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Myocardial glycophagy - a specific glycogen handling response to metabolic stress is accentuated in the female heart.

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Abstract

**Aims:** Cardiac metabolic stress is a hallmark of many cardiac pathologies, including diabetes. Cardiac glycogen mis-handling is a frequent manifestation of various cardiopathologies. Diabetic females have a higher risk of heart disease than males, yet sex disparities in cardiac metabolic stress settings are not well understood. Estrogen acts on key glycogen regulatory proteins. The goal of this study was to evaluate sex-specific metabolic stress-triggered cardiac glycogen handling responses.

**Methods and Results:** Male and female adult C57Bl/6J mice were fasted for 48hrs. Cardiac glycogen content, particle size, regulatory enzymes, signalling intermediates and autophagic processes were evaluated. Female hearts exhibited 51% lower basal glycogen content than males associated with lower AMP-activated-kinase (AMPK) activity (35% decrease in pAMPK:AMPK). With fasting, glycogen accumulated in female hearts linked with decreased particle size and upregulation of Akt and AMPK signalling, activation of glycogen synthase and inactivation of glycogen phosphorylase. Fasting did not alter glycogen content or regulatory proteins in male hearts. Expression of glycogen autophagy marker, starch-binding-protein-domain-1 (STBD1), was 63% lower in female hearts than males and increased by 69% with fasting in females only. Macro-autophagy markers, p62 and LC3BII:I ratio, increased with fasting in male and female hearts.

**Conclusions:** This study identifies glycogen autophagy ('glycophagy') as a potentially important component of the response to cardiac metabolic stress. Glycogen autophagy occurs in association with a marked and selective accumulation of glycogen in the female myocardium. Our findings suggest that sex-specific differences in glycogen handling may have cardiopathologic consequences in various settings, including diabetic cardiomyopathy.
1. Introduction

Metabolic stress plays an important role in diabetic cardiac pathology, characterised by a disturbance in the glucose-insulin balance [1]. The Framingham heart study reported a 5-fold increase in heart failure risk in diabetic females compared to a 2-fold increase in males with diabetes [2], suggesting that women exhibit a heightened vulnerability to diabetic heart disease. The mechanisms of sex-dependent metabolic stress-related cardiac pathology have not been elucidated. Experimentally, estrogen is known to modulate the insulin signalling pathway via upregulation of Akt in cardiomyocytes [3]. This pathway plays a crucial role in the metabolic stress response via regulation of glucose transport and storage as glycogen; sex-specific modulation of these processes has not been investigated.

Glycogen is a large intracellular particle consisting of thousands of glucose moieties, and glycogen mishandling is a key feature of metabolic stress. For over 80 years it has been known that glycogen deposition is increased in the human myocardium under pathologic circumstances associated with diabetic cardiomyopathy [4]. The basis for this is still not understood, but this is a replicated finding in a number of diabetic experimental models [5-7]. This occurrence of glycogen accumulation when glucose supply is restricted through impaired tissue uptake would appear to be a paradoxical metabolic stress response. In an experimental setting of acute metabolic stress induced by fasting, cardiac glycogen accumulation is also observed. Rodent studies have shown that the cardiac glycogen content increases in parallel with depletion of liver and skeletal muscle glycogen stores [8]. Glycogen-handling responses to an acute metabolic challenge are clearly tissue-specific. Understanding these metabolic processes of glycogen handling in metabolically stressed cardiac tissue may provide insight into the paradoxical accumulation of glycogen in the diabetic heart.
Glycogen storage diseases are characterised by an accumulation of glycogen-filled autophagosomes in the myocardium [9], demonstrating a close association of glycogen and autophagy. A distinct ‘glycogen autophagy’ process has been observed in neonatal hearts where membrane-bound vacuoles containing glycogen particles are abundant on electron microscopy images [10]. Thus autophagic degradation of excess glycogen may release free glucose, contrasting with the tightly regulated homeostatic degradation pathway catalysed by glycogen phosphorylase. The basis for shunting glycogen through autophagic degradation has not been established but it is speculated that this pathway is selective for glycogen particles with aberrant branching or abnormal structure that limits the accessibility of phosphorylase target sites [11].

Starch-binding domain-containing protein 1 (STBD1) is involved in binding glycogen and mediating membrane anchorage via interaction with the cognate autophagy protein GABA(A) receptor-associated protein like 1 (GABARAPL1). It is therefore evident that a specific autophagy pathway exists for degradation of glycogen, termed ‘glycophagy’ [12, 13]. This terminology distinguishes glycogen targeted autophagic processes from the more well characterised protein degradation pathways, described generally as 'macrophagy'. It is not known whether the glycogen autophagy pathway is operational in the heart. Interestingly GABARAPL1 is an estrogen-regulated gene [14] with the potential to mediate sex specificity of glycogen handling and autophagy, but to date, this mechanism has not been investigated.

Glycogen handling and autophagy share similar regulatory pathways. The insulin signalling (‘cell survival’) pathway promotes glycogen storage via Akt inhibition of glycogen synthase kinase 3β (GSK3β) and suppresses autophagy via mammalian target of rapamycin (mTOR) inhibition of unc51-like kinase 1/2 (ULK1/2) [15, 16]. In the heart, AMP-activated kinase (AMPK) indirectly promotes glycogen storage by increasing glucose uptake [17] leading to allosteric activation of glycogen synthase via glucose-6-phosphate. Unlike insulin signalling, AMPK promotes autophagy
via activation of ULK1/2 [18] thus providing a mechanism by which glycogen storage and autophagy may be simultaneously regulated. We have previously identified a role for macro-autophagic induction in the insulin resistant heart [19-21] and further work is now required to establish the mechanisms involved in cardiac metabolic stress-related autophagy. Delineating the macro-autophagic and glycogen-autophagic response to an acute metabolic challenge in male and female myocardium may provide mechanistic insight into the sex-specific characteristics of cardiac stress responses.

