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

Jingli Wang, Anna Alexanian, Rong Ying, Tinoy J. Kizhakekuttu, Kodlipet Dharmashankar, Jeanette Vasquez-Vivar, David D. Gutterman, Michael E. Widlansky

Objective—Hypoglycemia is associated with increased mortality. The reasons for this remain unclear, and the effects of low glucose exposure on vascular endothelial function remain largely unknown. We endeavored to determine the effects of low glucose on endothelial cells and intact human arterioles.

Methods and Results—We exposed human umbilical vein endothelial cells to low glucose conditions in a clinically relevant range (40–90 mg/dL) and found rapid and marked reductions in nitric oxide (NO) bioavailability (P<0.001). This was associated with concomitantly increased mitochondrial superoxide production (P<0.001) and NO-dependent mitochondrial hyperpolarization (P<0.001). Reduced NO bioavailability was rapid and attributable to reduced endothelial nitric oxide synthase activity and destruction of NO. Low glucose rapidly activated AMP kinase, but physiological activation failed to restore NO bioavailability. Pharmacological AMP kinase activation led to phosphorylation of endothelial nitric oxide synthase’s Ser633 activation site, reversing the adverse effects of low glucose, and this protective effect was prevented by nitro-L-arginine methyl ester. Intact human arterioles exposed to low glucose demonstrated marked endothelial dysfunction, which was prevented by either metformin or TEMPO.

Conclusion—Our data suggest that moderate low glucose exposure rapidly impairs NO bioavailability and endothelial function in the human endothelium and that pharmacological AMP kinase activation can inhibit this effect in an NO-dependent manner. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: endothelium ■ microcirculation ■ nitric oxide ■ vascular biology ■ vasodilation

The disappointing results of recent trials of tight glycemic control in type 2 diabetes highlight the potential risks of hypoglycemia. Hypoglycemia has been associated with excess mortality in critically ill patients, in patients presenting with acute coronary syndromes, and in diabetic patients. These data strongly suggest hypoglycemia increases the risk of morbidity and mortality in a wide variety of patients.

The mechanisms underlying the risks posed by hypoglycemia are unknown. However, there is considerable biological plausibility implicating hypoglycemia-triggered cardiac ischemia. Phenotypic endothelial dysfunction, characterized by inflammation, excessive oxidative stress, and a loss of bioavailable nitric oxide (NO), typically precedes the development of clinical cardiovascular events. Although there is ample evidence implicating hyperglycemia-induced increases in oxidative stress and reduced NO bioavailability as mechanistically important in the development of endothelial dysfunction in humans, few data exist as to the effects of clinically relevant levels of low glucose (LG) on oxidative stress, NO bioavailability, and endothelial function. Complete glucose deprivation stimulates the production of mitochondrial reactive oxygen species (ROS) and AMP kinase (AMPK) activation. Activation of AMPK would be expected to boost endothelial production of NO, although the response to AMPK activation may be delayed. The extent to which these responses occur in endothelial cells under acute, moderate, LG conditions (40–70 mg/dL) remains unknown.

In light of these data, we hypothesized that LG exposure at clinically relevant concentrations would lead to endothelial dysfunction, characterized by reduced NO bioavailability, increased oxidative stress, and impaired endothelium-dependent vasodilation. Furthermore, we endeavored to delineate the interactions between mitochondrial superoxide, NO, and AMPK that occur with acute exposure to LG concentrations.

