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High glucose induces mitochondrial dysfunction independently of protein O-GlcNAcylation

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Abstract

Diabetes is characterized by hyperglycaemia and perturbations in intermediary metabolism. In particular, diabetes can augment flux through accessory pathways of glucose metabolism, such as the hexosamine biosynthetic pathway (HBP), which produces the sugar donor for the β-O-linked-*N*-acetylglucosamine (O-GlcNAc) post-translational modification of proteins. Diabetes also promotes mitochondrial dysfunction. Nevertheless, the relationships among diabetes, hyperglycaemia, mitochondrial dysfunction and O-GlcNAc modifications remain unclear. In the present study, we tested whether high-induced increases in O-GlcNAc modifications directly regulate mitochondrial function in isolated cardiomyocytes. Augmentation of O-GlcNAcylation with high glucose (33 mM) was associated with diminished basal and maximal cardiomyocyte respiration, a decreased mitochondrial reserve capacity and lower Complex II-dependent respiration ($P < 0.05$); however, pharmacological or genetic modulation of O-GlcNAc modifications under normal or high glucose conditions showed few significant effects on mitochondrial respiration, suggesting that O-GlcNAc does not play a major role in regulating cardiomyocyte mitochondrial function. Furthermore, an osmotic control recapitulated high-glucose-induced changes to mitochondrial metabolism ($P < 0.05$) without increasing O-GlcNAcylation. Thus, increased O-GlcNAcylation is neither sufficient nor necessary for high-glucose-induced suppression of mitochondrial metabolism in isolated cardiomyocytes.

Keywords

cardiomyocytes; diabetes; hexosamine biosynthetic pathway; mitochondrial function

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AUTHOR CONTRIBUTION

Sujith Dassanayaka performed the experiments, analysed the data, generated the figures, wrote the draft and revised the manuscript. Ryan Readnower performed experiments, analysed data, wrote the draft and designed the experiments. Joshua Salabei performed the experiments and analysed data. Bethany Long performed the experiments and analysed data. Allison Aird performed the experiments and analysed the data. Yu-Ting Zheng performed the experiments and analysed the data. Senthilkumar Muthusamy performed and designed the experiments. Heberty Facundo performed and designed the experiments. Bradford Hill designed the experiments, revised the manuscript and provided funding. Steven Jones designed the experiments, wrote the draft, revised the manuscript and provided funding.

INTRODUCTION

Diabetes can promote cardiac dysfunction independently of coronary disease [1–3]. Although the mechanisms of diabetic cardiomyopathy [4,5] remain unknown, dysregulated cardiac metabolism is a likely contributor. Mitochondria, central elements in metabolism, become dysfunctional during diabetes [6,7] and the diabetic heart demonstrates lower levels of glucose utilization [8]. Despite decreases in glucose uptake and oxidation [9], diabetes may alter the fate of glucose and increase flux of glucose-derived carbons through accessory pathways, such as the hexosamine biosynthetic pathway (HBP). Indeed, this is commonly observed in diabetic tissues and in cells cultured under diabetic conditions [10–17]. Hence, some have argued that the HBP plays a causal role in diabetic pathophysiology of the cardiovascular system [18].

Under resting physiological conditions, a minor fraction of intracellular glucose is committed to the HBP, which culminates in the formation of UDP-GlcNAc. UDP-GlcNAc is the monosaccharide donor for the β-O-linked-*N*-acetylglucosamine (O-GlcNAc) post-translational modification of proteins [19–21]. O-GlcNAcylation of target proteins can induce functional changes and alter transcription and translation. In this evolutionarily conserved system, only two antagonistic enzymes catalyse O-GlcNAc modification of proteins: O-GlcNAc transferase (OGT) catalyses the O-linkage of GlcNAc to proteins at serine or threonine residues, whereas O-GlcNAcase (OGA) removes GlcNAc from proteins. Thus, hyperglycaemia, a hallmark of diabetes, may increase flux through the HBP and consequently promote protein O-GlcNAcylation [22–25]. Indeed, several studies suggest that protein O-GlcNAcylation contributes to various aspects of cellular dysfunction during diabetes [10–14,16– 18,25–31]. In contrast, acute protein O-GlcNAcylation confers protection in the context of cell stress [20,32–43].

Given the importance of understanding the mechanisms of diabetic pathophysiology, particularly metabolism and mitochondrial function, and the diverse roles of O-GlcNAc in various diseases, we investigated the influence of O-GlcNAcylation on mitochondrial bioenergetics. We used extracellular flux (XF) analysis and genetic/pharmacological manipulation of O-GlcNAc levels to address the potential relationship between mitochondrial dysfunction, high glucose and O-GlcNAc. Our results suggest that high-glucose-induced mitochondrial dysfunction can occur independently of increased protein O-GlcNAcylation.

MATERIALS AND METHODS

All animals were used in compliance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health. The experimental protocols for the present study have been reviewed and approved by the University of Louisville Institutional Animal Care and Use Committee.

Neonatal rat cardiomyocyte isolation and culture

Neonatal rat cardiomyocytes (NRCMs) were isolated as previously described [36,37,40,44–51] from 1–2-day-old Sprague–Dawley rats. NRCMs were plated at a density of 850 000 cells/ml in six-well plates for protein isolation or 75 000 cells per well in Seahorse plates for bioenergetic assay. For the first 4 days following isolation, NRCMs were cultured in medium containing BrdU (bromodeoxyuridine; 0.1 mM), 5% FBS, penicillin (100 units/ml), streptomycin (100 mg/ml) and vitamin B_{12} (2 μg/ml). On day 4 (post-isolation), NRCM medium was changed to Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM glutamine, 1 mM pyruvate and the corresponding treatment (5 mM D-glucose, 33 mM D-glucose or 5 mM D-glucose + 28 mM mannitol). A 5 mM D-glucose + 28 mM mannitol treatment served as the osmotic control. Cells were cultured in their respective treatment for 48 h prior to protein or bioenergetics assays.

Gene transfer and OGA inhibition

For gene transfer experiments, NRCMs were serum-starved overnight and then transduced with 100 multiplicity of infection (MOI) of replication-deficient adenoviruses carrying the OGT gene (Ad-OGT) or the OGA gene (Ad-OGA) or null virus (Ad-Null) in medium containing 5% FBS for 5 h [36,37,40,42,44,48]. After 5 h, the medium was replenished with medium lacking virus. Bioenergetics profiling and protein isolation occurred 48 h post-transduction.

To pharmacologically augment O-GlcNAc levels, we used Thiamet G (TMG; Cayman Chemicals), which inhibits OGA and increases protein O-GlcNAcylation. NRCMs were treated with 1 μM TMG or vehicle (DMSO). Bioenergetics profiling of intact NRCMs was conducted 48 h after TMG treatment. Bioenergetics profiling of permeabilized NRCMs was conducted 24 h after TMG treatment.

