ABSTRACT

Lipotoxic cardiomyopathy (LCM) is characterized by cardiac steatosis, including the accumulation of fatty acids, triglycerides and ceramides. Model systems have shown the inhibition of ceramide biosynthesis to antagonize obesity and improve insulin sensitivity. Sphingosine Δ4 desaturase (encoded by ifc in Drosophila melanogaster) enzymatically converts dihydroceramide into ceramide. Here, we examine ifc mutants to study the effects of desaturase deficiency on cardiac function in Drosophila. Interestingly, ifc mutants exhibited classic hallmarks of LCM: cardiac chamber dilation, contractile defects and loss of fractional shortening. This outcome was phenocopied in global ifc RNAi-mediated knockdown flies. Surprisingly, cardiac-specific ifc knockdown flies exhibited cardiac chamber restriction with no contractile defects, suggesting heart autonomous and systemic roles for ifc activity in cardiac function. Next, we demonstrated that ifc mutants exhibit suppressed Sphingosine kinase 1 (Sk1) expression. Ectopic overexpression of Sk1 was sufficient to prevent cardiac chamber dilation and loss of fractional shortening in ifc mutants. Partial rescue was also observed with cardiac- and fat-body-specific Sk1 overexpression. Finally, we showed that cardiac-specific expression of Drosophila inhibitor of apoptosis (dIAP) also prevented cardiac dysfunction in ifc mutants, suggesting a role for caspase activity in the observed cardiac pathology. Collectively, we show that spatial regulation of sphingosine Δ4 desaturase activity differentially affects cardiac function in heart autonomous and systemic mechanisms through tissue interplay.

KEY WORDS: Ifc, Sphingosine Δ4 desaturase, Dihydroceramide, Sphingolipid metabolism, Ceramide, Cardiac

INTRODUCTION

Sphingolipid metabolism is an important cellular pathway for the synthesis of key regulatory bioactive lipids including ceramide and sphingosine 1-phosphate. Sphingolipid intermediates and metabolites, beyond their structural role in membranes, can have a variety of molecular roles in cellular processes, including autophagy (Harvald et al., 2015), cell signaling (Smith and Merrill, 2002), apoptosis and proliferation (Mullen and Obeid, 2012). In recent years, myocardial sphingolipid metabolism has been shown to have a role in the pathogenesis of cardiac disease. Although ceramide, the metabolic hub of sphingolipid metabolism, has been shown to exhibit cardiotropic properties in the progression of lipotoxic (obesity and diabetic) cardiomyopathies (Bandet et al., 2019; Park et al., 2008; Walls et al., 2018), sphingosine 1-phosphate has been shown to be cardioprotective (Knapp, 2011; Ahmed et al., 2019).

However, less is known regarding the role of other sphingolipid intermediates, including dihydroceramide, in the etiology of these diseases. In Drosophila, the gene infertile crescent (ifc) encodes for the protein sphingosine Δ4 desaturase, which catalyzes the introduction of a double bond between C4 and C5 on the sphingoid backbone of dihydroceramide, the final step in de novo ceramide synthesis (Ternes et al., 2002). Sphingosine Δ4 desaturase has been implicated in a diverse set of biological processes, including photoreceptor cell maintenance (Huang et al., 2015), spermatogenesis (Castrillon et al., 1993) and spindle assembly in male meiosis (Basu and Li, 1998). Mutant female ifc flies also exhibit a myriad of reproductive defects, including malformed oocytes, follicle degeneration, egg retention and supernumerary spermathecae (Phan et al., 2007). However, the regulatory role of this protein in the heart is not completely understood.

More recent studies have shown that ifc mutants also exhibit classic hallmarks of obesity, including elevated triglyceride (TG) levels, fat body (adipocyte) hypertrophy and resistance to starvation-induced expiration (Walls et al., 2013). These studies suggest that the lipid accumulation reported in ifc mutants was directly associated with the loss of adipokinetic hormone (Akh)-mediated fat mobilization. Akh in Drosophila works as a functional ortholog, like β-adrenergic agonists or glucagon, and promotes the breakdown of glycogen and TGs for energy utilization (Gronke et al., 2007). Thus, ifc-encoded sphingosine Δ4 desaturase activity appears to be necessary for Akh-producing cell (Akhp) viability and function in flies.