Materials and Methods

Materials
Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD) and gifted to our laboratory by Dr
Results

Endothelial Cell NO Bioavailability, Mitochondrial Superoxide Production, and Cytosolic Superoxide Production Under LG Conditions

Acute exposure of HUVECs to graded reductions in glucose concentration from 90 to 40 mg/dL resulted in rapid impairment of stimulated NO bioavailability with 5 minutes of LG exposure (Figure 1a and 1b). Impaired NO production began to appear at 80 mg/dL and became significant at 70 mg/dL. Basal levels of NO production were not affected by LG (Figure I in the online-only Data Supplement). Concomitantly, mitochondrial superoxide significantly increased relative to baseline beginning at 80 mg/dL (Figure 1c). PEG-SOD completely prevented LG-induced increases in MitoSox fluorescence (Figure 1d), suggesting that almost all of the MitoSox fluorescence signal originates from mitochondrial superoxide production. Total cellular superoxide levels as measured using hydroethidium did not significantly increase with 60 minutes of exposure to LG (50 mg/dL) (Figure II in the online-only Data Supplement). However, cellular hydrogen peroxide concentration more than tripled with LG exposure and normalized with concomitant treatment with mito-TEMPOL (Figure 1e).

To determine whether free fatty acid supplementation would abrogate LG’s stimulation of mitochondrial superoxide production, we measured MitoSox fluorescence under NG (90 mg/dL) and LG (50 mg/dL) conditions with and without supplementation with 1% palmitate and t-carnitine, a mitochondrial antioxidant that can also improve free fatty acid utilization in endothelial cells to under LG conditions,11 We found that combined treatment of endothelial cells with 1% palmitate and t-carnitine reduced LG-induced mitochondrial superoxide production to normal levels (Figure IIIa in the online-only Data Supplement). However, when examined separately, mitochondrial superoxide production increased under LG conditions with the addition of 1% palmitate, whereas t-carnitine alone returned mitochondrial superoxide levels to those similar to the NG condition (Figure IIIb in the online-only Data Supplement).

To determine how rapidly LG reduced stimulated NO bioavailability, we performed time series experiments. We measured basal and stimulated NO production and mitochondrial superoxide production at 90 mg/dL (NG) and LG at 5, 30, and 60 minutes following exposure to glucose at either of these 2 concentrations. Impairment of stimulated NO production under LG conditions versus NG conditions was seen at all time points (n=6 for each glucose concentration, P<0.05 versus corresponding control for at all time points) (Figure 1e). Comparing measurements made at 50 mg/dL, there were no significant differences between stimulated NO measurements made at 5, 30, and 60 minutes.

To help delineate the potential mechanisms for reduced NO bioavailability with LG, we measured NO production from HUVECs under NG and LG conditions (30 minutes of exposure). 30 minutes of LG exposure significantly inhibited the conversion of L-arginine to L-citrulline by eNOS (Figure 1f). In a subsequent experiment, we supplemented HUVECs with BH₄, which failed to improve suppressed NO bioavailability of stimulated HUVECs, whereas treatment with 1 mmol/L TEMPOL, with or without BH₄, significantly improved NO bioavailability (Figure 1g).

Effect of LG on Mitochondrial Membrane Potential and Mitochondrial ROS Production: Regulation by NO

To determine whether LG-induced excess mitochondrial ROS production was associated with alterations in mitochondrial membrane potential, we measured mitochondrial membrane potential under both NG and LG conditions. LG induced mitochondrial hyperpolarization relative to NG based on a significant increase in the red/green ratio of JC-1 fluorescence (P<0.001).

Furthermore, spermine NONOate, an exogenous source of NO, reversed LG-induced mitochondrial hyperpolarization (P=0.97 versus NG alone, P=0.007 versus LG alone), whereas nitro-L-arginine methyl ester (L-NAME) significantly increased mitochondrial membrane potential in the setting of NG (P=0.004 versus NG alone). No significant differences in mitochondrial membrane potential were seen when comparing LG, LG+L-NAME, or NG+L-NAME (P=not significant for all post hoc comparisons). Our full results are summarized in Figure 2a. These findings were confirmed using a second mitochondrial membrane potential sensitive probe, TMRM (data not shown). CCCP (10 μmol/L) significantly reduced mitochondrial membrane potential relative to both NG and LG condition using JC-1, confirming the ability of JC-1 to measure changes in mitochondrial membrane potential (data not shown).