Bioenergetic profiling of intact adherent cells

The bioenergetics of intact adherent NRCMs that were seeded at 75000 cells per well was measured using a Seahorse Bioscience XF24 Flux Analyzer [42,47,50,52,53]. For these experiments, the treatment medium was replaced with 675 μl of assay medium: unbuffered DMEM supplemented with glucose (5 or 33 mM; or appropriate concentration of mannitol osmotic control), 4 mM glutamine and 1 mM pyruvate, and plates were placed in a non-CO_2 supplemented incubator 1 h before assay. Following microplate insertion, the XF24 automated protocol consisted of a 10 min delay followed by baseline oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements [3 × (3 min mixing, 2 min wait and 3 min measure)]. To interrogate mitochondrial function, the following compounds were injected following three baseline measurements: Port A, oligomycin; Port B, FCCP (carbonilcyanide p-triflouromethoxyphenylhydrazone); and Port C, antimycin A (AA). After each injection, one measurement was recorded, with each having a 3 min mixing, 2 min wait and 3 min measure cycle. Stocks (1 mM) of oligomycin (Sigma), FCCP (Sigma) and AA (Sigma) were prepared in 100% DMSO (Sigma). Prior to assay, stocks were diluted in assay medium to yield 0.01 mM oligomycin, 0.01 mM FCCP and 0.1 mM AA, which, after injection, yielded final concentrations of 1.0 μM oligomycin, 1.0 μM FCCP and 10.0 μM AA. All experiments were conducted at 37°C. Parameters of mitochondrial

function were calculated as previously described [47,54–56]. Protein concentration measured following XF analysis was not significantly different between groups.

Bioenergetic profiling of permeabilized, adherent cells

Bioenergetic profiling for electron transport chain activity was performed as recently described [57]. Prior to bioenergetic profiling, NRCMs were changed to mannitol and sucrose (MAS) medium {220 mM mannitol, 70 mM sucrose, 5 mM MOPS [3-(*N*-morpholino)propansulfonic acid] and 4% fatty-acid-free BSA, pH 7.2}. NRCMs were permeabilized following a Port A injection of 25 μg/ml saponin. Complex II-specific substrate [10 mM succinate, 1 μM rotenone (Rot) and 1 mM ADP] was also contained in Port A to support cellular respiration. Following State 3 rate measurements, oligomycin (1 μg/ml) was injected from Port B; following State 4_o measurement, AA (10 μM) and Rot (1 μM) were injected from Port C to inhibit mitochondrial oxygen consumption. Protein concentration measured following XF analysis was not significantly different between groups.

Cell fractionation

NRCMs ($n = 3$) were fractionated to assess O-GlcNAcylation of mitochondrial proteins following treatment with 5 or 33 mM glucose, osmotic control (5 mM glucose + 28 mM mannitol), TMG, Ad-Null or Ad-OGA. Cardiomyocytes were trypsinized with 0.25% trypsin/EDTA (Thermo Fisher) for 5 min at 37°C. Trypsin was neutralized and cells were counted. Cardiomyocyte fractionation was performed with a standard Cell Fractionation Kit (Abcam).

Immunoblotting

Total cellular protein was harvested and quantified from NRCMs using established protocols [20,36,37,39–42,45–48,50,53,58]. Standard Western blotting procedures were used to probe for O-GlcNAc (anti-O-GlcNAc, CTD110.6; Biolegend), OGT (DM-17; Sigma) and OGA (anti-NCOAT; Sigma). Antibodies for α-tubulin (Sigma) and ATP5A (Abcam) were used as loading controls for whole-cell and mitochondrial protein respectively. Protein expression levels were quantified by densitometric analysis. For O-GlcNAc Western blots, the entire lane was quantified to determine overall protein O-GlcNAcylation [20,36,37,39–42,44,45,48].

Statistical analyses

Results are shown as means + S.D. (not S.E.M.). Statistical analyses (GraphPad Prism 5.0d) were conducted using a paired Student's *t* test or by one-way ANOVA followed by Newman– Keuls Multiple Comparison Test, as appropriate. Each '*n*' represents a separate NRCM isolation; treatments/interventions were always performed with their respective controls. Differences were considered statistically significant if $P < 0.05$.

RESULTS

High glucose increases protein O-GlcNAcylation

To determine whether high-glucose promotes protein O-GlcNAcylation, NRCMs were cultured for 48 h in medium supplemented with 0, 5, 10, 20 or 33 mM glucose (the osmotic control group was 5 mM glucose + 28 mM mannitol) and O-GlcNAc levels were assessed via immunoblotting (Figure 1A). Quantification of protein O-GlcNAc levels in whole cells demonstrated that the 33 mM glucose treatment resulted in a significant increase in O-GlcNAcylation when compared with 5 mM glucose (Figure 1B); however, when we isolated the mitochondrial fraction from similarly treated cells and probed for mitochondrial protein O-GlcNAcylation, we observed a much smaller effect (Figure 1C). Because we confirmed that high glucose induced protein O-GlcNAcylation in our system (similar to others [17,59]), we next queried whether high glucose compromises mitochondrial function.

High glucose depresses mitochondrial bioenergetic reserve capacity

We performed XF analysis of NRCMs to determine whether high-glucose-mediated increases in O-GlcNAc could affect mitochondrial bioenergetics in the intact cell (Figure 1D, E). Protein concentration measured following XF analysis was not significantly different between groups. High glucose (33 mM, $n = 7$) depressed basal respiration (Figure 1F; $P < 0.05$), maximal respiration (Figure 1G; $P < 0.05$), reserve capacity (Figure 1H; $P < 0.05$) and proton leak (Figure 1J; $P < 0.05$) compared with the normal glucose control (5 mM, $n = 7$). ATP-linked respiration remained unaltered following hyperglycaemic treatment (Figure 1I). To address the contribution of an osmotic influence in the depression of mitochondrial function, we used an osmotic control (5 mM glucose + 28 mM mannitol, $n = 7$), which recapitulated the depression ($P < 0.05$) in basal respiration, maximal respiration, reserve capacity and proton leak (Figures 1E–1J). We also queried whether the osmotic stress also augmented O-GlcNAc levels (similar to the high glucose treatment) and found that the osmotic control (see lane ‘Osm’ in Figure 1A) did not affect O-GlcNAc levels. Similarly, mitochondrial O-GlcNAcylation was largely unaffected by the osmotic control treatment (Figure 1C). Collectively, these findings indicate that the high-glucose-induced suppression of basal respiration, maximal respiration and reserve capacity were associated with an increase in osmolarity and not the high-glucose-induced increase in O-GlcNAcylation (Figure 1A). Interestingly, much of the basal differences in OCR were attributable to reductions in proton leak, which was reduced in both the high glucose and the osmotic control groups ($P < 0.05$) (Figure 1J) compared with the normal glucose group. Despite this apparent improvement in mitochondrial coupling, cells that were treated with high glucose or exposed to osmotic stress showed diminished mitochondrial reserve capacity. Thus, we suggest that osmotic stress caused by high glucose instigates loss of mitochondrial reserve capacity.

