Cardiomyocyte glycophagy is regulated by insulin and exposure to high extracellular glucose

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Mellor KM, Varma U, Stapleton DI, Delbridge LM. Cardiomyocyte glycophagy is regulated by insulin and exposure to high extracellular glucose. Am J Physiol Heart Circ Physiol 306: H1240–H1245, 2014.—Disturbed systemic glycemic and insulinemic status elicits cardiomyocyte metabolic stress and altered glucose handling. In diabetes, pathological myocardial glycogen accumulation occurs. Recently, evidence of a specific myocardial autophagic degradation pathway for glycogen (“glycophagy”) has been reported, differentiated from the more well-characterized protein “macrophagy” pathway. The goal of this study was to identify potential mechanisms involved in cardiac glycogen accumulation, glycophagy, and macrophagy regulation using cultured neonatal rat ventricular myocytes (NRVMs). In NRVMs, insulin-induced Akt phosphorylation was evident with 5 mM-glucose conditions (~2.3-fold increased). Under high-glucose (30 mM) conditions, insulin-augmented phosphorylation was not observed. Accumulation of glycogen was observed in response to insulin only in high-glucose conditions (~2-fold increase). Increased expression of the glycophagy marker starch-binding domain-containing protein-1 (STBD1, 25% increase) was observed under high-glucose and insulin conditions. Expression levels of the macrophagy markers p62 and light chain protein 3BII:I were not increased by insulin at either glucose level. Preliminary results from hearts of streptozotocin-treated diabetic rats are supportive of the findings obtained in NRVMs, suggesting diabetes induced elevated expression of STBD1 and of an additional glycophagy marker GABA(A) receptor-associated protein-like-1. Confocal microscopy demonstrated that light chain protein 3B and STBD1 immunomarkers were not colocalized in NRVMs. These findings provide the first evidence that cardiomyocyte glycophagy induction occurs under the influence of insulin and is responsive to extracellular high glucose. This study suggests that the regulation of glycogen content and glycophagy induction in the cardiomyocyte may be linked, and it is speculated that glycogen pathology in diabetic cardiomyopathy has glycophagic involvement.

autophagy; glycogen; cardiac; heart; glucose

DISTURBED SYSTEMIC GLYCEMIC and insulinemic status elicits cardiomyocyte metabolic stress and altered glucose handling. In diabetes, myocardial glycogen accumulation is evident in humans and in a range of experimental models (8, 11, 14, 19). Cardiac glycogen excess has been linked to hypertrophy, arrhythmias, and contractile dysfunction (3). This cardiac-specific diabetic phenomenon appears paradoxical in a setting of impaired tissue glucose uptake. Recently, we provided the first evidence that a specific autophagic degradation pathway for glycogen (“glycophagy”) exists in the heart and is responsive to in vivo glycemic challenge (15). Glycophagy may be a crucial link between diabetes and glycogen mishandling in the heart.

Glycogen storage diseases are characterized by an accumulation of glycogen-filled autophagosomes in the myocardium (3), demonstrating a close association of glycogen and autophagy. Until recently, the term “autophagy” has been considered synonymous with the term “macroautophagy” or “macrophagy,” implying a single macromolecular degradative pathway. A new understanding of more nuanced forms of cellular phagic activity has emerged, and autophagy proteins specific for glycogen phagic processing have been recognized (4, 15). Starch-binding domain-containing protein-1 (STBD1) is involved in binding glycogen and mediating membrane anchorage via interaction with the cognate protein GABA(A) receptor-associated protein-like 1 (GABARAPL1) (4). This signaling distinguishes glycogen-targeted autophagic processes from the more well-characterized protein degradation macrophagy pathways.