To confirm that losses of NO production lead to increased mitochondrial ROS production, we performed additional experiments under NG and LG conditions with and without L-NAME (1 mmol/L) or spermine NONOate (10 μmol/L) and measured mitochondrial ROS production (Figure 2b).
L-NAME increased mitochondrial ROS production under NG conditions to the level of LG, whereas NONOate prevented LG-induced mitochondrial ROS production.

Effect of LG on AMPK Activity Relative to eNOS Phosphorylation at Serine 1177 and Serine 633 Activation Sites
To determine the time course of AMP activation following LG exposure, we measured pAMPK levels between 0 and 120 minutes following exposure to LG. As demonstrated in Figure 3a and 3b, the ratio of pAMPK to total AMPK increased 75% from time 0 to 15 to 30 minutes following LG exposure. Although pAMPK/total AMPK remained modestly elevated at 45 to 60 minutes of LG exposure compared with time zero (32% increase over baseline), the ratio was significantly lower than at 15 to 30 minutes.

In contrast to AMPK, the eNOS serine 1177 activation site was rapidly dephosphorylated (within 5 minutes of LG exposure).

Figure 1. Effect of low glucose (LG) exposure on human umbilical vein endothelial cell nitric oxide (NO) and mitochondrial superoxide production. 

A. Impairment of vascular endothelial growth factor (VEGF)-stimulated NO production relative to basal NO production appeared to begin at 80 mg/dL (n=8, P<0.001 overall), with significant decreases in stimulated NO production at concentrations ≥70 mg/dL (P<0.001 vs 90 mg/dL). B. Net NO production following VEGF stimulation was significantly suppressed at 5, 30, and 60 minutes following exposure to LG (n=6, P<0.001 overall, P<0.05 at all time points relative to normal glucose [NG]). C. Mitochondrial superoxide production as a percentage of control glucose concentration (90 mg/dL) was significantly increased at glucose concentrations of ≥80 mg/dL (n=8, P<0.001 overall, ≥80 mg/dL vs 90 mg/dL at all time points). D. MitoSox signal under LG conditions was completely eliminated in the presence of either PEG-SOD or PEG-catalase (n=8, P<0.001 overall, P<0.05 for LG vs all other conditions). E. LG induced a significant increase in cellular hydrogen peroxide levels as measured by DCF fluorescence intensity. This increase was completely suppressed by mitochondrial targeted antioxidant therapy with mito-TEMPOL (1 mmol/L) (P<0.01 overall, P<0.05 for LG vs all other exposure states). F. 30 minutes of LG exposure significantly inhibited NO production, as measured by the conversion of L-arginine to L-citrulline (n=12, 731±273 vs 206±62 fmol citrulline/mg protein per minute, P=0.026). G. The addition of BH4 (100 μmol/L) had no effect on LG-induced inhibition of stimulated NO production. TEM- 

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Effect of Pharmacological AMPK Activation on eNOS Phosphorylation at Activation Sites, Mitochondrial Superoxide Production, and NO Bioavailability

We exposed HUVECs under NG and LG condition to metformin (10 μmol/L) for 1 hour and measured eNOS phosphorylation at the Ser1177 and Ser633 activation sites. Metformin had no significant effect on LG-induced dephosphorylation at Ser1177 (Figure 3e and 3f). However, metformin significantly increased eNOS phosphorylation at Ser633 under both LG and NG conditions, whereas LG alone had no effect on Ser633 phosphorylation (Figure 3g and 3h).

Incubation with either metformin or AICAR for 1 hour blocked mitochondrial superoxide production during LG (Figure 4a). This was accompanied by an increase in NO bioavailability during AICAR and metformin exposure (Figure 4b). Reductions in mitochondrial superoxide produced by AICAR or metformin treatment were inhibited by concomitant exposure to L-NAME (Figure IV in the online-only Data Supplement). No effect of metformin or AICAR was seen on either mitochondrial superoxide production or NO bioavailability under NG conditions (Figure 4c and 4d).