OGA or OGT overexpression does not alter bioenergetic reserve

Although high-glucose-induced suppression of mitochondrial bioenergetics seemed O-GlcNAc-independent, we next asked whether it was possible for alterations in O-GlcNAc levels to influence mitochondrial bioenergetics. To this end, we transduced cells with Ad-OGT or Ad-OGA prior to normal glucose treatment for 48 h (Figure 2). Transduction

resulted in a corresponding increase in OGT and OGA protein expression (Figure 2A) and commensurate changes in protein O-GlcNAcylation (Figure 2B). Overexpression of either OGT (n = 5) or OGA (n = 5) induced a small but statistically significant depression in basal respiration (Figures 2C and 2D) and OGA overexpression decreased proton leak (Figure 2H; $P < 0.05$). Overexpression of neither OGT nor OGA affected maximal respiration or reserve capacity (Figures 2E and 2F). Importantly, none of these modest changes are consistent with the high glucose-induced suppression of mitochondrial reserve capacity (Figure 1). Thus, neither promoting nor antagonizing O-GlcNAcylation via genetic means is sufficient to recapitulate the high-glucose-induced bioenergetic defect.

Inhibition of OGA does not affect oxygen consumption in NRCMs

To further investigate whether protein O-GlcNAcylation is responsible for the bioenergetics depression observed with high glucose treatment, we used a potent pharmacological inhibitor of OGA, TMG, to increase robustly whole-cell protein O-GlcNAcylation (Figures 3A and 3B). Isolated mitochondrial fractions from similarly treated NRCMs showed no net significant increases in protein O-GlcNAcylation, although one band did appear to show elevated O-GlcNAc modification (n= 3; Figure 3C). Regardless, XF analysis in the intact cell preparations indicated that TMG did not significantly affect basal respiration, maximal respiration or reserve capacity (Figures 3D–3F). TMG treatment slightly but significantly decreased ATP-linked respiration (Figure 3H); however, proton leak (Figure 3I) and non-mitochondrial OCR (results not shown) were not different. Even inhibiting OGA with PUGNAc, a less potent inhibitor of OGA, or DON, an inhibitor of GFAT, the rate-limiting enzyme in formation of the O-GlcNAc moiety, did not alter mitochondrial bioenergetics (Supplementary Figure S1). Thus, inhibition of OGA (via TMG) increased O-GlcNAcylation but, unlike high glucose, did not suppress mitochondrial reserve capacity.

High glucose suppresses Complex II-dependent respiration

To determine the specific sites at which high glucose (and osmotic stress) might depress mitochondrial function, we subjected cardiomyocytes to conditions mimicking euglycaemia or hyperglycaemia for 48 h and then examined mitochondrial activity in permeabilized cells. We found Complex II-dependent respiration was depressed by high glucose (Figure 4). Succinate-supported State 3 and State 4_o respiration was approximately 40% lower in cardiomyocytes incubated in high glucose ($P < 0.05$; Figures 4A–4C). Similar to the findings in intact cells, an osmotic control recapitulated the high-glucose-induced suppression of State 3 respiration ($P < 0.05$; Figure 4B). No change in respiratory control ratio (RCR) was found after high glucose treatment or in the osmotic control (Figure 4D). These data are consistent with intact cell data, suggesting that high osmolarity mediates depression in mitochondrial function.

OGA overexpression does not rescue the high-glucose-induced bioenergetic defect

Next, we reasoned that, if enhanced protein O-GlcNAcylation were necessary (though clearly insufficient) for the high-glucose-induced depression in bioenergetic reserve capacity, then decreasing protein O-GlcNAcylation should rescue this defect. To this end, we overexpressed OGA, which as expected decreased O-GlcNAcylation (Figures 5A and

5B). OGA overexpression produced detectable OGA in mitochondrial fractions; however, there was not a significant difference in O-GlcNAcylation in the mitochondrial fraction (Figures 5C and 5D). OGA overexpression did not rescue suppression of mitochondrial reserve capacity induced by high glucose ($P < 0.05$; Figure 5H). In fact, in cells incubated with high glucose, overexpression of OGA depressed both maximal respiration ($P < 0.05$; Figure 5G) and reserve capacity ($P < 0.05$; Figure 5H). Overexpression of OGA did not affect ATP-linked respiration or proton leak (Figures 5I and 5J). These results suggest that an elevated level of O-GlcNAcylation is neither necessary nor sufficient to explain high-glucose-induced suppression of mitochondrial bioenergetics.

Inhibition of OGA increases Complex II-dependent State 3 respiration

Because high glucose impaired Complex II-dependent respiration (and induced epiphenomenal O-GlcNAcylation), we determined whether increasing O-GlcNAcylation with TMG (which inhibits OGA) might affect Complex II-dependent oxygen consumption in the permeabilized cell assay. Inhibition of OGA significantly increased protein O-GlcNAcylation (Figures 3A and 3B) and Complex II-dependent State 3 and 4_o respiration (Figures 6A–6C); however, OGA inhibition did not significantly change respiration stimulated by provision of pyruvate and malate (Complex I) and it did not affect Complex III + IV-dependent OCR (Supplementary Figure S2). Thus, augmenting protein O-GlcNAcylation pharmacologically is not sufficient to depress mitochondrial function and may actually increase Complex II-dependent mitochondrial respiration.

DISCUSSION

In the present study, we directly addressed an emerging question regarding the relationship between high glucose, mitochondrial dysfunction and O-GlcNAcylation. First, we confirmed that high glucose could induce mitochondrial dysfunction and increase protein O-GlcNAcylation. Although impaired mitochondrial function was evident in high glucose conditions, an osmotic control exerted a similar suppressive effect on bioenergetic reserve; yet, the osmotic control did not increase O-GlcNAcylation. Furthermore, increasing O-GlcNAcylation through either OGA inhibition or OGT overexpression was insufficient to recapitulate the deleterious effects of high glucose on mitochondrial function. Finally overexpressing OGA in the context of hyperglycemia to reduce protein O-GlcNAcylation did not rescue the hyperglycemia-induced suppression of reserve capacity. Thus, increased O-GlcNAcylation is neither sufficient nor necessary for high-glucose-induced mitochondrial dysfunction in isolated cardiomyocytes.

Considering the manifold changes in metabolism and metabolic signalling that occur in diabetes, there are numerous options for targeted investigations. In particular, protein O-GlcNAcylation has received growing attention as a candidate component of diabetic pathophysiology [18]. This idea holds that elevations in extracellular glucose floods accessory pathways of glucose metabolism, one of which is the HBP, which produces the sugar donor for the O-GlcNAc modification of proteins. Indeed, elevated extracellular glucose concentration enhances OGT activity [10] and promotes protein O-GlcNAcylation [10,16,59]. Moreover, O-GlcNAcylation is enhanced in patients with diabetes [29,60].

Animal models of diabetes, such as streptozotocin-treated mice, Zucker rats and *db*/*db* mice, all demonstrate elevated O-GlcNAcylation of at least some proteins in the heart [13,14,16]. Thus, the coincident observations of increased O-GlcNAcylation and cardiac dysfunction during diabetes are consistent with the notion that O-GlcNAc plays a role in diabetic cardiac dysfunction.

