Hypoglycemia Induced Mitochondrial Connexin-43 Accumulation Aggravates Diabetic Cardiomyopathy

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Abstract

Background: Diabetic cardiomyopathy (DCM) is a complex multifaceted disease responsible for elevated hospitalization and mortality in patients with diabetes mellitus (DM). DCM patients exhibit subclinical diastolic dysfunction, progression towards systolic impairment, and abnormal electrophysiology. Hypoglycemia events that occur spontaneously or due to excess insulin administration threaten the lives of DM patients – with the increased risk of sudden death. However, the molecular underpinnings of hypoglycemia-aggravated DCM remain to be elucidated.

Methods and Results: Here we used the established streptozotocin-induced type 1 diabetic cardiomyopathy (T1 DCM) murine model to investigate how hypoglycemia aggravates DCM progression. We showed that chronic hyper- or hypoglycemic challenges dampened cardiac diastolic function *in vivo* as well as myocardial contractility and calcium handling in isolated cardiomyocytes. Similar contractile defects were recapitulated using neonatal mouse ventricular myocytes (NMVMs) under glucose fluctuation challenges. Using immunoprecipitation mass spectrometry, we identified and validated that hypoglycemia challenge activates the MEK/ERK and PI3K/Akt pathways which results in Cx43 phosphorylation by Src protein in cardiomyocytes. Cx43 dissociation and accumulation at mitochondrial inner membrane was confirmed both in human and murine cardiomyocytes. To determine causality, we overexpressed a mitochondrial targeting Cx43 (mtCx43) using AAV2. At normal blood glucose levels, mtCx43 overexpression recapitulated cardiomyocytes

contractile deficiencies, cardiac diastolic dysfunction as well as aberrant electrophysiology both *in vitro* as well as *in vivo*.

Conclusions: Hypoglycemia challenges results in the accumulation of mtCx43 through the MEK/ERK/Src and PI3K/Akt/Src pathways. We provide evidence that Cx43 mislocalization is present in diabetes mellitus patient hearts, STZ-induced DCM murine model, and glucose fluctuation challenged NMVMs. Mechanistically, we demonstrated that mtCx43 is responsible for inducing aberrant contraction and disrupts electrophysiology in cardiomyocytes and our results support targeting of mtCx43 in treating DCM.

Translational perspective: Severe hypoglycemia drives cardiac dysfunction and aggressive ventricular arrhythmias in patients with DCM that leads to sudden cardiac death. Here we demonstrate that Cx43 mislocalization to mitochondria occurs upon hypoglycemic challenge and mtCx43 accumulation is responsible for cardiac diastolic dysfunction, cardiomyocyte contractile dysfunction, and aberrant electrophysiology *in vivo*. Our findings give support for therapeutic targeting of MEK/ERK/Src and PI3K/Akt/Src pathways to prevent mtCx43-driven DCM.

Keywords: hypoglycemia, diabetic cardiomyopathy, mitochondrial connexin 43, Src

68   **Background**

69   The IDF Diabetes Atlas 2019 estimated the number of people living with diabetes
70   mellitus (DM) would reach 693 million by 2045, representing 9.3% of the global adult
71   population (20–79 years) [1]. Myocardial dysfunction that progresses to heart failure
72   are present in patients with DM and is clinically defined as diabetic cardiomyopathy
73   (DCM) [2]. DCM hearts are characterized by cardiac remodeling, early onset of
74   diastolic dysfunction followed by systolic impairment, and eventually progresses to
75   heart failure with reduced ejection fraction (HFrEF) [3]. Compared to coronary artery
76   disease induced dilated cardiomyopathy, DCM has worse prognosis [4, 5]. Compared
77   to other cardiomyopathies, DCM exhibit metabolic dysfunction, electrophysiology, and
78   insulin resistance [6, 7] – making DCM management much more challenging. Moreover,
79   our understanding of the molecular underpinnings of DCM remains limited.

80
81   Hypoglycemia is a major challenge in DM management [8] that significantly increases
82   the mortality rate in both type 1 and type 2 DM patients [9, 10]. Severe hypoglycemia,
83   defined as blood glucose ≤ 3.0 mM, can result in cognitive confusion, loss of
84   consciousness, seizures, and even death in both young and elderly DM patients [11-
85   14]. Prolonged hypoglycemia induced neuroglycopenia are rare; most fatal
86   hypoglycemic episodes result in cardiac dysfunction, especially ventricular arrhythmias
87   [14, 15]. Experimentally induced hypoglycemic events in patients with type 1 or type 2
88   DM resulted in pro-arrhythmogenic cardiac repolarization with prolonged QT intervals
89   [16, 17]. In rodent models, severe hypoglycemia (blood glucose < 1.0 mM) leads to

prolonged QT interval, ventricular ectopy, and high-degree atrioventricular blockage [18]. How hypoglycemia can induce cardiac dysfunction and abnormal electrophysiology remains to be elucidated.