Effect of Acute LG Exposure on Endothelial Function

We isolated subcutaneous or mesenteric adipose arterioles from human volunteers without underlying atherosclerotic disease or diabetes for analysis of endothelium-dependent vasoreactivity under NG and LG conditions. Glucose concentration had no effect on the resting diameters of arterioles (100±13 versus 94±15 nmol/L for NG and LG, respectively; \( P=0.09 \)) As seen in Figure 5a, exposure to LG significantly blunted endothelium-dependent vasodilation to acetylcholine. LG with concomitant metformin or PEG-SOD exposure effectively inhibited the adverse effects of LG on endothelium-dependent vasodilation. No significant differences in endothelial function were found between NG, LG+metformin, and LG+PEG-SOD conditions. There were no significant differences in vasoreactivity to papaverine among exposure conditions (Figure 5a), and there was no difference in vasodilation to NONOate under LG compared with NG (Figure 5b). Using L-glucose (3 mmol/L) to equalize the osmolarity of the buffer had no effect on LG (2 mmol/L) impairment of arteriolar endothelial function (Figure V in the online-only Data Supplement).

Discussion

The present investigation has identified 3 novel findings. First, acute exposure of endothelial cells to LG concentrations in a clinically relevant range (40–80 mg/dL) induced a rapid reduction in NO bioavailability at least in part through dephosphorylation of at the serine 1177 site. Second, the loss of NO bioavailability was mechanistically linked to concomitant increases in mitochondria specific superoxide production, likely through mitochondrial hyperpolarization. Third, although native AMPK activation was not sufficient to maintain levels of activated eNOS and inhibit excessive mitochondrial superoxide production, pharmacological AMPK activation inhibited excessive mitochondrial oxidative stress through improved NO bioavailability that may involve phosphorylation at eNOS’s Ser633 activation site. Importantly, we demonstrated that our findings have relevance to human vascular endothelial function by showing that acute LG exposure profoundly impaired endothelial function in intact human adipose arterioles, whereas treatment with metformin or a superoxide scavenger designed to pass through membranes inhibited the adverse effects of LG. Taken together, these data suggest a new mechanism of inducing vascular mitochondrial oxidative stress and endothelial dysfunction, namely, brief hypoglycemia, typical of the clinical situation involving tight glycemic regulation.

The consequences of tight glycemic control include intermittent hypoglycemia in the range that we identify as capable of increasing mitochondrial oxidative stress and endothelial dysfunction. There have been few prior investigations of the effect of LG on endothelial function, and prior work in this area has been conflicting. Exposure of porcine aortic endothelial cells to 2-deoxyglucose in a glucose-free environment inhibits bradykinin-stimulated production of prostacyclin and possibly NO as well.16 In a study of rabbit aortas, inhibition of glycolysis with either 2-deoxyglucose or iodoacetate in buffer containing 16.6 mmol/L glucose failed to alter endothelium-dependent vasodilation to acetylcholine.17 A third study assessed the effect of moderate and severe insulin-induced hypoglycemia on cerebral blood flow in goats, finding an NO-dependent increase in cerebral blood flow.
under hypoglycemic conditions. These studies are significantly heterogeneous with respect to their study designs, including significant differences in the species studied, vascular beds tested, and methodologies used to induce LG states. The porcine and rabbit studies used inhibitors of glycolysis enzymes to mimic the effects of glucose deprivation, limiting their generalizability to physiologically relevant LG states. Our findings significantly expand our knowledge base on the effects of LG on human vascular endothelial function through the use of human tissues and clinically relevant concentrations of glucose.