Glycogen handling and autophagy share some similar regulatory pathways. Insulin signaling promotes glycogen storage via Akt inhibition of glycogen synthase kinase 3β and suppresses phagic activity distally via mammalian target of rapamycin inhibition of unc51-like kinase 1/2 (9). In the insulin resistant heart, we have demonstrated that macrophagy induction is coincident with downregulation of Akt and S6 phosphorylation (10), and the phosphoinositide 3-kinase (I)-Akt pathway suppression may act as a prominent upstream mediator of macrophagy. AMP-activated kinase (AMPK) indirectly promotes glycogen storage by increasing glucose uptake (23), leading to allosteric activation of glycogen synthase via glucose-6-phosphate. As AMPK also promotes macrophagy via activation of unc51-like kinase 1/2 (23), there exists potential for a mechanism by which glycogen storage and phagic activity may be simultaneously regulated. Thus extensive signaling cross talk between glycogen flux and induction of phagic activity occurs in the heart, but an understanding of how these processes operate in disturbed metabolic contexts is required.

Our goal was to delineate the mechanisms involved in glycogen, glycophagy, and macrophagy regulation in high glucose-cultured cardiomyocytes and diabetic hearts. We hypothesized that cardiomyocyte glycogen levels would be increased by sustained exposure to high glucose and provide signaling substrate for induction of glycophagy. In vitro experiments were undertaken using neonatal ventricular cardiomyocytes. At the neonatal stage phagic activity is pronounced (7), and this cell model is thus of particular relevance.

MATERIALS AND METHODS

**Cell culture of neonatal rat ventricular cardiomyocytes.** All experiments were conducted in accordance with the principles of laboratory animal care (National Institutes of Health: http://grants1.nih.gov/
the “Australian Code of Practice for the Care and Use of Animals for Scientific Purposes” and were approved by the University of Melbourne. Neonatal rat ventricular myocytes (NRVMs) were isolated from male and female 0–2-day Sprague-Dawley rats as previously described (13). Following overnight culture in minimum essential media with 10% newborn calf serum (Life Technologies) and 30 mM NaHCO₃, the media was switched to serum-free Dulbecco’s modified essential medium, supplemented with glucose or insulin (Sigma-Aldrich) to achieve designated final concentrations. Glucose (30 mM) was used to provide optimal contrast with the reference 5-mM glucose group and to represent the maximal reported levels of blood glucose attained in noncontrolled diabetes (22). After 24 h Dulbecco’s modified essential medium culture, NRVMs were incubated for 30 min in radioimmunoprecipitation assay buffer (Sigma-Aldrich) with protease and phosphatase inhibitors (Roche). Cells were scraped from wells and stored in sample buffer consisting of 50 mM Tris·HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 2.5% 2-mercaptoethanol for Western blot analysis.

Streptozotocin-induced diabetes. A single dose of streptozotocin (55 mg/kg, tail vein, Sigma-Aldrich) or equivalent volume of saline was administered to male Wistar rats at age 8 wk as previously described (2). After 8 wk of diabetes, rats were euthanized by cervical dislocation (isoflurane anesthesia), hearts were excised, and left ventricles were dissected and frozen at −80°C. Frozen tissue was homogenized in 100 mM Tris·HCl, 5 mM EGTA, and 5 mM EDTA (Sigma-Aldrich) buffer containing protease and phosphatase inhibitors (Roche, Switzerland). Heart homogenates were stored in sample buffer consisting of 50 mM Tris·HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 2.5% 2-mercaptoethanol for Western blot analysis.

Western blot analysis of protein expression. Sample protein concentrations were determined using a modified Lowry assay (12), and equal amounts of protein were loaded into the SDS-PAGE gel. Antibodies were sourced (Cell Signaling Technology) for phospho (p)Akt (Ser473; no. 9271), Akt (no. 9272), light chain protein 3B (LC3B; no. 2775), p62 (no. P0067, Sigma-Aldrich), STBD1 [custom], and for GABARAPL1 (no. 11010-1-AP, Proteintech Group) to assess protein expression in lysate and homogenate samples. Membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (GE Healthcare). The ECL Prime (Amer sham, GE Healthcare) chemiluminescent signal was visualized with a Chemi-XRS Imaging device and band intensity quantified using Quantity One imaging software (Bio-Rad). Equal protein loading was confirmed by Coomassie staining of polyvinylidene difluoride membranes (Coomassie Brilliant Blue R-250, Bio-Rad).