Given the critical role of mitochondria in metabolism in general and the centrality of high mitochondrial activity to cardiac function specifically, several groups have pondered the potential role of hyperglycaemia/high glucose in mitochondrial dysfunction [61]. Moreover, some have attempted to identify a connection between O-GlcNAcylation and mitochondrial dysfunction [59,62]. In studies of suspended permeabilized NRCMs, Hu et al. [59] identified high-glucose-induced O-GlcNAcylation of cardiac mitochondrial Complexes I, III and IV and associated it with impaired mitochondrial function. Overexpression of OGA (to reverse the high-glucose-induced increase in O-GlcNAcylation) largely rescued the observed defects in mitochondrial complex activities. Interestingly, the study by Hu et al. [59] and our present work share several similarities: (1) both focused on mitochondrial function in NRCMs and assessed it, in part, via respirometry; (2) both used mannitol as an osmotic control; (3) both attempted adenovirus-mediated rescue of high-glucose-induced mitochondrial dysfunction and (4) both studies (at least in part) assessed cell function in permeabilized cells. Nevertheless, there were key differences in methodology between the two reports: (1) we evaluated mitochondrial function in adherent cardiomyocytes (intact and permeabilized); (2) we recapitulated the increase in O-GlcNAcylation via genetic overexpression of OGT and pharmacological inhibition of OGA (in the absence of high glucose); and (3) we maintained the intact cells in the respective glucose conditions (i.e. the high glucose cells were subjected to the XF assay in high glucose conditions). Any number of these relatively subtle study differences may partially explain the discrepancies in our findings.

In a related and recent study, Tan et al. [62] used a proteomics approach to study the effect of altering O-GlcNAc on mitochondrial protein expression, morphology and function in neuroblastoma cells. They observed that overexpression of either OGT or OGA resulted in altered mitochondrial expression of proteins involved in transport, translation and respiratory activity [62]. Furthermore, these changes resulted in alterations in mitochondrial morphology and function. On face value, it may appear that the results of Tan et al. [62] also disagree with our present results; however, the differences may be fewer than they appear. We too found that overexpression of either OGT or OGA reduced OCR (including maximal OCR) in the context of high glucose. Yet, when we overexpressed OGT or OGA in euglycaemic conditions, only basal respiration was slightly depressed. Interestingly, TMG, which remarkably increases O-GlcNAc levels, did not negatively affect OCR. Thus, these data indicate that there is not a clear and deleterious relationship between O-GlcNAcylation and mitochondrial dysfunction.

The fact that high-glucose-induced O-GlcNAcylation had little effect on mitochondrial energetics suggests an alternative mechanism by which hyperglycaemia impairs mitochondrial function. Interestingly, the defect in reserve capacity caused by the hyperglycaemic condition was recapitulated with the osmotic control, mannitol. Although we did not study the mechanism by which osmotic stress may have mediated changes in the

mitochondrial function, this observation could be relevant for the response of myocytes to stress. For example, elevated by-products of anaerobic metabolism occurring during ischaemia create an osmotic load that has been estimated to be ∼60 mOsm [63–65]. This acute change in osmolality affects cell volume, cation/anion transport and other phenomena such as ischaemic pre-conditioning [66]. Hence, because cell volume is tightly linked with cation/anion transport and cation transport is a key regulator of ATP demand and mitochondrial activity, it is possible that the osmotic stress induced by high glucose reduces reserve capacity in a manner dependent on changes in these critical processes. Further studies are required to assess the full significance of osmotic stress to hyperglycaemia-induced myocyte stress.

A major question in the field relates to a mitochondrial O-GlcNAcylation system. Evidence of a mitochondrial OGT [(m)OGT] [67,68] would appear to suggest that a mitochondrial OGA [(m)OGA] should exist as well; however, our studies demonstrate that under normal conditions, mitochondria in myocytes have little, if any, detectable OGA and we only detected (m)OGA when we overexpressed OGA using adenoviruses. Because this is an overexpression system and fractionation procedures do not yield 100 % pure subcellular fractions, it is possible that the OGA we found in the mitochondrial fraction was a contaminant from the cytoplasm. Also, the transport mechanism for the essential substrate, UDP–GlcNAc, has never been identified in the mitochondrion. Hence, the nature of how mitochondrial O-GlcNAcylation occurs and whether it is due to a veritable mitochondrial O-GlcNAc-modification system, comprising a UDP–GlcNAc transporter, (m)OGA and (m)OGT, is still unclear. It is possible that O-GlcNAcylation of mitochondrial proteins predominately occurs in the cytosol prior to mitochondrial protein targeting.

In conclusion, several approaches, including overexpression of OGA or OGT, exposure to high glucose and pharmacological manipulation, were used to assess the role of O-GlcNAc in regulating cardiomyocyte bioenergetics. We demonstrate that O-GlcNAcylation is probably not responsible for mitochondrial dysfunction occurring during hyperglycaemic conditions and that osmotic stress due to high glucose appears to underlie depression of mitochondrial reserve capacity. Although these findings do not entirely rule out the involvement of O-GlcNAc in modulating bioenergetics in diabetes, they show that increasing O-GlcNAc levels are not sufficient or necessary to cause high-glucose-induced bioenergetic dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

(m)OGA	mitochondrial OGA
(m)OGT	mitochondrial OGT
AA	antimycin A
Ad-Null	null adeno virus
Ad-OGA	adenoviruses carrying OGA gene
Ad-OGT	adenoviruses carrying OGT gene
DMEM	Dulbecco's modified Eagle's medium
HBP	hexosamine biosynthetic pathway
MAS	mannitol and sucrose
NRCM	neonatal rat cardiomyocyte
OCR	oxygen consumption rate
OGA	O-GlcNAcase
O-GlcNAc	β-O-linked-*N*-acetylglucosamine
OGT	O-GlcNAc transferase
Rot	rotenone
TMG	Thiamet G
XF	extracellular flux

