SUPPLEMENT MATERIAL

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

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MATERIALS AND METHODS

Fluorescence Confocal Microscopy.

1. Mitochondrial membrane potential was determined by using rhodamine 123, (λex: 488 nm, λem: >505 nm long pass filter). 2. MitoSOX (λex: 405 nm, λem: >560 nm long pass filter) was used to measure mitochondrial ROS, specifically superoxide, based on the method reported by Robinson et al [1]. Several previous studies measured the MitoSOX fluorescence with λex: 488 nm, λem: >560 nm. However, recent studies by Robinson et al [1] provided evidence that MitoSOX fluorescence, when the fluoroprobe is excited at a wave length of λex: 405 nm and emission is captured at a wavelgth of λem: >560 nm, is specific for measurement of superoxide. 3. Fluo-4 AM (λex: 488 nm, λem: 505 nm long pass filter) was used to study $[Ca^{2+}]_i$. 4. 4-amino-5-methylamino- 2',7'-difluorofluorescein diacetate (DAF-FM, λex: 488 nm, λem: >505 nm long pass filter) was used to measure NO. The specificity of NO measurements was established by inhibition of NO generation by NOS with L-NAME.

Stock solutions of fluoroprobes were prepared in DMSO. The CMVECs were loaded in the dark with a 1:1 mixture of 5μmol/L fluo-4 AM or 5μmol/L DAF-FM and 20% (w/v) pluronic F-127 diluted in PBS containing glucose (1g/L) for 30 min at room temperature. For $[Ca^{2+}]_i$ measurements in arterial endothelial cells, freshly isolated rat posterior cerebral arteries were cut longitudinally, and pinned to a Sylgard block with the endothelium facing up as previously described. [2] Subsequently, fluo-4 AM was loaded, as described above, with the arterial segment pinned to the sylgard block, mounted in a vessel chamber with the endothelium facing the cover slip, and the objective on the inverted microscope. Cells were washed and incubated for a further 30 min to allow for complete de-esterification of intracellular AM esters and diacetates. Rhodamine 123 (5 μg/ml) or MitoSOX (5μmol/L) solutions were prepared in phenol-free

DMEM and CMVECs were loaded with fluoroprobes for 15 min at 37^{o}C. Confocal microscopy was performed using a laser scanning confocal system (LSM 510 and 7 Live; Zeiss, Jena, Germany) attached to an inverted microscope with a Zeiss C-Apochromat 63X /NA 1.2 water immersion objective. MitoSOX fluorescence was determined by Leica SP2 AOB laser confocal microscope. Fluorescence images were acquired before and after application of vehicle (dimethyl sulfoxide, DMSO) or BMS (50 μmol/L or 100 μmol/L) or diazoxide (100 μmol/L) for rhodamine 123, fluo-4 AM, and MitoSOX whereas DAF-FM images were acquired with or without a 30 min L-NAME pretreatment of cells. Imaging conditions such as gain levels and laser power were held constant. Offline analysis of images to determine the average pixel intensity of endothelial cells in each field (*n*=20-30) was performed using ImageJ software (National Institute of Health, Bethesda, MD) and the results were expressed in relative fluorescence units (RFUs) expressed as % change from the baseline images prior to administration of vehicle or BMS or diazoxide. For each coverslip with cells, 10-15 randomly selected fields containing 15-20 cells were imaged following vehicle or BMS or diazoxide treatment. Average fluorescence intensity of all the cells in the image was determined first followed by determination of the average of all images acquired from each cover slip. The 'n' value represents average fluorescence intensity of all the cells from 'n' number of cover slips that were included with each treatment.

NO Measurements

The NO measurements were performed using electron spin resonance (ESR) spectroscopy using the previously published methods [3, 4]

Colloid $Fe(DETC)_2$ spin trapping. DETC (7.2 mg/10ml) and $FeSO_4$ 7H2O (4.5mg/10ml) were separately dissolved under argon gas bubbling in two 10ml volumes of ice-

