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Myocardial glycophagy flux dysregulation and glycogen accumulation characterize diabetic cardiomyopathy

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ABSTRACT

Diabetic heart disease morbidity and mortality is escalating. No specific therapeutics exist and mechanistic understanding of diabetic cardiomyopathy etiology is lacking. While lipid accumulation is a recognized cardiomyocyte phenotype of diabetes, less is known about glycolytic fuel handling and storage. Based on in vitro studies, we postulated the operation of an autophagy pathway in the myocardium specific for glycogen homeostasis – glycophagy. Here we visualize occurrence of cardiac glycophagy and show that the diabetic myocardium is characterized by marked glycogen elevation and altered cardiomyocyte glycogen localization. We establish that cardiac glycophagy flux is disturbed in diabetes. Glycophagy may represent a potential therapeutic target for alleviating the myocardial impacts of metabolic disruption in diabetic heart disease.

1. Introduction

With the rising global prevalence of diabetes, the health burden of diabetic heart disease morbidity and mortality is escalating. Diabetic heart disease is recognized as a distinct cardiomyopathy for which there are no specific therapeutics [1]. Altered myocardial substrate metabolism in diabetes has been reported with a shift from glycolytic fuel (glucose) to enhanced fatty acid utilization. Accumulation and modifications of cardiomyocyte lipid stores (i.e. 'lipotoxicity') are well described features of diabetic heart disease [2,3]. In contrast, carbohydrate storage derangement is not well described. Post-mortem clinical histologic evidence of glycogen accumulation in the diabetic myocardium was first reported anecdotally >90 years ago [4] – yet strangely this phenomenon has been minimally pursued mechanistically [5]. Given the well-established literature identifying cardiac vulnerability in inherited glycogen storage diseases [6], investigation of the metabolic and structural aspects of myocardial glycogen accumulation in diabetes is surprisingly limited.

We have previously demonstrated that autophagy, a cellular macromolecular 'bulk' catabolic pathway, is involved in progression of diabetic heart disease [7]. In vitro work using non-cardiac cell lines first identified the properties of a glycogen-specific binding protein STBD1 (starch binding domain protein 1) known to interact with an Atg8 family protein GABARAPL1 (γ-aminobutyric acid receptor-associated protein-like 1, an LC3B homologue) with the potential to mediate a glycogen-selective autophagy [8,9]. We postulated the operation of an autophagy pathway in the myocardium specific for glycogen homeostasis (i.e. 'glycophagy') [10–12]. Our proof-of-concept in vitro studies have

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shown that glycophagy is operational in cardiomyocytes and is regulated by insulin and glucose in vitro [13].

A role for glycophagy in cardiopathology has not been previously investigated. Our goal in this study was to evaluate the occurrence of cardiac glycogen dysregulation in diabetes and seek evidence of glycophagy involvement in diabetic heart disease.

2. Materials and methods

2.1. Human myocardial samples

Right atrial appendage samples were obtained from non-diabetic and type 2 diabetic patients (aged 48–80 years) at the time of elective cardiac surgery either at the Royal Melbourne Hospital, Australia, or Dunedin Hospital, New Zealand (Table S1). Collection of clinical samples was approved by the Melbourne Health Human Research and Ethics Committee and the Human and Disability Ethics Committee of New Zealand (LRS/12/01/001), and all patients provided informed consent.

2.2. Animals – Type 1 (T1D) and type 2 (T2D) diabetes

All animal experiments were approved by Animal Ethics Committee at the University of Auckland and Cedars-Sinai Medical Center.