Gap junctions (GJs) are channels that directly connect two adjacent cells with two hemichannels, allowing passage of ions (Na^+ , K^+ , Ca^{2+}), and proteins and molecules less than 1.5 kDa [19] and are tightly regulated in response to intracellular and extracellular signals [20, 21]. Intercellular communication between cardiomyocytes through Cx43 allows for rapid electrical signal diffusion and synchronous ventricular contraction. Impaired gap junction-mediated arrhythmias are prevalent among patients with acute myocardial infarction and heart failure [22, 23]. Myocardial Cx43 are trafficked via three main pathways: (i) autophagosomal degradation via direct fusion with lysosomes [24] or phagosomes [25, 26]; (ii) lateralization to the lateral membrane; and (iii) translocation to the inner mitochondrial membrane (IMM) through heat shock protein (HSP) 90-dependent translocase [27]. Previously we have demonstrated that hypoglycemic challenge resulted in decreased Cx43 expression in hyperglycemia cultured H9c2 cells [28] suggestive an active role of Cx43 in DCM progression.

In this study, we investigated the role of Cx43 trafficking due to hypoglycemia challenge using streptozotocin (STZ)-induced DCM murine model. STZ animals exhibit diastolic dysfunction and showed increase arrhythmia susceptibility upon insulin-induced hypoglycemic challenge. Isolated DCM cardiomyocytes exhibit contractile

dysfunction when cultured under low glucose conditions. Using neonatal mouse ventricular myocytes (NMVMs), we show that glucose fluctuations can indeed result in contractile dysfunction and recapitulate Cx43 translocation to mitochondria. Molecularly, we demonstrate that activation of MEK/ERK/Src and PI3K/Akt/Src pathways are responsible for mitochondrial Cx43 (mtCx43) accumulation. Overexpression of mtCx43 is sufficient to recapitulate hypoglycemia-aggravated cardiac dysfunction and abnormal electrophysiology both *in vitro* and *in vivo*. Together, our study provides mechanistic insight into hypoglycemia-aggravated DCM with potential new avenues for therapeutic designs.

Materials and Methods

Ethics

For the collection and use of human cardiac tissue, informed consent was obtained from subjects and all protocols were reviewed and approved by the Ethics Review Committee at Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, China (SH9H-2020-TK238-1). All procedures performed in these studies were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants gave written informed consent. The general clinical characteristics of the patients are presented in the Additional file: Table S1.

For animal use, all protocols were approved by the Laboratory Animal Care Ethics

Review Committee at the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, China (SH9H-2020-A234-1). For adult mice, animals were euthanized by deep isoflurane (5%) and sacrificed by cervical dislocation. For neonatal mice (within 36 h) that are resistant to CO₂, animals were euthanized by decapitation. Other detailed methods are available in the methods section of Additional file.

Results

Hypoglycemia challenge worsens diastolic dysfunction and increases cardiac arrhythmia susceptibility

To study the effect of hypoglycemia on cardiac function, we first induced DCM (Fig. 1a) in mice using the established STZ model (150mg/kg). Two weeks post-injection, the mice exhibited an average blood glucose level of 6.06 ± 0.30 and 29.38 ± 1.15 mM in Control and DCM groups, respectively (Additional file: Figure S1a). Cardiac function was evaluated using echocardiography at 2- and 8-weeks post STZ injection (Fig. 1b). At 2-weeks post STZ injection, no significant differences in left ventricle (LV) ejection fraction (EF), fractional shortening (FS), early to late ventricular filling velocity ratio (E/A ratio), or early mitral inflow velocity to early diastolic mitral annulus velocity ratio (E/E' ratio) between DCM and Control mice (Fig. 1b, c) was observed. 8-weeks post STZ injection, DCM animals exhibited diastolic dysfunction marked by a significant decrease in E/A ratio and an increase in E/E' ratio, but no significant difference in EF and FS compared to Control animals (Fig. 1c). DCM animals were significantly emaciated and depilated after 10 weeks of continued STZ injection (Additional file:

Figure S1b, c).

We injected DCM animals with insulin to mimic hypoglycemic challenge (HDCM group; Fig. 1a). 10 DCM mice were randomly selected and injected with insulin to induce hypoglycemia for 120 min prior to sacrifice. Unlike DCM and Control animals, HDCM mice exhibited a significant decrease in blood glucose levels (Control: 6.35 ± 0.34 mM, DCM: 26.48 ± 1.30 mM, HDCM: 2.28 ± 0.20 mM; Additional file: Figure S1d). Moreover, HDCM animals exhibited a significant increase in EF and FS and a significant decrease in the E/A and E/E' ratios (Fig. 1b, d), suggesting compensatory systolic function coupled with worsened diastolic function. Electrophysiologically, we observed an increase in QT interval and JT interval in DCM animals compared to Control animals (Fig. 1e, f) in accordance with previous observations [29]. Furthermore, there was a further increase in QTc, QT interval, and JT interval in HDCM animals compared to DCM and Control animals (Fig. 1e, f). Together, these results suggest that hypoglycemic challenged DCM animals, have worsen diastolic cardiac dysfunction and are more susceptible to ventricular arrhythmias.