Our data demonstrated that LG exposure rapidly inhibits NO bioavailability at least in part through reducing the level of Ser1177 phosphorylated eNOS. Mechanistically, the loss of NO bioavailability may also relate to our observed increase in total cellular oxidative stress observed in this study by an increase in $\text{H}_2\text{O}_2$ under LG conditions. We were unable to show evidence of excessive total superoxide levels under LG conditions. Although we cannot exclude an effect on total superoxide concentrations for longer exposure periods, our data suggest that a 60-minute period of LG exposure does not induce excessive total superoxide levels. Our data are also

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**Figure 3.** Effect of low glucose (LG) exposure on AMP kinase (AMPK) phosphorylation. **a,** Raw results from Western blot for total AMPK (T-AMPK/tAMPK) and AMPK phosphorylation (p-AMPK/pAMPK). **b,** Exposure of human umbilical vein endothelial cells (HUVECs) to LG for 15 to 30 minutes resulted in an increase in pAMPK/T-AMPK levels. Although the ratio remained modestly elevated compared with baseline, pAMPK/T-AMPK dropped significantly by 45 to 60 minutes into LG and remained at this level at the 2-hour time point ($n=3-5$, $P<0.001$ overall, $^*P<0.05$ vs time 0, $\dagger P<0.05$ vs the 45–60-minute and 2-hour time points). **c,** Sample raw Western blot result for peNOS1177 and total endothelial nitric oxide synthase (teNOS). **d,** peNOS/eNOS ratio was significantly reduced within 5 minutes of LG exposure ($n=8$; $P=0.02$ overall; $^*P=0.03$ for the 5-, 45–60-, and 120-minute durations of LG exposure vs 0 minutes). **e,** Representative Western blot result for peNOS-1177 phosphorylation at the Ser1177 activation site ($n=7$, $P>0.01$ overall, $^*P<0.05$ vs normal glucose [NG]). **f,** Representative Western blot for peNOS-1177 phosphorylation at the Ser633 activation site under both NG and LG conditions. LG alone did not significantly alter eNOS phosphorylation at the Ser633 site ($n=10$, $P<0.001$ overall, $^*P<0.001$ for both LG and NG with metformin vs NG, $\dagger P<0.001$ LG vs LG/metformin).
consistent with prior work demonstrating that glucose deprivation inhibits the phosphatidylinositol 3-kinase/Akt signaling pathway intrinsic to the activation of eNOS. Our data significantly expand on this finding by (1) extending the prior study’s findings to clearly demonstrate an effect on eNOS and (2) demonstrating the potential clinical relevance of inhibition of this pathway at physiological levels of LG. Further work to delineate the mechanisms of eNOS inactivation by LG is necessary to determine potential targets for interventions.

NO is a key regulator of the mitochondrial electron transport chain (ETC), the major source of mitochondrial superoxide. NO modulates ETC activity through inhibition of complexes I and IV. Under normal homeostatic conditions, relatively high levels of NO in endothelial cells significantly inhibit ETC activity. Our data suggest that LG-induced losses of bioavailable NO remove homeostatic restrictions on mitochondrial ETC flux, leading to mitochondrial hyperpolarization and superoxide production. Mitochondrial hyperpolarization, as induced in our study by LG, is well known to be mechanistically related to increased mitochondrial ROS production. Our novel findings in the setting of LG are consistent with prior work demonstrating that the amount of glucose oxidation via the Krebs cycle, near dormant under NG conditions in endothelial cells, is significantly increased with LG exposure. Data from the neurological literature demonstrate hypoglycemia shifts mitochondria from state 3 to state 4 respiration in central neurons, an alteration well known for increasing mitochondrial superoxide production. Subsequent work also shows neuronal hypoglycemia induces marked increases in both mitochondrial superoxide and hydrogen peroxide production. Further work using inhibitors of the mitochondrial ETC and measurements of specific activity levels of components of the

