Fructose diet treatment in mice induces fundamental disturbance of cardiomyocyte Ca\(^{2+}\) handling and myofilament responsiveness

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Mellor KM, Wendt IR, Ritchie RH, Delbridge LM. Fructose diet treatment in mice induces fundamental disturbance of cardiomyocyte Ca\(^{2+}\) handling and myofilament responsiveness. Am J Physiol Heart Circ Physiol 302: H964–H972, 2012. First published December 23, 2011; doi:10.1152/ajpheart.00797.2011.—High fructose intake has been linked to insulin resistance and cardiac pathology. Dietary fructose-induced myocardial signaling and morphological alterations have been described, but whether cardiomyocyte function is influenced by chronic high fructose intake is yet to be elucidated. The goal of this study was to evaluate the cardiomyocyte excitation-contraction coupling effects of high dietary fructose and determine the capacity for murine cardiomyocyte fructose transport. Male C57Bl/6J mice were fed a high fructose diet for 12 wk. Fructose- and control-fed mouse cardiomyocytes were isolated and loaded with the fura 2 Ca\(^{2+}\) fluorescent dye for analysis of twitch and Ca\(^{2+}\) transient characteristics (4 Hz stimulation, 37°C, 2 mM Ca\(^{2+}\)). Myocardial Ca\(^{2+}\)-handling protein expression was determined by Western blot. Gene expression of the fructose-specific transporter, GLUT5, in adult mouse cardiomyocytes was detected by real-time and conventional RT-PCR techniques. Diastolic Ca\(^{2+}\) and Ca\(^{2+}\) transient amplitude were decreased in isolated cardiomyocytes from fructose-fed mice relative to control (16 and 42%, respectively), coincident with an increase in the time constant of Ca\(^{2+}\) transient decay (24%). Dietary fructose increased the myofilament response to Ca\(^{2+}\) (as evidenced by a left shift in the shortening-Ca\(^{2+}\) phase loop). Protein expression of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a), phosphorylated (P) phospholamban (Ser16), and P-phospholamban (Thr17) was reduced, and protein phosphatase 2A expression increased, in fructose-fed mouse hearts. Hypertension and cardiac hypertrophy were not evident. These findings demonstrate that fructose-diet-associated myocardial insulin resistance induces profound disturbance of cardiomyocyte Ca\(^{2+}\) handling and responsiveness in the absence of altered systemic loading conditions.

excitation-contraction coupling; cardiac; myocardial; cellular

IN WESTERN SOCIETIES, the use of added sweeteners has increased by 25% during the last 30 years, and it has been estimated that the average adolescent (North American) consumes over 30% of energy intake in the form of added sugars (17, 29). Fructose intake has been identified as a significant risk factor for insulin resistance and cardiovascular disease, independent of body mass index (45). Experimental models have demonstrated that a high fructose diet disturbs metabolic homeostasis, increases plasma fructose, and induces systemic insulin resistance in rodents (2, 24, 32, 35). The existence of an insulin-resistant cardiomyopathy, associated with perturbed cardiac metabolism and functional impairment, is generally recognized (33, 46, 48). In a previous study, we have demonstrated that excess fructose intake is associated with elevated blood glucose, glucose intolerance, and normal plasma insulin in the absence of hypertension and obesity (35). We have also shown that fructose feeding induces diffuse myocardial fibrotic infiltration linked with suppressed cardiomyocyte viability signaling (35). The impact of elevated dietary fructose intake on cardiomyocyte functional performance is yet to be demonstrated.