Next, to assess the contractile and relaxant function of individual cardiomyocyte, we measured myocardial contractility and Ca^{2+} transients in ventricular cardiomyocytes isolated from Control or DCM animals and cultured the cardiomyocytes in either normal (NG), high (HG) or low glucose concentration conditions (LG) (Fig. 2a, b; Additional file: Figure S2a). Since DCM cardiomyocytes

experienced a hyperglycemic environment prior to isolation, we expected that a HG culturing condition would be better tolerated by DCM cardiomyocytes while a low glucose culturing condition would mimic a hypoglycemia challenge. First, cardiomyocytes isolated from Control animals, HG or LG culturing conditions did not alter sarcomere shortening (Fig. 2c) or contraction peak height (Fig. 2d). HG medium significantly decreased contraction time to peak 90 (Fig. 2e) and contraction time to baseline 90 (Fig. 2f) in Control cardiomyocytes. Compared to NG medium, LG medium resulted in a significant decrease in contraction time to peak 90 (Fig. 2e), contraction time to baseline 90 (Fig. 2f), calcium-transient amplitude (Additional file: Figure S2b), but no difference in calcium-decay tau (Additional file: Figure S2c), calcium-time to peak 90 (Additional file: Figure S2d), and calcium-time to baseline 90 (Additional file: Figure S2e), suggesting LG medium inhibited contraction and relaxation with declined Ca^{2+} release in Control cardiomyocytes. Unlike Control cardiomyocytes, DCM cardiomyocytes were more susceptible to glucose fluctuations. Compared to HG condition, NG and LG treated DCM cardiomyocytes exhibited significant decreased sarcomere shortening (Fig. 2c) and contraction peak height (Fig. 2d), increased contraction time to peak 90 (Fig. 2e) and contraction time to baseline 90 (Fig. 2f) – indicative of slower contraction and relaxation in DCM cardiomyocytes upon hypoglycemic challenge. Moreover, LG treated DCM cardiomyocytes showed aberrant calcium handling including reduced calcium-transient amplitude (Additional file: Figure S2b) and increased decay tau (Additional file: Figure S2c), indicating a significantly slower Ca^{2+} handling. These data showed that changes on indices of contractile,

relaxant function, and Ca^{2+} handling caused by glucose concentration challenge were much greater in myocardial cells isolated from DCM mice than those from Control mice. Further, these data indicates that severe hyperglycemic or hypoglycemia challenge is well tolerated by healthy cardiomyocytes but is loss in DCM cardiomyocytes.

Recapitulation of aberrant contraction and electrophysiology in HLG challenged neonatal mouse ventricular myocytes

To further study the underlying mechanism that drives cardiac dysfunction upon hypoglycemic challenge, we established isolated NMVMs and treated the cardiomyocytes with either NG, HG, or high glucose following low glucose group (HLG) medium that mirrored *in vivo* blood glucose conditions (Fig. 2g). Our NMVM cultures contained $\geq 80\%$ cTnT⁺ cardiomyocytes evaluated by immunofluorescent staining (Additional file: Figure S3a). Using live cell imaging we measured the mean contraction velocity and beating frequency of NMVMs using a previously established algorithm [30, 31]. In a 4-hour time course study, we subjected NMVMs to either NG, HG, or HLG challenge and measured changes in contractility (Additional file: Figure S3b, c). Upon HG challenge, there was a slight decrease in mean velocity and beat rate compared to HG baseline (0 h) and NG (Additional file: Figure S3b, c). HLG treatment resulted in a significant reduction in both contraction velocity and beat rate compared to NG or HG treatment (Additional file: Figure S3b, c).

Next, we evaluated excitation-contraction coupling in NMVMs by measuring impedance (IMP; surrogate for contractile frequency) and extracellular field potential

(EFP, surrogate for cell surface voltage) under exogenous electrical stimulation (14 volts) at 1Hz frequency (Fig. 2h, i). Under continuous pacing, there was no difference in the IMP baseline among the three groups (Additional file: Figure S3d). Compared to NG, HG treatment did not induce any changes in IMP amplitude (Fig. 2j) or beat rate (Fig. 2k), but a significant decrease in EFP (Fig. 2l). In the HG phase of the HLG group, we observed no differences in IMP amplitude and beat rate but a significant decrease in EFP (Fig. 2j, l). Upon hypoglycemic challenge (media exchange at 2-hour mark), we observed a significant decrease in IMP amplitude, beating frequency and EFP compared to NG and Controls (Fig. 2j, l). These results suggest that hypoglycemia challenge can cause the significant decrease in contraction amplitude and beat frequency of NMVMs with or without external electrical stimulation, as well as reduced cell surface voltage. Together, we demonstrate that our NMVMs system can recapitulated our *in vivo* and *ex vivo* findings which can be used for subsequent molecular characterization.