Type 1 diabetic streptozotocin (STZ)-treated rats. Type 1 diabetes was induced in male Sprague Dawley rats at 8 weeks of age (55 mg/kg tail-vein injection of STZ, Sigma), and monitored for 8 weeks. STZ rats exhibited hyperglycemia (33.1 ± 0.2 vs. 6.45 ± 0.2 mM, $p < 0.05$) and smaller body weight (331 ± 15 vs. 490 ± 16 g, $p < 0.05$) relative to control rats at study endpoint. Echocardiography confirmed diastolic dysfunction (a hallmark of diabetic cardiomyopathy) in STZ rats. Animals were anaesthetized with isoflurane and transthoracic echocardiography was performed using the GE Vivid 9 Dimension echocardiography platform with a 15 MHz i13L linear array transducer (GE Healthcare). Pulse wave Doppler and tissue Doppler imaging were acquired from the apical 4 chamber view to assess velocity of early mitral inflow (E) and early diastolic velocity of mitral annulus (e') and E/e' ratio. Three consecutive cardiac cycles were sampled for each measurement taken, and analysis was performed in a blinded manner. In a sub-cohort of animals, STZ (8 weeks post-STZ injection) and control rats were injected with the lysosomal inhibitor, chloroquine (50 mg/kg, Sigma) or saline vehicle i.p., 4 h prior to tissue collection, for analysis of glycophagy flux.

Type 2 diabetic db/db mice. Male db/db mice (BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J) and heterozygote controls (db^{+}) were sourced from Charles River and evaluated at 10 weeks of age. db/db mice exhibit hyperglycemia, obesity, and hyperinsulinemia by 6 weeks of age [14].

Type 2 diabetic high fat high sugar diet rats. Obesity and type 2 diabetes were induced in male Sprague Dawley rats by a high fat high sugar dietary intervention commencing at 8–9 weeks of age. After a 1 week transitional feeding period, animals were fed a high fat diet with relatively high sugar (HFSD, 43% kcal from fat, 200 g/kg sucrose, SF04–001, Specialty Feeds) or control reference diet (18% kcal from fat, with no sucrose carbohydrate component, 'regular' chow, Harlan, USA) for 14–15 weeks. HFSD rats exhibited hyperglycemia (14.6 ± 1.1 vs. 10.7 ± 1.0 mM, $p < 0.05$) and larger body weight (704 ± 23 vs. 570 ± 22 g, $p < 0.05$) relative to control rats at study endpoint.

2.3. Glycogen content

Glycogen content was measured as reported previously [13]. Briefly, cardiac homogenate derived from atrial (human) or ventricular (rodent) tissues was digested with amyloglucosidase (#10102857001, Roche) then centrifuged at 16,000*g*, 4 °C for 2 min. Glucose was measured in the supernatant via colorimetric glucose assay (Sigma-Aldrich). Another aliquot was processed in parallel without amyloglucosidase to determine background glucose. Glycogen levels are presented as glucose units (nmol), normalized to protein (mg, determined by Lowry assay) and depicted as relative levels for comparative purposes.

2.4. Immunoblotting

Sample protein concentrations were determined using a Lowry assay and equal amounts of protein were loaded into the SDS-PAGE gel (confirmed by Coomassie staining, Bio-Rad) and transferred to PVDF membranes. Membranes were incubated with overnight at 4 °C and secondary antibody for 1 h (anti-rabbit, HRP-conjugated, GE Healthcare). Primary antibodies for the following proteins were used: phosphorylated (Ser641) glycogen synthase (ab81230, Abcam), glycogen synthase (3893, Cell Signaling), phosphorylated (Ser14) glycogen phosphorylase (gift from Dr. David Stapleton), glycogen phosphorylase (gift from Dr. David Stapleton), and GABARAPL1 primary antibody (Cell Signaling (#26632). The ECL Prime (Amersham, GE Healthcare) chemiluminescent signal was quantified.

2.5. Gene profiling & Jensens DISEASE classification

Gene profiling was performed on RNA extracted from type 1 diabetic rat heart tissue (STZ-treated), reversed transcribed to cDNA using the RT2 First Strand kit (Qiagen). RNA and cDNA quality was verified using the RT2 RNA Quality Control PCR Array (Qiagen). Custom PCR array plates were designed with 57 genes of interest and housekeeping gene β-actin (Table S3). Real time PCR was performed and differential gene expression was determined by ΔΔCt analysis and subjected to disease classification using the Jensen DISEASE resource (http://diseases.jensenlab.org/). The top 10 diseases most closely aligned with the differentially detected genes in T1D hearts were presented relative to their combined score calculated as ln(*p*-value) x odds ratio.