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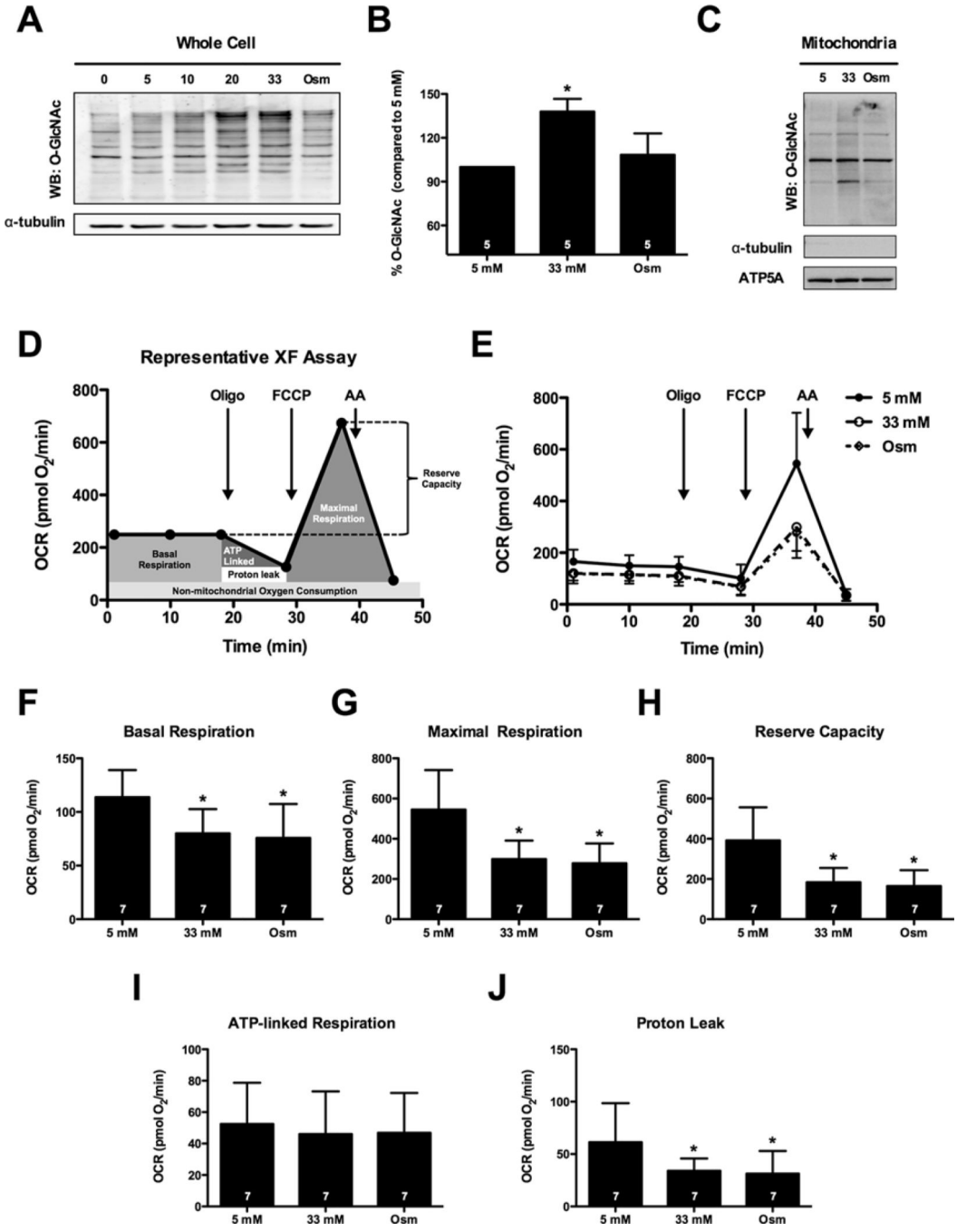

Figure 1. High glucose depresses the bioenergetic reserve capacity of cardiomyocytes

(**A**) Immunoblot of whole-cell protein O-GlcNAcylation following 0, 5, 10, 20 and 33 mM glucose treatment. An osmotic control (Osm, 5 mM glucose + 28 mM mannitol) was also used. (**B**) Densitometric measurement of O-GlcNAcylation in relation to 5 mM control demonstrated a significant induction of protein O-GlcNAcylation following 33 mM glucose. (**C**) Immunoblotting for mitochondrial protein O-GlcNAcylation. NRCMs were fractionated to isolate mitochondrial protein following treatment with 5 mM glucose, 33 mM glucose and the osmotic control. (**D**) Representative XF assay and diagram of how mitochondrial

measurements were calculated. (**E**) Mitochondrial function assay following 48 h of high glucose treatment. From (**E**) parameters of mitochondrial function were assessed (as described in **D**): (**F**) basal respiration, (**G**) maximal respiration, (**H**) reserve capacity, (**I**) ATP-linked respiration, and (**J**) proton leak. As indicated in bars, $n = 7$ independent experiments; $^{*}P < 0.05$, compared with 5 mM.

