOS with L-NAME pretreatment of CMVECs abolished enhanced DAF fluorescence in response to BMS (47.3±4%, n=7; *P*<0.05) and diazoxide (28.2±12%, n=6; *P*<0.05) compared with response in untreated CMVECs. L-NAME pretreatment reduced the DAF-FM fluorescence in vehicle-administered cells (50.3±4% and 33.3±11%, controls of BMS and diazoxide, respectively; n=6 each), however, this was a result of reduction in basal NO production that was sensitive to L-NAME. Thus, the increase in DAF fluorescence in response to BMS and diazoxide correlated with NOS-derived NO production (Figure 3C and 3F). Measurements of fluo-4 acetoxymethyl ester (fluo-4 AM)–loaded endothelial cells in freshly isolated cerebral arteries (Figure 4A and 4C) showed increase in fluorescence in response to BMS (172±20%, n=7; *P*<0.05) and diazoxide (185±23%, n=5; *P*<0.05) compared with vehicle (100±15%, n=6). Similarly, fluo-4 AM–loaded CMVECs (Figure 4B and 4C) showed increase in fluorescence in response to BMS (23.6±3%, n=6; *P*<0.05) and diazoxide (25.7±3%, n=6; *P*<0.05) compared with vehicle (14.5±2%, n=6). In addition, treatment of CMVECs with 100 μmol/L BMS increased fluo-4 AM fluorescence (196±14%, n=4; *P*<0.05), but did not affect MitoSOX (100±7%, n=4; *P*=NS) fluorescence compared with vehicle-treated cells (145±8%, n=4 and 102±5%, n=4; respectively; Figure II in the online-only Data Supplement). Thus, endothelial cells exhibited an increase in fluo-4 AM fluorescence in response to BMS and diazoxide, indicating elevation of cytosolic $[Ca^{2+}]_i$ in the cells.

NO Measurements by ESR

A characteristic NO–$Fe(DETC)_2$ signal with 3 peaks (g value ≈2.035) was detected in rat aortas incubated with $Fe(DETC)_2$ and various drugs. The magnitude of this signal was greatly increased by stimulation with the BMS (194±21%, n=6; *P*<0.05) or diazoxide (232±19%, n=6; *P*<0.05) compared with vehicle-treated aortas (100±0%, n=6; Figure 3D and 3G). Furthermore, the ESR signals from BMS- or diazoxide-stimulated aortas were abolished after addition of the NOS inhibitor, L-NAME.

Akt and eNOS Phosphorylation

Treatment of CMVECs with BMS (2.08±0.21, n=8; *P*<0.05) and diazoxide (1.83±0.17, n=8; *P*<0.05) led to increased phosphorylated-Akt/total-Akt ratio of immunoband intensity (arbitrary units), compared with vehicle treatment (1.12±0.13, n=8; Figure 5A and 5C). Similarly, treatment of arteries with BMS (1.31±0.03, n=5; *P*<0.05) and diazoxide (1.24±0.05, n=5; *P*<0.05) promoted increased phosphorylated-Akt/total-Akt ratio of immunoband intensity compared with vehicle-treated arteries (0.84±0.03, n=5; Figure 5A and 5C). Treatment of CMVECs with BMS (1.29±0.059, n=8; *P*<0.05) and diazoxide (1.29±0.06, n=8; *P*<0.05) led to increased phosphorylated-eNOS/total-eNOS ratio of immunoband intensity compared with vehicle-treated arteries (1.0±0.028, n=8; Figure 5A and 5D). Similarly, treatment of arteries with BMS (0.82±0.03, n=5; *P*<0.05) and diazoxide (1.01±0.06, n=5; *P*<0.05) induced increased phosphorylated-eNOS/total-eNOS ratio of immunoband intensity compared with vehicle-treated arteries (0.72±0.03, n=5; Figure 5A and 5D). In addition, the phosphorylated-eNOS/total-eNOS

