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Diversity of mitochondria-dependent dilator mechanisms in vascular smooth muscle of cerebral arteries from normal and insulin-resistant rats

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Katakam PV, Gordon AO, Sure VN, Rutkai I, Busija DW. Diversity of mitochondria-dependent dilator mechanisms in vascular smooth muscle of cerebral arteries from normal and insulin-resistant rats. Am J Physiol Heart Circ Physiol 307: H493–H503, 2014. First published June 14, 2014; doi:10.1152/ajpheart.00091.2014.—Mitochondrial depolarization following ATP-sensitive potassium (mitoKATP) channel activation has been shown to induce cerebral vasodilation by generation of mitochondrial reactive oxygen species (ROS), which sequentially promotes frequency of calcium sparks and activation of large conductance calcium-activated potassium channels (BKCa) in vascular smooth muscle (VSM). We previously demonstrated that cerebrovascular insulin resistance accompanies aging and obesity. It is unclear whether mitochondrial depolarization without the ROS generation enhances calcium sparks and vasodilation in phenotypically normal [Sprague Dawley (SD); Zucker lean (ZL)] and insulin-resistant [Zucker obese (ZO)] rats. We compared the mechanisms underlying the vasodilation to ROS-dependent (diazoxide) and ROS-independent [BMS-191095 (BMS)] mitoKATP channel activators in normal and ZO rats. Arterial diameter studies from SD, ZL, and ZO rats showed that BMS as well as diazoxide induced vasodilation in endothelium-denuded cerebral arteries. In normal rats, BMS-induced vasodilation was mediated by mitochondrial depolarization and calcium sparks generation in VSM and was reduced by inhibition of BKCa channels. However, unlike diazoxide-induced vasodilation, scavenging of ROS had no effect on BMS-induced vasodilation. Electron spin resonance spectroscopy confirmed that diazoxide but not BMS promoted vascular ROS generation. BMS- as well as diazoxide-induced vasodilation, mitochondrial depolarization, and calcium spark generation were diminished in cerebral arteries from ZO rats. Thus pharmacological depolarization of VSM mitochondria by BMS promotes ROS-independent vasodilation via generation of calcium sparks and activation of BKCa channels. Diminished generation of calcium sparks and reduced vasodilation in ZO arteries in response to BMS and diazoxide provide new insights into mechanisms of cerebrovascular dysfunction in insulin resistance.

calcium sparks; mitochondrial membrane potential; electron spin resonance; BMS-191095; superoxide

AGING, OBESITY, AND TYPE 2 diabetes (T2DM) are accompanied by metabolic insulin resistance. Clinical and animal studies have established a causative relationship between insulin resistance and the decline of higher cortical function accompanying aging, T2DM, and Alzheimer disease (8, 10, 58). Impairment of neurovascular coupling, which matches the demands of neuronal activity for oxygen and glucose with increased blood flow, has been implicated in age and T2DM related decline in cognitive function (20, 31, 56). Studies from our laboratory have demonstrated for the first time impaired cerebrovascular actions of insulin in aged Sprague Dawley (SD) and Zucker obese (ZO) rats with insulin resistance compared with young SD and Zucker lean (ZL) rats, respectively (35). Mitochondria act as the sensor of metabolic demand in the cells participating in the neurovascular coupling, and mitochondrial dysfunction has been implicated in the etiology of aging, Alzheimer disease, T2DM, and insulin resistance (11, 21, 47, 56, 57). Importantly, we reported diminished mitochondrial mediated vasodilation in cerebral arteries from ZO rats with insulin resistance (34).

Mitochondria responding to physical, physiological, pharmacological, and pathological factors appear to play an important role in the regulation of vascular tone in several regional circulations. Studies from our laboratory and others have clearly shown that mitochondrial mechanisms promote relaxation of endothelium-intact and endothelium-denuded cerebral arteries as well as isolated vascular smooth muscle (VSM) cells (7, 34, 36, 59, 60). Analysis of these studies has led to the concept that the integrated arterial response to mitochondrial activators such as diazoxide and BMS-191095 (BMS) involves direct VSM relaxation, which is modified by contributions of vasoactive factors such as nitric oxide and prostaglandins from endothelium (34, 36). In previous studies, Jaggar and colleagues (59) showed that diazoxide promotes reactive oxygen species (ROS) in cerebral VSM, which causes the sequential activation of ryanodine-sensitive calcium channels in sarcoplasmic reticulum, generation of calcium transients (otherwise known as ‘calcium sparks’), and the opening of adjacent large-conductance calcium activated potassium channels (BKCa) on the plasma membrane. The efflux of potassium leads to hyperpolarization, decreased global intracellular calcium of VSM, and vasodilation (7, 59).

