SUPPLEMENTAL MATERIAL

Post-ischemic Reperfusion Causes Smooth Muscle Calcium Sensitization and Vasoconstriction of Parenchymal Arterioles

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Supplemental Methods

Measurement of Smooth Muscle Calcium using Fura 2

Fura 2-AM (1 mmol/L stock dissolved in anhydrous dimethylsulfoxide, DMSO) was premixed with an equal volume of 25% pluronic acid dissolved in DMSO and then diluted in aerated physiologic saline solution (PSS) to yield a final concentration of 5μmol/L. Each vessel segment was cannulated in an arteriograph, pressurized to 10 mm Hg, and equilibrated at 37 °C for 10–15 min. The arteriolar segments were then incubated in the Fura 2-AM/PSS loading solution at room temperature in the dark for 60 minutes. This solution was changed for freshly aerated loading solution after 30 minutes into the incubation period to maintain pH = 7.4. Fura 2-loaded arterioles were washed two to three times with PSS and then continuously superfused at 3 ml/min with oxygenated PSS (10% O_2-5% CO_2-balanced N_2) at 37 °C. All experimental protocols were started after an additional 15-min equilibration period at 10 mm Hg to allow intracellular de-esterification of Fura 2-AM. Fura 2 fluorescence was measured using a photomultiplier system (IonOptix) in which background-corrected ratios of 510-nm emission were obtained at a sampling rate of 5 Hz from arteries alternately excited at 340 and 380 nm.

For the Fura 2 calibration procedure, the following solutions were used. Calcium-free calibration solution was composed of (in mmol/L): KCl 140.0, NaCl 20.0, HEPES 5.0, EGTA 5.0, $MgCl_2$ 1.0, 5 μmol/L nigericin, and 10 μmol/L ionomycin. Calcium-containing calibration solution was composed of (mmol/L): KCl 140.0, NaCl 20.0, HEPES 5.0, $MgCl_2$ 1.0, and $CaCl_2$ 10.0. Both solutions were adjusted to pH 7.15 at 37 °C with KOH. Ionomycin and nigericin were purchased from A.G. Scientific (San Diego, CA). Fura 2-AM and pluronic acid were purchased from Life Technologies (Grand Island, NY). Fura 2-AM was dissolved in dehydrated DMSO as a 1 mmol/L stock solution and frozen at -20 °C until use.

The concentration of intracellular calcium in vascular smooth muscle ($[Ca^{2+}]_i$) in intact (not permeabilized) arterioles was calculated using the following equation: $[Ca^{2+}]_i = Kd\beta(R - Rmin)/(Rmax - R)$, where K_d is the dissociation constant of Fura 2, R is experimentally measured ratio (340/380 nm) of fluorescence intensities, R_{min} is the ratio in the absence of $[Ca^{2+}]_i$, R_{max} is the ratio at Ca^{2+} saturation, and β is the ratio of the fluorescence intensities at 380-nm excitation wavelength at R_{min} and R_{max}. R_{min}, R_{max}, and β were determined using an in situ calibration procedure with nigericin (5 μmol/L) and ionomycin (10 μmol/L). These values were then used to convert the ratio values into a $[Ca^{2+}]_i$. The calibration procedure resulted in a β value of 4.43 ± 0.28 and R_{min} and R_{max} values of 0.55 ± 0.02 and 4.12 ± 0.26. The K_d was 282 nmol/L, as determined by using in situ titration of Ca^{2+} in Fura 2-loaded posterior cerebral arteries.[1] Arterial diameter, pressure, and ratio values were recorded using an Ion Wizard data acquisition program.

Measurement of sensitivity of parenchymal arterioles to calcium using S. aureus α-toxin

HEPES-buffered PSS contained (in mmol/L): NaCl 142.0, KCl 4.7, $MgSO_4$ 1.17, EDTA 0.5, $CaCl_2$ 2.79, HEPES 10.0, KH_2PO_4 1.2, glucose 5.5. pH was adjusted by 10 N NaOH to 7.4 at 37 °C. Relaxing solution contained (in mmol/L): potassium methanesulfonate 63.6, $MgCl_2$ 2.0, Mg-ATP 4.5, EGTA 2.0, phosphocreatine 10.0, and piperazine-N,N'-bis(2-ethanesulfonic acid) 30.0. *Relaxing solution* also contained 1.0 µmol/L carbonylcyanide p-trifluromethoxyphenyl-hydrazone, a mitochondrial blocker, and 1.0 µmol/L leupeptin, a protease inhibitor. pH was adjusted to 7.1 with 8 N KOH. The composition of the *activating solution* was similar to that of the relaxing solution, except it contained 10 mmol/L EGTA and 10 µmol/L GTP. The amount of $CaCl_2$ needed to yield the desired free ionic concentration of calcium in the activating solution was calculated by a web-based program Webmaxc Standard (http://www.stanford.edu/~cpatton/webmaxcS.htm). Ionic strength was kept at 200 mmol/L by adjusting the concentration of potassium methanesulfonate.

Data Calculations

Myogenic tone was calculated as a percent decrease in diameter from the fully relaxed diameter in calcium-free PSS with diltiazem or in calcium-free relaxing solution by the equation: $(1-(\varphi_{tone}/\varphi_{zero}))*100\%$; where φ_{tone} = inner diameter of vessel with tone and φ_{zero}= inner diameter in calcium-free PSS with diltiazem. Percent sensitivity to calcium was calculated from the equation: $((\varphi_{calcium}-\varphi_{start})/(\varphi_{6.0} - \varphi_{start}))$ x 100% where $\varphi_{7.00}$ is the inner diameter at –log 7.0 calcium, φ_{start} is diameter prior to giving the first concentration of calcium and $\varphi_{6.00}$ is the inner diameter at –log 6.0 calcium which was the highest concentration of calcium. Percent dilation to 9-phenanthrol, Y27632 and Gö6976 was calculated from the equation: $((\varphi_{start}-\varphi_{drug})/(\varphi_{start} - \varphi_{zero\ Ca}))$ x 100% where φ_{drug} is the inner diameter at a specific concentration of drug, φ_{start} is the inner diameter prior to giving the first concentration of drug and $\varphi_{zero\ Ca}$ is the diameter fully relaxed in zero calcium PSS. Effective concentrations that produced half maximal dilation (EC_{50}) were determined from individual plots of concentration-response curves then averaged per group.

Reference:

1. Knot HJ, Nelson MT. Regulation of arterial diameter and wall [Ca^{2+}] in cerebral arteries of rat by membrane potential and intravascular pressure. *J Physiol.* 1998; 508:199-209.

Supplemental Table I. Physiological Parameters of Sham control and MCAO Animals.

	Sham (n=6)	**MCAO (n=7)**
CBF % Drop vs. Basal	427 ± 46	340 ± 10
CBF % Reperfusion vs. Basal	----	-64.5 ± 4.3
Arterial blood gases and pH	----	11.4 ± 29.8
Start		
pH	7.49 ± 0.01	7.46 ± 0.02
$PaCO_2$ (mm Hg)	32.3 ± 1.9	39.2 ± 1.3
PaO_2 (mm Hg)	106 ± 9	117 ± 11
Middle		
pH	7.45 ± 0.02	7.46 ± 0.01
$PaCO_2$ (mm Hg)	39.4 ± 1.5	39.6 ± 1.4
PaO_2 (mm Hg)	95 ± 4	102 ± 10
End		
pH	7.45 ± 0.02	7.47 ± 0.01
$PaCO_2$ (mm Hg)	40.2 ± 2.2	39.1 ± 1.7
PaO_2 (mm Hg)	102 ± 8	117 ± 16