Hypoglycemia condition drive Cx43 redistribution

Cx43 has been shown to participate in synchronous calcium transient propagation in cardiomyocytes [32], and aberrant Cx43 expression and distribution has been speculated to participate in arrhythmia [32, 33]. Our previous study also showed that Cx43 expression was significantly decreased under HLG culture condition compared with NG and HG condition[28]. To further illustrate it, we used immunoelectron microscopy to examined Cx43 protein localization in Control, DCM and HDCM murine

cardiomyocytes (Fig. 3a). In Control cardiomyocytes, Cx43 localized to cell-cell junctions (Fig. 3a, white arrows). Interestingly, we observed mtCx43 aggregation in DCM and HDCM murine cardiomyocytes but not in Controls (Fig. 3a, yellow arrows). Compared to Controls, DCM and HDCM murine cardiomyocytes exhibited a significant decrease in number of Cx43 located at the cell-cell junction (Fig. 3b). There was a significant increase in Cx43 migratory distance away from cell membrane interface in HDCM cardiomyocytes compared to DCM and Control cardiomyocytes (Fig. 3c). Next, we quantified number of mtCx43 per mitochondrial cross-sectional area as a readout of mtCx43 density. We found although abnormal aggregation of mtCx43 could be observed in cardiomyocytes of both DCM and HDCM mice, mtCx43 density is significantly greater in HDCM cardiomyocytes compared to DCM cardiomyocytes (Fig. 3d). These data indicates that a certain amount of mtCx43 in DCM mice can maintain a compensatory balance, but too much mtCx43 in HDCM mice breaks this balance, resulting in a rapid decline in myocardial cell function.

To verify and quantify our immunoelectron microscopy findings, we performed immunofluorescence staining for N-cadherin (marking cell-cell junctions), translocase of outer mitochondrial membrane 20 (Tomm20; marking mitochondria), and Cx43 in NMVMs (Fig. 3e). Similar to our *in vivo* observations, Cx43 and N-cadherin co-localization in NMVMs decreased significantly post 4-hour HG treatment and was further exacerbated in the HLG group (Fig. 3f). A similar trend was observed for the mean Cx43 fluorescence intensity (Additional file: Figure S4a, b), while no change in

N-cadherin fluorescence intensity was observed (Additional file: Figure S4c). Notably, Cx43 and Tomm 20 co-localization significantly increased upon HLG treatment (Fig. 3g). Furthermore, we observed a loss of membrane Cx43 by immunoblotting of the hydrophobic fraction at 2-hour post-hypoglycemic challenge (Fig. 3h). To verify whether Cx43 dissociation and mitochondrial aggregation upon hypoglycemic challenge is conserved in humans, we performed a similar analysis on DM and Non-DM patient cardiac sections (Fig. 3i). Consistent with our results, we observed a significant decrease in Cx43 and N-cadherin co-localization (Fig. 3j), a significant increase in Cx43 and Tomm 20 co-localization (Fig. 3k), and a significant decrease in Cx43 mean fluorescence intensity (Fig. 3l) in DM patient cardiomyocytes compared to Non-DM patient cardiomyocytes. Together, these data demonstrate that upon hypoglycemic challenge, Cx43 proteins are lost from cardiomyocyte cell-cell junctions and aggregate in mitochondria.

Src mediated Cx43 phosphorylation drives Cx43 translocation to the mitochondria

To identify proteins responsible for Cx43 internalization and translocation to mitochondria upon hypoglycemia challenge, we performed co-immunoprecipitation coupled with mass spectrometry (Co-IP/MS) using antibody against Cx43 protein using NMVMs cultured under NG, HG and HLG conditions. Proteins mapped were used for downstream gene ontology enrichment analysis. Compared to those under NG and HG conditions, kinase activity, protein phosphorylation, cell-cell adhesion, and

cytoskeleton-associated proteins were significantly increased under HLG conditions (Fig. 4a). The percentage and propensity score matching coverage of mitochondria-associated proteins bound to Cx43 increased significantly upon HLG induction (Fig. 4b, c and Additional file: Figure S5a) These results are consistent with the increase in mtCx43 accumulation upon hypoglycemia induction (Fig. 4a-g) Furthermore, Src and Src-interacting proteins were enriched in our Cx43 Co-IP/MS analysis (Fig. 4d, e, Additional file: Figure S5b, c). In addition, immunofluorescence staining data showed that HLG culture could significantly increase the correlation coefficient between Src and Cx43 (Additional file: Figure S5d, e). To test if Cx43 translocation was mediated by Src, we treated HLG NMVMs with Saracatinib, a Src inhibitor (Fig. 4f). Saracatinib did not change the overall expression of Cx43 (Fig. 4g), which suggest that Saracatinib does not interfere with Cx43 turnover. Moreover, Saracatinib significantly increased Cx43 and N-cadherin co-localization (Fig. 4h) and decreased Cx43 and Tomm20 co-localization (Fig. 4i) in NMVMs cultured under HLG conditions. Lastly, Saracatinib restored Cx43 protein levels located at the membrane fraction (Fig. 4j).

