Acute Exposure to Low Glucose Rapidly Induces Endothelial Dysfunction and Mitochondrial Oxidative Stress: Role for AMP Kinase

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**Methods**

**Materials**

Culture medium M199, fetal bovine serum (FBS), penicillin/streptomycin/Glutamine solution, MitoSox™ Red, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), hydroethidine (HE), TMRM, Hanks' Balanced Salt Solution (HBSS, 1X) and Dulbecco's Phosphate-Buffered Saline (DPBS, 1X) were purchased from Invitrogen (Carlsbad, CA). Endothelial mitogen was obtained from Biomedical Technologies (Stoughton, MA). Antibodies against AMPK (anti-AMPK) and phosphorylated-AMPK (anti-pAMPK) were purchased from Cell Signaling Technology (Danvers, MA). Tetrahydrobiopterin (BH₄) (Schircks Laboratories, Jona, Switzerland) was generously provided by Jeanette Vasquez-Vivar, PhD (Medical College of Wisconsin). Spermine NONOate was obtained from Cayman Chemicals (Ann Arbor, Michigan). All other chemicals including vascular endothelial growth factor (VEGF), AICAR (5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside), metformin (1,1-dimethylbiguanide hydrochloride), L-carnitine, oxypurinol, TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy), acetylcholine chloride, endothelin-1, carbonyl cyanide m-chlorophenylhydrazone (CCCP), Nω-Nitro-arginine methyl ester (L-NAME), palmitate, and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). NADPH oxidase inhibitor gp91 ds-tat and control gp91 scrambled peptide were produced and obtained from the Blood Center of Wisconsin’s Protein Chemistry Core.

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) from passages 4 to 7 were used for all experiments. These cells were cultured in 1% gelatin-coated Petri dish with M199 medium supplemented with 20% FBS, 50 µg/ml EC-mitogen, 100 µg/ml heparin, and 100 U/ml Penicillin/100 µg/ml Streptomycin/2 mM Glutamine. The glucose level in
M199 medium was adjusted to 5 mmol/L. Cells were allowed to reach >90% confluence before doing experiments.

**NO Measurements**

HUVECs were incubated in either 5 mM (normal glucose, NG) or 2.2 mM (low glucose, LG) medium for 1 hour with or without concomitant exposure to TEMPOL (1 mM), metformin (10 µM), AICAR (100 µM), L-NAME (1 mM) or BH4 (100 µM). Following incubation, HUVECs were exposed for 30 minutes to L-arginine (25 µmol/L) in either the presence or absence of VEGF stimulation (50 ng/ml).

Basal and stimulated NO produced by HUVECs under all conditions were measured using a Model 280 NO Analyzer (Sievers Instruments, Boulder, CO, USA). All results were normalized with cellular protein level using DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Measurements of NO concentrations were also made following 1 hour of exposure to glucose concentrations of [in mg/dL (mM)] 80 (4.4), 70 (3.9), 60 (3.3), and 50 (2.8) to determine the gradient effect of lowering glucose concentrations on endothelial cell NO generation.

Measurements of eNOS activity were performed under normal and low glucose conditions using a NOS Activity Assay Kit (Cayman Chemical, Michigan, USA). Full details on this method are included in the online supplement.