Figure 4. a and b, Effect of pharmacological AMP kinase (AMPK) activation on mitochondrial reactive oxygen species (ROS) production and nitric oxide (NO) bioavailability. a and b, Both metformin (10 μmol/L) and AICAR (100 μmol/L) significantly suppressed low glucose (LG)-induced increases in mitochondrial ROS production (n=3–6, *P<0.001 overall, **P<0.05 vs LG) (a) and reversed LG-induced inhibition of NO production (b) (n=5, P<0.001 overall, **P<0.05 vs all other exposure groups except normal glucose [NG]). c, Neither metformin (MET) nor AICAR altered mitochondrial ROS production under NG conditions (n=6, P=0.46). d, Neither MET nor AICAR altered NO production under NG conditions (n=6, P=0.48).
availability, a concomitant increase in mitochondrial H₂O₂ this regulatory pathway, beginning with a loss of NO bioavailability by reacting with NO to form peroxynitrite. Peroxynitrite formation significantly impairs endothelial function relative to all 3 other exposure states (P < 0.001 for all comparisons). *P < 0.05 vs NG, LG + metformin, and LG + PEG-SOD at the indicated concentrations of acetylcholine. b, LG did not alter endothelium-independent responses to exogenous nitric oxide (NO) (NONOate, n = 3, P = acetylcholine [Ach], P = 0.20 for interaction between dose and exposure condition).

We found that LG exposure enhances endothelial cell H₂O₂ content with a corresponding increase in mitochondrial superoxide content. Increases in ROS production are linked to increased oxidative damage, reduced NO bioavailability, and endothelial dysfunction. In the context of our findings, the biological role of increased mitochondrial superoxide under LG conditions likely includes both the adverse sequelae of increased oxidative damage, as well as modulation of important pathological cell signaling cascades. Mitochondrial superoxide can reduce NO bioavailability by rapidly reacting locally with NO to form peroxynitrite. Peroxynitrite formation reduces NO bioavailability and can itself damage the ETC, antioxidant defenses, and mitochondrial proteins and nucleic acids. Our data are also consistent with the emerging concept of that mitochondrial ROS, particularly hydrogen peroxide (H₂O₂), are central regulators of homeostatic and pathological cell signaling in endothelial cells. Although superoxide can escape from mitochondria in modest quantities, the concentrations of more stable H₂O₂ (produced by rapid dismutation of superoxide to H₂O₂ in both the mitochondrial matrix and intermembrane space) far exceed that of superoxide in mitochondria. H₂O₂ can easily pass through mitochondrial membranes.

H₂O₂ has previously been reported to activate AMPK via calcium/calmodulin-dependent protein kinase kinase β. Prior work also establishes the ability of NO to reduce mitochondrial H₂O₂ production via suppression superoxide production from the mitochondrial ETC. Our data are entirely consistent with this previously established regulatory nexus of H₂O₂, AMPK, and NO. We have importantly extended these findings by showing exposure to LG at clinically relevant concentrations and exposure times triggers this regulatory pathway, beginning with a loss of NO bioavailability, a concomitant increase in mitochondrial H₂O₂ production, and an increase in AMPK activity.

Interestingly, we found that treatment with either metformin or AICAR, known pharmacological activators of AMPK through activation of tumor suppressor product LKB1, reversed the loss of NO bioavailability and inhibited excessive mitochondrial oxidative stress. Further, metformin reversed endothelial dysfunction in intact human arterioles acutely exposed to moderate LG concentrations. These data are consistent with prior work demonstrating AMPK activation of eNOS and NO suppression of mitochondrial ETC activity. Inhibition of eNOS using 1-NAME prevented the effects of metformin and AICAR, further suggesting that the ameliorative effects of pharmacological AMPK activation are mechanistically linked to increased NO bioavailability.

One potential paradox in our findings is that increased pAMPK with LG exposure alone failed to increase NO levels even out to 2 hours of LG exposure. Based on prior work and our data in Figure 3, we suspect that this initially puzzling finding relates to differences in the isoform of AMPK activated by metformin and AICAR (AMPKα2 via activation of LKB1) versus the isoform activated by H₂O₂ (AMPKα1 via activation of calcium/calmodulin-dependent protein kinase kinase β) and the relative importance of each isoform in regulating eNOS activity. Although AMPKα1 is more abundant in endothelial cells, AMPKα2 activity appears to be of significantly greater importance in eNOS regulation. In endothelial cells, pAMPKα2 plays a key role in activating eNOS and increasing NO bioavailability, whereas there is minimal to no role for AMPKα1 in eNOS regulation. Furthermore, these data would suggest that pharmacological AMPKα2 activation may have a protective effect against LG-induced endothelial dysfunction. Future work is clearly indicated to confirm this attractive hypothesis. Figure 6 provides a graphical synthesis of our findings with previously reported work in this area.