2.6. Electron microscopy

Left ventricular free wall heart segments (1 mm^3) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for >24 h at 4 °C. Fixed hearts were processed in 1% osmium tetroxide/1.5% potassium ferrocyanide in 0.1 M phosphate buffer for 90 min and dehydrated with increasing ethanol concentration followed by propylene oxide (PO; Merck-Millipore). Samples were infiltrated and embedded in pure resin at 60 °C for >48 h and cut (1 μm) and stained with 1% toluidine blue in 1% Na tetraborate prior to cutting of 80 nm sections on copper EM grids and staining with uranyl acetate and 0.3% lead citrate. Sections were imaged systematically through the grid locations using a Tecnai™ G^2 Spirit Twin transmission electron microscope.

2.7. Statistics

Data are presented as mean ± SEM (except clinical sample data presented as mean ± SD) and analyzed using Graphpad Prism V7.0. All datasets were tested for normal distribution using Shapiro-Wilk tests and for equal variances using F-tests. A 2-sided Student's *t*-test was used and *p*-value of <0.05 was considered statistically significant. For correlation analyses Spearman's non-parametric correlation coefficient method was used. The ROUT method for detection of outliers was used with the maximum false discovery rate set at 0.5%.

3. Results and discussion

3.1. Cardiac glycogen accumulation in diabetes

To evaluate the status of cardiac glycogen levels in various diabetic settings, a survey of glycogen content in myocardial tissues of diabetic patients and mature cardio-metabolic animal models was undertaken, including dietary (high fat high sugar diet rat), genetic (db/db mouse), and pharmacologically (STZ rat) induced diabetic states. This

investigation revealed that in both T1D and T2D diabetic conditions, elevation of myocardial glycogen levels relative to non-diabetic controls was a consistent feature, with varying levels to a maximal 4 fold increase (Fig. 1A, Table S1–2). In this study, human samples were derived from atrial tissue and rodent samples were derived from ventricular tissue, thus species and regional differences in glucose handling may comprise an element of variability. It is well established that atria operate with lower glucose uptake, higher glycogen content and lower metabolic reserve than ventricles [15,16]. Notwithstanding these possible sources of variation, diabetes-associated glycogen elevation was evident in both tissue and species settings (Fig. 1A). This is consistent with reported findings that atrial pathologies arise in patients with genetic glycogen storage diseases, in particular Wolff-Parkinson-White syndrome, where conduction abnormalities are present [6]. Further, in genetically-determined glycogen storage diseases, glycogen accumulation can be detected throughout cardiac tissues [6]. Accumulation of glucose in glycogen polymer form seems paradoxical in a context where cardiac glucose uptake is limited either by insulin deficiency or resistance, with downregulation of glucose transporter expression (GLUT1 & GLUT4) [17]. Redirection of glucose towards glycogen may be hypothesized to be a consequence of suppressed glucose metabolism, a well-described feature of diabetic cardiomyopathy. Glycogen processing and regulation has been extensively investigated in the context of exercise in skeletal muscle, but these findings cannot necessarily be extrapolated to cardiac tissue. While cardiac glycogen levels were found to be consistently elevated in diabetes, no elevation in glycogen was detected in skeletal muscle (quadriceps) of the same animals (Fig. 1A-B, Table S2).

We next examined the intracellular morphology associated with increased glycogen. A single branched glycogen molecule may comprise up to 120,000 glucose residues, and multiple molecules can be linked rendering these structures cytosolically identifiable using electron microscopy. In the myocardium, glycogen deposition was visualized as electron-dense particulates on transmission electron micrographs (optimized for glycogen opacity). In control hearts, glycogen particles were sparse. In contrast, marked glycogen infiltration was evident in diabetic myocardium (STZ rats), clustered near mitochondria and more dispersed throughout the myofilament architecture (Fig. 1C).

