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Depolarization of mitochondria in neurons promotes activation of nitric oxide synthase and generation of nitric oxide

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Katakam PV, Dutta S, Sure VN, Grovenburg SM, Gordon AO, Peterson NR, Rutkai I, Busija DW. Depolarization of mitochondria in neurons promotes activation of nitric oxide synthase and generation of nitric oxide. Am J Physiol Heart Circ Physiol 310: H1097–H1106, 2016. First published March 4, 2016; doi:10.1152/ajpheart.00759.2015.—The diverse signaling events following mitochondrial depolarization in neurons are not clear. We examined for the first time the effects of mitochondrial depolarization on neuronal nitric oxide synthase (nNOS) activation, and nitric oxide (NO) production in cultured neurons and perivascular nerves. Cultured rat primary cortical neurons were studied on 7–10 days in vitro, and endothelium-denuded cerebral arteries of adult Sprague-Dawley rats were studied ex vivo. Diazoxide and BMS-191095 (BMS), activators of mitochondrial KATP channels, depolarized mitochondria in cultured neurons and increased cytosolic calcium levels. However, the mitochondrial oxygen consumption rate was unaffected by mitochondrial depolarization. In addition, diazoxide and BMS not only increased the nNOS phosphorylation at positive regulatory serine 1417 but also decreased nNOS phosphorylation at negative regulatory serine 847. Furthermore, diazoxide and BMS increased NO production in cultured neurons measured with both fluorescence microscopy and electron spin resonance spectroscopy, which was sensitive to inhibition by the selective nNOS inhibitor 7-nitroindazole (7-NI). Diazoxide also protected cultured neurons against oxygen-glucose deprivation, which was blocked by NOS inhibition and rescued by NO donors. Finally, BMS induced vasodilation of endothelium denuded, freshly isolated cerebral arteries that was diminished by 7-NI and tetrodotoxin. Thus pharmacological depolarization of mitochondria promotes activation of nNOS leading to generation of NO in cultured neurons and endothelium-denuded arteries. Mitochondrial-induced NO production leads to increased cellular resistance to lethal stress by cultured neurons and to vasodilatation of denuded cerebral arteries.

Mitochondrial ATP-sensitive potassium channels; diazoxide; BMS-191095; membrane potential; superoxide anion

MTILD DISCHARGE

NEW & NOTEWORTHY

The current study provides evidence that pharmacological depolarization of mitochondria activates neuronal nitric oxide (NO) synthase (nNOS) in neurons and vascular wall. Generation of NO from nNOS activation affords protection against lethal stress in neurons and promotes cerebral vasodilation, thus indicating functional significance of mitochondria-nNOS signaling pathway.