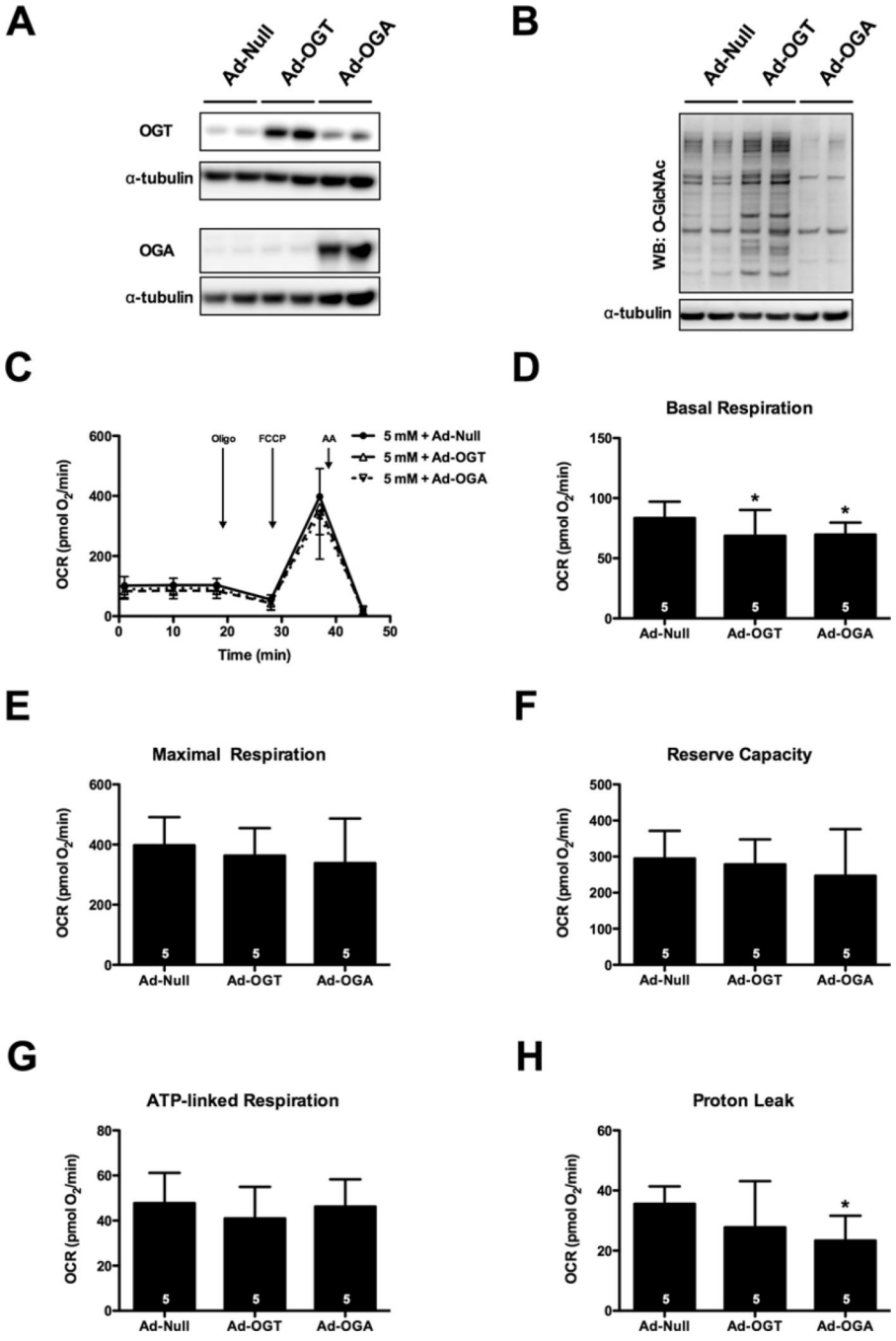

Figure 2. Overexpression of OGT or OGA does not affect bioenergetic reserve

NRCMs were subjected to adenoviral overexpression of OGT and OGA. (**A**) Immunoblot for OGT and OGA protein expression following adenoviral treatment. (**B**) Immunoblot for protein O-GlcNAcylation demonstrated an induction of O-GlcNAc in response to Ad-OGT and a reduction in response to Ad-OGA. (**C**) Mitochondrial function assay of NRCMs 48 h post-transfection. Parameters of mitochondrial function were calculated from (**C**), including: (**D**) basal respiration, (**E**) maximal respiration, (**F**) reserve capacity, (**G**) ATP-linked

respiration, and (**H**) proton leak. As indicated in bars, $n = 5$ independent experiments; $^{*}P < 0.05$, compared with Ad-Null.

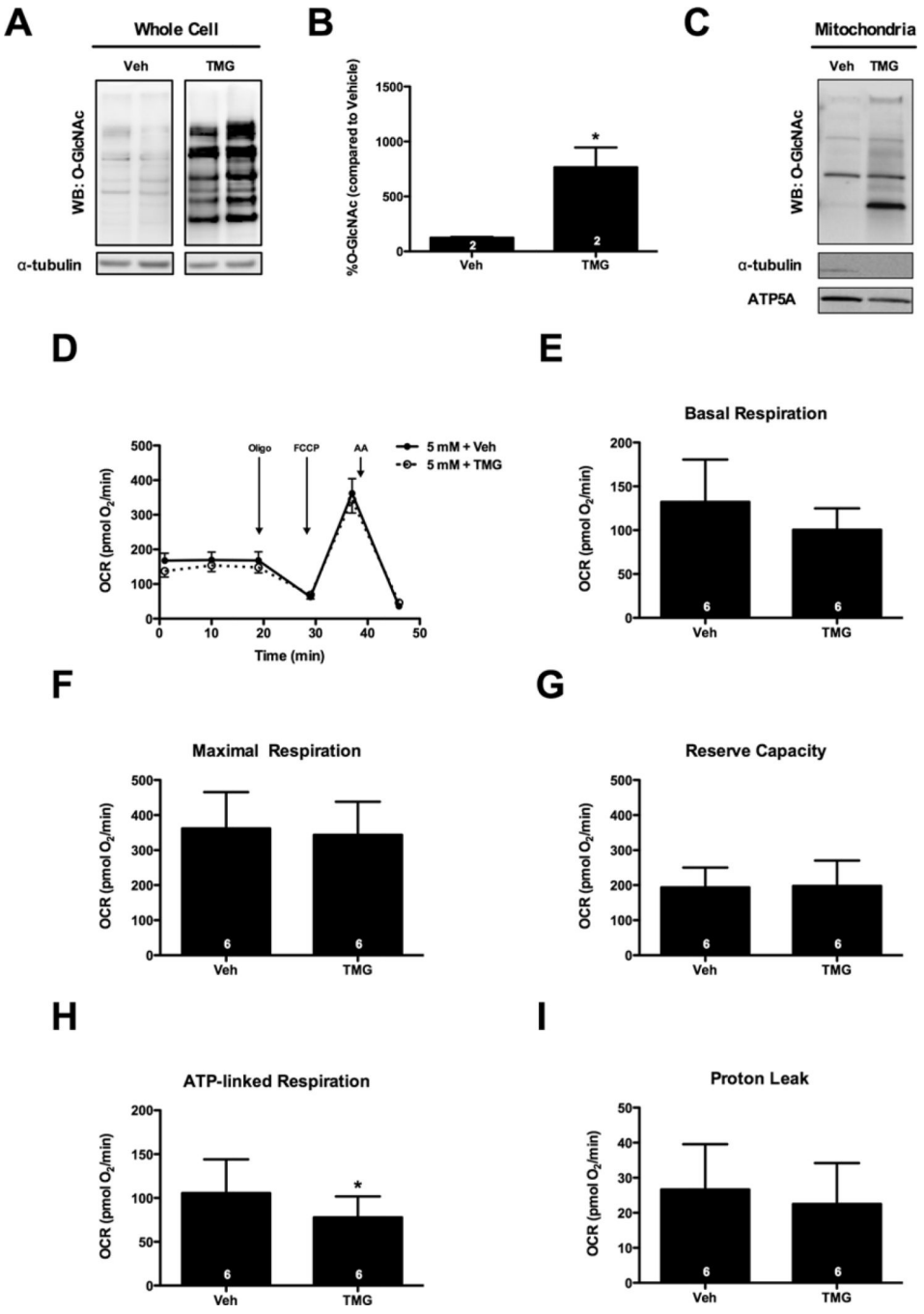

Figure 3. Inhibition of OGA has negligible effects on mitochondrial function

NRCMs were treated with TMG (1 μM) for 48 h to induce protein O-GlcNAcylation. (**A**) Immunoblot for whole-cell protein O-GlcNAcylation. (**B**) Densitometric measurement of O-GlcNAcylation. (**C**) Immunoblot for mitochondrial protein O-GlcNAcylation following treatment with 1 μM TMG. (**D**) Mitochondrial function assay of NRCMs following 48 h treatment with TMG. Parameters of mitochondrial function were measured from (**D**), including: (**E**) basal respiration, (**F**) maximal respiration, (**G**) reserve capacity, (**H**) ATP-

linked respiration, and (**I**) proton leak. As indicated in bars, $n = 6$ independent experiments; $^{*}P < 0.05$.