To gain insight into the disease relevance of the glycogen phenotype, we performed gene profiling on ventricular mRNA samples extracted from diabetic rodents exhibiting the most marked elevation in glycogen (a T1D β-cell ablation model). The DISEASES gene association analysis tool [18] was applied to a panel of 57 genes assembled to probe molecular signaling pathways involved in metabolic stress responses (Table S3). The differential gene expression profile was found to most closely align with a 'glycogen storage disease' state (Fig. 1D). This is a large group of genetic conditions relating to a range of mutations involved in glycogen catalysis [6]. These initial findings using a customized gene panel indicate a basis for more extensive pathway analysis (transcriptomic or proteomic) in future work to interrogate the proposition that diabetic cardiomyopathy may be a form of 'acquired' glycogen storage disease.

We next investigated the activation state of the canonical cytosolic glycogen enzymatic regulatory pathway which controls the availability of free glucose by addition (synthase) or removal (phosphorylase) of single glucose units of glycogen branch structures. Glycogen synthase was found to be either inhibited (increased phosphorylation at Ser641) or unchanged, and glycogen phosphorylase was either activated (increased phosphorylation at Ser14) or unchanged, in a survey of diabetic rodent hearts (Fig. S1A—B). Overall, these activation shifts would be consistent with suppressed glycogen synthesis and upregulation of cytosolic glycogen breakdown, certainly not instrumental as a causative mechanism of total myocardial glycogen elevation in diabetes. While these findings were not comprehensive and altered signaling involving other regulatory components of the canonical glycogen enzymatic processes could obviously not be precluded, the findings were indicative, prompting consideration of possible alternative (pathological) processing routes of glycogen.

3.2. Cardiac glycophagy flux disturbance in diabetes

Recently an enhanced understanding of lysosomal glycogen degradation has emerged, with identification of protein intermediaries involved in glycogen-selective autophagy ('glycophagy') [10,11]. Based on the general understanding of phagosomal mechanisms, the process of glycophagy can be understood to involve several stages – adaptor STBD1 attachment to glycogen (tagging) and binding to GABARAPL1 at the forming autophagosome membrane, membrane enclosure of glycogen cargo, and subsequent fusion with a glucosidase-containing lysosome (acid α-glucosidase, Gaa) to degrade glycogen, thereby releasing free glucose for local metabolic use (Fig. 2A). We have previously noted auto-phagosomal punctate inclusions in non-disease settings [12]. Using optimized methods of electron microscopy for glycogen visualization we were able to identify occurrence of glycogen-replete autophagosomes, which could be considered to reflect cargo-selective 'glycophagosomes'. These structures were identified in both control and diabetic cardiomyocytes (Fig. 2B). The electron micrographs show that glycogen accumulation occurs both within glycophagosomes and distributed throughout sarcomeric regions. While the stochastic nature of glycophagosome forming events discernible only at very high magnification limits quantitation, the representation of glycogen particles bundled into double membrane forming and de-forming structures provides a dynamic depiction of glycophagosome morphology (not achievable for protein macro-autophagy where the cargo is not so directly visualized in the same way).