Although both diazoxide and BMS are activators of mitochondrial ATP-sensitive potassium (mitoKATP) channels, an important difference between diazoxide and BMS is that the former is associated with production of ROS, whereas the latter is not (6, 36). The ROS production by diazoxide likely arises from inhibition of succinate dehydrogenase (9); however, effects are still limited to mitochondria. In contrast, we are unaware of any off-target effects of BMS. Although, the mechanisms of action of these two agents have recently been shown to be different in cerebral vascular endothelium (36), a
direct comparison has not been made in VSM. Moreover, nothing is known concerning the effects of BMS on calcium spark activity in cerebral VSM and how calcium spark activity is affected by insulin resistance. We have shown previously that dilation to diazoxide is impaired in cerebral arteries from insulin-resistant rats but the mechanisms have not been fully explored (34). Thus we hypothesized that mitochondrial depolarization enhances calcium spark activity in VSM cells independent of ROS generation. We further hypothesized that cerebral arteries with insulin resistance and mitochondrial dysfunction exhibit impaired calcium spark activity in response to mitochondrial activation.

In this study, we have examined the effects of mitochondrial depolarization induced by BMS in endothelium-denuded cerebral arteries on calcium sparks-mediated vasodilation in normal and insulin-resistant cerebral arteries. Our results have allowed us to confirm our earlier findings in cerebral vascular endothelium (36) that ROS-dependent and ROS-independent mechanisms share key elements of calcium signaling, which link mitochondrial depolarization with cellular events. Thus, our findings validate the concept that calcium spark activity subsequent to mitochondrial depolarization can occur without ROS production and thereby have illustrated the diversity of pathways linking mitochondrial activation, calcium sparks, and vasodilation in health and disease.

MATERIALS AND METHODS

The animal protocol was approved by the Institutional Animal Care and Use Committees of Tulane University School of Medicine. The investigation complies 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). Young, male SD (n = 28) and ZL and ZO rats (n = 14 each) were obtained at 10–12 wk of age. Rats were housed in the animal care facility and received standard rat chow and tap water ad libitum. Studies were performed on arteries isolated after euthanizing the animals.

ZO rat model. The ZO rat with a leptin receptor mutation, which is homozygous for the mutation (Fa/fa), and is genetically appropriate controls (5). Previous data from our laboratory (18, 19, 35) and others (48, 50, 54) have shown that ZO rats develop insulin resistance with a metabolic profile similar to that observed in Zucker rats. Average MitoSOX fluorescence measurements of vehicle (DMSO) or BMS (50 μmol/l) were determined. Endothelium was removed by injecting a stable myogenic tone, and cumulative concentration responses to drugs were determined. Endothelium was removed by injecting a bolus of 1 ml of air through the arteries. Endothelial denudation was verified by lack of dilator response to bradykinin (10 μmol/l), and viability was tested by intact vasodilator response to nitroprusside (10 μmol/l). We have validated this method by demonstrating endothelial denudation using electron microscopy (43) and have successfully used it in many of our previous studies (33–36, 44).

Vascular responses to 10, 50, and 100 μmol/l BMS were determined in endothelium-denuded arteries. In addition, responses to 50 μmol/l of BMS or diazoxide were evaluated in endothelium-denuded arteries pretreated with 100 μmol/l manganese(III) tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP; a cell-permeant SOD mimetic) (1, 34, 37), and 100 mmol/l ibetioxin (a selective inhibitor of BKCa channels) (1, 59).