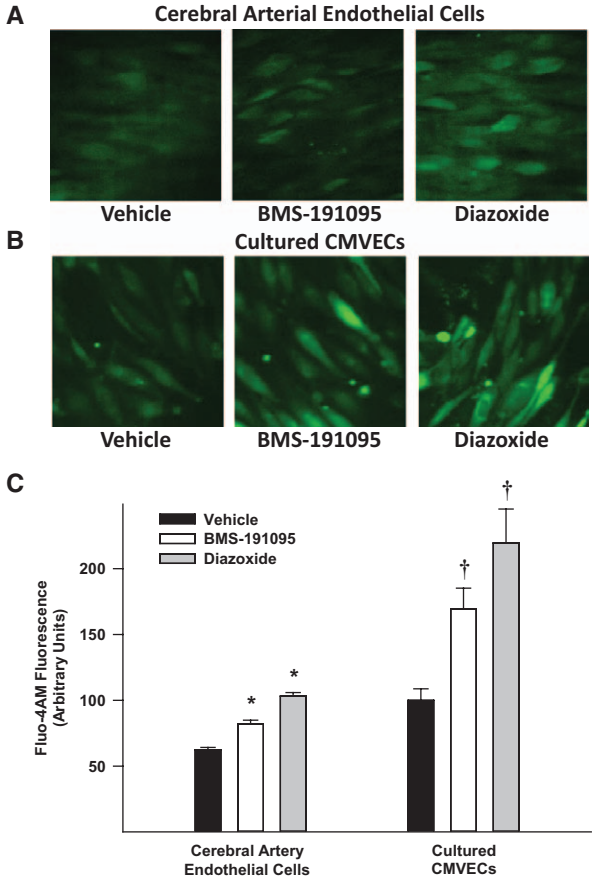

Figure 4. Fluorescence images of cerebral artery endothelial cells (**A**) and cultured primary rat brain microvascular endothelial cells (CMVECs; **B**) loaded with fluo-4 AM, a calcium-sensitive fluoroprobe, and treated with vehicle (DMSO) or 50 μmol/L BMS-191095 (BMS). The cumulative data of fluorescence intensity from the images were determined and plotted as a bar graph in **C**. * and † indicate significant difference in response to BMS or diazoxide vs vehicle, in arterial endothelial cells or CMVECs, respectively (*P*<0.05).