Next, we investigated in vivo cardiac glycophagy throughput ('flux') in diabetic rats. Autophagy is a dynamic process involving Atg8 protein movement in and out of autophagosomes during the phagosome-lysosome cycle as cargo is captured. Thus snapshot measures of total protein levels do not necessarily correlate with autophagy activity, whereas flux measurements provide a more informative indicator of autophagosome functionality [19]. To quantify cardiac glycophagy throughput in diabetes in vivo, we measured the extent of glycogen accumulation (relative to control) over 4 h in response to acute lysosomal inhibition in diabetic rodents (T1D, STZ-induced, 8 weeks duration). As previously established, acute in vivo chloroquine administration (i.p.) arrests lysosome fusion and quantification of the accumulated Atg8 markers (e.g. LC3BII) is an established method for monitoring macro-autophagy flux [20]. By quantifying an increase in glycophagy 'cargo' (i.e. glycogen) following lysosomal blockade, we provide a method for monitoring glycogen-specific autophagic throughput over a defined time period (to approximate glycophagy flux). As expected, chloroquine treatment increased cardiac glycogen in the myocardium of control animals by 20 glycosyl units over 4 h (~5 units/h, Fig. 2C). In diabetic animals where cardiac glycogen levels were elevated (note extended y-axis range, and see Fig. 1A, Table S2), no effect of chloroquine was evident, indicating an already impaired cardiac glycophagy flux in diabetes (Fig. 2C). Similarly, chloroquine-induced GABARAPL1 protein accumulation was absent in diabetic rat hearts (Fig. 2D). Taken together, the findings in this diabetic model (STZ rat) suggest that reduced glycogen degradation linked with disrupted glycophagy overrides the observed enzyme shifts in glycogen synthase and phosphorylase (Fig. S1) to result in a dramatic 4-fold higher level of myocardial glycogen (Fig. 1A). These in vivo studies confirmed a role for glycophagy flux disturbance in cardiac glycogen accumulation in chronic diabetic conditions in the STZ rat model, and further work is required to validate these findings in T2D models.

Our findings indicate that, in addition to lipid dysregulation, glycogen dysregulation is a significant phenotype of the diabetic myocardium. A significant correlation between diastolic function (echocardiography E/e' ratio) and cardiac glycogen is evident (Fig. S2), providing suggestive evidence of functional impact of cardiac glycogen accumulation in diabetes. The nature of the link between glycogen