Mitochondrial membrane potential is a critical regulator of cellular activity and survival and is controlled by a variety of factors including the ATP-sensitive potassium (mitoK\textsubscript{ATP}) channels located on the inner mitochondrial membrane (2). Pharmacological activators of mitoK\textsubscript{ATP} channels, such as BMS-191095 (BMS) and diazoxide, decrease the mitochondrial membrane potential by facilitating the influx of potassium from cytosol into the matrix (2). Signaling events following mitochondrial depolarization appear to be diverse in the various cell types comprising the neurovascular unit (cerebral vascular endothelium and smooth muscle, perivascular neurons, parenchymal neurons, and glia). Both BMS and diazoxide applications result in dilation of isolated cerebral arteries; however, individual effects on vascular smooth muscle and endothelium, which contribute to the overall vascular effect, are completely different (20, 22, 35, 42). Thus BMS and diazoxide relax cerebral vascular smooth muscle cells via generation of localized calcium sparks by sarcoplasmic reticulum linked to mitochondrial depolarization, resulting in decreased global intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) (20, 42). Conversely, mitochondrial depolarization in cerebral endothelial cells leads to activation of endothelial nitric oxide synthase (eNOS) and production of nitric oxide (NO) by increasing [Ca\textsuperscript{2+}]\textsubscript{i} as well as by increasing phosphorylation of eNOS via the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) pathway (22). Endothelial NO then diffuses to adjacent vascular smooth muscle and enhances the intrinsic relaxant effects through a cGMP-dependent mechanism (33). Little is known about the relationships between mitochondrial membrane potential and NOS activation in other cell types of the neurovascular unit such as cortical neurons and perivascular nerves. Substances such as NO could be produced by cortical neurons or perivascular nerves and promote relaxation of cerebral vascular smooth muscle (41). Cerebral arteries are heavily invested by NOS-containing perivascular nerves and are closely associated to parenchymal neurons (1, 39, 40). Diazoxide and BMS depolarize mitochondria in neurons resulting in enhanced cellular protection in the form of pre- and postconditioning (9, 16, 23, 24). However, the signaling events following mitochondrial depolarization are not fully elucidated and no previous studies have examined the effects of mitochondrial depolarization on neuronal NOS (nNOS) activity.
In this study, we examined the effects of mitochondrial depolarization on nNOS activation and NO production in primary cultures of rat neurons as well as subsequent signaling events. Specifically, we determined the effects of diazoxide and BMS on mitochondrial membrane potential, mitochondrial reactive oxygen species (ROS) production, mitochondrial oxygen consumption rate (OCR), [Ca\(^{2+}\)]\(_i\), and NO production. Furthermore, we examined the functional role of nNOS resulting from diazoxide application in promoting cellular protection of cultured neurons. We also examined the effects of BMS on relaxation of endothelium-denuded cerebral arteries, which contain intact perivascular innervation, to demonstrate that our studies in cultured neurons are supported by results from naturally occurring neurons. nNOS is located in perivascular nerves supplying the cerebral vasculature (39), and NO from both the perivascular (7, 8, 39) and parenchymal neurons (31) has access via diffusion to the cerebral vasculature. Although several investigators have indicated that nNOS or other NOS isoforms are present in mitochondria as well as in the cytosol, the prevailing evidence does not support a mitochondrial localization of NOS under basal conditions in neurons or cardiac cells (25, 44).

**MATERIALS AND METHODS**

The animal protocols were approved by the Institutional Animal Care and Use Committee of Tulane University School of Medicine and comply with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996, Bethesda, MD). Timed-pregnant dams (n = 14; for culturing neurons) and 10-wk-old male rats (n = 16; for vascular studies) Sprague-Dawley (SD) rats were obtained from Harlan (Indianapolis, IN). Rats were housed in the animal care facility and received standard rat chow and water ad libitum. Vehicle for drugs were either H\(_2\)O, DMSO, or NaOH.

**Rat primary cortical neuronal cell culture.** Timed-pregnant rat dams with embryonic day 18 fetuses were anesthetized with 5% isoflurane (VetOne, Boise, ID) and decapitated. Rat primary cortical neurons were isolated and cultured as previously described (9, 24). Briefly, cortical neurons were isolated and plated onto poly-\(\)lysine-coated dishes, multiwell plates, or coverslips and maintained in a humidified 5% CO\(_2\) incubator. After cell attachment, plating medium was replaced with Neurobasal Medium (Waltham, MA) supplemented with B27 (2%), l-glutamine (0.5 mM), 2-mercaptoethanol (55 \(\mu\)M), and KCl (25 mM). ARA-C (10 \(\mu\)M) was used to inhibit astrocyte growth. Positive immunostaining for microtubule-associated protein-2 and negative immunostaining for glial fibrillary acidic protein confirmed the cultures consisted of more than 99% of neurons. Neurons were studied on 7–11 days in vitro (DIV).