Measurement of cardiac glycogen. Lysates were digested with amyloglucosidase (Sigma-Aldrich) at 50°C for 20 min with 0.1 M sodium acetate buffer (pH 6.0). Following centrifugation (14,000 g, 2 min), aliquot glucose levels were determined by a two-enzyme, colorimetric assay (Sigma-Aldrich). Periodic acid–Schiff staining was used to visualize intracellular glycogen deposits in NRVMs fixed (100% methanol, 20 min, −20°C) on coverslips. Fixed cells were oxidized (1% periodic acid for 5 min), incubated in Schiff’s reagent (10 min, 25°C), counterstained (Mayer’s hematoxylin 10 s), dehydrated, and DPX mounted. Images were acquired using an Imager D1 microscope with AxioCam MRc5 camera (Carl Zeiss Germany).

Immunohistochemistry and confocal microscopy. NRVMs on coverslips were fixed (100% methanol, 20 min, −20°C) and incubated (1 h) with image-iT FX signal enhancer (Life Technologies). After primary antibody incubation (14 h, at 4°C) with rabbit-LC3B (no. CS2775, Cell Signaling Technology) and mouse-STBD1 (no. CAB-2027MH, Creative Biomart), secondary antibody was applied (1 h, 25°C; anti-rabbit Cy3 (no. 711-165-152, Jackson Immunoresearch), anti-mouse Alexa488 (no. A11029, Life Technologies), and 4′,6-diamidino-2-phenylindole, dihydrochloride (Life Technologies) staining used to label nuclei. Coverslips were mounted onto slides with ProLong Gold anti-fade reagent (Life Technologies) and imaged using an LSM Meta laser-scanning confocal microscope and Zen 2012 Imaging software (Carl Zeiss Germany) at × 63 magnification.

Statistical analyses. Data are presented as means ± SE. Data were analyzed by two-way ANOVA with post hoc Fisher’s least significant difference tests where appropriate (SPSS v. 20, SPSS) with P value < 0.05 considered as statistically significant.

RESULTS

Insulin stimulates glycogen production in cardiomyocytes exposed to high glucose. In response to insulin treatment (10 mM, 24 h), NRVMs incubated in 5 mM glucose (physiological reference condition) exhibited activation of Akt (Fig. 1A, ~46% increase pAkt/Akt vs. control, P < 0.05). Myocytes cultured in high glucose (30 mM) did not show augmented

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**Fig. 1.** Glycogen content and Akt signaling in cultured neonatal rat ventricular myocytes (NRVMs). A: ratio of phosphorylated Akt (pAkt) to total Akt increased by insulin exposure in 5 mM but not 30 mM glucose (n = 7–11 culture wells/group). B: cardiomyocyte glycogen content increased in the presence of insulin in 30 mM glucose (n = 3 culture wells/group). C: periodic acid–Schiff-stained (pink) glycogen deposits most evident in hyperglycemic insulin-treated NRVMs. Con, control; Ins, insulin treated; Gluc, glucose; Arb U, arbitrary units. *P < 0.05, 2-way ANOVA, annotated with least significant difference (LSD) post hoc tests; means ± SE.
In vitro and in vivo diabetic cardiomyocyte glymphagic induction. To determine whether insulin-stimulated glycogen elevation in myocytes cultured in high glucose could be linked to activation of glymphagy, expression levels of the STBD1 and GABARAPL1 markers were evaluated. STBD1 has been identified to tether glycogen to the autophagosome via binding to the cognate protein GABARAPL1 (4). NRVMs cultured with high glucose exhibited higher expression of STBD1 when insulin treated (Fig. 2A, control vs. insulin, 0.95 ± 0.05 arbitrary units) or high glucose (Fig. 2B, control vs. insulin, 0.99 ± 0.05 vs. 0.99 ± 0.13 arbitrary units) or with high glucose (Fig. 2B, control vs. insulin, 0.89 ± 0.08 vs. 0.84 ± 0.12 arbitrary units). Thus expression of the glymphagy cargo protein STBD1 appeared to be linked to myocyte glycogen content, but the STBD1 expression shift was not matched by GABARAPL1 response in this treatment period.