cold modified Krebs–Hepes buffer (K-H) containing (in mmol/L) 99.01 NaCl, 4.69 KCl, 1.87 CaCl2, 1.20 MgSO4, 25 NaHCO3, 1.03 K2HPO4, 20 sodium-HEPES, and 11.1 d-glucose, pH 7.35. These were rapidly mixed to obtain a pale yellow-brown opalescent colloid $Fe(DETC)_2$ solution (0.4mmol/L) which was used immediately. Aortas were placed separately in 12-well plates, each filled with 1 ml K-H buffer, in the presence and absence vehicle (DMSO), BMS (50 μmol/L), diazoxide (100 μmol/L), or spermine NONOate [10 μmol/L, N-(2-Aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine] to stimulate NOS. Colloid $Fe(DETC)_2$ was then added to each well (0.4 mmol/L)) and incubated at 37 °C for 90 min. Separate experiments were completed to examine the effects of NOS inhibition by addition of NG-nitro-L-arginine methyl ester (L-NAME) to the incubation medium (final concentration 1mM). Incubations were also performed with colloid Fe(DETC)2 alone (without aortas) to correct for any background signals.

ESR spectroscopy. After incubation, the aortic segments were removed and frozen in liquid nitrogen, and placed in the middle of a column of K-H buffer as follow: The needle-end was cut from a 1 ml syringe barrel, the plunger retracted 1.5 cm from the cut end, and aortic segments were placed into the syringe barrel, which was then filled with K-H buffer and frozen in liquid nitrogen. After freezing, the syringe was removed from the liquid nitrogen and warmed for 5 s; the plunger was then removed, and the remaining barrel was filled with K-H buffer and frozen in liquid nitrogen. The syringe was then re-warmed for 5 s, and the syringe plunger was used to push the frozen column out of the syringe barrel directly into a finger dewar (Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany) containing liquid nitrogen. The ESR spectra were obtained using a benchtop X-band EMX series ESR spectrometer (Bruker Biospin GmbH, Karlsruhe, Germany) using a high sensitivity SHQ microwave cavity in a finger dewar

filled with liquid nitrogen. The ESR spectrometer settings were as follows: microwave power 40 mW, modulation amplitude 8 G center-field 2.03 g, sweep width 80 G, conversion time 80 msec, time constant 20 msec, sweep time 10.24 s, with 60 scans. Signals were quantified by measuring the total amplitude after correction of baseline. The amount of NO was determined by measuring the total amplitude of the ESR signal for each treatment and comparing it with the ESR signal amplitude of the signal following NO-donor spermine NONOate. Finally, diazoxide and BMS induced NO production in aortas are expressed as % change from basal levels (vehicle treated).

A characteristic NO-$Fe(DETC)_2$ signal with three peaks (g value approximately 2.035) was detected in rat aortas incubated with $Fe(DETC)_2$ and various drugs. The amount of NO was determined by measuring the total amplitude of the ESR signal for each treatment and comparing with the ESR signal amplitude of the signal following NO-donor spermine NONOate. Finally, diazoxide and BMS induced NO production in aortas are expressed as % change from basal levels (vehicle treated) (Figure II). Furthermore, the ESR signals from BMS or diazoxide-stimulated aortas were abolished after addition of the NOS inhibitor, L-NAME.