Mitochondrial membrane potential measurements. Mitochondrial membrane potential was determined by using 100 μmol/l tetramethylrhodamine ethyl ester (TMRE; λex: 532 nm, λem: >580 nm long pass filter). TMRE (1 mg/ml) stock solution was prepared in methanol and a final solution (100 mmol/l) prepared in phenol-free DMEM with 10 mmol/l HEPES was used to load the arteries for 15 min at 37°C. Fluorescence images were acquired both before and after application of vehicle (DMSO) or BMS (50 μmol/l) or diazoxide (100 μmol/l) for TMRE fluorescence measurements. Imaging conditions such as gain levels and laser power were held constant. Imaging studies of Zucker rats were performed with paired ZO and ZL arteries in an alternating sequence. Offline analysis of images to determine the average pixel intensity of smooth muscle cells in each field (n = 20–30) was performed using ImageJ software (NIH, Bethesda, MD), and the results were expressed in relative fluorescence units (RFUs). Fluorescence measurements in RFUs were expressed as percent change from the baseline images before administration of vehicle or BMS or diazoxide. Representation of TMRE data as percent change from baseline is consistent with previous reports and the concentration of TMRE used. The n value represents the average fluorescence intensity of all the cells from the number of arteries from each treatment group. We did not use different arterial approaches for the color presentation of the representative images to aid the reader in the assessment of the data.

Mitochondrial ROS measurements. Mitochondrial ROS, specifically superoxide, was determined by using 5 μmol/l MitoSOX (λex: 405 nm, λem: >550 nm), based on the method reported by Robinson et al. (52). MitoSOX (5 mmol/l) stock solution was prepared in DMSO, and final solution prepared in phenol-free DMEM with 10 mmol/l HEPES was used to load the arteries for 15 min at 37°C. MitoSOX fluorescence was captured by Leica SP2 AOB laser confocal microscope with a C-Apochromat 63×/NA 1.2 oil immersion objective. We also used an alternate method for measuring MitoSOX fluorescence with λexcitation of 405 nm and λemission >550 nm. Fluorescence images were acquired both before and after application of vehicle (DMSO) or 50 μmol/l BMS or 100 μmol/l diazoxide, maintaining imaging conditions such as gain levels and laser power constant. Offline analysis of images was performed using ImageJ software (NIH). Fluorescence images were captured from each artery at an average 6–9 nonoverlapping fields of view with each containing 40–50 VSM cells. Average MitoSOX fluorescence measurements were determined by choosing region of interest (ROI) of all the VSM cells in each image. The average fluorescence measurements from all arterial segments were expressed as means ± SE RFU for each treatment.

Calcium spark imaging. Fluo-4 AM (λex: 488 nm, λem: 505 nm long pass filter) was used to study [Ca2+]i. The arteries were loaded in the dark with a 1:1 mixture of 5 μmol/l Fluo-4 AM and 20% (wt/vol) pluronic F-127 diluted in HEPES-buffered PSS (in mmol/l) of 134 NaCl, 6 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 0.026 EDTA, and

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MgSO$_4$, 25 NaHCO$_3$, 1.03 K$_2$HPO$_4$, 20 sodium-HEPES, and 11.1
96-well plate containing 150
aorta were cut and 2 segments were placed in a single well of a
minimum F/F$_0$ of 1.2 was required for an event to be considered a
D-glucose, at pH 7.35. Thoracic aortas from SD rats were rapidly
performed using a laser scanning confocal system (7 Live; Zeiss, Jena,
cannulation as previously described (59). Confocal microscopy was
These values were then averaged for all arteries in the study.