These findings correlate with previous studies which showed that apoptosis-induced cell death of Akhp suppressed TG mobilization from Drosophila fat-body cells (Gronke et al., 2007). Similarly, Akhp-specific knockdown of ifc led to fewer Akhp in 3rd instar larvae relative to control. By adulthood, Akhp and akh transcripts were nearly undetectable in Akhp—ifc RNA interference (RNAi) knockdown flies (Walls et al., 2013). Predictably, these flies also exhibited increased TG levels, which could be alleviated by concomitant Akhp-specific overexpression of Drosophila inhibitor of apoptosis (dIAP; also known as Diap1).

Thus, sphingosine Δ4 desaturase deficiency induced lipotoxicity and cell death in Akhp, leading to a loss of Akh-mediated TG mobilization, accumulation of lipotoxic sphingolipid intermediates and an obese phenotype (Walls et al., 2013). Given the established associations between obesity and heart disease, we sought to characterize the role of sphingosine Δ4 desaturase in cardiac function.

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In this study, we found that ifc mutants, which accumulate excess TG and an aberrant sphingolipid profile (Walls et al., 2013), develop severe heart dysfunction including diastolic and systolic cardiac dilation and loss of fractional shortening. Similar cardiac phenotypes are also observed in flies with global RNAi-mediated knockdown of ifc mRNA. Unexpectedly, when we knockdown ifc specifically in the heart, the hearts of these flies are not dilated but instead exhibit diastolic and systolic cardiac chamber restriction. The restricted cardiac phenotype is reminiscent of serine palmitoyl transferase (lace) and ceramide synthase (schlank) global and heart-specific knockdowns (Walls et al., 2018), suggesting that this cardiac restriction phenotype might be due to genetic suppression of de novo ceramide synthesis.

To gain further insight into the cause of the disparate phenotypes exhibited between global versus cardiac-specific knockdown of ifc, we examined the expression levels of other sphingolipid metabolic genes in ifc mutants. Indeed, ifc mutant flies exhibit decreased global expression of Sphingosine kinase 1 (Sk1), which appears to account, in part, for the differential phenotype exhibited by global versus cardiac-specific ifc knockdown flies, as Sk1 is not expressed in the heart. Specifically, we show that global overexpression of Sk1 in ifc mutants prevents cardiac dilation and normalizes contractility. Next, we show that overexpression of Sk1 specifically in the heart or fat body partially rescues the ifc mutant cardiac phenotypes, suggesting that cardiac function might be regulated by sphingolipid metabolite modulation both systemically and in the heart. Restoration of Akhpcs, through the Akhpc-specific overexpression of dIAP, also partially restored ifc mutant cardiac function, presumably by reactivation of TG utilization in the heart and periphery. Finally, cardiac-specific overexpression of dIAP was sufficient to rescue the ifc mutant...
phenotype, suggesting that the cardiac phenotype is at least partially caspase dependent. These results correlate strongly with the previously reported cardiac-specific dIAP overexpression rescue of ceramide dietary-induced cardiac dilation and contractile dysfunction (Walls et al., 2018).

RESULTS

Sphingosine Δ4 desaturase ifc-4 mutants exhibit cardiac defects

Mutant ifc-4 flies have been reported to exhibit obese phenotypes with the accumulation of TG and sphingolipid subspecies, including dihydroceramide, dihydrospingosine and ceramide/sphingosine dienes, which correlated with a global loss of ifc mRNA expression (Walls et al., 2013). To determine the effects of this shift in lipid profile on cardiac function, we employed high-speed video microscopy on semi-intact 3-week-old female ifc-4 fly heart preparations (Fink et al., 2009; Ocorr et al., 2009). Relative to wild-type flies, ifc-4 mutants exhibited dilated diastolic and systolic diameters (Fig. 1A,B) with a concomitant loss in fractional shortening (Fig. 1C). However, no changes in heart beat length (heart period) or rhythmicity were observed, as illustrated by a representative M-mode (Fig. 1D). Similar phenotypes were observed in ifc in trans to a deficiency of the locus [Df(2L)AP1], thus substantiating the hypothesis that it is indeed the loss of ifc gene function that causes severe cardiac dilation and compromised contractility. This finding correlated with an observed loss of ifc expression in the hearts of ifc-4 mutants (Fig. 1). Taken together, these data suggest that sphingosine Δ4 desaturase mutants exhibit a lipotoxic cardiomyopathy-like phenotype.