REFERENCES

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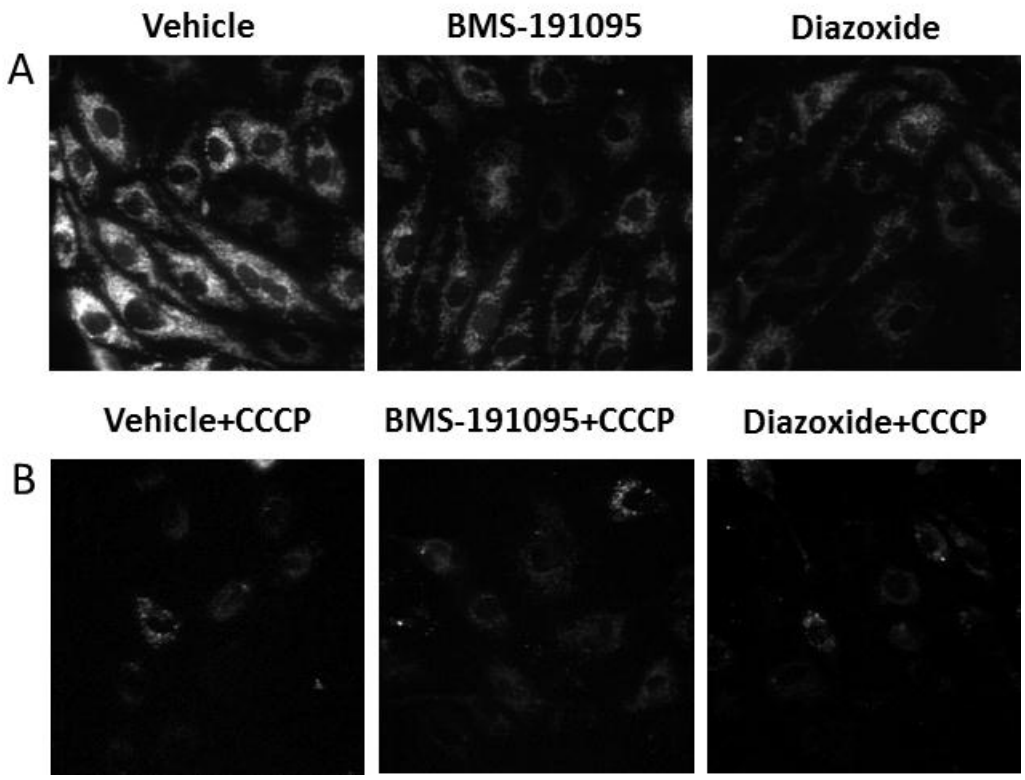
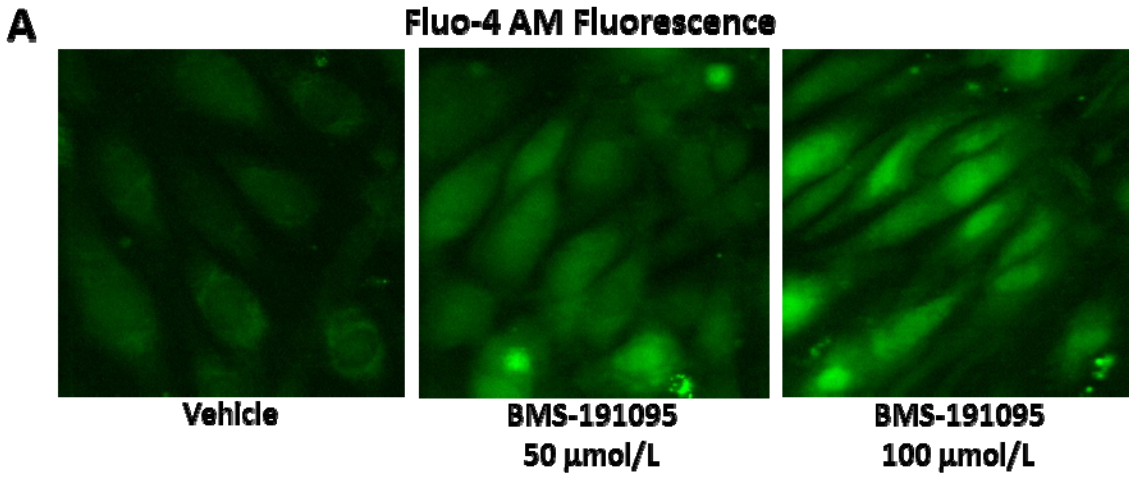