Cellular fructose uptake is mediated by insulin-independent transporters. Fructose is rapidly phosphorylated by fructokinase and bypasses the glycolytic rate-limiting enzyme, phosphofructokinase, proceeding through glycolysis to produce pyruvate and lactate in a less-controlled manner than glucose (30). The close association of glycolytic enzymes with Ca\(^{2+}\), related transporters provides indication that specific excitation-contraction coupling processes are particularly reliant on glycolytically produced ATP (38, 50). There is some evidence that high fructose intake suppresses cardiac output in vivo (9), but cardiomyocyte functional effects of dietary fructose have not been directly assessed. The combined insults of myocardial insulin resistance-induced metabolic alterations and direct fructose-induced glycolytic dysregulation may have specific consequences for cardiomyocyte function. Rodent models of insulin-resistant cardiomyopathy (e.g., db/db mice, ob/ob mice, high-fat-fed rodents) exhibit cardiomyocyte excitation-contraction coupling disturbances coincident with hypertension and/or obesity (5). The load-independent myocardial functional effects of insulin resistance are not yet delineated.

We have recently demonstrated that fructose feeding in mice increases myocardial reactive oxygen species (ROS) production and downregulates the insulin-responsive phosphoinsitide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, independent of hypertension and obesity (31, 35). This signaling dysregulation was linked with myocardial structural pathophysiology. Other studies have previously identified a role for Akt in cardiomyocyte Ca\(^{2+}\) regulation, specifically phosphorylating key Ca\(^{2+}\)-handling proteins, the L-type Ca\(^{2+}\) channel (8, 27) and phospholamban [PLB; the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a) regulator] (7). Activity of Ca\(^{2+}\) flux proteins is tightly regulated by both kinase and phosphatase activities, and there is some evidence that dephosphorylation of PLB and ryanodine receptors (14) plays a role in diabetic cardiomyopathy (39). No study to date has sought a link between increased fructose intake and cardiomyocyte Ca\(^{2+}\) dysregulation in the insulin-resistant myocardium.

Given the regulatory associations between insulin signaling, glycolysis, ROS, and cardiomyocyte excitation-contraction coupling, we hypothesized that fructose dietary intervention would directly modulate cardiomyocyte Ca\(^{2+}\) handling and contractile performance, in the absence of systemic loading disturbance. To examine this contention, mice were subjected...
to a high fructose diet intervention for 12 wk. Isolated cardiac myocytes were loaded with a Ca\(^{2+}\)-sensitive fluorophore for functional assessment. Expression and signaling studies of key transporters involved in Ca\(^{2+}\) flux regulation were undertaken. This investigation provides the first demonstration that a relatively short-term elevation of fructose intake in mice induces profound disturbance of cardiomyocyte Ca\(^{2+}\) handling and Ca\(^{2+}\) responsiveness.

MATERIALS AND METHODS

Dietary treatments and in vivo measurements. Male C57Bl/6J mice were housed in temperature-controlled conditions in a 12:12-h 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 Australian 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. At the commencement of the dietary treatment period, mice aged 4–5 wk were allocated to either a control (54% starch, 10% sucrose, 7% total fat, and 19.4% protein) diet, as previously detailed (31). The diets were based on the American Institute of Nutrition standard rodent growth diet (40), were of equal digestible energy, and were micro- and macronutrient matched (AIN93G, SF03–018; Specialty Feeds, WA, Australia). Measurement of blood glucose was performed following a 6-h fast in the final week of dietary intervention. A small blood sample was obtained by tail prick, and glucose was determined by a blood glucometer (ACCU-CHEK Advantage; Roche, Mannheim, Germany). We have previously demonstrated that 12 wk of 60% fructose feeding induces significant glucose intolerance (~14% increase in area under the glucose tolerance curve), with maintenance of normal blood pressure (35).

Tissue recovery. At the completion of the 12-wk feeding period (age 16 wk), all mice were fasted for 3–4 h, heparinized (100 IU ip), and anesthetized by sodium pentobarbitone (100 mg/kg ip). Hearts were excised and weighed for measurement of cardiac weight indexes and anesthetized by sodium pentobarbitone (100 mg/kg ip). Hearts were excised and weighed for measurement of cardiac weight indexes (cardiac weights normalized to tibia length). Hearts were utilized for myocyte isolation or assigned to molecular analysis (Westerns, real-time RT-PCR).