Systemic versus heart-specific knockdown of ifc differentially affects cardiac function

To determine whether the observed cardiac defects occurred specifically from loss of sphingosine Δ4 desaturase function in the heart or through the systemic effects of sphingosine Δ4 desaturase loss, we utilized a Gal4/UAS RNAi-mediated knockdown (KD) approach (Brand and Perrimon, 1993). As in ifc-4 mutants, flies with global KD of ifc using an actin-Gal4 driver exhibit significant loss of ifc mRNA expression (Walls et al., 2013). Global ifc KD fly hearts also showed increased diastolic and systolic diameters (Fig. 2A,B). A significant loss in fractional shortening was also observed.

Next, we examined the effects of heart-specific KD of ifc on cardiac function using the Hand-Gal4 driver (Han and Olson, 2005). Strikingly, flies with heart-specific KD of ifc exhibited constricted rather than dilated cardiac chamber width, with a reduction in both diastolic and systolic diameter, but with no significant change in fractional shortening (Fig. 2D-F). Based on myofibrillar staining, heart-specific ifc KD hearts also exhibited sarcomeric gaps, compared with control (w1118>ifc RNAi KK) hearts (Fig. 2G). This constricted heart phenotype is reminiscent of the KD of two other ceramide biosynthetic genes, lace and schlank (Walls et al., 2018). Importantly, cardiac-specific KD of ifc does not affect global TG levels, as observed in both ifc-4 mutants and global ifc-4 KDs (Walls et al., 2013). These cardiac effects correlated with a loss in cardiac ifc mRNA expression (Fig. S1) relative to UAS-ifc RNAi controls. The opposing phenotypes in heart function upon global versus heart-specific ifc loss-of-function suggests that the systemic effects of sphingosine Δ4 desaturase perturbation versus cardiac-

Fig. 3. The ifc-4 mutant cardiac phenotypes are Sk1 dependent. (A) The ifc-4 mutants exhibit a reduction in both ifc and Sk1 expression compared with Canton-S (wild-type) controls. (B-D) The ifc-4 mutants were compared with global (ifc-4; Act5c-G4/UAS-Sk1), heart-specific (ifc-4; Tinc4-G4/UAS-Sk1) or fat-body-specific (ifc-4; lsp-G4/UAS-Sk1) Sk1-overexpressing lines in an ifc-4 mutant background. Diastolic diameter (B), systolic diameter (C) and fractional shortening (D) were assessed. Controls: w1118. Drivers: ifc-4; Act5c-G4/+; ifc-4; tinc4Δ4/+; ifc-4; lsp-G4/. UAS: (ifc-4; UAS-Sk1/+). Multiple comparison two-tailed Student’s t-test. *P<0.05; **P<0.01; ***P<0.001. Error bars=s.e.m. n=30 flies.
specific perturbation differentially affect cardiac function. This observation might result from a variety of possible changes in either systemic and/or cardiac-specific sphingolipid, fatty acid and/or triglyceride metabolism.

**Mutant ifc^4 flies exhibit reduced Sk1 expression**

We previously reported that ifc^4 mutants exhibit reduced C_{14:1} ceramide levels, but accumulated larger amounts of C_{14:0} dihydroceramide and C_{14:2} ceramide dienes, with total ceramide levels in these flies slightly decreased relative to wild-type flies (~10%) (Walls et al., 2013). Interestingly, we had previously observed that Sk2 KD flies accumulate ceramide, dihydroceramide, sphingosine and dihydrosphingosine, but flies with KD of Sk1 preferentially accumulate ceramide, ceramide dienes, sphingosine and sphingosine dienes. These results suggest that Sk1 and Sk2 exhibit differential activity regarding the metabolism of specific ceramide and sphingosine subspecies based on the degree of sphingoid base chain saturation. Hence, we sought to determine whether lipidomic profiles in ifc^4 mutants are accompanied by differential expression of genes involved in sphingolipid metabolism. We compared lace, ifc, Sk1, Sphingosine kinase II (Sk2) and Sphingosine 1-phosphate lyase (Sply) mRNA expression in ifc^4 mutants with wild-type controls (refer to fig. 1A in Walls et al., 2018).