**Electron spin resonance studies.** Our previous studies using dihy-
droethidine and MitoSOX fluorescence in endothelial cells (36) and
neurons (23, 24) have clearly demonstrated that diazoxide induces ROS generation, whereas BMS fails to promote ROS generation. In
the present study, electron spin resonance (ESR) spectroscopy was
used to provide additional evidence of diazoxide-induced ROS gen-
eration and lack of ROS induction in response to BMS. Endothelium
denuded aortic segments were used instead of cerebral arteries be-
cause of the greater quantity of tissue required for the ESR experi-
ments. We believe that VSM cells in the endothelium denuded aorta
are comparable with the VSM cells from cerebral arteries in terms of
mitochondrial responses (36). ESR spectroscopy measurements of
superoxide in aortic homogenates in place of cerebral arteries have
been reported in animals with cerebrovascular oxidative stress (40).
Arterial ROS production was measured using ESR as described
previously (14) using the spin probe 1-hydroxy-3-methoxybenzyl-
2,2,5,5-tetramethyl-pyrrolidine (CMH). Diethyldithiocarbamate
(DETC; 2.5 mmol/l) and 25 mmol/l desferoxamine were dissolved
under nitrogen gas bubbling in ice-cold modified Krebs-Hepes (KH)
solution of KH buffer containing DETC and desferoxamine. Later, the
incubated CMH containing KH buffer solutions were then taken in 50
mg/l/ml). Cell lysates were incubated with SDS/
mercaptoethanol sample buffer at 100°C for 5 min. Protein samples
were separated by electrophoresis on a 4–20% SDS-PAGE gradient
gel, and proteins were transferred onto a polyvinylidene difluoroide
(0.22 µm) or nitrocellulose membrane. Membranes were then incu-
bated in a blocking buffer (Tris-buffered saline, 0.1% Tween 20, and
1% skimmed milk powder) for 1 h at room temperature followed by
incubation with primary antibodies against markers of endoplasmic
reticulum (ER) stress, overnight at 4°C in the blocking solution.
Antibodies against total and phosphorylated eukaryotic initiation
factor 2α (38 kDa, 1:400; Cell Signaling), 78 kDa glucose regulated
protein also known as Binding Protein (1:1,000; Cell Signaling),
C/EBP-homologous protein that inhibits C/EBP (27 kDa, 1:1,000;
Cell Signaling), and X box binding protein 1 (42 kDa, 1:1,000;
abcam) were used. The membranes were washed three times in
Tris-buffered saline with 0.1% Tween 20 and incubated for 1 h in the
blocking buffer with goat anti-rabbit IgG (1:5,000; Santa Cruz) or
goose anti-mouse IgG (1:5,000; Santa Cruz) conjugated to hors eradish
peroxidase. The final reaction products were visualized using en-
hanced chemiluminescence (SuperSignal West Pico; Pierce, Rock-
ford, IL) and developed on an X-ray film. For quantitative analysis,
the bands were scanned and band densities were quantified using
Imagel 1.3.1 software. The band intensities were normalized to that of
β-actin.

**RESULTS**

**Mitochondrial depolarization.** Treatment with BMS depo-
larized the mitochondria of VSM cells from SD rats indicated
by reduction of TMRE fluorescence compared with vehicle-
treated arteries (Fig. 1, A and B). The decrease in TMRE
fluorescence (percent change from baseline; 1.00) in VSM
4%,
17, in endothelium-denuded; 142 ± 19, n = 6 in iberiotoxin treated; and 142 ± 15,
17, in endothelium-denuded; 56 ± 2% n = 6 in iberiotoxin treated; and 52 ± 4%, n = 5 in
MnTBP treated) upon pressurization.

The BMS elicited a dose-dependent vasodilation in endo-
thelium denuded cerebral arteries with 8.1 ± 2.3%, 31.6 ±
2.1%, and 39.5 ± 3.2% relaxation in response to 10, 50, and
100 µmol/l, respectively (n = 6–14; P < 0.05) (Fig. 1C). Scavenging of the ROS with MnTBP did not affect vasodi-
lation to 50 µmol/l BMS (34.9 ± 6.7%, n = 5) confirming that
BMS-induced vasodilation was independent of ROS genera-
tion (Fig. 1D). Inhibition of BK$_{Ca}$ channels with iberiotoxin
decreased vasodilation to 50 µmol/l BMS in endothelium-
denuded arteries (17.7 ± 1.9%, n = 6; P < 0.05) (Fig. 1D),
suggesting that BK$_{Ca}$ channels mediate approximately one-half
of endothelium-independent vasodilation induced by BMS.

The mechanisms underlying the mitochondria mediated vaso-
dilation that is independent of BKCa channels have to be determined in future studies.