Cardiomyocyte isolation. Ventricular cardiomyocytes were isolated from fructose- and control-fed mice (18). Following heart excision, the aorta was cannulated, and the heart was retrogradely perfused with Ca\(^{2+}\)-free HEPES-buffered Krebs (in mM: 150 NaCl, 5 KCl, 0.33 NaH\(_{2}\)PO\(_{4}\), 1 MgCl\(_{2}\), 25 HEPES, 20 d-glucose, 3 sodium pyruvate, and 1 sodium lactate, pH 7.4) at 2 ml/min at 37°C for 10 min. Addition of Type II Collagenase (0.66 mg/ml, 295 U/mg; Worthington Biochemicals), CaCl\(_{2}\) (50 μM), and trypsin (33 μg/ml; Sigma-Aldrich) to the perfusate facilitated enzyme digestion. The heart was then removed from the cannula, atria were dissected away, and the ventricles were gently teased apart. Cells were dispersed in a high-potassium HEPES-buffered Krebs solution (in mM: 30 KCl, 90 KOH, 30 KH\(_{2}\)PO\(_{4}\), 3 MgSO\(_{4}\), 50 glutamate, 20 taurine, 0.5 EGTA, 10 d-glucose, and 10 HEPES, pH 7.4) and resuspended in Ca\(^{2+}\)-free HEPES-buffered Krebs with trypsin inhibitor (25 μg/ml; Sigma-Aldrich).

Cardiomyocyte Ca\(^{2+}\) handling and twitch analysis. Cardiomyocytes were loaded with the Ca\(^{2+}\)-fluorescent dye fura 2-AM (1 μM, 20-min incubation at 25°C; Invitrogen). The fura 2 loading conditions provided an optimal signal-to-noise ratio without compromising myocyte inotropic state and responsiveness. Myocytes were superfused with 2 mM Ca\(^{2+}\)-HEPES buffer (in mM: 146.2 NaCl, 4.69 KCl, 11 d-glucose, 0.35 NaH\(_{2}\)PO\(_{4}\), 1.05 MgSO\(_{4}\), 7.5 H\(_{2}\)O, and 10 HEPES) and stimulated to establish steady-state contractile performance at 4 Hz (>5 min, 37°C). At these near-physiological conditions, myocyte contractions were stable, and no evidence of spontaneous Ca\(^{2+}\) release was observed. Myocyte Ca\(^{2+}\) signals were measured by microfluorimetry (360:380 nm fluorescence ratio, 1,000 Hz sampling; IonOptix) (41). The indexes used to describe the Ca\(^{2+}\) transient were amplitude [ratio of fluorescence at 360 to 380 nm (F\(_{360}/F_{380}\)), diastolic Ca\(^{2+}\) (F\(_{500}/F_{380}\)), systolic Ca\(^{2+}\), time constant of decay (tau, ms), and the area of the Ca\(^{2+}\) transient. All fluorescent signals were corrected for background. Cardiomyocyte twitch properties were assessed by video-based edge-detection (IonOptix) and analyzed for peak shortening normalized to diastolic cell length, maximum rate of shortening normalized to diastolic cell length (%MRS), maximum rate of lengthening normalized to diastolic cell length (%MLR), and twitch cycle duration (time from stimulus voltage to 90% return to resting cell length). All indexes were analyzed off-line using IonWizard (IonOptix) and were determined after averaging 10 steady-state transients for each myocyte. Representative Ca\(^{2+}\)-shortening phase-loop plots were constructed as described (3, 44).