**Mitochondrial respiration of neurons.** Mitochondrial OCR in cultured cortical neurons was determined using a Seahorse Bioscience XF24 Analyzer as described previously (9, 35). The Seahorse Bioscience XF24 Analyzer determines mitochondrial OCR by measuring the rate of change in oxygen and proton concentrations in the medium surrounding the neurons that were cultured in poly-\(\)lysine coated 24-well plates. For the experiments, Neurobasal Medium was replaced with Seahorse XF Assay Medium (no. 102365-100; Seahorse Bioscience), containing 5.0 mmol/l glucose and 2.0 mmol/l pyruvate at pH 7.4. Experiments were conducted at 37°C. The neurons were exposed to medium alone, medium containing vehicle, or medium containing 500 \(\mu\)mol/l diazoxide. Individual components of mitochondrial respiration were evaluated using serial injections of the drugs oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenoxymethylhydrazone (FCCP), and antimycin A plus rotenone. Mathematical calculations were made using the raw OCR values. Nonmitochondrial respiration equals the minimum value of the five OCR measurements after antimycin A and rotenone injection. Basal respiration equals the values for OCR measurements before the first injections minus nonmitochondrial respiration. Proton leak equals OCR measurements after oligomycin injection before FCCP injection minus nonmitochondrial respiration. ATP production equals basal respiration minus proton leak. Maximal respiration equals OCR values after FCCP injection before antimycin A injection minus nonmitochondrial respiration. Spare respiratory capacity equals maximal respiration minus basal respiration. For mitochondrial function, we expressed OCR data in picomoles per minute.

**Fluorescence confocal microscopy.** We determined the effects of vehicle, diazoxide (100 \(\mu\)mol/l), and BMS (50 \(\mu\)mol/l) on mitochondrial membrane potential, NO levels, mitochondrial ROS production, or levels of [Ca\(^{2+}\)], on neurons cultured on glass coverslips using protocols described previously (18, 20–22). In some experiments, we also coapplied 7-nitroindazole (7-NI, a selective nNOS inhibitor; 100 \(\mu\)mol/l). The concentrations of diazoxide, BMS, and other drugs were chosen based on our prior studies (9, 16, 20, 22, 23, 35, 36).

Mitochondrial membrane potential was determined using rhodamine-123 (20, 22). Mitosox was used to measure mitochondrial ROS, specifically superoxide anion (34). Fluo-4 AM was used to determine [Ca\(^{2+}\)] (20). Diaminorhodamine-4M (DA-4M) was used to determine NO (18, 22). All fluorophores were obtained from Molecular Probes (Eugene, OR). Confocal microscopy and imaging were performed using a laser scanning confocal system (7 Live; Zeiss, Jena, Germany) or a Leica SP2 AOBS laser confocal microscope attached to an inverted microscope with optics and filters specific to the fluorophore. Imaging conditions such as gain levels and laser power were held constant for each protocol. Offline analysis of images was performed using ImageJ software (NIH) to determine the average pixel intensity of neurons in each defined field (10–20 fields per coverslip), and the results are expressed in relative fluorescence units. Fluorescence measurements in relative units are expressed as percent change from the baseline images before administration of vehicle, BMS, or diazoxide. The \(n\) value represents the number of cover slips containing neurons for each treatment.

**Electron spin resonance spectroscopy for NO measurements.** The NO measurements were performed using electron spin resonance (ESR) spin trapping, as previously described (9, 11, 22). Cultured neurons were incubated in 1 ml K-H buffer in the presence and absence of vehicle, diazoxide (100 \(\mu\)mol/l), or N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate, a NO donor; 10 \(\mu\)mol/l). After incubation with vehicle or drugs, neurons were washed and collected for analysis. The ESR spectra were obtained using a benchtop X-band EMX series ESR spectrometer (Bruker Biospin, Karlsruhe, Germany) using a high-sensitivity SHQ microwave cavity in a finger Dewar filled with liquid nitrogen. The ESR spectrometer settings included the following: microwave power: 40 mW; modulation amplitude: 8-G center-field 2.03 g; sweep width: 80 G; conversion time: 80 ms; time constant: 20 ms; and sweep time: 10.24 s, with 60 scans. For calculation of baseline, signals were quantified by measuring the total amplitude after correction. The amount of NO was determined by measuring the total ESR signal amplitude for each treatment and comparing it with the ESR signal amplitude following NO donor spermine NONOate. Diazoxide-induced NO production in neurons was expressed as percent change from basal levels (vehicle treated). The \(n\) value represents the sample size for normalized ESR signal amplitude of neurons grown to confluence in a 35-mm culture dish for each treatment.