Given that women appear to be more susceptible to metabolic stress-related cardiac pathology, and that estrogen upregulates signalling intermediates involved in promotion of glycogen storage (and potentially glycogen autophagy), we hypothesised that the myocardial glycogen handling response to metabolic stress would be accentuated in female myocardium. An acute, experimental pathophysiologic metabolic challenge was employed to interrogate the sex-specific characteristics of glycogen-related storage and breakdown processes. Male and female mice were subjected to 48hr fasting and cardiac glycogen content, particle size, regulatory enzymes, signalling intermediates and autophagic processes were evaluated. We demonstrate that female hearts contain less glycogen than male hearts under basal conditions. Fasting selectively induced a marked accumulation of glycogen linked with a decrease in average glycogen particle size in female hearts. Upregulation of Akt and AMPK signalling and induction of macro- and glycogen-autophagy is implicated. This study provides the first demonstration that glycogen autophagy is operational in the heart and reports the novel finding that glycogen management in female hearts is especially responsive to metabolic stress. Our findings indicate that sex-specific differences in glycogen handling may have cardiopathologic consequences in various settings, including diabetic cardiomyopathy.
2. Materials and Methods

2.1 Animals and dietary treatments

C57Bl/6J male and female mice were purchased from the Animal Resources Centre, Western Australia and were acclimatised within the University of Melbourne Animal Facility for 1 week in temperature-controlled conditions in a 12hr light/dark cycle, and were cared for in accordance with the ‘Principles of laboratory animal care’ (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) and the National Health and Medical Research Council of Australia Code of Practice for the Care and Use of Animals for Scientific Purposes, and procedures were approved by the Animal Ethics Committee of the University of Melbourne. A specific low-soy diet was chosen to minimise the impact of phytoestrogens on study outcomes (Barastoc, Agriproducts, Australia). At 8 weeks of age, mice were fasted for 48hrs, a time point that has been previously established to induce metabolic stress involving upregulation of macro-autophagy [22]. Equal numbers of At the 48hr time point, mice were anaesthetised (sodium pentobarbital, 70mg/kg i.p.), hearts excised, rinsed in HEPES-Krebs buffer, blotted and weighed for determination of cardiac weight index and snap frozen for molecular analysis. Tibia length was measured and blood glucose concentrations determined (ACCU-CHEK® Advantage glucometer). Equal numbers of mice from all groups were assessed in parallel and all heart tissue was collected between 3pm and 5pm. Cardiac dimensions were also determined echocardiographically (see Supplement for details).

2.2 Western blot analysis of myocardial protein expression

Frozen ventricular tissue was homogenised and protein expression was analysed by western blot as previously described [23]. Briefly, tissue was homogenised in Tris-HCl 100mM, EGTA 5mM, EDTA 5mM (Sigma Aldrich, MO, USA) buffer containing protease and phosphatase inhibitors
Heart homogenates were stored in sample buffer (50mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 2.5% 2-mercaptoethanol). Sample protein concentration was determined by a modified Lowry assay [24] and equal amounts of protein were loaded into the SDS-PAGE gel. Antibodies for pAMPK (Thr172; Cell Signalling #2535), AMPK (Cell Signalling #2532), pAkt (Ser473; Cell Signalling #9271), Akt (Cell Signalling #9272), glycogen synthase (GS; Cell Signalling #3893), pGS (Ser641; Epitomics #1919-1), glycogen phosphorylase [25], glycogen branching enzyme (GBE; [26]), glycogen debranching enzyme (GDE; [25]), light chain protein 3B (LC3B; Cell Signalling #2775), p62 (American Research Products #03-GP62-C), STBD1 [27] and GABARAPL1 (Protein Tech #11010-1-AP) were utilised to assess protein expression in crude homogenate samples. Protein expression was determined by SDS-PAGE and western blotting. Equal protein loading was confirmed by staining the PVDF membranes with Coomassie dye (Coomassie Brilliant Blue R-250; Biorad, CA, USA).

Phospho-glycogen phosphorylase (pGP (Ser14)) polyclonal antibodies were raised in rabbits against the peptide CKRKQISPO4 VRGLA-amine (GP (9-21) C9 phospho-S14) and affinity purified through the dephosphorylated peptide first to remove antibodies recognising epitopes to the GP peptide itself, followed by the phosphorylated peptide. Both peptides were immobilised on Sulfolink gel (Pierce, Rockford, IL, USA). Bound GP phospho-specific antibodies were eluted with 200 mM glycine pH 3 into aliquots of 100 mM TRIS pH 8 to immediately adjust the pH and dialysed overnight at 4°C against 4 L PBS.