ifc^4 mutants exhibited a >65% global reduction in ifc expression (Fig. 3A). Interestingly, these flies also exhibit reduced Sk1 expression (Fig. 3A). We previously reported that ifc^4 mutants exhibit an accumulation of C_{14:2} ceramide dienes (Walls et al., 2013) and that global Sk1 KD flies also exhibit an accumulation of C_{14:2} ceramide dienes. Thus, the accumulation of ceramide dienes in ifc mutants might be due to concomitant reduction in Sk1 expression. No change in the mRNA levels of lace, Sk2 or Sply was observed (Fig. 3A).

**Inducing expression of Sk1 reversed the cardiac dilation in ifc^4 mutants**

Although Sk1 is widely expressed throughout various fly tissues, it is not expressed in the heart (Chintapalli et al., 2007; Leader et al., 2018). Additionally, global but not heart-specific reduction in Sk1 function causes cardiac dilation, which correlates with a respective loss of global Sk1 expression (Walls et al., 2018). This observation suggests that the concomitant reduction of systemic Sk1 in ifc^4 mutants might override the constriction phenotype observed in heart-specific ifc KD and lead to cardiac dilation (Fig. 1).

As ifc^4 mutants exhibited reduced expression of Sk1, we sought to determine whether expression of Sk1 could rescue the dilation phenotype. Indeed, global overexpression of Sk1 using a UAS-Sk1 construct (Herr et al., 2004) and global actin driver UAS-Sk2 in ifc
mutants reversed the dilation and normalized contractility (Fig. 3B-D). Next, we ectopically expressed Sk1 specifically in the heart of ifc4 mutants to determine whether cardiac-specific expression of Sk1 was sufficient to confer cardioprotection from the effects of systemic ifc4 mutants. Cardiomyocyte-specific Sk1 expression with the TnCa4-Gal4 driver (Lo and Frasch, 2001) in the ifc4 mutant background partially reversed the dilation and contractility phenotype of these mutants (Fig. 3B-D).

As the fat body has a major role in global lipid homeostasis and performs roles comparable to both adipose tissue and liver in mammals (Banerjee et al., 2012), we utilized the fat-body-specific lsp2-Gal4 driver to determine whether Sk1 overexpression in the fat body could also prevent the phenotype observed in ifc4 mutants. Fat body expression of Sk1 also partially rescued the dilation phenotype, but not the reduced fractional shortening of ifc4 mutants. (Fig. 3B-D). Taken together, these data suggest that suppression of Sk1 expression might underlie the lipotoxic cardiac phenotype observed in ifc4 mutants.

Preventing Akh loss in ifc mutants improves cardiac function

Akhpc (Fig. 4A) have a glucagon/β-adrenergic-type role in regulating glycogen and TG storage in flies (Gronke et al., 2007). Akhpc-specific expression of dIAP in ifc4 mutants reversed the loss of Akhpc (Walls et al., 2013). Thus, we tested if Akhpc restoration also has a protective effect on cardiac function. Indeed, ifc4-induced dilation was partially reversed upon Akh cell restoration, but loss of fractional shortening was not significantly improved (Fig. 4B-D). The partial rescue is probably achieved through the restoration of Akhpc-mediated TG utilization, thus leading to a reduction in both systemic and heart TGs. However, this finding also suggests that systemic loss of sphingosine A4 desaturase activity might still have a role in the induction of moderate cardiac dilation and loss of shortening, despite preventing Akhpc ablation in ifc4 mutants (see Walls et al., 2013 for dIAP-mediated prevention of Akhpc loss in ifc4 mutants). Taken together, systemic factors involving ifc4 loss of function, independent of Akhpc viability, contribute to the observed cardiac defects.