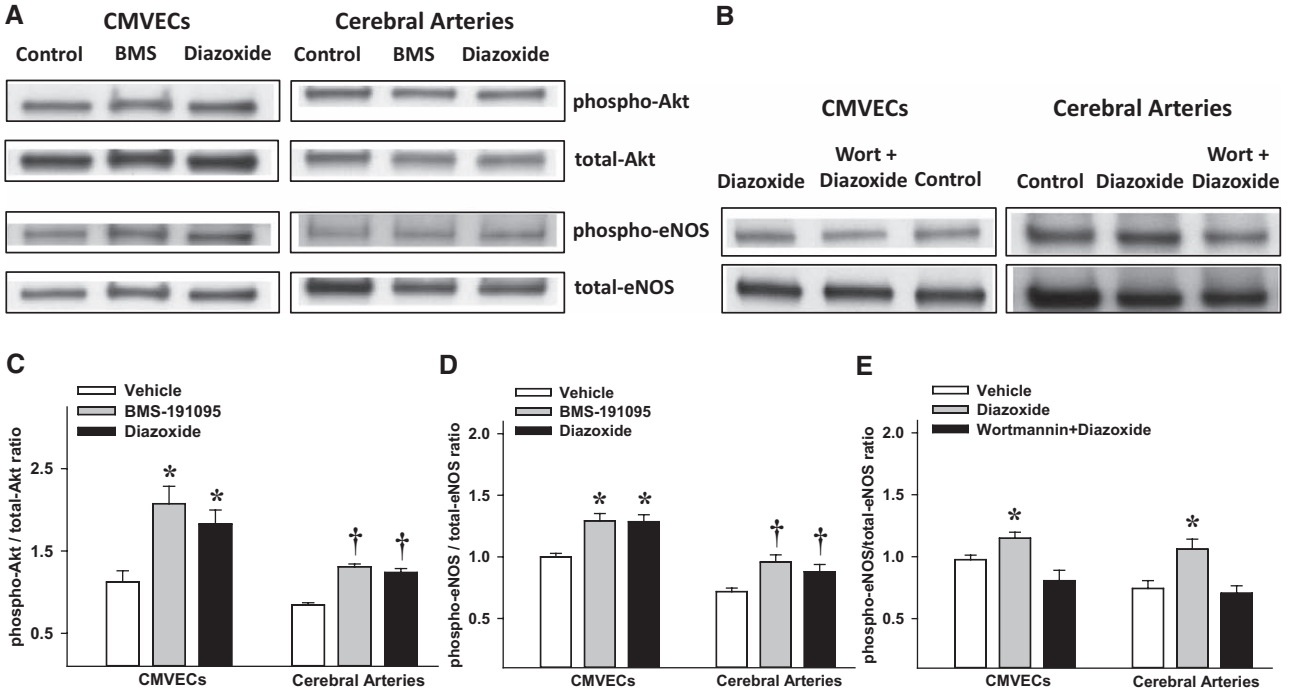

Figure 5. Western blots of total and phosphorylated forms of Akt and endothelial nitric oxide synthase (eNOS) in homogenates of cultured primary rat brain microvascular endothelial cells (CMVECs) and cerebral arteries treated with vehicle (control) or BMS-191095 (BMS) or diazoxide are shown (**A**). In **B**, Western blots of total and phosphorylated forms of eNOS in homogenates of CMVECs and cerebral arteries treated with vehicle or diazoxide or diazoxide+wortmannin are shown. The cumulative data (mean±SEM) from the immunoband intensities were expressed as ratio of phosphorylated Akt to total Akt (**C**), and phosphorylated eNOS to total eNOS (**D** and **E**). * and † indicate significant difference compared with corresponding vehicle-treated CMVECs and arteries, respectively ($P<0.05$). Wort indicates wortmannin.

ratio of immunoband intensities showed an increase in response to diazoxide (1.15±0.05, n=6; $P<0.05$) compared with vehicle-treated CMVECs (0.98±0.04, n=6; P=NS), and wortmannin pretreatment abolished the diazoxide response (0.81±0.08, n=6; $P<0.05$; Figure 5B and 5E). Similarly, in cerebral arteries, the phosphorylated-eNOS/total-eNOS ratio of immunoband intensities showed an increase in response to diazoxide (1.06±0.08, n=3; $P<0.05$) compared with vehicle-treated arteries (0.74±0.06, n=3; P=NS), and wortmannin pretreatment abolished diazoxide response (0.7±0.06, n=3; $P<0.05$; Figure 5B and 5E). Thus, pretreatment of CMVECs and arteries with wortmannin eliminated the increase in eNOS phosphorylation in response to diazoxide.

Discussion

There are 4 major, new findings of the study. First, mitochondrial depolarization in the presence as well as in the absence of ROS generation is a potent initiator of dilator responses of cerebral arteries. Second, mitochondrial depolarization-induced relaxation involves contributions from endothelium as well as VSM. Third, the endothelium-dependent vasodilation induced by mitochondrial depolarization is mediated by NO resulting from activation of eNOS via increased endothelial $[Ca^{2+}]_i$ and activation of PI3K leading to Akt phosphorylation. Fourth, both mitochondrial ROS-dependent and -independent mechanisms are capable of promoting vasodilation by mitochondrial depolarization. Our observations indicate that mitochondrial factors specific to depolarization can have major influences on the regulation of cerebral vascular tone.

Our finding that activation of mitoK_{ATP} channels elicited vasodilation is consistent with previous studies from our laboratory[3] and others,[2] and in addition, demonstrates the importance of the endothelium. Scavenging of ROS reduced the vasodilation response to diazoxide alone, whereas BMS-induced vasodilation was unaffected. Thus, the ability of mitochondrial depolarization by BMS to promote vasodilation independent of ROS generation is a novel observation. Measurements of mitochondrial ROS in endothelial cells provided additional evidence that, unlike diazoxide, BMS does not enhance the generation of mitochondrial ROS. Furthermore, reduced vasodilation in response to BMS and diazoxide after endothelial denudation confirmed the important contribution of endothelium to mitochondria-mediated vasodilation. Notably, the greater endothelial contribution to vasodilation in response to BMS versus diazoxide suggests that the ROS production by diazoxide may interfere with NO-dependent mechanisms.

Mitochondria-mediated vasodilation was also sensitive to inhibition of mitoK_{ATP} channels confirming their role in mitochondrial depolarization. Studies in our laboratory and others have demonstrated the role of mitoK_{ATP} channels in mediating the effects of diazoxide[2,14,22] and BMS[3,12,13,16,18,19,21,23,24] in isolated mitochondria, neurons, vasculature, and cardiomyocytes. Also, our laboratory has characterized the distinct ROS-independent mechanism of actions of BMS compared