Western blot analysis of myocardial protein expression. Frozen cardiac tissue was homogenized in 100 mM Tris-HCl, 5 mM EGTA, 5 mM EDTA (Sigma-Aldrich), protease inhibitors, and phosphatase inhibitors (Roche, Basel, Switzerland). Sample protein concentration was determined by a modified Lowry assay (37). Protein expression was determined by SDS-PAGE and Western blotting. Equal protein loading was confirmed by staining the polyvinylidene difluoride membranes with Ponceau-S (Sigma-Aldrich). Membranes were incubated with primary antibody overnight at 4°C. Primary antibodies were sourced as follows: SERCA2a (no. A010-20) and phospho-(Thr\(^{17}\))-PLB (no. A010-13) (Badrilla, Leeds, UK); phospho(Ser\(^{16}\))-PLB (no. 07-052) and PLB (no. 05-205) (Upstate); phospho-calmodulin kinase (CaMK) II (no. ab32678) (Abcam, Cambridge, UK); β-tubulin, protein phosphatase 2A (PP2A)-A subunit, and PP2A-C subunit (Cell Signaling Technology). Membranes were incubated for 1 h with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK). The ECL Plus (Amersham; GE Healthcare) chemiluminescent signal was imaged and analyzed using Quantity One software (Bio-Rad).

RT-PCR. RNA was extracted from frozen cardiac tissues using the TRIzol reagent in conjunction with the PureLink Micro-to-Midi Total RNA Purification kit (Invitrogen) as per the manufacturer’s instructions and was reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time RT-PCR was used to determine the relative gene expression levels of β-myosin heavy chain (β-MHC), α-myosin light chain (α-MHC), sodium/hydrogen exchanger (NHE), solute carrier family 2 facilitated glucose/fructose transporter member 5 (Slc2a5, GLUT5), and the housekeeper gene 18S in ventricular tissue. Real-time RT-PCR primer sequences for each gene were as follows: β-MHC: 5′-TTCTCCTGTCTTATTCTAAGTGA-3′ and 5′-GATCTCCTCTGTAGGGCTCTC-3′; α-MHC: 5′-GCCCAAGCTCTCCAGAGTCT-3′ and 5′-GCTTAACTAATCTTCTCC-3′; NHE: 5′-AGCTGAGAGAGAAGAGATCCAACA-3′ and 5′-AGGGTCTGATGCTTATGCA-3′; GLUT5 (Slc2a5): 5′-CCCAATTTGGAACAGTAGGTCG-3′ and 5′-GCGGCAAGTGAAGGCTCTATT-3′; and 18S: 5′-TCGAGGCGGCTTGAATTGGA-3′ and 5′-CCTCCTCAATGATCTCCCT-3′. The comparative ΔΔCt method was used to analyze the genes of interest as described (26, 31). To confirm the presence of the fructose-specific transporter, GLUT5 (Slc2a5), in adult mouse isolated cardiomyocytes and heart homogenate, conventional RT-PCR was performed using Platinum Tag Polymerase (Invitrogen) with two consecutive runs of 30 PCR cycles to allow visualization of low-expression genes. Conventional RT-PCR primer sequences, designed to obtain an amplicon size of 492 bp, were as follows: GLUT5, 5′-CCAATATGGTGCAACAGCTGCTG-3′ and 5′-AAGGGGACGGCCTCCTCCTTT-3′. Statistical analyses. Data are expressed as means ± SE and were analyzed with Student’s unpaired t-test. A P value <0.05 was considered statistically significant. Graphs were created, and statistical analyses were performed using GraphPad Prism version 5.02 (Graph-
Table 1. Cardiac and somatic characteristics of control- and fructose-fed mice