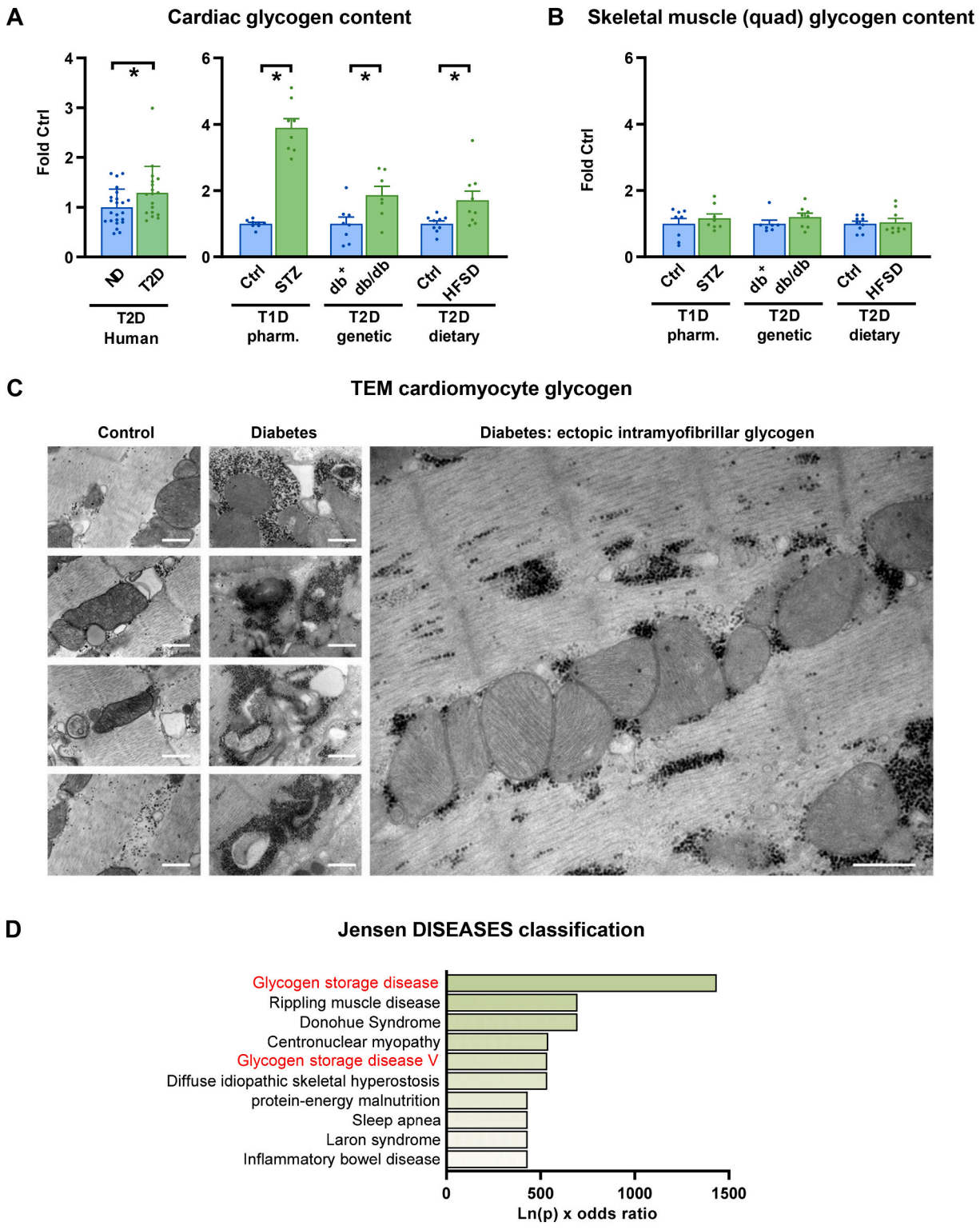

Fig. 1. Cardiac glycogen accumulation in diabetes. (A) Cardiac glycogen content is increased in human patients with T2D (n = 18–23 patients/group, mean ± SD) and animal models of diabetes (T1D, type 1 diabetes; T2D, type 2 diabetes; mean ± SEM). STZ, (streptozotocin-treated rat 8 weeks); db/db (leptin receptor mutant mouse age 10 week, db + littermate control); HFSD (high fat high sugar diet-fed rat 14 weeks), $n \geq 7$ animals/group, analyzed by 2-sided Student's *T*-Test. **(B)** No change in skeletal muscle glycogen content in diabetic animal models (STZ rat; db/db mice, high fat high sugar diet rats (HFSD), n ≥ 7 animals/group). **(C)** Cardiomyocyte transmission electron micrographs (TEM) show electron-dense glycogen particulate deposits (black puncta) in control and diabetic (STZ, 8 weeks) rat hearts (scale bar 500 nm). **(D)** Jensen DISEASES classification analysis identified 'glycogen storage disease' as characteristic of differentially expressed genes detected by gene profiling diabetic rat hearts (STZ, n = 6 rats/group, n = 57 genes). Data presented as mean ± s.e.m. *$p < 0.05$. See also Tables S1–3.