Figure I. Fluorescence images of CMVECs loaded with rhodamine 123, a mitochondrial membraine potential sensitive fluoroprobe and treated with vehicle (DMSO) or 50 μmol/L BMS followed by depolarization with carbonyl cyanide 3-chlorophenylhydrazone (CCCP, a protonophore and uncoupler of oxidative phosphorylation in mitochondria). Treatment with 5 μmol/L CCCP abolished the rhodamine 123 fluorescence confirming the localization of the fluoroprobe to mitochondria and also specificity of changes in the rhodamine 123 fluorescence to changes in mitochondrial membrane potential in response to BMS and diazoxide.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Fluo-4 AM Fluorescence
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BMS-191095
50 µmol/L
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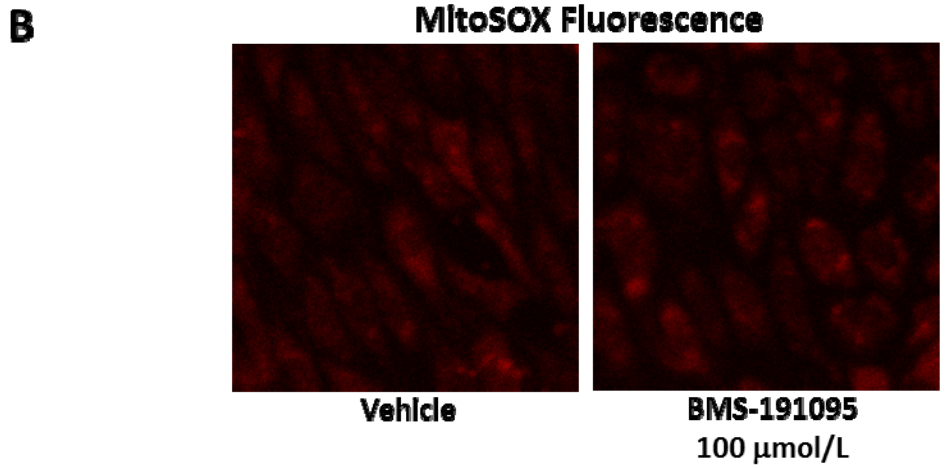
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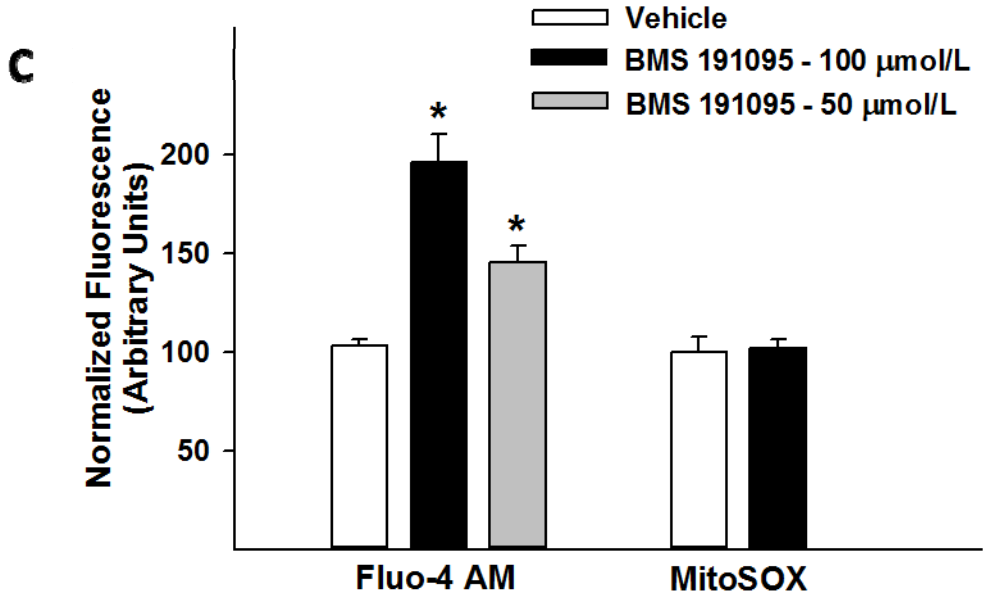
C
Vehicle
BMS 191095 - 100 µmol/L
BMS 191095 - 50 µmol/L
Normalized Fluorescence
(Arbitrary Units)
200
150
100
50
*
*
Fluo-4 AM
MitoSOX

Figure II. Fluorescence images of CMVECs loaded with various fluorescent probes and treated with vehicle (DMSO) or 50 μmol/L and 100 μmol/L BMS. Fluorescence images of CMVECs loaded with fluo-4 AM, fluoroprobe for calcium, are shown in panel A. Fluorescence images of MitoSOX, a mitochondrial ROS sensitive dye, are shown in panel B. The cumulative data of fluorescence intensity from the images were determined and plotted as a bar graph for Fluo-4 AM and MitoSOX in panel C. (*****) indicates significant difference in response versus vehicle for corresponding fluorescent probes ($p<0.05$). BMS promoted dose-dependent increase in fluo-4 AM fluorescence compared with vehicle treated cells indicating elevation of intracellular Ca^{2+}. However, BMS treatment even at 100 μmol/L concentration fail to increase MitoSOX fluorescence in endothelial cells compared with vehicle treated cells.

Thus, it appears that even though diazoxide and 100 μmol/L BMS elevate intracellular Ca^{2+} to a similar degree, diazoxide promoted mitochondrial ROS elevation in endothelial cells whereas BMS did not. Mitochondrial ROS generation may be triggered by both mitochondrial Ca^{2+} -dependent and -independent pathways. This elevation of mitochondrial Ca^{2+} in response to increase in intracellular Ca^{2+} by BMS may not be sufficient to induce detectable mitochondrial ROS generation in endothelial cells. Alternatively, BMS may have a different impact on mitochondrial Ca^{2+} uptake compared with diazoxide, which may explain the differences in the mitochondrial ROS generation in BMS and diazoxide treated cells.