<table>
<thead>
<tr>
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<th>Control</th>
<th>Fructose</th>
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<tr>
<td>Blood glucose, mM</td>
<td>7.26 ± 0.58</td>
<td>8.68 ± 0.26*</td>
</tr>
<tr>
<td>Heart wt, mg</td>
<td>149.0 ± 6.9</td>
<td>153.1 ± 3.3</td>
</tr>
<tr>
<td>Ventricle wt, mg</td>
<td>132.4 ± 6.7</td>
<td>135.6 ± 3.2</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>26.0 ± 0.4</td>
<td>22.7 ± 0.7*</td>
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<tr>
<td>Tibia length, mm</td>
<td>17.8 ± 0.2</td>
<td>17.4 ± 0.1</td>
</tr>
<tr>
<td>Heart wt/tibia length, mg/mm</td>
<td>8.39 ± 0.41</td>
<td>8.78 ± 0.17</td>
</tr>
<tr>
<td>Ventricle wt/tibia length, mg/mm</td>
<td>7.46 ± 0.40</td>
<td>7.78 ± 0.17</td>
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Data presented as means ± SE; n = 6–8 mice in each group. *P < 0.05.

RESULTS

Morphological characteristics of fructose-fed mice. At the completion of the 12-wk fructose-feeding intervention, blood glucose concentrations were increased by 20% in fructose-fed mice relative to control. No significant differences were observed in total heart mass or ventricular mass between treatment groups (Table 1). Because fructose-fed animals showed a small relative reduction in body weight gain during the treatment period, cardiac tissue measures were normalized to tibial length, which was similar for both groups. Cardiac weight index and ventricular weight index were not altered by fructose feeding. These findings are consistent with our previous report that cardiac hypertrophy is not evident at the whole heart or cellular level (35) and confirm the absence of myocardial tissue hypertrophy with an identical mouse fructose feeding regime.

Fig. 1. Ca2+ handling parameters from isolated cardiomyocytes of control- and fructose-fed mice. A: representative traces of myocyte Ca2+ transients. Diastolic Ca2+ levels have been normalized to demonstrate Ca2+ transient amplitude differences. F360:380, ratio of fluorescence at 360 to 380 nm. B: Ca2+ transient amplitude. C: diastolic Ca2+ levels. D: peak (systolic) Ca2+ levels. E: time constant of Ca2+ transient decay (tau). F: area under the Ca2+ transient trace. Data are presented as means ± SE, 19–24 cells/group. *P < 0.05.
Increased myofilament Ca\textsuperscript{2+} responsiveness in fructose-fed mouse cardiomyocytes. Evidence of altered Ca\textsuperscript{2+} responsiveness in cardiomyocytes of fructose-fed mice was sought. Examination of individual myocyte shortening-Ca\textsuperscript{2+} relationships was undertaken using “phase-loop” plots (44). These plots map myocyte Ca\textsuperscript{2+} levels against cell length (normalized to diastolic cell length) throughout the contraction and relaxation phases of the activation cycle. During the relaxation phase, the descending portion of the “loop” provides a dynamic index of myofilament Ca\textsuperscript{2+} sensitivity (3, 44). Exemplar myocyte Ca\textsuperscript{2+}-shortening phase-loop plots, presented in Fig. 3A, show a left shift in the relaxation phase in the fructose group compared with control, indicative of increased myofilament responsiveness to Ca\textsuperscript{2+} (44). The phase-loop shift was quantified by assessment of intracellular Ca\textsuperscript{2+} at 50% myocyte relaxation. Fructose-fed mouse cardiomyocytes exhibited a 22% decrease in Ca\textsuperscript{2+} levels at 50% myocyte relaxation relative to controls (Fig. 3B). These data are consistent with increased myofilament Ca\textsuperscript{2+} responsiveness.

Myofilament shortening response to Ca\textsuperscript{2+} is substantially determined by myosin isoform type (α- vs. β-MHC) and by intracellular pH. An increase in the relative expression of α- to β-MHC isoform is associated with increased myofilament Ca\textsuperscript{2+} sensitivity (36). The mRNA expression levels of MHC isoforms were compared in fructose- and control-fed mouse hearts. A marked and significant increase in the α-MHC-to-β-MHC mRNA ratio (Fig. 3C) was detected in fructose-fed mouse myocardium (2.8-fold increase), which would indicate increased myosin responsiveness to Ca\textsuperscript{2+}.