**Measurement of eNOS activity**

eNOS activity of HUVECs under NG and LG pre-treatment was determined by using NOS Activity Assay Kit (Cayman Chemical, Michigan, USA; Catalog No. 781001), measuring the rate of conversion to L-[³H]-citrulline from L-[³H]-arginine. HUVECs on passage 4 were seeded on 100 x 15 mm petri dishes and grown to >95 confluence. The
cells were incubated with either NG (90 mg/dL) or LG (40 mg/dL) medium for 1 hour,. [3H]-Arginine monohydrochloride (45 – 70 Ci/mmol) was purchased from Perkin Elmer (Catalog No. NET1123250 UC). nNOS and L-NNA supplied with the kit were used as positive and background control, respectively. Radioactivity of eluted [3H]-citrulline was quantified using a liquid scintillation counter. Data is expressed as fmol citrulline/mg protein/minute. Normalization to protein content was performed following testing by lysing cells in MOPS lysis buffer containing (in mM) 20 MOPS, 2 EGTA, 5 EDTA, 30 Sodium Fluoride, 40 β-glycerophosphate, 10 sodium pyrophosphate, 2 sodium orthovanadate, and 0.5% NP (pH 7.4). A protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) were added to lysis buffer in the ratio of 100 µl:10 ml prior to use. The lysate was freeze-dried at -80°C for overnight and thaw on ice the next day. After centrifuging at 14000 rpm for 15 min at 4°C, the supernatant was collected and the protein concentration in the supernatant was measured using DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

**Measurement of Mitochondrial Superoxide Production**

Following 1 hour of incubation in either NG or LG medium, HUVECs were exposed to 10 µM MitoSox™ red (a specific fluorescent probe for mitochondrial superoxide) and were incubated at 37°C for 30 min. Cells were subsequently washed twice with DPBS and fluorescence intensity was measured by fluorescent microscopy. (Ex/Em 510/ 580 nm, Eclipse TE 200, Nikon, Japan). Due to known issues with the specificity of hydroethidium for superoxide in intra-cellular work,¹ we measured MitoSox™ fluorescence under NG and LG conditions in the presence and absence of PEG-superoxide dismutase (SOD, 150 U/mL) to verify the fluorescence originated from superoxide production.
Mitochondrial superoxide production was also measured following 1 hour of exposure to glucose concentrations of [in mg/dL (mM)] 80 (4.4), 70 (3.9), 60 (3.3), and 50 (2.8) to determine the gradient effect of lowering glucose concentrations on endothelial cell mitochondrial superoxide generation.

Measurements of mitochondrial superoxide production were also made in the setting of palmitate (0.1 mmol/L) with and without concomitant L-carnitine (50 µM) under LG (50 mg/dL) conditions. A similar 1 hour exposure time was used for these experiments.

**Measurement of Total Superoxide Levels**

HUVECs were cultured in 60 mm or 100mm Ф Petri dish until confluent. After washing once with DPBS, the cells were randomly divided into two groups and treated with either NG (90 mg/dL) or LG (40 mg/dL) medium for 60 minutes. 10 µM of hydrehidium (HE) with 50 ng/ml VEGF in a 2% FBS medium was subsequently added to the cells. The cells were incubated at 37C for 20 minutes, washed with DPBS, and scraped in 1 ml DPBS. The cell suspension solution was pelleted 3000 rpm for 5 minutes and stored at -80C until HE measurement measurement by HPLC.

**Measurement of Hydrogen Peroxide Levels**

HUVECs were cultured as above. The cells were first treated with either NG (90 mg/dL) or LG (40 mg/dL) cell culture medium for 1 hour with or without pre-treatment of PEG-catalase (500 units/ml) or Mito-TEMPOL (1mM)(Sigma). One hour later, 10 µM of 2’7’-dichlorofluorescein-diacetate (DCFH-DA, Sigma) was added to each well and incubated at 37°C for 30 min. After incubation, the cells were washed twice and the DCF
fluorescence intensity measured by microplate reader (SpectraMax Gemini EM, Molecular Devices) with Ex/Em wavelengths of 490/530 nm.

**Measurement of Mitochondrial Membrane Potential**

HUVECs were exposed to NG or LG conditions for one hour. A portion of the cells exposed to low glucose were concomitantly treated with CCCP (1 μM) during this 1 hour period as a negative control. Cells were subsequently washed twice and incubated at 37°C with 2 μM JC-1 for 20 minutes. JC-1 red (Ex/Em 485/590) and green (Ex/Em 485/528) fluorescent intensity were measured using a fluorescent plate reader (Synergy HT, BIO-TEK Instruments, Vermont, USA).