**Western blotting for nNOS.** Cultured neurons were treated with vehicle, 25 \(\mu\)mol/l BMS, or with 100 \(\mu\)mol/l diazoxide at 37°C for 15–20 min. Cells were washed and homogenates were prepared from the cells in lysis buffer containing protease and phosphatase inhibitors. Subsequently, cellular signaling was determined by Western blotting, as previously described (19). We used primary antibodies directed against phosphorylated nNOS (serine 1417, 160 kDa, Ab-
cam; and serine 847, 160 kDa, Abcam) and total nNOS (160 kDa, BD Transduction Laboratories, San Jose, CA). Each immunoband intensity of phosphorylated nNOS was normalized to the corresponding immunoband intensity of total nNOS. The n value represents the sample size for normalized intensity of immunobands from the lysate of neurons grown to confluence in a 35-mm culture dish for each treatment.

**Preconditioning experiments.** Primary cortical neurons were cultured in 96-well plates for 7 DIV. On the seventh day, cells were treated with 1) vehicle, 2) 500 μmol/l diazoxide, 3) diazoxide plus 100 μmol/l nitro-L-arginine methyl ester (L-NAME), and 4) diazoxide plus L-NAME and the NO donors, sodium nitroprusside (SNP; 1 μmol/l), or diethylamine nitric oxide (DEANO; 5 μmol/l), for 3 consecutive days. On 10 DIV, neurons were subjected to either oxygen glucose deprivation (OGD) or maintained at normoxia as previously described (23, 24). For OGD, 96-well cell culture plates and dishes were rinsed with PBS and the culture medium was replaced with glucose-free DMEM and placed in a Shel Lab Bactron Anaerobic Chamber (Sheldon Manufacturing, Cornelius, OR) containing anaerobic mixed gas (AMG; 5% CO₂ -5% H₂-90% N₂) at 37°C for 3 h. The 5% H₂ in the chamber removed remaining traces of oxygen-forming water on a platinum catalyst. The oxygen level was continuously monitored with an infrared gas analyzer (Illinois Instruments, Ingle- side, IL) and maintained at <0.1% during the experiments. Control cell cultures were treated identically and incubated in glucose-containing DMEM (4.5 mg/ml) in a 5% CO₂ cell culture incubator.

**Vascular reactivity.** Vaseoreactivity of isolated cerebral arteries following endothelial denudation was measured as described previously (20, 22). Briefly, rats were killed under deep anesthesia and posterior cerebral arteries were isolated from rat brains. Endothelium was removed by injecting a 1-ml bolus of air through the arteries. Arteries were transferred to a vessel bath filled with oxygenated, warm physiological salt solution, cannulated with glass pipettes, and secured with 10-0 ophthalmic suture. Intraluminal diameter was measured by video dimension analyzer (Living Systems Instrumentation, Burlington, VT). After an equilibration period of ~30 min, arteries were slowly pressurized to 70 mmHg with PSS until they developed a stable myogenic tone (40–45% of passive diameter). Subsequently, drugs were administered abuminally via the bath solution and cumulative concentration responses to drugs were determined. Endothelial denudation was verified by lack of dilator response to Bradykinin (10 μmol/l) and viability was tested by intact vasodilator response to SNP (10 μmol/l) and by contraction to KCl. Vascular responses to 50 μmol/l BMS were determined in endothelium denuded arteries pretreated with and without 7-NI (a selective nNOS inhibitor; 100 μmol/l) or tetrodotoxin (5 μmol/l). The n value represents the sample size for each segment of isolated cerebral artery for each treatment.

**Electron microscopy.** For identification of perivascular nerves in cerebral arteries, rats were euthanized by deep anesthesia with 5% isoflurane and perfused with a PBS solution containing 2% of glutaraldehyde and 3% of formaldehyde. Arteries were removed and kept in the perfusion solution for 1 h and were fixed in a PBS solution containing 2% of glutaraldehyde and 3% of formaldehyde. Later, arteries were postfixed in 1% osmium tetroxide and embedded in Spurr’s resin. Ultrathin sections (80–90 nm) were mounted on formvar-coated copper grids (200 mesh), air dried, and stained with uranyl acetate and lead citrate (at 7 and 7 min, respectively). The sections were put on grids and viewed at a magnification of ×11,000 using a FEI Tecnai BioTwin 120 keV TEM with a digital imaging setup (Wake Forest University Health Sciences, Winston-Salem, NC).

