Published in final edited form as:

Exp Gerontol. 2015 October ; 70: 157–162. doi:10.1016/j.exger.2015.08.002.

Elevated Oxygen Consumption Rate in Response to Acute Low-Glucose Stress: Metformin Restores Rate to Normal Level

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Abstract

Cardiovascular Disease (CVD) continues to be the leading cause of mortality among all age demographics in the United States, with the highest occurrence in populations aged 65 and older. Glucose levels, particularly hyperglycemia, are associated with the premature onset of age-related diseases including CVD. A major challenge in the treatment of elderly patients with chronically elevated blood glucose is the frequency of hypoglycemic episodes. Molecular mechanisms of hypoglycemia remain unclear, but are associated with premature onset of age-related-diseases. Here we report a mitochondrial metabolic profile assessing short-term (up to six hours) and longer-term (12–24 hours) durations of low-glucose stress. We observed that the antidiabetic biguanide and mitochondrial complex I inhibitor, metformin, can lower and restore the elevated oxygen consumption rate during shorter-term glucose stress to levels similar to that of cells cultured in normal glucose. This effect appears, in part, to involve activation of the 5' AMP-activated protein kinase (AMPK).

Introduction

Cardiovascular disease (CVD) and stroke together continue to account for nearly one-half of all deaths in the United States in those aged 65 and older (1–3). A significant factor that contributes to the age-associated increase in cardiovascular disease is impaired glycemic control (insulin resistance) in the elderly (4). A challenge providers and patients often face in the management of diabetes mellitus are the complications associated with hypoglycemia (<60 mg/dL). Approximately 90% of older patients who take exogenous insulin experience at least one clinically significant episode of hypoglycemia (5–7). It has been observed that hypoglycemia induces oxidative stress (8).

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Many nutrient-sensing pathways are vital to the aging process, including the mammalian target of rapamycin (mTOR) and the AMP kinase (AMPK) pathways (9, 11). A central element in the AMPK-mTOR axis (and many nutrient sensing pathways) is the respiratory chain of the mitochondria, including complex I (NADH:ubiquinone oxidoreductase). Complex I dysfunction is the most common oxidative phosphorylation (OXPHOS) disorder in humans (12). Moreover, the biguanides (which includes metformin) in part confer protection by suppression of complex I of the respiratory chain (13, 14), which is likely mediated through AMPK and mTOR. A high glucose level (>300 mg/dL) leads to an increased oxygen consumption rate (OCR), as more energetic fuel is available for ATP production (15). Metformin decreases OCR in many cells, including cancer cell lines in the fasted state (14).

Taken together, we sought to better understand mitochondrial function during short (up to six hours) and longer-term (12-to 24 hours) glucose deprivation and postulated that metformin may have an effect via its inhibition on complex I of the mitochondrial respiratory chain.

Materials & Methods

Cell Cultures

Pooled human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD) and cultured as previously described (16). Mouse myoblast cells (C2C12) were obtained from ATCC (ATCC CRL-1772) and cultured as previously described (16).

Low-Glucose Stress

HUVECs were cultured in 100 mg/dL, after which cells were incubated in normal glucose (100 mg/dL), followed by low glucose (30 mg/dL), for six hours each; this alternating pattern was continued for 96 continuous hours. C2C12 cells were cultured in clinically relevant low glucose (30 mg/dL) for six different durations from five minutes through six hours.

Western Blotting

Western blot protocol was followed as previously described (16).

RNA Isolation

Total cellular RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA) as previously described (16, 17).

Cell Viability

Analysis of viable cells was performed by Trypan Blue exclusion. This method is based on the count with a Burker hemocytometer of both living cells and stained dead cells. Dead cells were reported as a percentage of the total number of cells in 5 different fields of each low glucose time point (up to 24 hours) in two separate experiments.

Mitochondrial Glycolytic and Oxygen Consumption Analysis

C2C12 cells were seeded at 15,000 cells per well in XF 96 Well plates (Seahorse Bioscience, Billerica, MA). Cells were incubated for one, three, and six hours continuously in 30 mg/dL glucose 1X DMEM. After low glucose stress, the cells were subjected to mitostress and glycolysis stress test(s), using the Mito Stress kit and Glycolytic Stress kit, respectively (Seahorse Biosciences Billerica, MA). Rotenone, oligomycin, antimycin A and FCCP were prepared in filtered ultrapure DMSO (Sigma Chemical Co., St. Louis, MO) at 2.5 mM. The XF Assay media (Seahorse Biosciences) was adjusted to pH = 7.4 using NaOH. Oligomycin, glucose and 2-D-glucose (2-DG) were prepared according to manufacturer's instructions that were supplied in the XF glycolysis test kit.