ROS measurements by ESR. A characteristic ESR signal with three peaks (12, 13) was detected in rat aortas from SD rats incubated with CMH and various drugs. The magnitude of this signal (arbitrary units normalized to dry weight of tissue) was greatly increased by stimulation with diazoxide (1.69 × 10^6 ± 0.6 × 10^6, n = 17; P < 0.05) and rotenone + antimycin A combination (1.96 × 10^6 ± 0.27 × 10^6, n = 8; P < 0.05) compared with vehicle-treated aortas (0.56 × 10^6 ± 0.6 × 10^6, n = 15), indicating increased generation of ROS. However, treatment with BMS failed to enhance the ROS compared with vehicle-treated aortic segments (0.49 × 10^6 ± 0.57 × 10^6, n = 15), confirming that BMS-induced mitochondrial depolarization did not result in generation of ROS in vascular wall (Fig. 2, A and B). Cotreatment with cell-permeable ROS scavenger SOD diminished baseline (vehicle treated) (0.35 × 10^6 ± 0.61 × 10^6, n = 6; P < 0.05) and diazoxide-induced (0.73 × 10^6 ± 0.55 × 10^6, n = 8; P < 0.05) increase in ESR signal amplitude confirming the generation of superoxide. However, ESR signal amplitude was unchanged by SOD and BMS cotreatment (0.33 × 10^6 ± 0.43 × 10^6, n = 7; P = not significant), suggesting that BMS did not promote superoxide generation in the vascular wall (Fig. 2B).

Mitochondrial ROS generation. We observed that MitoSOX fluorescence was uniform along the length of the arterial segment. Treatment with diazoxide, our positive control, increased MitoSOX fluorescence from the mitochondria of VSM cells, indicating increased mitochondrial ROS generation compared with vehicle-treated arteries (Fig. 2, C and D). In contrast, MitoSOX fluorescence was not changed by BMS when compared with vehicle-treated arteries. MitoSOX fluorescence in cerebral artery segments expressed as RFU was 71 ± 3 in response to 100 μmol/l diazoxide (n = 5; P < 0.05) and 40 ± 2 in response to 50 μmol/l BMS (n = 5) vs. 42 ± 1 in response to vehicle-treated arterial segments (n = 4). N represents the number of arterial segments.

Calcium spark generation. To determine whether mitochondrial depolarization activates calcium spark generation independent of ROS, arteries from SD rats were treated with ROS-independent BMS or ROS-dependent diazoxide and calcium spark frequency was assessed. Representative traces of calcium spark activity under basal (vehicle) and BMS-stimulated conditions are shown in Fig. 3A. Both diazoxide and BMS robustly increased the frequency of calcium sparks in VSM cells. Calcium spark frequency (in sparks/cell/s) were 0.08 ± 0.01 at basal levels (vehicle, n = 6 arteries) compared with 0.22 ± 0.03 in diazoxide (n = 8 arteries; P < 0.05) and...
0.26 ± 0.02 in BMS (n = 8 arteries; P < 0.05)-treated arteries (Fig. 3B). N represents the number of arterial segments, and 1 to 2 arterial segments from each rat was used for each experiment.

**ROS-independent effects of mitochondrial depolarization in ZO arteries.** Our previous studies demonstrated impaired diazoxide-induced mitochondrial depolarization dependent on ROS generation (35). To determine whether mitochondrial depolarization independent of ROS generation is altered by insulin resistance, we determined the responses to BMS in the cerebral arteries of Zucker rats. BMS-induced mitochondrial depolarization was diminished in VSM cells from ZO arteries compared with ZL arteries similar to diazoxide (Fig. 4A). Mitochondrial depolarization indicated by the decrease in TMRE fluorescence (in percentage) was 10.7 ± 2, 12.1 ± 0.5, and 18.5 ± 0.5 in ZL arteries in response to 25 μmol/l BMS, 50 μmol/l BMS, and 100 μmol/l diazoxide, respectively (n = 4, 1 artery per animal). In contrast, the decrease in TMRE fluorescence (in percentage) was 0.92 ± 0.7, 3.7 ± 1.5, and 9.2 ± 1.3 in ZO arteries in response to 25 μmol/l BMS, 50 μmol/l BMS, and 100 μmol/l diazoxide, respectively (Fig. 4B).

However, consistent with our previous observations (35) baseline TMRE fluorescence was similar in both ZO and ZL arteries (data not shown).