To help determine the effects of NO on mitochondrial membrane potential, HUVECs were incubated in LG for one hour, then incubated with JC-1 and Spermine NONOate (1 μM) for 20 minutes prior to measurement of fluorescent intensity. HUVECS were also exposed to L-NAME (1 mM) for 2 hours under NG and LG conditions and subsequently incubated in JC-1 to facilitate measurement of mitochondrial membrane potential.

**Western Blots**

HUVECs pre-treated with NG and LG for time periods up to 2 hours were lysed in MOPS lysis buffer containing (in mM) 20 MOPS, 2 EGTA, 5 EDTA, 30 Sodium Fluoride, 40 β-glycerophosphate, 10 sodium pyrophosphate, 2 sodium orthovanadate, and 0.5% NP (pH 7.4). Protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) were added to lysis buffer prior to use. Supernatant protein concentration was measured using DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA). 25-30 μg proteins were separated on 10% Tris-HCL CRTGEL (Bio-Rad Laboratories) and transferred from the gel onto a nitrocellulose membrane (Bio-Rad
The membrane was blocked with 5% of non-fat dry milk for one hour. After blocking, the membrane was washed with PBS containing 0.1% Tween 20 (PBST) and cut into two parts along the level of 50 KDa for p-AMPK and 75 kDa for p-eNOS. The upper membrane was probed with anti-pAMPK (1:1000) or anti-p-eNOS (Ser1177 or Ser633, 1:1000) and the lower one was probed with anti-β-actin antibody overnight at 4°C. Membranes were subsequently washed with PBST and incubated with both goat anti-rabbit IgG-HRP conjugated secondary antibody (Santa Cruz Biotechnology) and goat anti-mouse-HRP conjugate secondary antibody (Bio-Rad Laboratories). Blots were developed with ECL Plus western blotting detection system (GE Healthcare Biosciences, Pittsburgh, PA). To detect the expression of total AMP Kinase (tAMPK) and total eNOS (t-eNOS), the membrane with pAMPK was stripped and re-probed with anti-AMPK antibody (1:1000) and anti-eNOS antibody (1:1000). Band density was measured with Image J 1.42.

Vessel Preparation and Assessment of Endothelial Function by Videomicroscopy

All fat samples obtained were placed immediately in cold (4°C) HEPES buffer following removal from the study subject. Human adipose arterioles (60–150 µm internal diameters) were carefully isolated from the fat samples, and carefully cleared of fat and connective tissue. Arterioles were then suspended in a micro-organ chamber and vasomotor activity was measured using video microscopy as described below.

Isolated arterioles were transferred to an organ bath and cannulated with tapered glass micropipettes (internal tip diameter ~ 40 - 60 µm) filled with cold Krebs buffer consisting of (in mM) 123 NaCl, 4.7 KCl, 2.5 CaCl₂, 0.026 EDTA, 1.2 MgSO₄, 20 NaHCO₃, 1.2 KH₂PO₄, and 5 glucose. Vessel ends were secured with 10-0 nylon Ethilon monofilament suture (22 µm diameter; Look, Norwell, MA), and side branches, if present, were tied off with 2-0 silk suture (Ethicon; Somerville, NJ). After mounting, the arteriole
was stretched to approximate its *in situ* length and transferred to the stage of an inverted microscope (magnification x200) attached to a video camera, videomicrometer, video monitor, and a video-measuring device (model VIA-100; Boeckeler). Prior to vasoreactivity testing, arterioles were allowed to equilibrate at an intraluminal pressure of 20 mmHg followed by 60 mmHg (30 minutes at each pressure). Each preparation was continuously superfused with Krebs buffer bubbled with a gas mixture of 21% O₂-5% CO₂-74% N₂.