**Data analysis and statistics.** Results are expressed as mean ± SE; n indicates the number of independent experiments. Means were compared by one-way ANOVA. Post hoc analysis was done by Tukey’s test. A P < 0.05 was considered as statistically significant.

**RESULTS**

**Mitochondrial membrane potential and ROS measurements.** Diazoxide and BMS depolarized mitochondria in cultured neurons as indicated by the decreased rhodamine 123 fluorescence intensity (relative units) from 142 ± 13 following vehicle treatment to 82 ± 4.5 and 61 ± 2.7 following diazoxide and BMS, respectively (n = 6 each; P < 0.05, compared with vehicle; Fig. 1, A and B). On the other hand, BMS (33 ± 1.1, n = 5; P = NS) did not increase mitochondrial ROS levels whereas diazoxide increased ROS levels (53 ± 2.4, n = 8; P < 0.05, compared with vehicle), indicated by the increase in MitoSOX fluorescence intensity, compared with vehicle (35 ± 0.7, n = 6) in neurons (Fig. 1, C and D).

**Mitochondrial OCR.** Diazoxide had no significant effects on raw values for OCR as well as for the calculated values for basal respiration, maximal respiration, spare respiratory capacity, or nonmitochondrial respiration (Fig. 2). However, diazoxide had statistically significant but modest effects on ATP production and proton leak (Fig. 2, n = 5 for each group; P < 0.05, DZ compared with vehicle, P < 0.05, DZ compared with medium, P < 0.05, vehicle compared with medium).

**Cytosolic Ca²⁺.** Diazoxide and BMS increased [Ca²⁺], indicated by the increased fluo-4AM fluorescence intensity (relative units) to 24.1 ± 0.9 (n = 8; P < 0.05) and 24.8 ± 0.9 (n = 8; P < 0.05, compared with basal).

**nNOS phosphorylation.** Treatment of neurons with diazoxide (1.1 ± 0.8, n = 6; P < 0.05) and BMS (1.09 ± 0.06, n = 5; P < 0.05) led to an increased phosphorylated serine 1417-nNOS total-nNOS ratio of immunoband intensity compared with vehicle treated neurons (0.66 ± 0.06, n = 6; Fig. 4, A and B). In contrast, treatment of neurons with diazoxide (0.87 ± 0.04, n = 6; P < 0.05) and BMS (0.84 ± 0.1, n = 5; P < 0.05) induced a decreased phosphorylated-nNOS total-nNOS ratio of immunoband intensity compared with vehicle-treated neurons (1.1 ± 0.04, n = 6; Fig. 4, A and B).

**NO production.** Diazoxide (125 ± 2, n = 7) and BMS (122 ± 3, n = 8) increased NO levels in cultured neurons indicated by the increased DAR-4M fluorescence intensity (relative units) compared with vehicle (100 ± 2 and 99 ± 2 for respective controls, n = 12 for each; Fig. 5, A–C; P < 0.05, compared with vehicle). Inhibition of nNOS with 7-NI pretreatment blocked NO increases to diazoxide (83 ± 2, n = 6; P = NS) and BMS (82 ± 2, n = 6; P = NS) compared with vehicle (83 ± 2 and 84 ± 2 for respective controls, n = 5 for each; Fig. 5, A–C). NO levels (relative units) also increased in the presence of diazoxide (507 ± 39, n = 5) or spermine NONOate (834 ± 172, n = 4) compared with basal levels in the presence of vehicle (177 ± 33, n = 5) using the ESR method (Fig. 6, A–D; P < 0.05, compared with basal).
Preconditioning. None of the treatments in DMEM containing glucose during normoxia significantly affected neuronal survival compared with untreated neurons (data not shown). After OGD, untreated neurons showed a significant decrease in viability (Fig. 7A). Diazoxide preconditioning improved neuronal survival significantly after OGD, compared with untreated neurons (Fig. 7A, P < 0.05, compared with OGD). Coadministration of l-NAME during preconditioning abolished the protection.
tive effects of diazoxide (Fig. 7A). Exogenously supplying NO using donors such as SNP and DEANONOate along with the combined diazoxide and l-NAME treatment rescued the protective effects of diazoxide (Fig. 7A, P < 0.05, compared with OGD).