Although we identified Src as the key player mediating Cx43 dissociation from cell membrane, but upstream activators remain elusive. In our Co-IP/MS, we identified MAPK1, MAP2K1, PI3K, and Akt, which belong to the MEK/ERK and PI3K/Akt pathways (Fig. 4a) as potential Src interacting partners (Fig. 5a). Compared to NG and HG group, HLG NMVMs exhibited ERK1/2 up-regulation (high p-ERK1/2 signal) and Akt down-regulation (low p-Akt signal) (Fig. 5b). To elucidate the regulatory role of

MEK/ERK1/2 and PI3K/Akt pathways in Src-mediated Cx43 translocation, we treated NMVMs cultured under HLG conditions with either MEK inhibitor (U0126), ERK activator (Ceramide C6), Akt inhibitor (Tricirbine) or Akt activator (Sc79) and measured Cx43-N-cadherin and Cx43-Tomm 20 co-localization. Inhibition of the MEK pathway (U0126) and activation of the Akt pathway (SC79) significantly reduced Cx43 dissociation from cell-cell junctions (Fig. 5c-e), decreased Cx43 mitochondrial aggregation (Fig. 5c, f, g), and increased Cx43 fluorescence intensity (Fig. 5c, h, i) in HLG NMVMs. Conversely, ERK activation (Ceramide C6) or Akt inhibition (Tricirbine) significantly increased Cx43 dissociation from cell-cell junctions (Fig. 5c-e), increased Cx43 mitochondrial aggregation (Fig. 5c, f, g), and decreased Cx43 fluorescence intensity (Fig. 5c, h, i) in HLG NMVMs. In isolated cell membrane fraction, we detected increased Cx43 protein levels when HLG NMVMs were treated with MEK inhibitor (U0126) or Akt activator (Sc79) while ERK activator (Ceramide C6) and Akt inhibitor (Tricirbine) treatments did the reverse (Fig. 5j, k). Together, these data suggest that HLG challenge activates MEK/ERK pathway and/or inhibits PI3K/Akt pathway to mediate Cx43 membrane dissociation and translocation to mitochondria.

Building on the HLG NMVMs model, MEK inhibitor (U0126), and Akt activator (Sc79), which blocked Cx43 dissociation, did not alter IMP baseline; however, ERK activator (Ceramide C6) and Akt inhibitor (Tricirbine) significantly lowered IMP baseline (Fig. 6a) prior to low glucose switch. Functionally, MEK inhibition (U0126) and Akt activation (Sc79) prevented loss of IMP Amplitude (Fig. 6b), beat rate (Fig. 6c), and

EFP (Fig. 6d) upon low glucose switch. Conversely, ERK activator (Ceramide C6) and Akt inhibitor (Tricirbine) failed to confer protection upon HLG challenge (Fig. 6b-d). Western blot data showed that inhibiting MEK/ERK pathway or activating PI3K/Akt pathway could inhibit HLG-induced Src activation (Additional file: Figure S5f). Similarly, live cell contractility assay faithfully recapitulated our Nanlon CE96 findings (Additional file: Figure S5g, h). Together, these data demonstrate that hypoglycemic challenge activates MEK/ERK and PI3K/Akt pathways that converge at Src to drive Cx43 mitochondrial translocation.

Overexpression of mitochondrial Cx43 results in worse cardiac dysfunction and risk of arrhythmia susceptibility

To determine whether mtCx43 is sufficient to increase the risk of cardiac dysfunction and susceptibility of arrhythmias, we constructed an AAV overexpression vector containing a mitochondrial localization sequence fused to either Cx43 or EGFP (Fig. 7a; Additional file: Figure S6a). First, we confirmed that overexpression of mtCx43 in NMVMs showed an increase in the Cx43 signal at mitochondria by immunofluorescence (Additional file: Figure S6b, yellow arrows). Functionally, overexpression of mtCx43 in NMVMs resulted in a significant increase in base IMP (Fig. 7b) and decrease in IMP (Fig. 7c) as well as beat rate (Fig. 7d), but no change in EFP (Fig. 7e) compared to untreated or mtEGFP Controls. We also observed a significant decrease in the contraction velocity and beat rate of mtCx43 overexpressing NMVMs under no pacing stimulation (Additional file: Figure S6c-e).

354

355   To further validate whether overexpression of mtCx43 can lead to cardiac
356 dysfunction, wild-type animals were injected with AAV2 viruses overexpressing
357 mtCx43, expression was confirmed by immunoelectron microscopy (Additional file:
358 Figure S6f). Cardiac functions were evaluated by echocardiography (Fig. 7f). In
359 mtCx43 overexpression animals, we did not observe any differences in EF, FS, or the
360 E/A ratio (Fig. 7g-i) compared to mtEGFP Controls; however, we observed a significant
361 decrease in the E/E' ratio in the mtCx43 overexpression group compared to that in the
362 mtGFP group in week 2 (Fig. 7j), suggestive of mild diastolic function. As expected,
363 mtCx43 overexpression induced QTc, QT interval, and JT interval prolongation (Fig.
364 7k, l, m, n), suggestive of ventricular arrhythmia susceptibility.