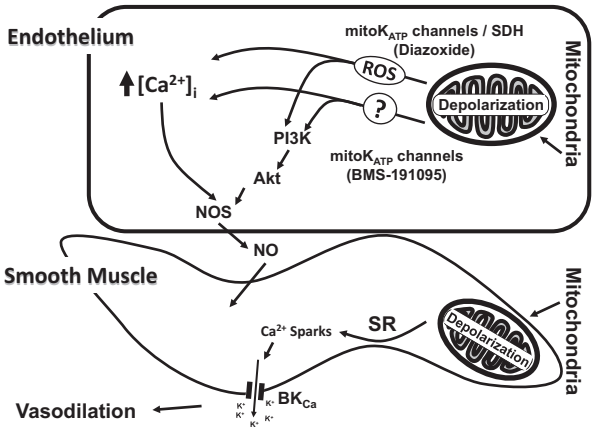

Figure 6. A schematic of the proposed mechanisms underlying the vasodilation induced by the mitochondrial depolarization in the endothelium. Depolarization of mitochondria leads to elevation of endothelial $[Ca^{2+}]_i$ by both reactive oxygen species (ROS)-dependent and -independent mechanisms. Elevation of global endothelial $[Ca^{2+}]_i$ promotes activation of endothelial nitric oxide synthase (eNOS), which, in turn, leads to generation of nitric oxide (NO). Mitochondrial depolarization in endothelial cells also promotes activation of phosphoinositide-3 kinase (PI3K), phosphorylation of Akt, and subsequently phosphorylation of eNOS resulting in $[Ca^{2+}]_i$-independent activation of eNOS leading to NO generation. Finally, mitochondrial depolarization-induced endothelium-dependent vasodilation is primarily mediated by NO, and this effect enhances vascular smooth muscle (VSM)-specific relaxation. Mitochondrial depolarization in VSM cells sequentially causes the activation of ryanodine-sensitive Ca^{2+} channels on sarcoplasmic reticulum, generation of calcium transients, calcium sparks, and the opening of adjacent large-conductance calcium-activated potassium channels (BK_{Ca}) on the plasma membrane. The efflux of K^+ thus leads to hyperpolarization, decreased global intracellular Ca^{2+} of smooth muscle, and vasodilation.

with diazoxide in isolated mitochondria and cultured cortical neurons.[12,16] The present study confirms our previous findings of ROS-independent actions of BMS in promoting mitochondria-mediated vasodilation.

Inhibition of NOS diminished vasodilation to BMS and diazoxide in endothelium-intact arteries indicating that part of the mitochondria-mediated vasodilation was NO-dependent vasodilation. This finding is consistent with our previous observations, which showed that diazoxide-induced vasodilation involved endothelium and NOS.[3] Moreover, fluorescence measurements of NO in CMVECs confirmed the ability of BMS and diazoxide to enhance NO generation sensitive to NOS inhibition. Furthermore, measurements of NO by ESR provided additional evidence supporting the ability of BMS and diazoxide to promote NO generation in vascular tissues. Two potential mechanisms underlie the activation of eNOS in response to BMS and diazoxide: First, fluorescence measurements of Ca^{2+} showed elevation of intracellular Ca^{2+} in response to mitochondrial depolarization in CMVECs and also in endothelial cells of intact cerebral arteries, thus implicating $[Ca^{2+}]_i$ in the production and release of endothelial-relaxing factors. Second, administration of wortmannin resulted in diminished vasodilation in response to BMS and diazoxide, implicating the PI3K pathway in vasodilation. Mitochondrial depolarization initiates key elements of cellular signaling, such as activation of Akt or increases in $[Ca^{2+}]_i$, events which are associated with direct effects on VSM and production of endothelium-dependent relaxing factors, such as NO and prostacyclin.[3] For example, we have recently shown that insulin dilates cerebral arteries after activation of the PI3K and Akt system.[25] Our findings showing that wortmannin reduced vasodilation to BMS and diazoxide are consistent with this mechanism, and similar findings have been reported in heart.[21] In addition, BMS and diazoxide treatment of CMVECs and arteries promoted phosphorylation of Akt consistent with involvement of PI3K–Akt pathway in vasodilation.[26] Our previous studies reported for the first time that mitochondrial depolarization leads to the activation of PI3K–Akt signaling pathway in cultured neurons,[12] as opposed to PI3K–Akt pathway activation leading to alteration of mitochondrial membrane potential.[27] Mitochondrial depolarization has been shown to increase localized calcium transients in the form of Ca^{2+} sparks in VSM[2] without significant change in global $[Ca^{2+}]_i$, however, the exact mechanisms by which mitochondrial depolarization leads to an increase in $[Ca^{2+}]_i$, and activation of PI3K in endothelial cells needs further investigation. We believe that the mechanisms underlying the release of NO are likely to promote the release of non-NO factors, such as hydrogen peroxide, prostacyclin, and so on, contributing to mitochondria-mediated vasodilation. Furthermore, treatment of cerebral arteries and CMVECs with BMS and diazoxide resulted in increased phosphorylation of eNOS, further confirming the activation of eNOS by mitochondrial depolarization. Finally, wortmannin abolished the diazoxide-induced increase in phosphorylation of eNOS in both CMVECs and arteries confirming the role of the PI3K pathway in mitochondria-mediated vasodilation.