Membranes were incubated with anti-rabbit HRP-conjugated secondary antibody (GE Healthcare UK Ltd, Buckinghamshire, UK). The ECL Plus (Amersham™, GE Healthcare UK Ltd, Buckinghamshire, UK) chemiluminescent signal was imaged and analysed using Quantity One software (Biorad, CA, USA).
2.3 Glycogen Assay

Cardiac glycogen concentration was measured by digesting an aliquot of mouse ventricular crude homogenate with amyloglucosidase (Sigma-Aldrich, St. Louis, MO, USA) at 50°C for 20mins in a buffer containing 0.1 M sodium acetate pH 6.0. Following centrifugation at 14,000g for 2mins, an aliquot was removed and glucose levels determined using a two enzyme, colorimetric glucose assay (Sigma-Aldrich, St. Louis, MO, USA). Thus glycogen levels were measured in glucose units (nmol), normalised to protein (mg, determined by Modified Lowry Assay [24]) and depicted as relative levels for comparative purposes.

2.4 Periodic Acid-Schiff (PAS) Staining

Heart mid-sections were fixed in 10% formalin (Sigma-Aldrich, St. Louis, MO, USA) for 24hrs, washed with phosphate-buffered saline and stored in 70% ethanol at 4°C. Tissue segments were then embedded in paraffin and cut into 4μm sections. Following dewaxing, sections were oxidised with 1% periodic acid for 5mins, washed and incubated in Schiff’s reagent for 10mins at room temperature. Sections were then washed and counterstained with Mayer’s haematoxylin for 30secs, washed, dehydrated and mounted in DPX. Selected sections were incubated with 5mg/ml α-amylase for 5mins at 37°C (Sigma-Aldrich, MO, USA) to confirm distinct glycogen and glycoprotein stain localisation. Images were acquired using a Zeiss Imager DI microscope with a Zeiss AxioCam MRc5 colour camera (Carl Zeiss, Oberkochen, Germany). Image analysis was performed using Image Pro Plus (V4.5.1, Media Cybernetics, MD, USA).

2.5 Glycogen purification

Ventricular tissue was homogenised in glycogen isolation buffer (in mM: 50 HEPES, 150 NaCl, 2 EDTA, protease inhibitor tablet (complete EDTA-free, Roche Cat#04693132001) and phosphatase inhibitor tablet (PhosSTOP, Roche Cat#04906837001), pH 7.4) and centrifuged at
7000g for 10mins at 4°C. The supernatant was collected, pellet resuspended in glycogen isolation buffer and centrifugation repeated. The resulting supernatant was pooled with the supernatant from the first spin and the sample was subsequently centrifuged at 150,000g for 45mins at 4°C. The membrane/glycogen rich pellet was resuspended in glycogen isolation buffer and homogenised at low speed. The sample was then layered over a sucrose density gradient (75, 50, 25 % (w/v) in glycogen isolation buffer) and centrifuged at 300,000g for 120mins at 4°C. The glycogen fraction pelleted through all three layers whilst microsomal/membranes were retained within the 25-50% sucrose fraction. To assure glycogen purity, the pellet was resuspended in 50 mM HEPES, pH 7.5, layered over 200μl, 75% sucrose in glycogen isolation buffer and centrifuged at 300,000g for 30mins at 4°C. The resulting pellet was resuspended in 50 mM HEPES, pH 7.5 and frozen at -80°C until required.

2.6 Transmission Electron Microscopy

Cardiac glycogen was prepared for TEM as previously described for rat liver and human skeletal muscle glycogen [25] and diluted 1 in 40 initially to determine the appropriate concentration that provided an even distribution of glycogen particles. Following this, glycogen particles were examined using a Tecnai 12 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands) at an operating voltage of 120KV as previously described [28]. Images were recorded using a Megaview III CCD camera and AnalySIS camera software (Olympus) at a magnification between 30,000 and 150,000 times. Analysis of the glycogen particle size (particle diameter) was performed using ImageJ software as previously described [28].

2.7 Statistical Analyses

Data are presented as mean ± standard error of the mean (S.E.M). Statistical analyses were performed using SPSS v20 (SPSS Inc., IL, USA). Data were analysed by two-way analysis of variance (ANOVA) with post-hoc Fisher’s LSD tests where appropriate. As required, data were
log$_{10}$-transformed to achieve a normal distribution. Non-parametric testing (Mann Whitney U) was used when normal distribution could not be achieved by data transformation. A p-value <0.05 was considered statistically significant. Figure statistical annotation (*) specifies post-hoc analysis outcomes. For instances where the 2-way ANOVA revealed a significant overall ‘factor’ effect in the absence of significant post-hoc individual group comparisons, this information is included in the text, in the figure legends, and annotated on each panel (†).
3. Results

3.1 Systemic and cardiac morphological characteristics of male and female fasted mice

After 48 hours of fasting, extent of body weight reduction was greater in male than in female mice (22% vs. 16% decrease, relative to pre-fasting weight, respectively; p<0.05 fasted male vs. fasted female, Table 1). Fasting-induced ~61% and ~67% decrease in blood glucose levels in males and females respectively (p<0.05 fasted vs. control). Male and female mice exhibited similar fasting-induced reduction in heart weight (~10-13% decrease; p<0.05 fasted vs. control) and cardiac weight index (heart weight normalised to tibia length, (~10-12% decrease; p<0.05 fasted vs. control, Table 1). Echocardiographic assessment of a subset of animals revealed a fasting associated reduction in ventricular wall dimensions (LVIDd; p<0.05 fasted vs. control) with no indication of a sex differential in the response (see Supplement Figure S2). Thus, although somatic weight decrease in response to fasting was modestly higher in male mice, systemic glycemic and cardiac weight parameters were not different.