To determine whether impaired mitochondrial depolarization independent of ROS generation elicits diminished vasodilation in cerebral arteries from ZO rats, we compared the vasodilator responses to BMS with those to diazoxide in...
arteries from Zucker rats. BMS-induced vasodilation was diminished in endothelium denuded arteries from ZO arteries compared with ZL arteries similar to diazoxide (Fig. 5, A and B). BMS induced an increase in diameter (in \( \mu m \)) from 105 \( \pm \) 5.6 at baseline (preconstricted) to 156 \( \pm \) 10 in ZL arteries (\( n = \)

Fig. 3. Calcium sparks generation in response to BMS-191095 and diazoxide. A: selected fluorescence images of VSM cells loaded with 5 \( \mu mol/l \) Fluo-4AM from time series image stacks are shown. Region of interest sites of calcium sparks are shown (arrow heads) from vehicle (DMSO) and BMS (50 \( \mu mol/l \))-treated endothelium-denuded cerebral arteries from SD rats. B: bar graph showing the cumulative data of calcium sparks frequency in response to vehicle, diazoxide, and BMS. *Significant difference in response vs. vehicle (\( P < 0.05 \)).

Fig. 4. Mitochondrial depolarization in Zucker rat arteries. A: representative images of VSM cells loaded with TMRE are shown from BMS (50 \( \mu mol/l \)) and diazoxide (100 \( \mu mol/l \)) treated cerebral arteries of Zucker lean (ZL) and Zucker obese (ZO) rats. The color range from red to yellow indicates the range of TMRE fluorescence intensity from fully polarized (red) to depolarized (yellow) mitochondria. BMS and diazoxide elicited robust mitochondrial depolarization in ZL arteries indicated by greater yellow relative to red. In contrast, BMS and diazoxide elicited relatively diminished mitochondrial depolarization in ZO arteries compared with ZL arteries, indicated by less yellow relative to red suggestive of impaired mitochondrial depolarization. B: bar graph showing cumulative data of percent decrease in TMRE fluorescence in response to BMS (25 and 50 \( \mu mol/l \)) and diazoxide (100 \( \mu mol/l \)) from baseline before the application of drugs. Data are means \( \pm \) SE of 6–14 experiments. *Significant difference in response to corresponding treatment in ZL arteries (\( P < 0.05 \)). An \( n \) represents the number of arterial segments and single arterial segment per experiment from each rat was used.
diameters increases in ZO arteries were significantly decreased when compared with responses in ZL arteries ($P < 0.05$; Fig. 5, A and B). $N$ represents the number of arterial segments and single arterial segment from each rat was used for the experiment.

To determine whether mitochondrial depolarization independent of ROS generation elicits diminished calcium spark generation in VSM cells of cerebral arteries from ZO rats, we compared the calcium spark generation in response to BMS with that of diazoxide in arteries from Zucker rats. Both BMS and diazoxide promoted increased frequency of calcium sparks in VSM cells from ZL arteries. Calcium spark frequency increased from $0.12 \pm 0.01$ at basal levels (vehicle, $n = 9$) to $0.2 \pm 0.01$ in diazoxide ($n = 9$; $P < 0.05$) and $0.24 \pm 0.02$ in BMS ($n = 4$; $P < 0.05$)-treated ZL arteries (Fig. 5C). In contrast, calcium spark frequency did not significantly increase in VSM cells from ZO arteries. Calcium spark frequency was $0.12 \pm 0.01$ at basal levels ($n = 9$), $0.134 \pm 0.03$ following diazoxide ($n = 8$) and $0.124 \pm 0.01$ in response to BMS ($n = 5$) in ZO arteries (Fig. 5C). $N$ represents the number of arterial segments and single arterial segment from each rat was used for the experiment. Thus, similar to diazoxide, BMS-induced mitochondrial depolarization failed to elicit an increase in calcium spark generation in ZO arteries with impaired mitoK_{ATP} channel function.

$ZO$ arteries exhibit ER stress. Immunoblot analysis of various markers of ER stress showed increased phosphorylated and total eukaryotic initiation factor 2 ratio and protein levels of binding protein/78 kDa glucose regulated protein, C/EBP-homologous protein that inhibits C/EBP, and X box binding protein 1 in ZO arteries compared with ZL arteries (Fig. 6).