As glycogen accumulation is a reported characteristic of the diabetic myocardium (11, 14, 19), we sought to identify evidence of glymphagy activity in left ventricular homogenate tissue from streptozotocin (STZ)-induced diabetic rats. STBD1 expression and GABARAPL1 expression were observed to be higher in diabetic rat hearts (~60 and ~20% increased, respectively, as depicted; Fig. 2C). These findings provide indication of activation of the glymphagy degradation pathway in the myocardium of STZ-treated versus nondiabetic controls.

Macroautophagy is suppressed by insulin in cardiomyocytes exposed to high glucose. A role for macroautophagy in the diabetic heart has also been demonstrated (10), but it is not known how cardiomyocyte macropagy is influenced by insulin and glucose conditions. To determine whether the macrophagy response is dependent on glycemic status, p62 and LC3BII-to-LC3BI expression was evaluated in NRVM lysates. When elongating the autophagosome membrane, LC3B is transformed into the phosphatidylethanolamine-conjugated form (LC3BII). LC3B is the inactive, unconjugated form. The ratio of LC3BII to LC3B is thus used as a measure of macropagy “packaging” activity. p62 is an adaptor molecule which functions to tether proteins and organelles to the autophagosome via binding to LC3B.

p62 levels were not different in insulin-treated myocytes in either reference glucose (insulin vs. control, 5 mM glucose: 1.00 ± 0.07 vs. 1.03 ± 0.11 arbitrary units) or high glucose (insulin vs. control, 30 mM glucose: 0.92 ± 0.07 vs. 0.99 ± 0.07 arbitrary units) conditions (Fig. 3A). The LC3B II-to-LC3B I ratio was not different between insulin-treated and control NRVMs cultured under reference glucose conditions. When cultured with high glucose, insulin elicited a differential in the NRVM LC3B II-to-LC3B I ratio (Fig. 3B, ~28% difference, insulin vs. control, 30 mM glucose; P < 0.05), reflecting a modest ratio increase with high glucose in the absence of insulin. These findings indicate that insulin-induced modulation of cardiomyocyte macropagy activity in a high-glucose environment may occur without alteration in the level of Akt activation.

Examination of macropagy marker expression in ventricular tissues of STZ-treated animals provided an interesting contrast—p62 expression was not changed relative to control (similar to NRVM finding), whereas the LC3B II-to-LC3B I ratio was significantly higher in diabetic compared to nondiabetic hearts (~60 and ~20% increased, respectively, as depicted; Fig. 2C).

**Fig. 2. Influence of insulin and high-glucose exposure on glymphagy marker protein expression (in vitro and in vivo).** A: starch-binding domain-containing protein-1 (STBD1) protein expression in NRVMs: significantly higher with insulin treatment in 30 mM glucose, trend for insulin-induced increased STBD1 in 5 mM glucose (P < 0.05) (n = 7–11 culture wells/group). B: GABA(A) receptor-associated protein-like 1 (GABARAPL1) protein expression in NRVMs not responsive to insulin (n = 7–11 culture wells/group). C: immunoblots of protein expression in ventricular homogenate from streptozotocin-treated (diabetic) and control rats. Glymphagy markers STBD1 and GABARAPL1 upregulated in diabetic myocardium (n = 2 hearts/group in duplicate; no statistical analysis applied). *P < 0.05, 2-way ANOVA, annotated with LSD post hoc tests; means ± SE.
To establish that cardiomyocyte glycophagy and macrophagy operate as distinct pathways, a coimmunostaining approach was used to assess the localization of STBD1 and LC3B (Fig. 4, STBD1 green, LC3B red, and 4′,6-diamidino-2-phenylindole dihydrochloride-stained nuclei blue) as evidenced by occurrence of discrete fluorescent puncta.