3.2 Sex-specific effects of fasting on cardiac glycogen content and regulation

Cardiac glycogen content and regulation were evaluated in male and female mice under control (fed) conditions and subjected to 48hr fasting. In the control state, the glycogen content of female myocardial tissues was substantially and significantly lower compared with male (~50% lower; p<0.05 control male vs. control female; Fig. 1a). This was associated with a higher level of inactive glycogen synthase (GS; ~2.9-fold increase in phosphorylated-GS: total GS ratio; p<0.05 control male vs. control female; Fig. 1b) and a higher level of active glycogen phosphorylase (GP; ~5.2-fold increase in phosphorylated-GP: total GP ratio; p<0.05 control male vs. control female; Fig. 1c) relative to male mouse hearts. In response to fasting, female hearts exhibited a marked elevation of glycogen (~135% increase; p<0.05 fasted female vs. control female) associated with activation of
glycogen synthase (~95% reduction in phosphorylated-GS: total GS ratio; p<0.05 fasted female vs. control female; Fig. 1b) and inactivation of glycogen phosphorylase (~78% reduction in phosphorylated-GP: total GP ratio; p<0.05 fasted female vs. control female, Fig. 1c). In contrast, male hearts exhibited no changes in cardiac glycogen content, glycogen synthase, or glycogen phosphorylase in response to 48hr fasting. To visualize the cellular localization of glycogen deposits, tissue PAS staining was evaluated (Fig 1d). This staining was notably prominent in the fasted female myocardial sections (Fig 1d, lower right panel). Sections were also treated with amylase to confirm intracellular site of glycogen occurrence. After amylase digestion, only residual extracellular glycoprotein staining was evident (Fig 1e, sample section).

Thus, in female hearts, it appeared that lower glycogen synthesis combined with higher glycogen degradation was associated with lower basal (control) glycogen content relative to males. Fasting induced a female-specific activation of glycogen synthesis and inactivation of glycogen degradation coincident with accumulation of glycogen stores in females only.

To determine whether glycogen accumulation in fasted female hearts was associated with alteration in glycogen structure, cardiac glycogen particles were purified and their diameters determined by transmission electron microscopy. Mean cardiac glycogen particle size was ~11% greater in control females than control males (p<0.05; Fig. 2a), and fasting induced a ~15% decrease in mean particle size in female mice (p<0.05 fasted female vs control females). Mean glycogen particle size in fasted females was ~4% smaller than that in fasted males (p<0.05 fasted female vs fasted male; Fig. 2a). Based on size, glycogen particles can be characterised as small β particles (<40nm), or as larger α particles (>40nm), the latter particle type considered to comprise aggregated β particles [25]. The data appearing in Figure 2a are depicted in Figure 2b, to provide alternative visual representation, emphasizing the relative increase in the proportion of β-particles (i.e. <40nm) in fasted females only.
(compared non-fasted controls). In males, fasting did not modify either mean particle size or the proportion of particle types. Figure 2C provides representative electron micrographs depicting the particle size distribution in each group.

To assess how the disparate sex effects of fasting on cardiac glycogen content and structure could reflect underlying differences in enzymatically-regulated structural shaping of glycogen (extension and degradation), expression levels of glycogen branching and debranching enzymes (GBE and GDE respectively) were evaluated. In control mice, cardiac GBE expression was ~4.1-fold higher in females compared with males (p<0.05, Fig. 3a) consistent with larger average particle size seen in females. Cardiac GBE expression in fasted female mice was not statistically different to fasted male mice. Male mice did not exhibit changes in GBE expression with fasting, consistent with the lack of change in glycogen content and glycogen particle size. GDE expression was higher overall in females than males (p(sex)<0.05, 2-way ANOVA factor effect; Fig. 3b), but was not modified by fasting for either sex.

3.3 Sex-specific signalling mechanisms underlying the fasting-induced cardiac glycogen response

Glycogen formation and degradation is regulated by a myriad of signalling pathways involving PI3K/Akt and AMPK signalling, most well characterised in non-cardiac tissues. Activation of the Akt signalling pathway relieves GSK3β inhibition of glycogen synthase. To determine the role of Akt signalling in mediating the sex-specific alterations of cardiac glycogen content with fasting, the total Akt expression and level of phosphorylation (pAkt) was examined. In control mice, pAkt was ~2.2-fold higher in females than males (p<0.05 control male vs. control female; Fig. 4a). With fasting, in females there was a 2-fold increase in pAkt (p<0.05 fasted female vs. control female). Fasted females exhibited ~142% higher pAkt level than fasted males (p<0.05 fasted female vs.
fasted male; Fig. 4a). Total Akt was overall higher in females than males (control female vs. control male p<0.05, fasted female vs. fasted male p<0.05), and not modified by fasting (Fig. 4b). The ratio of phosphorylated Akt to total Akt was increased by ~89% with fasting in females (p<0.05 fasted female vs. control female) whereas the observed trend for increased pAkt:Akt in fasted males (vs. control males) did not reach statistical significance (Fig. 4c). Control males and females exhibited similar pAkt:Akt expression ratio (Fig. 4c). These data are consistent with female-specific stimulation of glycogen synthesis by fasting-induced Akt activation.