365

366   In summary, we demonstrated that hypoglycemic challenge causes diastolic
367 cardiac dysfunction and increases the risk of ventricular arrhythmia in an STZ-induced
368 T1D murine model. Cellularly and molecularly, we demonstrated that hypoglycemia
369 activates MEK/ERK and PI3K/AKT pathways that converge on Src protein, which
370 drives Cx43 dissociation and translocation to the mitochondria. Further, by
371 overexpressing mitochondrial localizing mtCx43, we demonstrated that mtCx43
372 aggregation can result in contractile dysfunction at the cellular level and causes cardiac
373 dysfunction and increases the risk of ventricular arrhythmia *in vivo*. Our results provide
374 novel molecular insights into the pathogenesis of hypoglycemia-aggravated diabetic
375 cardiomyopathy and identify novel targets for future therapeutic designs.

376

377 **Discussion**

378 We provide evidence for Src driven mtCx43 responsible for DCM defects in human
379 and murine cardiomyocytes. This is remarkable, as in absence of hypoglycemic
380 challenge, AAV2-mtCx43 overexpression was capable of inducing diastolic dysfunction
381 and arrhythmia susceptibility marked by prolonged QT intervals. In isolated adult and
382 neonatal mouse ventricular cardiomyocytes, we show glucose manipulation is a great
383 way to mimic hypoglycemia events and allow the precise characterization of Cx43
384 localization. Mechanistically, we demonstrate that MEK/ERK and PI3K/Akt pathways
385 are upstream of Src activation. Using pharmacological means, we showed that
386 MEK/ERK and PI3K/Akt modulation can enhance or prohibit Cx43 translocation.

387

388 Clinically, DCM patients treated with insulin exhibited left ventricular hypertrophy
389 and greater diastolic dysfunction compared to diabetic patients without insulin
390 prescription – possibly due to acute hypoglycemia induced by insulin therapy [34]. In
391 keep with previous finding [35], we find that acute hypoglycemic shock greatly
392 dampens myocardial function. The observed prolongation of QT interval and QRS
393 interval – events that lead to ventricular arrhythmias – have been highly correlated with
394 increased mortality during severe hypoglycemic events [36]. Similar to Cx43
395 remodeling that happens prior to cardiac remodeling in large animal model of
396 nonischemic heart failure [37], we too find that mtCx43 overexpression is sufficient for
397 inducing cardiac dysfunction in absence of cardiac fibrosis.

398

399 Cx43 is the most ubiquitously expressed gap junction protein found in almost all
400 tissue types. Cx43 expression levels have been implicated in mitochondrial
401 homeostasis [38], autophagy regulation [39], intracellular trafficking [40], and long-
402 distance communication mediated *via* extracellular vesicles (EVs) [41]. In accordance
403 to our previous observation [28], we find that hypoglycemia activates Src via MEK/ERK
404 and PI3K/Akt pathways which results in mtCx43 accumulation. Interestingly, Src has
405 been implicated in Cx43 dysregulation in the heart [42-45] but most studies attributed
406 cardiac dysfunction to loss of cell-cell junction. Here we demonstrate that in presence
407 of intact cell-cell junctions and absence of hypoglycemia, overexpression of mtCx43
408 induced diastolic dysfunction and increase the risk of arrhythmia susceptibility. Apart
409 from being used to treat cancer [46], Src inhibitor Saracatinib (AZD0530) has been
410 shown to confer ~50% improvement in cardiac conduction velocity and lowered overall
411 arrhythmia episodes by stabilizing membrane Cx43 [45]. Based on our results, we
412 would argue that the efficacy of Saracatinib is less likely due to the increase of
413 membrane Cx43 but rather the prevention of mtCx43 accumulation.

414

415 Our mass spectrometry data show that Cx43 binds to IMM [38], yet the role and
416 function of mtCx43 remains controversial [47-51]. The observed large Cx43 nanogold
417 aggregates are suggestive to the formation of mtCx43 hemichannels. MtCx43
418 hemichannels could potentially act as large conductance channels that dissipate
419 mitochondrial membrane potential and disrupt ATP synthesis [38]; in open states, Cx43

hemichannels could elicit myocardial death [48]. In addition to metabolic homeostasis, mitochondria participate in calcium handling and impaired mitochondria Ca^{2+} handling plays a key role in the development of the cardiac diastolic dysfunction characteristic of early diabetic cardiomyopathy [2]. Although the percentage of mtCx43 hemichannels in DM patients remains to be determined, disruption of mitochondrial membrane potential and induction of aberrant calcium handling aligns with DCM progression – a notion which our *in vivo* and *in vitro* mtCx43 overexpression results support.

In summary, our study provides mechanistic insights by which mitochondrial Cx43 translocation aggravates DCM. Based on our findings, we boldly speculate that mtCx43 accumulation may be compensatory adaptation of DCM, yet acute hypoglycemic challenges may lead to Cx43 hemichannel opening and disrupt cardiac electrophysiology. Restoration of membrane Cx43 and prevention of mtCx43 accumulation offers a new therapeutic possibility for the prevention of sudden cardiac death and DCM progression.