Although this is a relatively new field of study, it appears that diverse physiological, pathological, and pharmacological factors can depolarize mitochondria in vascular cells. Depolarization of mitochondria in other nonvascular cells within the neurovascular unit, such as astroglia and neurons, might also lead to the production and release of substances, which could affect cerebral vascular tone. Thus, increased shear stress in coronary arteries, possibly through filamentous connections with the glycocalyx, can distort and activate mitochondrial-driven events resulting in production of dilator agents, such as hydrogen peroxide in mitochondria.[28] Similarly, decreased oxygen levels can affect mitochondrial membrane potential in blood vessels.[2,10] Furthermore, we have shown that drugs such as rosuvastatin also depolarize mitochondria in neurons.[29] Thus, our findings have demonstrated for the first time the ability of mitochondrial depolarization to promote the release of endothelial-relaxing factors via increased $[Ca^{2+}]_i$ and the PI3K–Akt–eNOS pathway.

Limitations

Our studies used pharmacological approaches to promote mitochondrial depolarization, and agents such as diazoxide have been shown to exhibit some nontarget and nonspecific mitochondrial effects. Many physiological/pathological stimuli (hypoxia)[30,31] that promote mitochondrial depolarization are plagued by similar, but significantly greater

nonspecific effects. Importantly, diazoxide is a widely used well-characterized mitochondrial agent. We and others have studied diazoxide and BMS in variety of cell types. Despite recent evidence,[32] there is a lack of consensus on the molecular identity of the mitoK$_{ATP}$ channel, specificity of its blockers, and their role in mitochondrial depolarization. Our purpose of using pharmacological agents was to achieve mitochondrial depolarization with minimal nontarget effects. The primary mechanisms of elevation of endothelial global $[Ca^{2+}]_i$, phosphorylation of Akt, and eNOS in response to diazoxide and BMS were qualitatively similar, implicating mitochondrial depolarization as the common activator of these pathways.

In summary, our study has uncovered a novel mitochondria-mediated mechanism of vasodilation in cerebral arteries, which involves the release of endothelium-derived relaxing factors in response to mitochondrial depolarization. Activation of ROS-dependent and ROS-independent signaling pathways after mitochondrial depolarization leads to complex cellular events involving release of endothelial factors, which lead to a vascular response to match cerebral blood flow with metabolism.

Acknowledgments

We thank Nancy Busija, MA, SLP, for the help with editing the manuscript. In addition, we thank Dan Liu for technical assistance with Western blot experiments.

Sources of Funding

This work was supported by the National Institutes of Health grant numbers HL-077731, HL-030260, HL093554, and HL-065380 (D.W.B.), and the Ja´nos Bolyai Research Scholarship of the Hungarian Academy of Sciences and National Scientific Research Fund of Hungary grant number OTKA K68976 (F.D.).

Disclosures

None.

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Significance

Mitochondria play an important role in the regulation of vascular tone. Recent, limited evidence shows that mitochondrial depolarization promotes relaxation of intact or endothelium-denuded arteries or isolated vascular smooth muscle cells. Mitochondrial depolarization occurs in physiological (metabolic demand) and pathological (hypoxia-ischemia) conditions. However, the exact mechanisms by which mitochondrial depolarization promotes the release of endothelial vasoactive factors have not been adequately studied. In the present study, we induced endothelial mitochondrial depolarization by 2 mechanistically different selective activators of mitochondrial ATP sensitive potassium channels. We demonstrated that mitochondrial depolarization results in activation of endothelial NO synthase by dual pathways, involving increased intracellular calcium as well as by phosphoinositide-3 kinase-protein kinase B-induced endothelial NO synthase phosphorylation. Both mitochondrial reactive oxygen species-dependent and –independent mechanisms mediate activation of endothelial NO synthase by endothelial mitochondrial depolarization. Thus, present study provides the first evidence of the mechanism by which mitochondria may match metabolic demand with blood flow.