A previous study by our laboratory demonstrated the involvement of Akt/mammalian target of rapamycin (mTOR)/S6K in diazoxide induced preconditioning, and we extended these studies by examining effects of l-NAME treatment. Administration of diazoxide enhanced the levels of phosphorylated/total of Akt, mTOR, and S6K and coadministration of l-NAME reversed this effect. We found that phosphorylated Akt protein/total protein expression was elevated from 16.8 ± 2.1 arbitrary units in the vehicle group to 59.4 ± 70.9 arbitrary units with diazoxide preconditioning and that l-NAME coapplication with diazoxide attenuated this increase (42.1 ± 7.1 arbitrary units; data not shown; P < 0.05, for diazoxide preconditioned vs. vehicle or diazoxide plus l-NAME). Similarly, we found that phosphorylated mTOR protein/total protein expression was elevated from 41.9 ± 4.2 arbitrary units in the vehicle group to 77.6 ± 6.8 arbitrary units with diazoxide preconditioning and that l-NAME coapplication with diazoxide attenuated this increase (42.1 ± 7.1 arbitrary units; data not shown; P < 0.05, for diazoxide preconditioned vs. vehicle or diazoxide plus l-NAME). Lastly, we found that S6K protein expression was elevated from 53.4 ± 11.4 arbitrary units in the vehicle group to 362.7 ± 70.9 arbitrary units with diazoxide preconditioning and that l-NAME coapplication with diazoxide attenuated this increase (70.3 ± 3.9 arbitrary units; data not shown; P < 0.05, for diazoxide preconditioned vs. vehicle or diazoxide plus l-NAME). Data are derived from 4–18 independent neuronal cultures per treatment.

**Intraluminal diameter measurements.** The basal diameters of the cerebral arteries were similar for each group of experiments (data not shown). Administration of 50 μmol/l BMS elicited a robust vasodilation in endothelium-denuded cerebral arteries with 31.6 ± 2.1% relaxation (n = 14; Fig. 7B). Coadministration of 7-NI diminished vasodilation to 20.8 ± 2% in response to 50 μmol/l BMS (n = 11; P < 0.05, compared with baseline) and tetrodotoxin reduced vasodilation to 23.3 ± 3.1% (n = 11; P < 0.05, compared with vehicle; Fig. 7B).

**Demonstration of perivascular nerves.** Electron microscopy identified perivascular nerve terminals and neuromuscular junctions on the surface of the cerebral arteries; a representative image is shown in Fig. 7C. Furthermore, fluorescence images of cerebral arteries loaded with a calcium binding fluoroprobe, fluo-4-AM, showed perivascular nerve terminals morphologically consistent with previous reports by Segal et al. (38) and Yokomizo et al. (43) (Fig. 7D).

**DISCUSSION**

There are four major, new findings of the study. First, mitochondrial depolarization is a potent activator of nNOS in neurons and mitochondrial depolarization-induced NO generation in neurons is associated with increased [Ca2+]i, and activating nNOS phosphorylation. Second, mitochondrial dep-
polarization has minimal effects on mitochondrial respiration indicating that mitochondria can maintain energy production despite perturbations in mitochondrial status. Third, NO is an important mediator of diazoxide-induced preconditioning in cultured neurons demonstrating that NO is an important signaling agent for inducing cellular protection against potentially lethal stresses. Fourth, endothelium-independent vasodilation induced by mitochondrial depolarization is substantially mediated by NO, probably derived from the nNOS in perivascular nerves, establishing that perivascular and cultured parenchymal neurons respond similarly to mitochondrial activation. Our observations provide strong support for the view that the
mitochondria-nNOS-NO signaling pathway represents an important, previously unknown, determinant of diverse responses in the neurovascular unit (Fig. 8).