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Availability data

All data are available from the corresponding author on reasonable request.

Supplementary material

Details of materials and methods are provided in SI Materials and Methods.

Ethics approval and consent to participate

This study was approved by Ethics Review Committee at Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, China (SH9H-2020-TK238-1).

Author's contributions

X. Wei, A. C. Y. Chang and Q. Y. Zhang conceived the study; X. Wei carried out the experiments and the data analysis; M. Lei, A. C. Y. Chang and Q. Y. Zhang contributed to the design of the experiments, supervised analysis and interpretation; A. C. H. Chang conducted experiments and modified the language of the article; H. S. Chang,

466 Y. K. Liang contributed to sample preparation and immunoelectron microscopy; Y. L.
467 Xue and Y. X. Zhang helped in data analysis of immunofluorescence staining images
468 and immunoelectron microscopy images. X. Wei wrote the manuscript in consultation
469 with A. C. Y. Chang and Q. Y. Zhang who supervised the project. All authors discussed
470 the results and contributed to the final manuscript.

471

472 **Consent for publication**

473 Not applicable.

474

475 **Conflict of interest**

476 The authors declare no competing interests.

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Fig. 1 HDCM mice display worsen diastolic dysfunction and increased cardiac arrhythmia susceptibility.

a. Schematic diagram of mice modeling. Mice were divided into three groups: Control (n = 10 mice), DCM (n = 10 mice), HDCM (n = 10 mice). DCM and HDCM mice were injected with streptozotocin (STZ) (150 mg/kg), whereas Control mice were injected with sodium citrate buffer *via* intraperitoneally injection (ip). On week 10, HDCM mice were injected with insulin (100 IU) to induce severe hypoglycemia then sacrificed. b. M-model and tissue doppler flow in Control, DCM and HDCM mice on week 10. c. Echocardiography analysis of ejection fraction (EF), fractional shortening (FS), E/A and E/E' ratios reveals that DCM (n = 20 mice) display diastolic dysfunction at both week 2 and 8 post STZ injection compared to Control (n = 10 mice). d. Presence of systolic and diastolic dysfunction of 10-week HDCM (n = 10 mice) compared to DCM (n = 10 mice) evaluated by echocardiography. e. Representative surface electrocardiographs (ECGs) of Control (black, n = 5 mice), DCM (gray, n = 8 mice), and HDCM (red, n = 8 mice). f. Summary of QTc, QRS interval, and QRS amplitude. Data are shown as mean $\pm$ SEM. One-way ANOVA test was used. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Parentheses indicate nonsignificant P values.

Fig. 2 Contractility and electrophysiology disorder in LG-cultured cardiomyocytes from DCM mice and HLG-treated NMVMs.

a. Cardiomyocyte isolation schematic diagram. b. Average sarcomere length of Control and DCM mice cardiomyocytes transient in different medium condition under 1 Hz, 14 V stimulation. c-f. Calculated parameters: sarcomere shortening (c), peak height (d), time to peak 90 (e), and time to baseline 90 (f) of cardiomyocytes from Control (n = 4 mice) and DCM (n = 4 mice, each mice had 10 myocardial cells measurements) cultured in NG, HG and LG media. g. Isolation and culture of neonatal mouse ventricular myocyte (NMVMs) diagram. h-i. Impedance and electric field potential of NMVMs after 4 hours of pacing under different media condition. j-l. Analysis of impedance amplitude (j, n = 15 wells for NG, n = 30 wells for HG, n = 29 wells for HLG), beat rate (k, n = 15 wells for NG, n = 30 wells for HG, n = 32 wells for HLG) and surface voltage (l, n = 8 wells per condition) changes after 4 hours treatment. Data are shown as mean $\pm$ SEM. One-way ANOVA test was used. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Parentheses indicate nonsignificant P values.

Fig. 3 Displacement and degradation of Cx43 caused by hypoglycemia.

a. Immunoelectron microscopy images of Cx43 in myocardial tissue of Control, DCM and HDCM (green arrow: cell-cell interactions; scale bars, 1 nm; white arrow: Cx43 at cell-cell interactions; scale bars, 500 nm; yellow arrow: aberrant Cx43 in mitochondria; scale bars, 500 nm, n = 4 mice per group). b. Number of Cx43 along gap junction normalized to gap junction distance. c. Average distance of Cx43 to gap junction. d. Number of aggregated mtCx43 clumps averaged to mitochondria surface area (n = 100 mitochondria per group). e. Immunofluorescence staining of Cx43, N-cadherin and Tomm 20 in NMVMs (yellow arrow: aberrant Cx43 in mitochondria; scale bars, 60 μ m). f-g. Analysis of colocalization of Cx43 and N-cadherin (f, n = 12 field views per

condition), Cx43 and Tomm 20 (g, n = 7 field views per condition). Data are shown as mean $\pm$ SEM. One-way ANOVA test was used. * P <0.05, ** P <0.01, **** P <0.0001. h. Western blot of Cx43 in hydrophilic cell membrane of NMVMs after hypoglycemia change. i. Immunofluorescence staining of Cx43, N-cadherin and Tomm 20 in myocardial tissue of patients with or without DM (yellow arrow: aberrant Cx43 in mitochondria, n = 3 patients per group; scale bars, 60 μ m). j-l. Analysis of colocalization of Cx43 and N-cadherin (j, n = 12 field views per group), Cx43 and Tomm 20 (k, n = 9 field views per group) and mean intensity of Cx43 (l, n = 12 field views per condition). Data are shown as mean $\pm$ SEM. Student two-tailed t-test was used. ** P <0.01, *** P <0.001, **** P <0.0001. Parentheses indicate nonsignificant P values.