Cardiac expression of dIAP is cardioprotective in ifc4 mutants

Previously, we provided evidence that ceramide-associated lipotoxic cardiomyopathy was in part mediated by caspase activation through interactions with Annexin X (Walls et al., 2018). Furthermore, we observed that cardiac-specific KD of Annexin X (a caspase activator) or cardiac-specific overexpression of dIAP (a caspase inhibitor) protected the heart against ceramide-associated lipotoxicity. Importantly, flies with cardiac-specific expression of dIAP alone exhibit no cardiac phenotype (Walls et al., 2018). We sought to determine whether caspase activation specifically in the heart was important in the induction of the dilated lipotoxic cardiomyopathy observed in ifc4 mutants. Hence, we expressed dIAP in ifc4 mutant flies. Strikingly, cardiomyocyte-specific expression of dIAP exhibited a strong cardioprotective effect: both diastolic and systolic diameters were normalized in ifc4 flies upon dIAP expression and fractional shortening increased, indicative of substantially improved cardiac function. These data suggest that cardiac-specific inhibition of caspase-activation through dIAP overexpression in ifc4 mutants prevents the deleterious effects of mutation on cardiac function.

DISCUSSION

Our results obtained using a unique combination of spatially controlled genetic interactions provide evidence that co-regulation of the genes ifc and Sk1 can differentially affect cardiac function in a tissue-specific manner. Specifically, the ifc4 mutation leads to systemic reduction in Sk1 expression. We propose that this phenotype is associated with lipotoxicity generated in part by systemic changes in either sphingolipid, fatty acid and/or TG metabolism, which we have previously reported (Walls et al., 2013; Fyrst et al., 2009; Fyrst et al., 2008; Struckhoff et al., 2004). Additionally, global ifc KD adversely affects cardiac function with similar, albeit less severe, lipotoxic cardiomyopathy phenotypes. This finding is consistent with other ceramide-associated models of lipotoxic stress in the fly heart (Walls et al., 2018).

We also previously reported that global Sk1 KD induced the accumulation of ceramide and sphingosine dienes (Walls et al., 2013), which correlated with a dilated lipotoxic cardiac phenotype in these flies (Walls et al., 2018). Cardiac-specific knockdown of Sk1 produced no phenotype, which was expected as Sk1 is not expressed in the heart (Walls et al., 2018). Here, we showed that ifc4 mutants global Sk1 expression is suppressed, which might account in part for the observed dilated phenotype in these flies. Importantly, global ectopic expression of Sk1 in an ifc4 mutant background conferred a slightly restricted phenotype relative to control flies. Additionally, heart-specific ectopic expression of Sk1 in ifc4 mutants mitigates the dilated phenotype observed in ifc4 mutants. Similar effects were observed when Sk1 was ectopically overexpressed in the fat body. These data suggest that modulation of genes regulating both ceramide synthesis and degradation, specifically in the heart as well as the periphery, affect cardiac structure and function. Hemolymph-mediated transport of cardiotoxic sphingolipid metabolites between the fat body and heart might underlie these processes, although the effects of sphingosine A4 desaturase activity and its effects on general lipid metabolism are also likely to be major contributing factors (Matsuo et al., 2019). Interestingly, cardiac-specific ifc KD confers a restricted phenotype. This is consistent with previously reported
models, where cardiac-specific KD of the ceramide biosynthesis genes *lace* and *schlank* also conferred a restricted phenotype (Walls et al., 2018). This outcome probably occurs through the heart autonomous reduction of ceramide production, without a concomitant reduction in global *Skf* expression, and through changes in *Skf* target sphingolipid intermediates.

However, it has yet to be determined directly whether the observed cardiac dysfunction in *ifc* mutants is associated with cardiac accumulation of ceramide and sphingolipid species that originated in the periphery (fat body, etc.). The development of reliable analytical methods for measuring sphingolipidomes in the fruit fly heart will be needed to determine whether these cardiotoxic sphingolipids are changed in the heart when modulated systemically, although our genetic data suggest that this is probably the case, together with probable changes in other lipotoxic lipid metabolites including TG. Nevertheless, tissue dysfunction, in either the fat body or Akhpc, is also likely to contribute to cardiac dysfunction when improperly regulated. Collectively, our data show that an important interplay exists between the heart, fat body and Akhpcs in regulating cardiac function under conditions of lipotoxicity in the fruit fly (Fig. 5). Similar observations have been made in mammalian models, in which interplay occurs between the heart, liver, adipose tissue and neuroendocrine regulatory organs.

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Competing interests
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