The current findings on neurons complement and extend our previous studies on other cell types and demonstrate the heterogeneity of mitochondrial potential-linked signal pathways in the neurovascular unit (14, 16, 23, 24). Thus nNOS activation in neurons following mitochondrial depolarization is similar in some respects to eNOS activation in cerebral vascular endothelium but is completely different from the NO-independent signaling pathways involving mitochondria that we have observed in cerebral vascular smooth muscle. Many structural, regulatory, and functional similarities exist between eNOS and nNOS isoforms (13). For example, both isoforms exhibit similar regulation of their activities by caveolins (37). Similarly, both isoforms are catalytically activated by increased [Ca\textsuperscript{2+}]. In addition, both isoforms have similar phosphorylation sites containing serine residues. We have shown that the PI3K-Akt pathway is activated by BMS and diazoxide to eNOS phosphorylation (22). Diazoxide also activates the PI3K-Akt signaling pathway in neurons (28). We observed that mitochondrial depolarization in neurons induces elevation of [Ca\textsuperscript{2+}]. In addition, we found that mitochondrial depolarization not only promotes nNOS phosphorylation at serine 1417 (positive regulatory site) but also diminishes phosphorylation at serine 847 (the negative regulatory site). The phosphorylation of nNOS at serine 1417 is likely to be due to activation of PI3K/Akt pathway. Notably, we previously have shown that mitochondrial depolarization activates PI3K/Akt pathway in neurons and endothelial cells (16, 22). In contrast, diminished phosphorylation at serine 847 has been shown to be due to promotion of protein phosphatase activity (45). It appears that mitochondrial depolarization promotes phosphatase activity in neurons that activates nNOS by reducing negative regulatory phosphorylation at serine 847. Further studies are required to confirm the identity and the role of mitochondria-activated phosphatases. Thus, for the first time, we found that mitochondrial depolarization in neurons leads to activation of nNOS and generation of NO. Although several studies have suggested the idea that NOS isoforms are present normally in mitochondria, we and others have been unable to find evidence for this concept in quiescent neurons and cardiomyocytes (25, 44). Nonetheless, mitochondrial as well as nuclear localization of NOS isoforms may occur following chronic stress such as inflammation (6). In contrast to endothelium and neurons, the predominant early event following mitochondrial depolarization in vascular smooth muscle cells is the localized generation of calcium sparks from sarcoplasmic reticulum resulting in a decrease in [Ca\textsuperscript{2+}] (20, 42).

BMS has selective effects on mitoK\textsubscript{ATP} channels; however, it appears that diazoxide has dual effects on mitochondria, which involve activation of mitoK\textsubscript{ATP} channels as well as...
generation of ROS from inhibition of complex II (2). We have documented that mitochondria are the source of superoxide anion in response to diazoxide application using the selective fluoroprobe MitoSOX. Based on our earlier studies, the formation of ROS after diazoxide application is probably via the inhibition of succinate dehydrogenase. Thus, although diazoxide produces two different effects, these effects are still limited to mitochondria. These findings confirm and extend our original observations with BMS and diazoxide in cerebral endothelial and vascular smooth muscle cells as well as in cultured neurons and astroglia and isolated mitochondria (20, 22). The consistent results from our studies as well as several investigations by others clearly demonstrate our original finding that mitochondrial depolarization with agents such as BMS can occur without increasing intracellular or mitochondrial ROS (2, 3, 16, 20, 22, 23). It is interesting that although diazoxide and BMS differ in their effect on mitochondrial ROS generation in various cell types we studied, they activate identical signaling events downstream of mitochondrial depolarization (3, 15, 16, 20, 22). Further studies are needed to examine the ROS-dependent and -independent effects of mitochondrial depolarization. Furthermore, it is noted that the only difference that we observed between diazoxide and BMS actions was the generation of acute production of superoxide only by diazoxide. Diazoxide and BMS elicit identical responses in activation of every signaling event that we measured. With regards to ONOO⁻, we have previously demonstrated in isolated mitochondrial from brain and heart that ONOO⁻ formed in the mitochondria is an endogenous opener of mitoKATP channels (26, 27). Thus it is very likely that simultaneous formation of NO and superoxide in the mitochondria could lead to peroxynitrite formation and subsequent effects on neurons. However, diazoxide-induced superoxide is formed in the mitochondria and is therefore mostly segregated in the mitochondrial compartment whereas diazoxide-induced nitric oxide is formed in the cytosol. Therefore, diazoxide-induced formation of peroxynitrite in the mitochondria is a possible but unlikely event. However, future studies will measure the peroxynitrite formation following diazoxide treatment in mitochondria and the cytosol.