Fig. 4 Activation of Src protein regulates the entry of Cx43 into mitochondria. a. Gene ontology pathway analysis of activated genes under HLG treatment. b-c. Coverage and propensity score matching (PSM) analysis of mitochondria relative proteins (n = 162 proteins per condition). Data are shown as mean $\pm$ SEM. One-way ANOVA test was used. **** P <0.0001. d-e. Coverage and PSMs analysis of Src interacting proteins (n = 131 proteins per condition). Data are shown as mean $\pm$ SEM. One-way ANOVA test was used. ** P <0.01, *** P <0.0001. f. Colocalization of Cx43 and Tomm 20 in HLG and Saracatinib-treated HLG NMVMs (Scale bars, 60 μ m). g-i. Analysis of mean intensity of Cx43 (g, n = 12 per condition), Cx43 and N-cadherin (h, n = 12 per condition), colocalization of Cx43 and Tomm 20 (i, n = 7 per condition). Data are shown as mean $\pm$ SEM. Student two-tailed t-test was used. * P <0.05, **** P <0.0001. j. Western blot of Cx43 in hydrophilic cell membrane of HLG and Saracatinib-treated HLG NMVMs. Parentheses indicate nonsignificant P values.

Fig. 5 MEK/ERK and PI3K/Akt pathways regulate Cx43 transfer into mitochondria through Src. a. Schematic diagram of proposed abnormal Cx43 aggregation in mitochondria mechanism. b. Activation of MEK/ERK and inhibition of PI3K/Akt pathways under HLG condition. c. Immunofluorescence staining of Cx43 of HLG group under drug stimulation (Scale bars, 60 μ m). d-i. Analysis of colocalization of Cx43 and N-cadherin (n = 12 field views per condition), Cx43 and Tomm 20 (n = 7 field views per condition), and mean intensity of Cx43 (n = 12 field views per condition). j-k. Western blot of Cx43 in hydrophilic cell membrane of HLG with activators or inhibitors cultured NMVMs. Data are shown as mean $\pm$ SEM. One-way ANOVA test was used. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001.

Fig. 6 Electrophysiology function of NMVMs after treatment with activators or inhibitors of MEK/ERK and PI3K/Akt pathways. a-d. Analysis of base impedance (a, n = 8 wells per condition), amplitude (b, n = 8 wells per condition), beat rate (c, n = 7 wells per condition) and voltage changes (d, n = 8 wells per condition) in drug stimulated HLG NMVMs. Data are shown as mean $\pm$ SEM. One-way ANOVA test was used. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001.

Fig. 7 Mitochondrial Cx43 overexpression causes aberrant cardiac contraction, electrophysiological abnormalities, and increased fatal arrhythmias susceptibility. a. Recombinant p-AAV vectors of mito-GFP-FLAG and mito-Cx43-FLAG. b-e. Base impedance (b, n = 12 wells per group), amplitude (c, n = 12 wells for Untreated group, n = 12 wells for mtEGFP group, n = 8 wells for mtCx43 group), beat rate (d, n = 12 wells for Untreated group, n = 12 wells for mtEGFP group, n = 8 wells for mtCx43 group) and voltage changes (e, n = 5 wells for Untreated group, n = 11 wells for mtEGFP group, n = 11 wells for mtCx43 group) of Untreated (black), mtEGFP (shaded teal) and mtCx43 overexpression (solid teal) under electrostimulation. Data are shown as mean $\pm$ SEM. One-way ANOVA test was used. * P <0.05, ** P <0.01, *** P <0.001. f. M-model and tissue doppler flow in mtGFP and mtCx43 overexpression mice on week 2. g-j. Echocardiography analysis of EF (g), FS (h), E/A (i), and E/E' (j) (n = 4 mice for mtEGFP group, n = 7 mice for mtCx43 overexpression group). Data are shown as mean $\pm$ SEM. Student two-tailed t-test was used. * P <0.05. k. Representative surface electrocardiographs (ECGs) of mtGFP (shaded teal) and mtCx43 overexpression (solid teal). l-n. Summary of QTc (l), QRS interval (m) and QRS amplitude (n) of mtGFP and mtCx43 overexpression (n = 7 mice per group). Data are shown as mean $\pm$ SEM. Student two-tailed t-test was used. *** P <0.001, **** P <0.0001.

Fig. 1

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Fig. 5

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