Activation of AMPK signalling can promote glycogen formation by increasing glucose-6-phosphate, an allosteric activator of glycogen synthase [17]. The sex-dependent role of AMPK signalling in cardiac glycogen handling was evaluated. Myocardial AMPK phosphorylation levels were similar for control, non-fasted male and female mice (Fig. 4d). With fasting, females but not males, exhibited increased pAMPK levels (~39%, p<0.05 fasted female vs. control female). Thus pAMPK levels tended to be higher in fasted females relative to fasted males (~33%, p=0.052; Fig. 4d). Females exhibited ~47% higher total AMPK expression than males under control conditions (p<0.05 control male vs. control female; Fig. 4e). Males and females exhibited a similar reduction in total AMPK expression with fasting (~19% and ~25% decrease respectively, p<0.05 fasted vs. control). Fasted females exhibited ~36% higher total AMPK than fasted males (p<0.05 fasted female vs. fasted male; Fig. 4e). The ratio of pAMPK to AMPK was lower in control females compared to control males (~35%, p<0.05 control male vs. control female; Fig. 4f), and increased robustly by ~89% in fasted females (p<0.05 fasted female vs. control female). Males did not exhibit changes in pAMPK:AMPK ratio with fasting (Fig. 4f). Thus, lower basal AMPK activity may underlie lower basal cardiac glycogen content observed in control females. In fasting, accumulation of cardiac glycogen may involve a dual activation of glycogen synthase by Akt (via relieving
GSK3β inhibition) and AMPK (via increasing G6P). These data support a female-selective role for AMPK regulation of cardiac glycogen content under fasting-induced metabolic stress conditions.

3.4 Differential glycogen- and macro-autophagy signalling in male and female hearts

Recent evidence suggests that glycogen content is regulated by a specific ‘glycogen autophagy’ pathway, acting to degrade glycogen particles for release of free glucose [11, 12]. To establish that glycogen autophagy proceeds in heart tissue, and to determine whether sex differences in glycogen storage may be associated with autophagic adaptations in fasting, specific glycogen autophagy markers were assessed in ventricular homogenate samples. STBD1 has been identified to tether glycogen to the autophagosome via binding to the cognate protein GABARAPL1 [12]. Indeed, expression of both STBD1 and GABARAPL1 was evident in myocardial tissue. Under control conditions cardiac STBD1 expression was found to be significantly lower in females compared with males (~63% reduction, p<0.05 control male vs. control female; Fig. 5a). In response to fasting, female STBD1 levels were elevated significantly (~69% increase, p<0.05 fasted female vs. control females), while in contrast fasted male STBD1 protein expression was unaltered. In fasted state, STBD1 expression remained lower in females than males, but the differential was apparently less marked and did not reach significance (p=0.088 fasted female vs. fasted male). GABARAPL1 expression was higher in control females relative to males (p<0.05 Fig. 5b). With fasting, GABARAPL1 was significantly increased in both males (~73%, p<0.05 fasted vs. control) and females (~55%, p<0.05 fasted vs. control; Fig. 5b). Thus a disconnection between STBD1 and GABARAPL1 was apparent. Female myocardial expression of STBD1 was lower, yet GABARAPL1 levels higher than male. With fasting, STBD1 and GABARAPL1 increased in parallel in females, but in males, only GABARAPL1 increased.
To distinguish fasting-induced macro-autophagy and glycogen autophagy responses, markers of macrophagy were evaluated. When elongating the autophagosome membrane, LC3B is transformed into the phosphatidylethanolamine (PE)-conjugated form (LC3BII). LC3BI is the inactive, un-conjugated form. The ratio of LC3BII to LC3BI is thus used as a measure of autophagic activity. p62 is an adaptor molecule which functions to tether proteins and organelles to the autophagosome via binding to LC3B. A trend towards overall elevated p62 expression in females relative to males was observed (p(sex)=0.082, 2-way ANOVA factor effect; Fig. 6a). In both sexes, fasting significantly increased p62 levels (control male vs. fasted male p<0.05, control female vs. fasted female p<0.05).

In females, LC3BI (inactive) was ~3.4 fold higher than in males under control conditions (p<0.05 control female vs. control male), and fasting induced a ~77% reduction in expression (p<0.05 fasted female vs. control female; Fig. 6c). In males, no effect of fasting on LC3BI was evident. No difference in LC3BI between fasted males and females was observed. Fasting induced very marked increases in LC3BII (active) in both males and females (~9.4 fold and ~3.5 fold respectively, p<0.05 fasted vs. control; Fig. 6d). LC3BII expression was similar for males and females. The ratio of active: inactive LC3B (LC3BII:I) was ~57% lower in females relative to males under control conditions (p<0.05; Fig. 6b) and was increased ~17-fold by fasting in females (p<0.05 fasted female vs. control female), and ~9.8-fold in males (p<0.05 fasted male vs. control male; Fig. 6b). Fasted female LC3BII:I ratio levels were not statistically distinguishable from fasted males.
4. Discussion

To our knowledge, this study provides the first evidence that metabolic stress induces a female-specific cardiac glycogen response. This study demonstrates that glycogen autophagy (‘glycophagy’) is operational in adult hearts and presents the novel finding that glycogen autophagy is modulated by a pathophysiologic stimulus (fasting) in a sex-specific manner. Basally, females exhibit lower cardiac glycogen content than males, which is stored in the form of larger average particle size. With fasting, glycogen accumulation in female hearts is evident, driven by Akt and AMPK signalling activation, and associated with upregulation of glycogen autophagy proteins STBD1 and GABARAPL1. These findings suggest that female hearts operate with lower glycogen stores under basal conditions and have a selective capacity to switch on a glycogen response in a setting of metabolic stress - a response which is potentially maladaptive.