Results

Oxygen Consumption Rate (OCR) Increases in response to Low Glucose Stress without Increased ATP Production

Incubation of C2C12 cells for periods of 6-hour duration of low-glucose stress caused the cells to have a higher basal metabolic rate or oxygen consumption rate (OCR), with a 24% increase compared to those in normal glucose. However, the low-glucose exposure generated comparable amounts of ATP. (Fig. 1, left panel). Low-glucose exposure increased the oxygen maximal respiration more than that in normal glucose. We also evaluated the rate of glycolysis, measured by extracellular acidification rate (ECAR), in response to six-hour low glucose and found no significant differences in glycolytic function, glycolysis, glycolytic capacity, or glycolytic reserve in low-glucose-treated cells compared with that in normal glucose (Fig. 1, right panel).

Metformin Restores Oxygen Consumption Rate Levels to Normal Levels During Short-term Low-Glucose Stress

Our observation that low glucose increases OCR raised the question of whether metformin could restore OCR during glucose deprivation. The C2C12 cells were treated with low glucose, with and without metformin for 24-hours, and underwent OCR determination at short (1 and 6 hours) and longer (12 and 24 hours) time points. After one and six hours (Figs. 2a and 2b, respectively) of low-glucose exposure, there was a higher basal respiration rate compared to that in cells cultured in normal glucose. However, cells treated with metformin exhibited a lower basal respiration rate and reduced maximal respiration under low glucose, similar to that in the normal-glucose-treated groups (Figs. 2a and 2b).

As the low-glucose incubation was continued to 12 and 24 hours (Figs. 3a and 3b, respectively), there appeared to be a loss of the "protective" effect of metformin. At the 12 hour time point, metformin reduced maximal respiration less than that in C2C12 cells incubated in normal glucose, normal glucose + metformin and low glucose without metformin (Fig. 3a, Table 1). At 24 hours, the effects of metformin at 6 hours were no longer seen (Fig. 3b).

Metformin Supplementation Attenuates Low-Glucose-Induced Cell Death

We performed a Trypan-Blue Exclusion Assay, in which dead cells are shown as a distinctive blue color under a microscope. Our data show that metformin resulted in a nearly 50% reduction in cell death (Fig. 5). At 9 hours of low glucose stress, we observed a reduction in cell death of almost 40% from approximately 29% to approximately 15% cell mortality in cells supplemented with metformin (Figs. 5 and 6). The 9-hour metformin-supplemented cells did start to exhibit morphological characteristics of C2C12 cell undergoing cell death, most notably a spherical, detached phenotype. At 12 hours, metformin did not offer protection and in fact, appeared to result in slightly more cell death.

Glucose Deprivation Leads to Activation of 5-AMP-Activated Protein Kinase (AMPK)

We proposed that the two pathways of AMPK and mTOR potentially played a role in the low-glucose stress response. Our data show that while the levels of AMPK did not change over the 12-hour course of low-glucose stress, the activation of AMPK positively correlated with the low glucose duration (Figure 7). After six hours of low glucose stress, p-AMPK levels increased 1-fold and was increased by almost 3-fold after 12 hours of low-glucose stress (Fig. 7d). Shortterm low-glucose stress did not appear to affect expression of mTOR (Figure 8).

Discussion

Hypoglycemic episodes pose a significant hurdle in achieving blood glucose control and are associated with higher morbidity and mortality compared with hyperglycemia, especially in the elderly. Studies aimed at understanding signaling pathways could improve our understanding of the optimal nutrient restrictions.

In vitro Low-Glucose Models: C2C12 Cells and HUVECs

Using an *in vitro* system, we studied both HUVECs and C2C12 cells to assess glucose stress and how it affects vascular aging. HUVECs are an endothelial model that we have observed to be useful for studying the effects of glucose flux on aging cells (advancing PDLs. As endothelial cells increase in passage level, they become more fragile. Multiple attempts to perform extracellular flux studies and cell viability assays in late passage HUVEC cells in several laboratories have remained unsuccessful. Cells of the mouse myoblast (C2C12) cell line tends to be hardier than HUVECs, do not demonstrate replicative senescence, and use of the flux analyzer to assess mitochondrial function has been well-cited with the use of C2C12 cells (18).