DISCUSSION

The novel findings from our studies establish an important new concept that both ROS-independent and ROS-dependent mechanisms can mediate the mitochondrial depolarization-induced generation of calcium sparks in cerebral VSM and subsequent vasodilation. First, VSM mitochondrial depolarization induced by BMS, which is not associated with ROS generation, elicits enhanced calcium spark activity and vasodilation of endothelium-denuded cerebral arteries. Second, both ROS-independent and -dependent vasodilation induced by the mitochondrial depolarization is mediated by activation of BK_{Ca} channels in the VSM. Third, vascular mitochondrial dysfunction accompanying insulin resistance displays impaired mitochondrial depolarization, reduced calcium spark activity, and associated attenuated vasodilation to both diazoxide and BMS, thereby demonstrating shared signaling pathways. Thus our findings demonstrate that 1) increased calcium spark frequency subsequent to mitochondrial depolarization can occur without ROS production and 2) pathways linking and/or impairing mitochondrial activation, calcium sparks, and vasodilation in health and disease are diverse.

Mitochondrial depolarization not accompanied by ROS generation. Studies in our laboratory and others have demonstrated the role of activation of mitoK_{ATP} channels in mediating the effects of diazoxide (39, 51, 59) and BMS (2, 6, 24, 26, 27, 34, 36, 38, 42, 45) in isolated mitochondria, neurons, endothelium, vasculature, and cardiomyocytes. Scavenging of ROS failed to reduce BMS-induced vasodilation in endothel-
Cerebral arteries from Zucker rats. Fig. 6. Increased levels of endoplasmic reticulum (ER) stress markers in cerebral arteries of ZL and ZO rats are shown. C/EBP (CHOP), and X box binding protein 1 (XBP1) in the cellular lysates of Zucker Lean Zucker Obese

\[
\text{Immunoband Intensity Normalized to } \beta\text{-actin}
\]

\begin{tabular}{|c|c|}
\hline
\textbf{Zucker Lean} & \textbf{Zucker Obese} \\
\hline
\textbf{p-eIF2} & * \\
\hline
\textbf{BiP/GRP78} & * \\
\hline
\textbf{CHOP} & * \\
\hline
\textbf{XBP1} & * \\
\hline
\textbf{\(\beta\)-actin} & * \\
\hline
\end{tabular}

\[n = 6 \text{ animals per group. IF2}, \text{ initiation factor 2a.}\]

\[\text{IF2, initiation factor 2a.}\]

\[p<0.05; n = 6 \text{ animals per group. IF2a, initiation factor 2a.}\]

Understanding on the species, tissues, and cell types studied, we confirmed the inability of BMS to promote ROS production in VSM cells by using complementary methods of ESR spectroscopy and fluorescence microscopy. Consistent with a previous report by Jaggar et al. (59), we observed increased mitochondrial and cellular ROS generation in response to diazoxide in our Mitosox fluorescence and ESR experiments.