4.1 Cardiac glycogen accumulation is female-specific

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) [5-7, 29], yet surprisingly, the processes of glycogen turnover in the heart have received only modest attention. Clinical studies have reported that diabetic females have a higher risk of cardiac complications than diabetic males [2]. Sex differences in cardiac glycogen handling have not been previously investigated. In the present study, as a first step towards understanding the basis for glycogen mis-handling in energy stress, we employed a non-pathological metabolic challenge (fasting) to evaluate the fundamental differences in glycogen-related storage and access responses in male and female hearts. Cardiac glycogen was lower in females compared to males under control conditions, coincident with lower glycogen synthase activity and higher glycogen phosphorylase activity. Branching enzyme expression was markedly higher (~4.1 fold) in control female hearts relative to
males and associated with larger average particle size. Thus, although overall cardiac glycogen content was lower in control females, the glycogen particles are apparently more branched and exist in the form of aggregate α particles. In response to fasting, females, but not males, increased glycogen synthase activity and reduced glycogen phosphorylase activity resulting in increased cardiac glycogen content. Interestingly, the accumulation of glycogen in females was coincident with a reduction in average particle size suggesting that new glycogen is formed as smaller β particles. Sex differences in cardiac glycogen content in fasted mice were not linked to differential fasting-induced cardiac atrophy (a similar cardiac weight index reduction was observed in males and females, 10-12% decrease, Table 1; consistent with previous reports [30, 31]). Earlier reports of cardiac glycogen levels in hearts of male fasted rodents have been inconsistent (unchanged, decreased, elevated levels observed) [32-35]. In female rats, no change in cardiac glycogen with fasting has been previously observed [32], however the use of a soy-based protein diet (known to contain relatively high phytoestrogen content) in this previous study may have influenced the sex differences observed. A 48hr fasting intervention appears more severe in mice than in rats, inducing ~62-68% reduction in blood glucose levels in mice (present study) vs. only 10% reduction [33], ~30% reduction [32] or no change [35] in blood glucose levels in rats, thus direct comparison of fasting studies in different species is difficult. It is possible that in the present study involving mice, the 48hr fast was optimal for discriminating the sexually dimorphic response, and that with prolonged energy stress, a male glycogen storage response may become apparent. It may be important that the significant sex differences in the energy stress response involving glycogen handling are evident in a setting where phytoestrogen dietary constituents are maintained at a low level (as in the present study utilizing a low-soy diet). Glycogen content was evaluated in crude heart homogenate samples. Whether fasting induces myocardial regional- or epi-/endo-cardial-specific changes in glycogen content is yet to be determined. To date, estrogen response elements in the genes which encode the enzymes involved in glycogen regulation have not been reported.
(glycogen synthase (GYS1), glycogen phosphorylase (PYGM), glycogen branching enzyme 19 (GBE1) or glycogen debranching enzyme (AGL) [36]. Given that the expression profiles of glycogen regulatory proteins were remarkably different in male and female hearts in the present study, a detailed investigation into the influence of sex steroids on the expression of glycogen regulatory genes is now warranted. Interestingly, cardiac glycogen accumulation is reported in females during pregnancy [37]. Thus a female-specific evolutionary adaptation may underlie the observation in the present study that females, but not males, have the capacity to increase cardiac glycogen stores in response to metabolic stress.

4.2 Sex-dependent induction of glycogen handling signalling with fasting

The PI3K/Akt signalling pathway is known to promote glucose storage via relieving GSK3β inhibition of glycogen synthase. Expression of Akt is modulated by estrogen and consistently reported to be higher in female hearts [3, 38-40]. Indeed phosphorylated and total Akt is higher in female hearts under basal conditions in the present study. Given that Akt phosphorylation of GSK3β leads to activation of glycogen synthase, higher Akt expression in females might be expected to promote higher levels of cardiac glycogen in females. But surprisingly, female mice exhibited less cardiac glycogen content than males under basal conditions. Thus, glycogen downregulation in female hearts may be driven by a different signalling pathway. More recently, AMPK signalling has been shown to increase G6P-allosteric activation of glycogen synthase in the heart and in skeletal muscle by increasing glucose uptake [17, 41]. In the present study, lower AMPK signalling drive (as evidenced by lower pAMPK(Thr172):AMPKα ratio, Fig. 4f) may provide a mechanism by which cardiac glycogen content is maintained at a lower basal level in female hearts compared with male hearts. With fasting, both Akt and AMPK signalling pathways are activated in females coincident with marked accumulation of glycogen. Given that plasma insulin levels are decreased with fasting [8, 42-44], the mechanism of fasting-induced activation of
the PI3K/Akt ‘insulin’ signalling pathway in females is likely to be ligand-independent and may involve intracellular modulation of signalling intermediates. Further investigation is required to establish the mechanism of female-specific Akt response to metabolic stress.

In some settings of acute metabolic stress, cardiac glycogen accumulation appears to be an adaptive response. Male mice overexpressing AMPKγ exhibited improved post-ischemic outcomes relative to wildtypes. This protection was abrogated when glycogen stores were depleted prior to the ischemic insult [45]. In other settings however, evidence suggests that glycogen accumulation is maladaptive. Constitutive activation of the γ2 regulatory subunit of AMPK in humans (arising due to a mutation of the PRKAG2 gene) resulted in accumulation of glycogen-filled vesicles and hypertrophic cardiomyopathy [46]. In chronic ischemic-hibernating myocardium and in dilated cardiomyopathy, glycogen accumulation has been shown to occur in association with a derangement of myofibrillar and cytoskeletal proteins [47, 48]. Pompe disease is characterised by a progressive skeletal and cardiac pathology in association with glycogen accumulation. X-ray diffraction studies have linked this glycogen deposition with reduced interaction between actin and myosin [49]. The cardiac phenotype of glycogen storage diseases, such as Pompe’s disease, supports the contention that excess glycogen storage plays a causal role in cardiac dysfunction. Whether excess glycogen storage directly mediates contractile abnormalities in other pathological settings is yet to be established. The findings from the present study suggest that cardiac glycogen handling is distinctive, and there is a sex specificity to the regulatory processes which may confer cardiopathology vulnerability.