Low Glucose-induced Increase in Oxygen Consumption Rate without a Concomitant Increase in ATP Production

In the present study, we observed that the oxygen consumption rate (OCR) was increased in response to low glucose stress, similar to the OCR levels that occurred with elevated glucose levels. Unlike high glucose, however, there was not an increase in ATP production after low-glucose exposure (compared to cells cultured in normal glucose; data not shown). It is plausible that this could potentially be due to partial respiratory uncoupling. Under nutrient stress, it is not atypical for cells to switch from an oxidative phosphorylation means to a

glycolytic means of ATP production in the presence of oxygen; this is the basis for the Warburg Hypothesis to partially explain cancer-associated cellular metabolism (19). However, the measurement of glycolysis via the extracellular acidification rate (ECAR) in the present study showed no differences in glycolytic capacity or function after low glucose compared to that of cells cultured in normal glucose. We also did not observe any increase in proton leakage, but this could potentially be explained by the expression of various uncoupling proteins (e.g. UCP-1, 2, and 3) that would allow the influx of protons from the cytoplasm to the inner mitochondrial matrix. Our assay evaluates only protons that leaked out from the mitochondria into the cytoplasm, not those that may have leaked back inside the mitochondrial matrix. Other proteins that may also play a role in this phenomenon include the phosphate translocase that shuttle protons into the mitochondrial matrix and the anti-port adenine nucleotide translocase (ANT-1 and 2).

Low Glucose-Induced Activation of AMPK and Oxygen Consumption Rate

In models of caloric restriction and nutrient deprivation, AMPK is thought to serve as the “master regulator”, activating a number of effector proteins and transcription factors. Indeed, we also observed an increase in AMPK activation (p-AMPK), but no significant change in overall AMPK. AMPK has been reported to also inhibit oxidative phosphorylation and promote glycolytic ATP production. This is in contrast to the increased oxygen consumption that was observed in our studies. What could potentially account for this phenomenon is that AMPK also promotes oxidation and break down of fatty acids in the mitochondria. Since our cellular model was cultured in 10% new-born calf serum, it is possible that the lipid content in the serum could possibly have served as a transient metabolic substrate in episodes of glucose deprivation. It is estimated that the lipid content of cell culture grade serum can range from 10–100µg/L, depending on the pooled sources (20).

Metformin Protects Against Low Glucose-Induced Elevated Oxygen Consumption; a process that may involve reducing oxidative stress

The elevated oxygen consumption rate is associated with an increased production of free radicals, which creates an oxidative burden on the cells. While the present studies did not undertake quantitation of ROS/RNS, the uncoupled respiration and the elevated oxygen consumption most likely did lead to an increased production of free radicals. Complex I and complex III are the two major ROS/RNS producers in the respiratory chain, together accounting for over 50% of the free radicals produced in the cell. Metformin acts on the mitochondrial respiratory chain by inhibition of complex I. Our experiments did not include complex III studies, but its potential contribution would be of future interest. Our data showed that metformin supplementation lowered oxygen consumption rates in glucose-deprived cells to levels similar to that of cells exposed to normal glucose, thereby likely reducing the oxidative stress.

Metformin's Protection Extends Beyond the Mitochondria and Preserves Cellular Viability: The Little Mermaid Effect

The fact that metformin's protective effect was time-limited and appeared to dissipate after 12 hours, brings to mind the old fairy tale of the Little Mermaid, written by the Danish

author, Han Christian Andersen in 1837, in which the little mermaid traded a short term benefit (i.e., human identity) with her subsequent long life in the sea. It may be that this “little mermaid effect” of metformin was the apparently enhanced survival in the short term, but with the trade-off of reduced cell survival after prolonged low-glucose stress (similar to the stipulation that if the little mermaid did not get “rescued” by the prince, then she would perish at sunrise and ascend to the sky as small bubbles, somewhat similar to the dying cells lifting off from the bottom of the culture dish).

Metformin: Potential Cross-Talk Among Various Nutrient Sensitive Pathways

Metformin may also achieve the restoration of oxygen consumption rate indirectly through cross-talk among multiple nutrient sensing pathways (21, 22). We measured AMPK and mTOR levels to see a pattern, if any. Interestingly, our data showed no overall increase in AMPK, but rather an increase in activated AMPK (p-AMPK). Activated AMPK could account for the lowering of oxygen consumption, as it functions to inhibit oxidative phosphorylation and upregulate β -oxidation of fatty acids.

Low-glucose stress did not appear to increase or decrease levels of mTOR in the C2C12 cells. Other potential mechanisms that may also be involved in metformin’s effect during short-term, low-glucose exposure may include epigenetic modulation, including transcription activation and/or repression. In addition, a compensatory upregulation of complex II to offset complex I inhibition may also be plausible. It has been reported that metformin and other biguanides tend to reduce complex I and may increase complex II activity via GPDH (23, 24). This potential link could partially explain the “uncoupling” effect that our data seem to suggest. The possible involvement of p49/STRAP in this uncoupling effect and the role of metformin is intriguing, and future studies in this area would be of interest.