Our findings with respect to fatty acid and l-carnitine suggest mechanistically that free fatty acid utilization in vivo would not be sufficient to suppress LG-induced NO suppression and excessive mitochondrial superoxide production, particularly in diabetic patients who have significant impairment in free fatty acid utilization and reduced basal plasma and tissue carnitine levels. Interestingly, our data does suggest that pharmacological supplementation with l-carnitine may be able to inhibit the adverse effects of LG on mitochondrial superoxide production and perhaps improve endothelial dysfunction. l-Carnitine plays a key role in both transporting and intracellular handling of fatty acids and l-carnitine supplementation has been shown to improve NO bioavailability and improve free fatty acid–induced endothelial dysfunction. The mechanism of the vascular benefits of l-carnitine remains unclear, and further work is necessary to delineate the efficacy and mechanism of action of l-carnitine in the setting of LG.

Our data have limitations. First, HUVECs may not be representative of other vascular endothelial cells. However, we tested arteriolar endothelial function of intact human vessels and verified that LG induces a state of endothelial dysfunction reversible with metformin and antioxidant therapy, suggesting our findings have physiological relevance to human arteriolar endothelial function. Second, the mecha-
Figure 6. Low glucose (LG)-induced endothelial dysfunction: role of mitochondrial (Mito) superoxide production and prevention by AMP kinase (AMPK) activation. Acute LG exposure leads to rapid inhibition of endothelial nitric oxide synthase (eNOS) and a reduction in bioavailable nitric oxide (NO). This leads to a subsequent increase in mitochondrial electron transport chain activity, mitochondrial membrane hyperpolarization, and increased mitochondrial superoxide production. Increased mitochondrial superoxide production can further reduce NO bioavailability and leads to increased cellular hydrogen peroxide levels that activate AMPK isoform α1 through a calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ)–dependent pathway. LG-induced losses of NO bioavailability could be prevented/reversed by treatment with AMPKα2 activating agents acting through an LKB1-dependent activation pathway.

Figure 6 continued

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Disclosures

None.

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Hypoglycemic symptoms often do not occur until systemic levels drop below 55 mg/dL. Our data support the biological plausibility that acute hypoglycemia may result in the acute development or worsening of endothelial dysfunction, triggering cardiovascular events. Further, our data suggest the potential for pharmacological AMPK activation, and perhaps L-carnitine, to inhibit these effects. Further work is needed to determine whether in vivo hypoglycemia, similar to hyperglycemia, is also a significant risk factor for endothelial dysfunction. Such confirmation would help explain the observed detrimental effect of tight glycemic control on cardiovascular events and have important ramifications in the treatment strategies for glucose control in diabetes.


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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 Φ 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 37ºC 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 37ºC 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...
Laboratories). 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 \textit{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\% O$_2$-5\% CO$_2$-74\% N$_2$.

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/cm$^2$), 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).

\textbf{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 pre-treated 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 Material
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Supplemental Figure I

Glucose (mg/dL)

Baseline NO concentration (pmol/mg protein)
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Supplemental Figure III

(a) Fluorescence intensity (A.U.)

- NG
- LG
- NG+Palm
- LG+Palm
- L-Carn

(b) Fluorescence intensity (A.U.)

- NG
- NG+Palm
- LG
- LG+Palm
- LG+LC
Supplemental Figure IV

(b) MitoSox Fluorescence Intensity vs. LG alone

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

* indicates significant difference.
Reference List