4.3 Sex-specificity of distinct macro- and glycogen-autophagic pathways

Cellular glycogen exists in both the cytosol and in vacuolar/autophagosomal bodies [50]. Cytosolic glycogen is broken down by the combined actions of glycogen phosphorylase and glycogen
debranching enzyme. Lysosomally-derived glycogen-hydrolyzing acid α-glucosidase (GAA) is required for autophagosomal glycogen degradation [50]. Pompe disease, caused by deficiency in (GAA), impacts most severely on cardiac and skeletal muscle tissue [51] underscoring the importance of glycogen autophagy in these organs. In a similar manner to the p62 tagging of ubiquinated proteins to LC3B [52], STBD1 binds glycogen and anchors it to pre-autophagosome membranes by binding to GABARAPL1 [13], a protein which was originally isolated from endometrial cells and identified through its transcriptional induction by estrogen [14]. Indeed, consistent with this estrogenic link, in the present study we find that GABARAPL1 expression is higher in female hearts relative to male hearts (Fig. 5b). Under basal conditions female hearts exhibit markedly lower STBD1 expression than males, contrasting with the GABARAPL1 findings. Very little is known about the relationship between GABARAPL1 and STBD1 expression regulation, and our findings provide new insights. Female hearts may exhibit a ‘reserve’ capacity for glycogen autophagy via GABARAPL1, whereas STBD1 expression levels follow the shift in intracellular content of its ‘ligand’ - glycogen. Given that fasting increases glycogen content in female hearts, it is surprising that the glycogen autophagic degradation route is also increased. It would appear that in fasted female hearts, even though glycogen autophagic degradation is upregulated, elevation of the glycogen content is still observed. This suggests that induction of glycogen autophagy is secondary to glycogen accumulation in female fasted hearts.

This study provides a simultaneous evaluation of macro-autophagy and glycogen autophagy markers in cardiac tissue. The importance of macrophagy (often referred to more generally as ‘autophagy’) as a process involving primarily bulk phagic degradation of protein macromolecules is increasingly recognised and more pronounced susceptibility to autophagy induction in females has been reported in non-cardiac cell types [53, 54]. We demonstrate that female hearts exhibit an enhanced ‘reserve’ for LC3B induction (i.e. higher basal levels of ‘inactive’ LC3B-I) but
interestingly, fasting activated LC3B similarly in males and females, at least at the 48hr timepoint. A trend for increased p62 expression was observed in female hearts (p=0.082) and p62 increased with fasting similarly in both sexes (Fig. 6a). STBD1 may be considered to be the ‘glycophagy’ equivalent to p62 (i.e. the adaptor molecule which mediates membrane localisation of phagic target). Intriguingly, there is a sex-specificity in the expression response of these molecules. STBD1 is lower in control females while p62 is slightly higher, and fasting increases STBD1 expression in females only but p62 is induced by fasting in both sexes). These findings provide further evidence that the macro-autophagy and glycogen autophagy pathways are distinct and may be subject to dissimilar regulatory influence. A novel field of investigation is now apparent – new understanding of the role of sex steroids (systemic and locally produced) in the co-regulation of glycophagy and macrophagy is required and experimental approaches which use selective pharmacologic and genetic interventions to manipulate androgen and estrogen levels will be informative.

Although little is known about the signalling pathways which regulate glycogen autophagy, knowledge of the regulation of macro-autophagy is further advanced (although is mostly extrapolated from studies in non-cardiac tissues). PI3K/Akt signalling has been shown to inhibit macro-autophagy via promoting mTOR inhibition of ULK1/2 [15, 16]. In this study, simultaneous upregulation of Akt activity and autophagy markers with fasting is observed, presenting an alternative mechanistic context. In the fasting context it appears that the autophagic upstream signalling drive is not via Akt but via AMPK activation of ULK1/2 [55, 56] indicating a complex relationship between mTOR and AMPK signalling actions on autophagic processes.
5. Conclusions

In conclusion, this study provides the first evidence that through regulation of key glycogen handling proteins, female myocardial glycogen store levels are lower than males under basal conditions, and female hearts display a unique glycogen accumulation response to fasting. We identify paradoxical (insulin-independent) Akt activation in response to fasting, consistent with increased glycogen in females. Importantly, in female hearts the anti-autophagic actions of Akt appear to be overridden by AMPK signalling in response to acute energy stress, resulting in both macrophagy and glycophagy induction. Glycogen autophagy appears to be an adaptive response in this setting and further work is now required to establish the specific role of glycogen autophagy in cardiac metabolic stress. Our findings of female-specific cardiac glycogen handling response to metabolic stress may have important implications for cardiac pathologies such as diabetes in which females are identified to be at higher risk than males. Further work directed towards understanding female specific glycogen handling dysregulation in such pathologic settings is now warranted.

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Disclosures: none declared.
References

[31] Samarel AM, Parmacek MS, Magid NM, Decker RS, Lesch M. Protein synthesis and degradation during starvation-induced cardiac atrophy in rabbits. Circ Res. 1987;60:933-41.