**DISCUSSION**

This study provides the first evidence that cardiomyocyte glycophagy induction occurs under the influence of insulin and is responsive to high extracellular glucose. The novel finding that insulin-induced glycogen accumulation and glycophagic “tagging” in cardiomyocytes, under high glucose conditions, occur without coincident insulin-augmented Akt activation provides important new insight into the glycogen storage stimulus in the insulin-resistant diabetic heart. Expression shifts of the cardiomyocyte glycophagic cargo protein STBD1 were concomitant with augmented glycogen levels in high-glucose settings. These findings suggest that the regulation of glycogen content and glycophagic induction in the cardiomyocyte may be linked. Elevated glycogen content in the diabetic heart may constitute a heightened vulnerability to autophagic cell death via induction of excess glycophagic activity.

**Insulin-induced cardiomyocyte glycogen production is not linked to augmented Akt activation.** The occurrence of cardiac glycogen accumulation in human diabetic patients and experimental animal diabetic models has been known for some time (i.e., more than 60 years) (19), yet surprisingly, the processes of glycogen turnover in the heart have received relatively modest attention. Acute insulin (15–20 min) stimulation of glycogen production via activation of glycogen synthase in cardiomyocytes has been demonstrated (21), but whether sustained insulin elevation modulates cardiomyocyte glycogen content has not been previously reported. In the present study, 24 h insulin treatment induced glycogen accumulation in cardiomyocytes cultured in high glucose (30 mM) media, but not under normal (5 mM) glucose conditions. Interestingly, insulin-induced glycogen production was not coincident with further activation of Akt at this 24-h time point. These findings suggest that with maintained high glucose and with insulin present, the mechanism of glycogen promotion may be independent of the insulin signaling pathway, although the possibility of an early increased Akt response to insulin during the 24 h period cannot be precluded. Future experiments employing the use of Akt inhibitors are required to clarify the role of Akt pathway involvement. The current findings are consistent with the in vivo diabetic setting where suppression of insulin signaling and stimulation of glycogen storage occur in parallel.
A possible non-phosphoinositide 3-kinase/Akt mechanism of insulin-induced cardiomyocyte glycogen production may involve AMPK signaling. In skeletal muscle, it has been demonstrated that AMPK induces allosteric activation of glycogen synthase via glucose-6-phosphate (23). However, acute AMPK activation by AICAR has been shown to inactivate glycogen synthase in the perfused rat hindlimb muscle (20). In the diabetic heart, inconsistent findings relating to the role of AMPK have been reported (5, 18) and the question of whether cardiac AMPK activity is elevated or diminished in diabetes remains controversial. Further investigation, particularly in relation to AMPK regulation of glycogen, will be informative.

Differential regulation of glycophagy and macrophagy. Localization of glycogen to double membrane-bound structures (identified as “autophagosomes”) was first observed in 1972 in the skeletal muscle of neonatal rats (16) and subsequently reported in the neonatal rat heart (6). Recent identification of specific proteins involved in glycophagic processing has facilitated new mechanistic investigations of particular relevance for the diabetic heart. In the present study, cardiomyocytes exposed to high glucose exhibited elevated expression of STBD1 in response to insulin. STBD1 binds glycogen and anchors it to preautophagosome membranes by binding to GABARAPL1. Interestingly, under high glucose and insulin conditions, increased STBD1 was not matched by elevated expression of GABARAPL1. Conversely, the macrophagy cargo protein p62 was unaffected by high-glucose and insulin exposure. In the high glucose setting, a differential between control and insulin-treated LC3B II-to-LC3B I ratio was detected, and this appears to reflect a relatively higher level of macrophyagy packaging activity in the absence of insulin, the upstream signaling basis for this response requires more investigation.