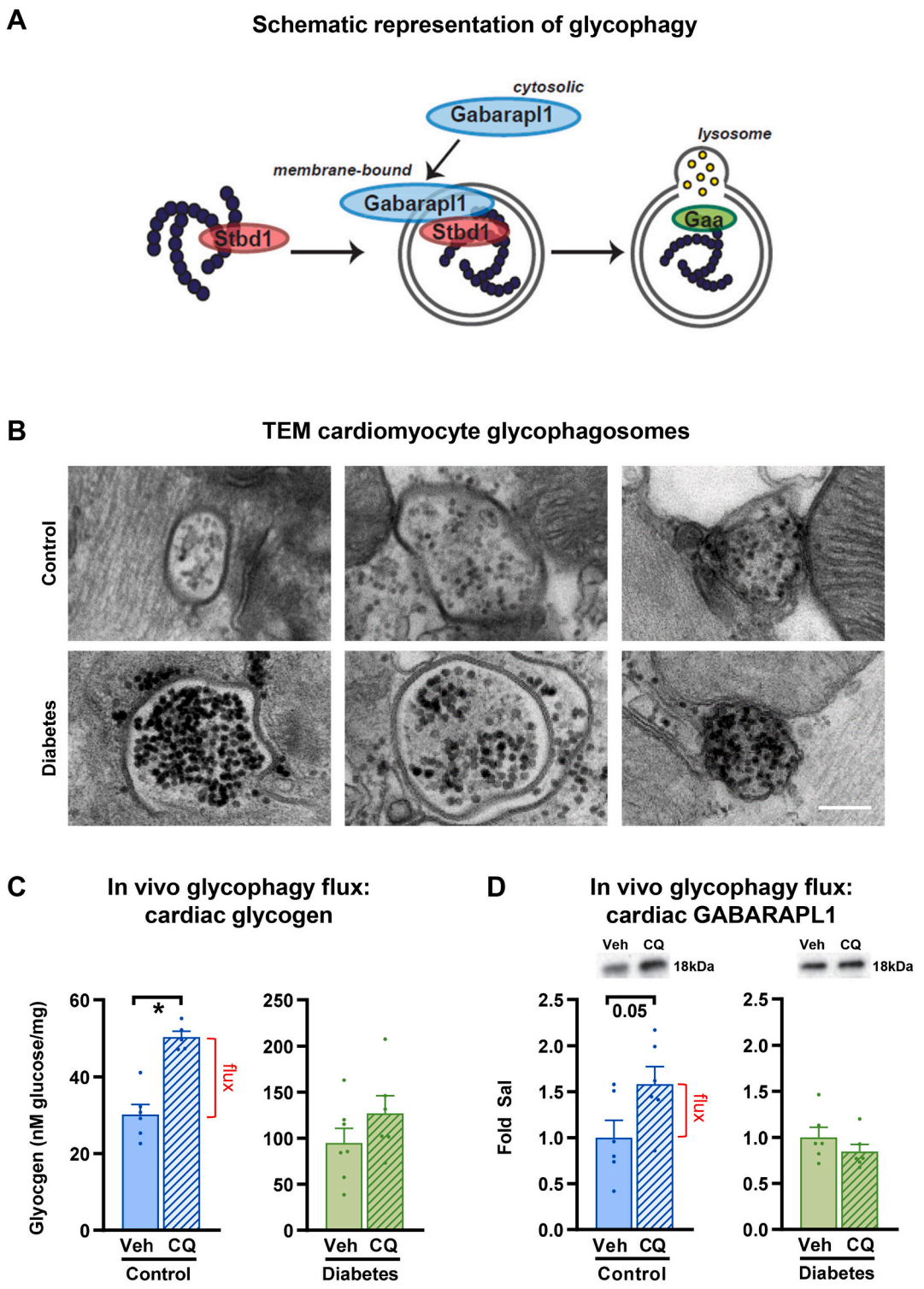

Fig. 2. Cardiac glycophagy disturbance is evident in diabetes. (A) Schematic illustrating three stages of glycophagy. Stbd1 tags glycogen and binds to the Atg8 protein, Gabarapl1 (GABA receptor-associated protein-like 1), at the forming glycophagosome membrane. The mature glycophagosome fuses with a lysosome where Gaa (acid α-glucosidase) degrades glycogen to free glucose for metabolic recycling. **(B)** Cardiomyocyte transmission electron micrographs (TEM) show electron-dense glycogen particulate deposits (black puncta) within autophagosome structures in both control and diabetic (STZ, 8 weeks) rat hearts (scale bar 200 nm). **(C)** In vivo glycophagy flux is impaired in diabetic hearts. In control rats, cardiac glycogen is accumulated in response to lysosomal inhibition (chloroquine (CQ). In diabetic animals where cardiac glycogen levels are elevated, no significant effect of chloroquine is evident. 50 mg/kg i.p. CQ 4 h prior to tissue collection vs vehicle (Veh, saline) in diabetic rats (STZ 8 weeks), n = 6–7 rats/group, analyzed by 2-sided Student's *T*-Test. **(D)** In control rats, a trend for CQ-induced GABARAPL1 protein accumulation is detected ($p = 0.05$). In diabetic rats, a GABARAPL1 response to chloroquine is absent. 50 mg/kg i.p. CQ 4 h prior to tissue collection vs saline (Sal) in diabetic rats (STZ 8 weeks), n = 6–7 rats/group, analyzed by 2-sided Student's T-Test. Data presented as mean ± s.e.m. *$p < 0.05$.