Figure Legends

**Fig. 1.** Effect of 48hr fasting on glycogen handling proteins in male and female mouse cardiac tissue. (A) Total cardiac glycogen content. (B) Ratio of phosphorylated (Ser641) to total glycogen synthase (GS). See supplementary Figure S1A & B for representative blots. (C) Ratio of phosphorylated (Ser14) to total glycogen phosphorylase (GP). See supplementary Figure S1C & D for representative blots. (D) PAS-glycogen stained (pink) paraffin-embedded formalin-fixed ventricular sections. Relative glycogen levels evaluated by image segmentation analysis (arbitrary pixels/field: control male (326432 arb. pixel units), control female (215483 arb. pixel units), fasted male (375630 arb. pixel units), fasted female (416108 arb. pixel units). (E) PAS-stained, amylase digested sample section from fasted female mouse heart (cell glycogen enzymatically removed by amylase, leaving residual extracellular glycoprotein traces, confirming that PAS stain evident in panel D represents intracellular sites of digestible glycogen deposits).

Panel A, B, C: data presented as mean ± s.e.m, n=6, analysed by 2-way ANOVA, annotated with LSD post-hoc tests, *(p<0.05).

**Fig. 2.** Effect of 48hr fasting on cardiac glycogen particle size in male and female mouse cardiac tissue. (A) Average glycogen particle size. (B) Proportion of glycogen particles less than 40nm (i.e. β particles) vs. greater than 40nm (i.e. α particles). (C) Representative electron microscopy images of glycogen particles depicting the differences in particle size in male and female control and fasted mouse hearts.

All panels: data presented as mean ± s.e.m, n=6, analysed by 2-way ANOVA, annotated with LSD post-hoc tests, *(p<0.05).
**Fig. 3.** Effect of 48hr fasting on glycogen particle regulatory proteins in male and female mouse cardiac tissue.

(A) Glycogen branching enzyme protein expression. (B) Glycogen debranching enzyme protein expression, *(p<0.05) overall sex factor effect.

All panels: data presented as mean ± s.e.m, n=6, analysed by 2-way ANOVA, annotated with LSD post-hoc tests, *(p<0.05).

**Fig. 4.** Effect of 48hr fasting on Akt and AMPK signalling in male and female mouse cardiac tissue.

(A) Expression of phosphorylated Akt (Ser473). (B) Total Akt protein expression. (C) Ratio of phosphorylated (Ser473) to total Akt protein expression. (D) Expression of phosphorylated AMPKα (Thr172). (E) Total AMPKα protein expression. (F) Ratio of phosphorylated (Thr172) to total AMPKα.

All panels: data presented as mean ± s.e.m, n=6, analysed by 2-way ANOVA, annotated with LSD post-hoc tests, *(p<0.05).

**Fig. 5.** Effect of 48hr fasting on cardiac glycogen autophagy markers in male and female mice. (A) Starch Binding Domain 1 (STBD1) protein expression. (B) GABA(A) receptor-associated protein-like 1 (GABARAPL1) protein expression.

All panels: data presented as mean ± s.e.m, n=6, analysed by 2-way ANOVA, annotated with LSD post-hoc tests, *(p<0.05).

**Fig. 6.** Effect of 48hr fasting on cardiac macro-autophagy markers in male and female mice. (A) p62 protein expression, overall sex factor effect p=0.082. (B) Ratio of active (II) to inactive (I) LC3B protein expression. (C) LC3BI (inactive) protein expression. (D) LC3BII (active) protein expression.
All panels: data presented as mean ± s.e.m, n=6, analysed by 2-way ANOVA, annotated with LSD post-hoc tests, *(p<0.05).
Myocardial glycophagy - a specific glycogen handling response to metabolic stress is accentuated in the female heart.

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Research Highlights

- female basal cardiac glycogen stores are markedly lower than male
- females exhibit cardiac glycogen accumulation in response to metabolic stress
- fasting activates myocardial Akt and AMPK signalling in females selectively
- myocardial glycophagy signalling is evident, with potential for estrogen regulation
- glycogen dysregulation may underlie increased risk in female diabetic hearts
Figure 1
Figure 2
Figure 3

A  Glycogen branching enzyme

B  Glycogen debranching enzyme

( arb. units )

Figure 3
Figure 4
Figure 5
Figure 6
Table 1
Systemic and cardiac morphological characteristics of control & fasted, male & female mice (48hr treatment period).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td><strong>Body Weight (BW, g)</strong></td>
<td>27.2 ± 0.6</td>
<td>19.9 ± 0.3#</td>
</tr>
<tr>
<td><strong>BW change over treatment period (%) change relative to initial</strong></td>
<td>1.3 ± 0.9</td>
<td>-0.9 ± 0.6</td>
</tr>
<tr>
<td><strong>Heart Weight (HW, mg)</strong></td>
<td>141 ± 3</td>
<td>101 ± 3#</td>
</tr>
<tr>
<td><strong>Tibia Length (TL, mm)</strong></td>
<td>17.6 ± 0.4</td>
<td>16.8 ± 0.2#</td>
</tr>
<tr>
<td><strong>HW:TL (mg/mm)</strong></td>
<td>8.00 ± 0.12</td>
<td>-5.99 ± 0.16#</td>
</tr>
<tr>
<td><strong>Blood Glucose (mM)</strong></td>
<td>10.3 ± 0.8</td>
<td>10.3 ± 1.7</td>
</tr>
</tbody>
</table>

Data presented as mean ± s.e.m, n=5-6/group, 2-way ANOVA, LSD post-hoc tests.
*p<0.05 vs. respective Control
#p<0.05 vs. respective Male
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