Confocal microscopic immunofluorescence examination determined that STBD1 did not colocalize with the macrophyagy marker, LC3B, demonstrating that glycophagy and macrophagy are separable processes in cardiomyocytes. Our evidence suggests that glycophagy and macrophagy pathways are differentiated types of selective autophagy with distinct localization and regulatory responses.

A role for cardiac glycophagy in diabetic pathophysiology. In insulin resistant settings, where tissue glucose uptake is impaired, intracellular glucose storage as glycogen is stimulated linked with a reduction in glucose oxidation (14). Intriguingly, glycogen accumulation is not explained by an increase in production: glycogen synthase activity is lower and the rate of glycogen synthesis is reduced (measured by 13C-NMR) (8). There is some evidence to suggest that phosphorylase-mediated glycogen breakdown is impaired (1), but other studies report no effect of diabetes on basal phosphorylase activity (8). Our new observations show for the first time that glycogen accumulation is not linked to reduced glycophagic breakdown; on the contrary, our preliminary results in the STZ-induced diabetic heart exhibits upregulation of both STBD1 and GABARAPL1 (Fig. 2C). From our in vitro and in vivo investigations, it is apparent that glycophagy marker levels (STBD1, in particular) follow the shifts in intracellular content of the ligand glycogen. If the conventional homeostatic route of glycogen formation (via synthase) and degradation (via phosphorylase) is impaired in diabetic cardiomyocytes, upregulation of glycophagy may be an adaptive response to provide a non-phosphorylase degradation route to remove excess glycogen. In the diabetic setting, this alternative glycogen degradation route may gain more significance. It is well established that excess recycling of cellular constituents by autophagy leads to programmed cell death; thus, it is possible that enhanced glycophagic activity in diabetic hearts is linked to heightened myocyte viability vulnerability.

The STZ-induced rat model of diabetes is characterized by low systemic insulin and marked hyperglycemia (2). With diminished insulin receptor activation, an expected downregulation of Akt phosphorylation was observed (Fig. 3C). The STZ-induced systemic insulin/glucose status could be notionally referenced to the “no insulin, high glucose” group in our in vitro study. In each of these treatment groups, p62 expression was unaffected relative to control, whereas in both instances some evidence of LC3BII-to-LC3BI ratio activation was apparent. These observations indicate congruent NRVMs and preliminary in vivo responses in relation to macrophyagy. In contrast, the observed increased expression of STBD1 and GABARAPL1 in STZ-treated rat hearts appears to be inconsistent with our in vitro finding that these glycophagy markers are not affected by hyperglycemia in the absence of insulin (Fig. 2, A and B). Although cultured neonatal cardiomyocytes provide a useful setting to investigate the regulation of glycogen and phagic activity, full recapitulation of the in vivo adult diabetic setting is not possible in this in vivo environment. Nonetheless, collectively, these findings suggest that hyperglycemia per se does not induce glycophagy, and different/additional factors yet to be identified play a role in the chronic in vivo diabetic state.

In conclusion, this study identifies the occurrence of cardiomyocyte glycophagy and provides novel evidence that these cellular processes may be enacted differentially. Extracellular glucose, in association with insulin, has a role in influencing glycophagic induction, and the regulatory mechanism appears not to rely on Akt activation augmentation. While modest in scope, this study provides a compelling impetus for further work to develop a more complete understanding of myocardial glycophagy regulation. Evaluation of glucose handling under the high-glucose, high-insulin culture conditions (e.g., Glut4 translocation, glucose oxidation), and an insulin dose-response analysis would be informative. In particular, based on these limited in vivo findings, new investigations of the role of glycophagy in mediating diabetic cardiopathology are warranted.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

K.M.M., U.V., and L.M.D. conception and design of research; K.M.M., U.V., and D.I.S. performed experiments; K.M.M., U.V., and D.I.S. analyzed...

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