Acknowledgments

Supported in part by the Claude D. Pepper Older American Independence Center grant (1P30AG28718-01A2). We thank Laura Gocio for assistance with manuscript preparation.

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Highlights

1. Acute low-glucose stress causes an increase in oxygen consumption rate. (74 characters w/spaces)
2. Metformin lowers oxygen consumption rate in low glucose-stressed cells. (73 characters w/spaces)
3. Metformin's protection extends beyond mitochondria to include cell viability. (79 characters w/spaces)
4. Metformin's protective mechanism in low glucose may entail AMPK pathway. (74 characters w/spaces)

Figure 1. Low glucose and acute glycolytic function

(left) After 6 hours of low glucose incubation, oxygen consumption rate (OCR) was measured after sequentially adding to each well 25 μ L of oligomycin (1 μ M) in “Port A” and 25 μ L of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) at a final concentration of 0.3 μ M to “Port B”, (0.3 μ M) and rotenone/antimycin to Port D (4 μ M).

(right) After 6-hour low glucose stress, glycolytic function was measured after sequentially adding 25 μ L of 1.5 mM glucose in “Port A”, 1.0 μ M Oligomycin to “Port B” and 100 mM of 2-DG to “Port C.

Figure 2. Oxygen consumption rate (OCR) after 1 and 6- hour low glucose stress with metformin supplementation

After 1(a) and 6 (b) hours of low glucose +/- metformin, OCR was measured as described in Figure 1. LG + MET = low glucose with metformin supplementation (Blue); LG = low glucose (Red); NG = normal glucose (Green). Error bars, $\pm$ SE (percentage).

Figure 3. Oxygen consumption rate (OCR) after 12 and 24-hour low glucose stress with metformin supplementation

After 12 (a) and 24 (b) hour of low glucose +/- metformin, OCR was measured as described in Figure 1. Error bars, $\pm$ SE (percentage).

Figure 4. Low and normal glucose 24-hour metabolic profile with and without metformin
C2C12 Myoblasts were incubated in low and normal glucose with or without metformin for up to 24 hours, at which oxygen consumption rate (OCR) was measured and compared.

Figure 5. Low Glucose with Metformin's Effect of on C2C12 Cell Viability

C2C12 cells were subjected to continuous 6, 9, and 24-hour low glucose stress, respectively, with and without metformin supplementation. After which, cells were assayed for viability using trypan blue (described in Materials and Methods section).

Figure 6. Quantitative Analysis of Low Glucose with Metformin's Effect of on C2C12 Cell Viability

C2C12 cells were subjected to continuous 6, 9, and 24-hour low glucose stress, respectively, with and without metformin supplementation. After which, cells were assayed for viability using trypan blue (described in Materials and Methods section). Note: Data means are the $\pm$ SEM of 5 different depths of field analysis in two different experiments. * $p < 0.05$, LG vs. LG + Met; ** $p < 0.01$, LG vs. LG + Met.

Figure 7. AMPK expression and activation during 12-hour low-glucose stress

(a) Overall AMPK expression in C2C12 cells exposed to six and 12-hours of low glucose as described in details in materials and methods. (b) p-AMPK expression during six and 12-hours of low-glucose stress. (c) Time course of Low glucose-induced AMPK protein expression quantitation. (d) Time course of Low glucose-induced p-AMPK protein expression quantitation. Data expressed as means $\pm$ SE of two experiments. * $p < 0.05$.

Figure 8. mTOR expression during 12-hour low-glucose stress

(a) mTOR expression in C2C12 cells exposed to six and 12-hours of low glucose. (b) Time course of Low glucose-induced mTOR protein expression quantitation.. Data expressed as means $\pm$ SE of two experiments. * $p < 0.05$.

Table 1

Comparative Effects of Short-term Glucose Stress with Metformin on Oxygen Consumption Rate, p49/STRAP Expression, and Cell Viability in C2C12 Cells

Treatment	Oxygen Consumption Rate	Cell Viability
6 Hours Normal Glucose *	—	—
6 Hours Normal Glucose (MET)	No change	No Change
6 Hours Low Glucose	↑ (36%)	↓ (10%)
6 Hours Low Glucose (MET)	No change	↑ (4%)

Treatment	Cell Death % Change (vs. 9-hr LG Time Point)
6 Hours Low Glucose (MET)	↓ (88%)
9 Hours Low Glucose (MET)	↓ (36%)

Normal Glucose = 100 mg/dL; Low Glucose = 35 mg/dL. MET = Metformin.

* Reference control for OCR and p49/STRAP expression in 6-hour glucose exposure times.