We previously showed that BMS-induced mitochondrial depolarization had minimal effects on ATP production in cultured neurons (23). We have extended these results by a more comprehensive examination of the effects of diazoxide on mitochondrial respiration in cultured neurons. Diazoxide did not affect basal respiration, maximal respiration, spare respiratory capacity, or nonmitochondrial respiration. Diazoxide had only minimal, but statistically significant, effects on ATP production and proton leak. Although mitochondrial effects underlie the neuroprotection afforded by diazoxide, acute treatment of diazoxide has minimal effects on the mitochondrial respiration in neurons. It appears that diazoxide does not affect the normal mitochondrial functioning in neurons. In contrast, we have previously demonstrated that diazoxide treatment increases basal, ATP synthesis-linked, and maximal respiration in neurons subjected to oxygen-glucose deprivation (9). Thus diazoxide-afforded neuroprotection does not involve alteration of mitochondrial function under normal conditions but utilize other mitochondrial-initiated signaling pathways as previously described (9). We can conclude that mitochondrial respiratory dynamics are robust and are only minimally affected by depolarization with BMS or by depolarization and succinate dehydrogenase inhibition by diazoxide.

We also previously demonstrated that exposure of isolated cerebral arteries to diazoxide or BMS leads to relaxation that involves distinct and overlapping mechanisms originating from vascular smooth muscle and endothelium (20, 22, 36). Our current study extends these observations with the demonstration that enhanced NO production by perivascular nerves in the adventitia and its diffusion to vascular smooth muscle also contributes to the integrated arterial response to diazoxide and BMS. Large cerebral arteries are heavily invested with many types of nerves (1). Furthermore, it is likely that NO from parenchymal neurons can promote dilation of cerebral resistance vessels, as previously shown, with application of glutamate receptor agonists to the intact cortical surface (32). Notably, nNOS has been identified in the vascular smooth muscle (12) and thus may also contribute to the mitochondrial depolarization induced vasodilation in endothelium-denuded arteries. However, the exact mechanisms underlying the activation of vascular smooth muscle nNOS are unclear and need further study. Mitochondria in various cell types within the neurovascular unit could be depolarized simultaneously by global events such as ischemia, but individual cells types may be activated by more restricted events. For example, increased shear stress has been shown to selectively activate mitochondria in endothelium and secondarily promote relaxation of smooth muscle of human coronary arteries (29, 30). In addition, cortical spreading depression depolarizes mitochondria in neurons but not in astroglia and we have shown
that NO is an important mediator of vasodilation (7, 8, 16). Thus we propose that cell specific mitochondrial depolarization in the neurovascular unit during changes in physiological status, such as occurs during neuronal activation, will lead to appropriate blood flow changes due to production of NO.

Our results showing a critical role of endogenous NO in diazoxide-induced protection of cultured neurons is consistent with our earlier findings showing that NO is critical for in vivo preconditioning following cortical spreading depression (16, 17). An important role for NO in the development of neuronal protection has also been shown by other investigators but has not been linked to mitochondrial membrane potential (5). Together with our earlier findings (9), it appears that diazoxide induces preconditioning via a NO-S6K signaling pathway.

In summary, our study has uncovered a novel mitochondrial-mediated mechanism of NO generation in neurons and cerebral arteries that involves the activation of nNOS in response to mitochondrial depolarization. Activation of both ROS-dependent and ROS-independent signaling pathways following mitochondrial depolarization leads to complex cellular events, which appear to match neuronal activity and cerebral blood flow with metabolism.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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