\[BK_{Ca} \text{ channels and calcium sparks. } BK_{Ca} \text{ channels have been shown by us (34) and others (59) to mediate part of the mitochondria-induced vasodilation. The mechanisms contributing to the remaining mitochondria-mediated vasodilation are still not completely known. Inhibition of } BK_{Ca} \text{ channels diminished vasodilation to } BMS \text{ in endothelium-denuded arteries, indicating that part of the mitochondria-mediated vasodilation was } BK_{Ca} \text{ channel dependent. This finding is consistent with previous observations from us and others, which showed that diazoxide-induced vasodilation partly involved } BK_{Ca} \text{ channels (34, 36, 59). Interestingly, previous studies in our laboratory failed to detect the activation of whole cell } K^+ \text{ currents in isolated VSM cells in response to } BMS-191095 \text{ (42). It is possible that } BK_{Ca} \text{ channels were more readily activated in the isolated pressurized arteries used in our study than those in patch clamp experiments in which VSM cells were isolated by enzymatic digestion. Myogenic response in cerebral arteries has been shown to be regulated by } BK_{Ca} \text{ channels through hyperpolarization of VSM membrane potential and inactivation of voltage-gated calcium channels (25, 30, 46). Generation of calcium sparks is the primary mechanism of activation of cerebral artery } BK_{Ca} \text{ channels; however, the exact mechanisms underlying the increased frequency of calcium sparks in VSM cells are not fully understood. Recent evidence has implicated mitochondrial depolarization and subsequent ROS formation in increased generation of calcium sparks via redox regulation of RyR channels (59). Consistent with previous reports, we observed that mitochondrial depolarization by diazoxide enhanced generation of calcium sparks in VSM cells of endothelium-denuded arteries. In addition, we observed that mitochondrial depolarization by } BMS \text{ was capable of enhancing the generation of calcium sparks in VSM cells independent of } ROS. Thus } BMS \text{ and diazoxide share many vascular actions including depolarization of } VSM \text{ mitochondria, increased frequency of calcium sparks, activation of } BK_{Ca} \text{ channels, and promotion of vasodilation in cerebral arteries (Fig. 6). However, the exact mechanism by which mitochondrial depolarization independent of } ROS \text{ activates calcium sparks is not clear and needs further investigation. Many studies have identified close physical and functional communication between ER/ sarcoplasmic reticulum (SR) and mitochondria, which facilitates the transfer of ions, nucleotides, radicals, and yet unknown factors that aid in the bidirectional regulation of organelle (15, 41). We speculated that close SR-mitochondrial communication may facilitate mitochondrial depolarization to activate RyR through a mechanism that involves electrical coupling of the organelle (Fig. 7). Further studies are needed to identify the nature of interorganelle communication leading to the increased frequency of calcium sparks.}\]

\[ROS\text{-independent mitochondria mediated vasodilation in insulin resistance. We further evaluated the } ROS\text{-independent effects of mitochondrial depolarization by comparing the } BMS \text{ and diazoxide induced mitochondrial depolarization, calcium spark generation, and vasodilation in cerebral arteries of } ZO\]

\[H500 \text{ SUPEROXIDE-INDEPENDENT MITOCHONDRIA MEDIATED VASODILATION}\]
by mitochondrial KATP channels leading to impaired generation of mitochondrial ROS was found to be the cause of impaired vasodilation in ZO arteries. It is apparent that impaired activation of mitochondrial K<sub>ATP</sub> channels by two mechanistically different activators resulted in identical deficits in vasodilation in ZO arteries, suggesting that shared signaling pathways mediate ROS-dependent and ROS-independent effects of mitochondrial depolarization. Furthermore, findings from ZO and ZL arteries validate the hypothesis that ROS-independent actions of BMS similar to ROS-dependent generation of calcium sparks rely on mitochondria-SR functional coupling.

Cerebral arteries from ZO rats also displayed increased levels of ER stress markers compared with that of ZL arteries. Many studies have demonstrated impaired calcium filling of ER/SR and abnormal calcium release from intracellular stores as a result of ER stress (3, 4). Although we have not measured ER/SR calcium dynamics in the arteries, ER stress in ZO arteries may likely contribute to impaired calcium spark generation. However, further studies are needed to establish this potential mechanism of impaired mitochondrial-mediated calcium spark generation in ZO arteries.

Limitations. Our studies used only pharmacological approaches to promote mitochondrial depolarization without ROS formation, and this approach reflects the stage of development of this field. Nonetheless, there are advantages to this approach. For example, we and others have extensively studied diazoxide and BMS in a variety of cell types and experimental conditions and we are aware of potential nonspecific effects. Diazoxide has been shown to exhibit some nontarget and nonspecific mitochondrial effects, especially at higher doses than those we used, and, despite thorough investigations, we are unaware of BMS causing any effects other than activation of mitochondrial K<sub>ATP</sub> channels. Many physiological/pathological stimuli (hypoxia) (28, 53) that promote mitochondrial depolarization are similarly plagued by multiple sites of action. Unfortunately, there are no other pharmacological agents currently available to induce mitochondrial depolarization not accompanied by ROS generation other than BMS. In addition, the present studies provide evidence supporting ROS-independent communication between mitochondria and SR; however, the nature of this communication needs further investigation.
REFERENCES


AUTHOR CONTRIBUTIONS


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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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