In NG buffer, arterioles were pre-constricted with endothelin-1 to approximately 50% of maximum diameter under resting condition. Endothelium-dependent vasodilation was determined by adding acetylcholine (Ach) into organ bath to reach concentrations of \(10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}\) and \(10^{-5}\) M. Following these initial measurements, vessels were washed twice and re-equilibrated for 15 minutes in NG buffer. Subsequently, the buffer was changed to LG and either 1) 10 µM of metformin or 2) 150 U/ml of PEG-SOD or 3) LG buffer alone. Prior to testing, these solutions were circulated through the arterial lumen for 30 minutes at a low rate (less than 5 dynes/cm²), rendering the effects of shear negligible. Solutions were circulated through the lumen throughout the testing period. Endothelium-dependent vasodilation was subsequently re-measured by using acetylcholine as described above. At the end of each series of study, endothelium-independent vasodilation was examined by adding papaverine (0.2 mM).

**Statistical analyses**

For comparisons between two groups, unpaired Student’s t-test or Wilcoxon rank sum tests were applied as appropriate depending on the distribution of the data. For comparisons of NO production, mitochondrial superoxide production, and apoptosis percentage with more than two experimental groups, we tested for overall differences between groups using one-way ANOVA or ANOVA on ranks as applicable. If statistical
significance was detected, post-hoc testing was performed to determine the groups driving the observed differences. For comparisons of human arteriole endothelial function, two-way ANOVA was applied. P-values of <0.05 was considered significant. All data are presented as mean±SE unless otherwise specified.

Supplemental Figure Legends:

Figure I: Basal Nitric Oxide Production Is Not Significantly Changed Under Low Glucose Conditions. The median (black horizontal bar within gray bars), 95% confidence interval (gray bars), and range (black dots) for basal NO production at each concentration of glucose is shown. P>0.05 by repeated measures ANOVA.

Figure II: Short term LG exposure does not increase total superoxide levels a: 60 minutes of low glucose exposure did not significantly alter total superoxide levels as measured by the sum of the concentrations of the 2OHE+ and E+ components of HE (N=8, p=0.21).

Figure III: Mitochondrial Superoxide Production with Free Fatty Acid and/or L-Carnitine Supplementation in Combination with LG: The addition of both 1% palmitate and L-carnitine (50 µM) completely abrogated LG-induced excessive mitochondrial superoxide production (N=5, P<0.001 overall, *P<0.001 for LG versus all other exposures) (b) The addition of L-carnitine reduced mitochondrial superoxide production to the level of LG alone but remained higher than NG (N=3-6, P<0.001 overall, *P<0.05 vs. NG, †P<0.05 vs. LG+Palm).
Figure IV: Reductions in Mitochondrial Superoxide Production by Pharmacological AMP Kinase Activation Under LG are NO-dependent. Both metformin (10µM) and AICAR (100 µM) significantly suppressed LG-induced mitochondrial superoxide production. The addition of L-NAME (1 mM) to cells pretreated with metformin or AICAR blunted the protective effects of both agents. (n=3-6, P<0.001 overall, *-P≤0.002 vs. LG NG).

Figure V: Low Glucose Induced Endothelial Dysfunction is not Secondary to Osmotic Differences. Human subcutaneous adipose arterioles were exposed to 1) 30 minutes of 5 mM D-glucose buffer and 2) 30 minutes of 2 mM D-glucose + 3 mM L-glucose and Ach induced vasodilation was tested. Test periods were separated by a 30 minute washout period at 5 mM glucose. Endothelial function remained impaired by low glucose despite the addition of 3 mM L-glucose to equalize osmolarity. (n=3, P=0.009 overall, *-P<0.006 at the indicated Ach doses). Ach- acetylcholine
Supplemental Figure IV

MitoSox Fluorescence Intensity vs. LG alone

- LG
- LG+ LNAME
- LG+ MET
- LG+ MET+ LNAME
- LG+ AICAR
- LG+ AICAR+ LNAME

*(b)*
Reference List