accumulation and cardiomyopathy remains to be resolved. Given the severe cardiac pathology observed in patients with glycogen storage diseases, it could be speculated that glycogen accumulation may be linked to cardiac dysfunction in diabetes. More investigation is now required to build on this novel finding. Deranged glycolysis relating to impaired glycogen flux has potential to disturb key components of cardiomyocyte Ca^{2+} handling, integral to electro-mechanical coupling. Ultrastructurally, increased intramyofibrillar localization of glycogen may cause mechanical/ physical disturbance to sarcomeric force transmission. Further investigation is required.

4. Conclusion

In conclusion, our study has provided the first evidence that dysfunctional glycophagy is a feature of diabetic cardiac metabolic pathology. We show that the diabetic myocardium (type 1 and type 2) is characterized by marked glycogen elevation and altered cellular localization of glycogen - a paradoxical metabolic pathology given suppressed cardiomyocyte glucose uptake in diabetes. Gene profiling analysis indicates that the diabetic cardiac glycogen pathology may be perceived as a form of 'acquired' glycogen storage disease. In left ventricular samples from STZ-induced diabetic rats, we demonstrate that glycogen accumulation is linked with glycogen-selective autophagy pathway ('glycophagy') flux defect in this cardio-metabolic disease state. As a first 'proof-of-concept' step to determine the translational potential of this work, we confirmed that diabetes-associated myocardial glycogen accumulation was evident in a clinical setting (using atrial biopsies from diabetic patients). Regional tissue differences in glycophagy activity are yet to be explored, and diabetic atrial pathology may involve nuanced changes in glycogen metabolism. An extensive survey of chamber-specific glycogen and glycophagy dysregulation in diabetes is warranted in the future. Collectively, the findings from this study reveal that cardiac glycophagy is a key metabolic homeostatic process perturbed in diabetes and offers potential as a therapeutic target to treat diabetic heart disease.

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CRediT authorship contribution statement

Kimberley M. Mellor: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Upasna Varma:** Investigation, Formal analysis, Data curation. **Parisa Koutsifeli:** Investigation, Formal analysis, Data curation. **Lorna J. Daniels:** Investigation, Formal analysis, Data curation. **Victoria L. Benson:** Investigation, Formal analysis, Data curation. **Marco Annandale:** Investigation, Formal analysis, Data curation. **Xun Li:** Investigation, Formal analysis, Data curation. **Yohanes Nursalim:** Investigation, Formal analysis, Data curation. **Johannes V. Janssens:** Writing – review & editing, Conceptualization. **Kate L. Weeks:** Writing – review & editing, Conceptualization. **Kim L. Powell:** Resources. **Terence J. O'Brien:** Resources. **Rajesh Katare:** Resources. **Rebecca H. Ritchie:** Resources. **James R. Bell:** Writing – review & editing, Conceptualization. **Roberta A. Gottlieb:** Resources, Methodology, Conceptualization. **Lea M.D. Delbridge:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Correspondence and requests for materials should be addressed to L.M.D.·D (lmd@unimelb.edu.au).

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Declaration of generative AI and AI-assisted technologies in the writing process.

The authors did not use generative AI or AI-assisted technologies in the development of this manuscript.

Appendix A. Supplementary data

Supplementary material.

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