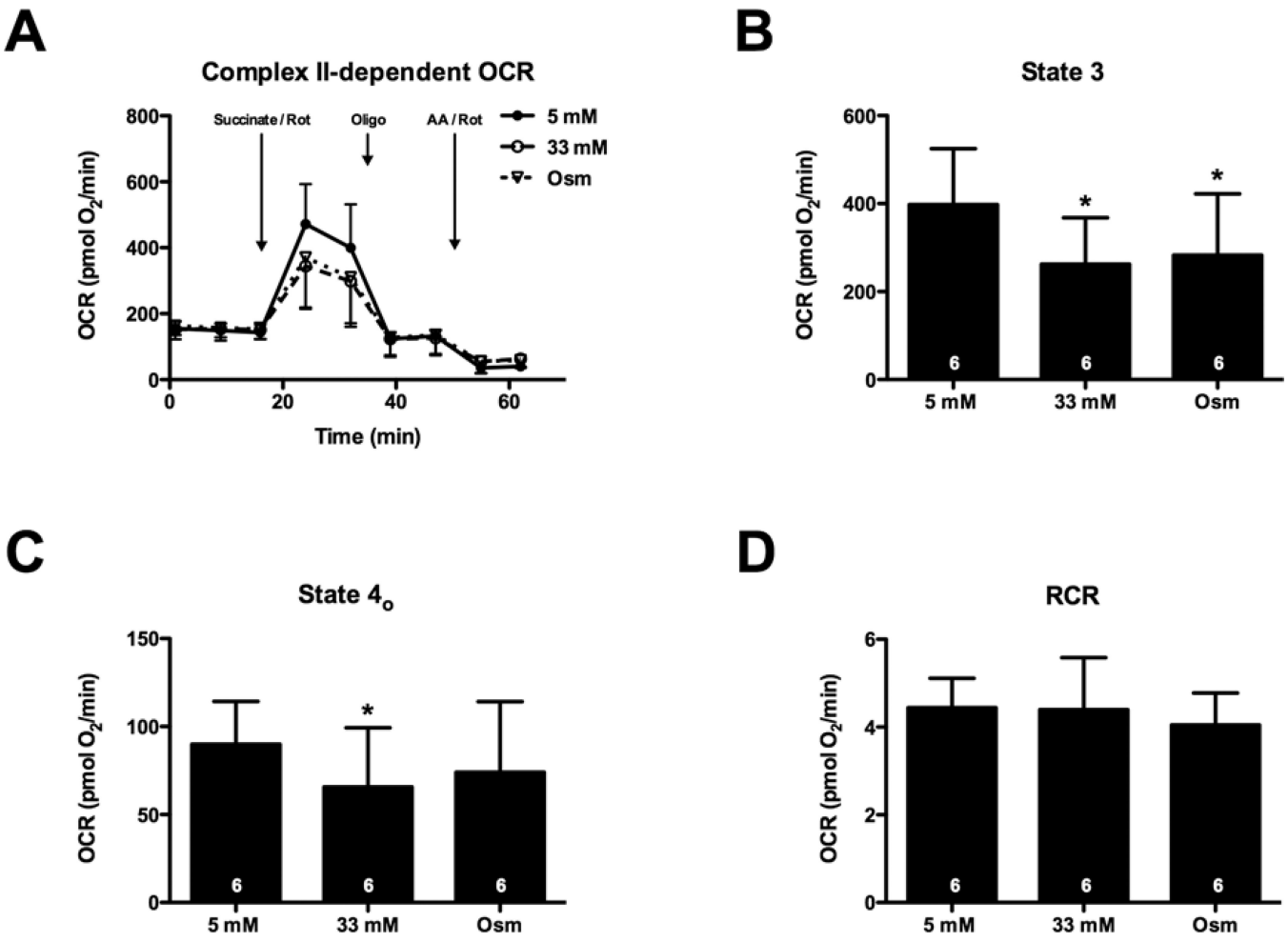

Figure 4. High glucose depresses Complex II-dependent State 3 and 4_o respiration

NRCMs were treated for 48 h with 5 or 33 mM glucose or an osmotic control. During XF analysis, NRCMs were permeabilized and provided with succinate (to support Complex II-dependent respiration) as well as Rot (to inhibit Complex I activity). (**A**) XF assay of Complex II-dependent respiration: following three baseline OCR measurements in MAS buffer, the permeabilization agent, saponin, and succinate + Rot were injected. After two measurements, oligomycin (Oligo), then AA + Rot (AA–Rot) were injected sequentially, with measurements recorded after each injection. (**B**) State 3 OCR: The AA + Rot rate was subtracted from the succinate-stimulated rate to determine the State 3 rates. (**C**) State 4_o OCR: The AA + Rot rate was subtracted from the oligomycin rate to obtain State 4_o rates. (**D**) RCR: State 3/State 4_o. As indicated in bars, $n = 6$ independent experiments; $^{*}P < 0.05$, compared with 5 mM glucose.

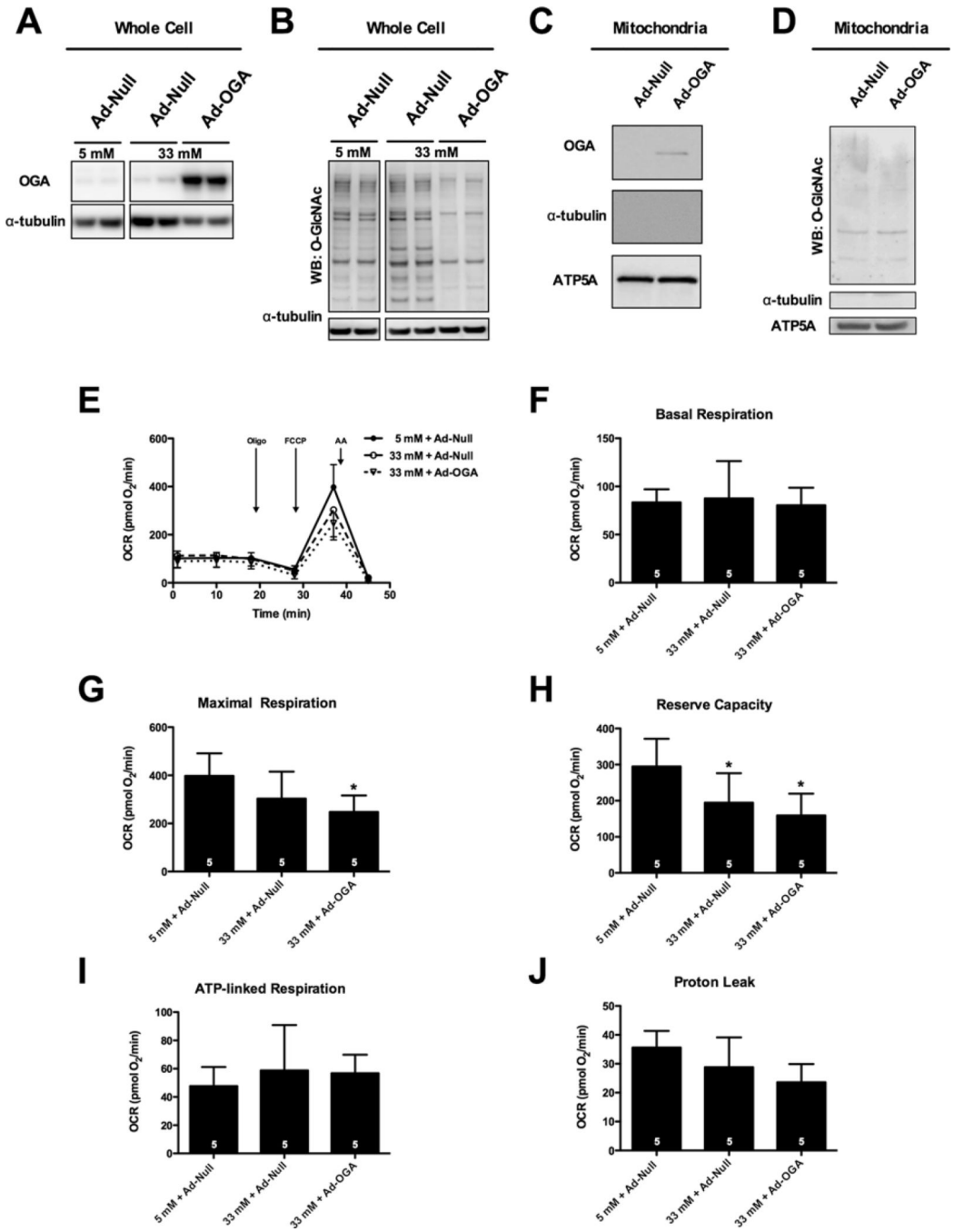

Figure 5. Overexpression of OGA does not rescue high-glucose-induced suppression of bioenergetic reserve

NRCMs were transduced with virus to overexpress OGA prior to treatment with hyperglycaemia. Ad-Null was used as a vector control. (**A**) Immunoblot for whole-cell OGA protein expression following viral transduction. (**B**) Whole-cell protein O-GlcNAcylation levels following adenoviral transduction. (**C**) Immunoblot for mitochondrial OGA following adenoviral overexpression. (**D**) Immunoblot for mitochondrial protein O-GlcNAcylation following adenoviral overexpression of OGA. (**E**) Mitochondrial function assay following

48 h of hyperglycaemic treatment. From assay in (**E**) parameters of mitochondrial function were measured: (**F**) basal respiration, (**G**) maximal respiration, (**H**) reserve capacity, (**I**) ATP-linked respiration, and (**J**) proton leak. As indicated in bars, $n = 5$ independent experiments; $^{*}P < 0.05$, compared with 5 mM glucose + Ad-Null.

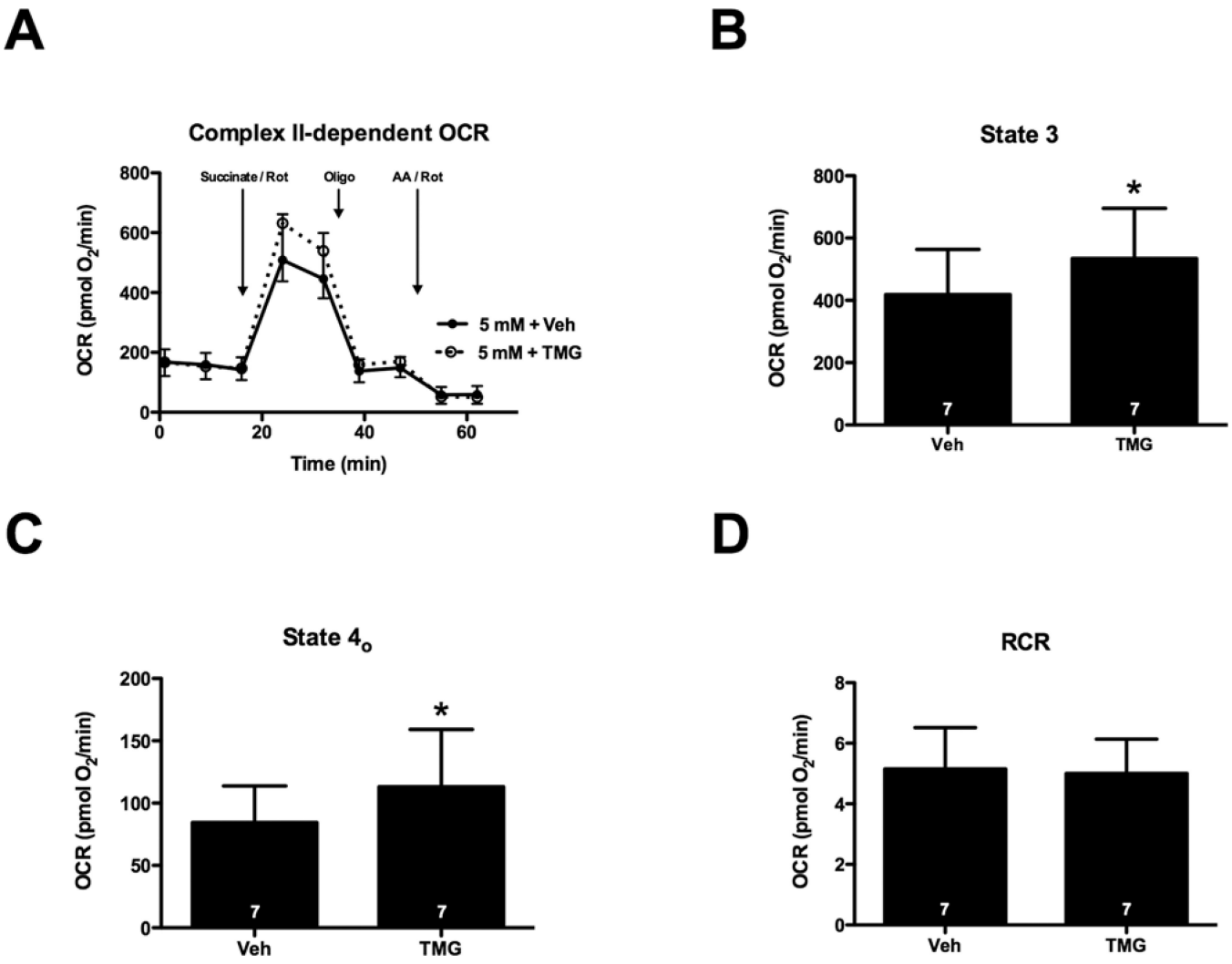

Figure 6. Inhibition of OGA increases Complex II-dependent State 3 respiration

NRCMs were treated for 24 h with 1 μM TMG to induce protein O-GlcNAcylation. During XF analysis NRCMs were permeabilized and provided with succinate (to support Complex II-dependent respiration), as well as Rot (to inhibit Complex I activity). (**A**) XF assay of Complex II-dependent respiration: following three baseline OCR measurements in MAS buffer, the permeabilization agent, saponin, and succinate + Rot were injected. After two measurements, oligomycin (Oligo) and AA + Rot (AA–Rot) were injected sequentially, with two measurements recorded after each injection. (**B**) State 3 OCR: the AA + Rot rate was subtracted from the succinate-stimulated rate to determine the State 3 rates. (**C**) State 4_o OCR: the AA + Rot rate was subtracted from the oligomycin rate to obtain State 4_o rates. (**D**) RCR: State 3/State 4_o. As indicated in bars, $n = 7$ independent experiments; $^*P < 0.05$.