Fig. 2. Contractile parameters from isolated cardiomyocytes of control- and fructose-fed mice. A: representative traces of cardiomyocyte shortening profiles of control- and fructose-fed mice. B: resting (diastolic) myocyte length (L\textsubscript{d}). C: %extent of shortening normalized to cell length (L\textsubscript{0}). D: %maximum rate of shortening (MRS) normalized to cell length (L\textsubscript{0}). E: %maximum rate of lengthening (MRL) normalized to cell length (L\textsubscript{0}). F: duration of contractile cycle. Data are presented as means ± SE, 19–24 cells/group. *P < 0.05.

Fig. 3. Regulation of myofilament Ca\textsuperscript{2+} responsiveness by myosin heavy chain (MHC) isoform expression. A: representative Ca\textsuperscript{2+}-shortening phase loops from control- and fructose-fed mouse cardiomyocytes. Broken arrow indicates progression of contractile cycle. Arrow on top indicates left shift in phase loop in fructose-fed mouse cardiomyocytes. B: intracellular Ca\textsuperscript{2+} level at 50% myocyte relaxation (n = 17–24 cells). C: ratio of α-MHC/β-MHC mRNA expression in ventricular tissue from control- and fructose-fed mice (n = 7–9 hearts). D: mRNA expression of Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE-1) in ventricular tissue from control- and fructose-fed mice (n = 7–9 hearts). Data are presented as means ± SE. *P < 0.05.
Intracellular pH modulates myofilament Ca\textsuperscript{2+} sensitivity (10), and altered proton efflux through upregulation of NHE-1 has been previously observed in association with myocardial insulin resistance (13). The effect of fructose feeding on NHE-1 expression was evaluated, and no difference between treatment groups in relative transcript levels for this transporter was detected (Fig. 3D). These findings suggest that myofilament MHC isoform, but not NHE-1-mediated proton flux, plays a role in increasing the Ca\textsuperscript{2+} responsiveness of cardiomyocytes of fructose-fed mice.

Myocardial Ca\textsuperscript{2+}-handling proteins are downregulated in fructose-fed mice. To evaluate the basis for the fructose-induced alterations in cardiomyocyte Ca\textsuperscript{2+} transients, additional molecular analyses were undertaken. Ca\textsuperscript{2+} removal from the cytosol during relaxation is primarily mediated by SERCA2a in cardiomyocytes. As depicted in Fig. 4A, myocardial protein expression of SERCA2a was significantly decreased in fructose-fed mice, relative to control. Phosphorylation of the SERCA2 regulator, PLB at Ser\textsuperscript{16} or Thr\textsuperscript{17} residues [by protein kinase A (PKA) or CaMKII, respectively], relieves SERCA2 inhibition. Fructose-fed mice exhibited a significant reduction in the ratio of phosphorylated (Ser\textsuperscript{16}) to total PLB. The ratio of phosphorylated (Thr\textsuperscript{17}) to total PLB was also significantly decreased (Fig. 4C), and this was associated with a nonsignificant reduction in the level of phosphorylated CaMKII (Thr\textsuperscript{286}) (P = 0.09, Fig. 4D). No effect of fructose on total PLB protein content was observed (fructose: 0.98 ± 0.16-fold change control, P > 0.05). PP2A dephosphorylates PLB at both Ser\textsuperscript{16} and Thr\textsuperscript{17} phosphorylation sites (28). PP2A subunits A (structural) and C (catalytic) were upregulated in fructose-fed mouse hearts (Fig. 5, A and B). These findings indicate that the myocardial protein density and activity of SERCA2a was downregulated in response to high fructose intake, consistent with the observation that the removal of Ca\textsuperscript{2+} from the cytosol is delayed (increased tau, Fig. 1E).

GLUT5 fructose transporter is expressed in cardiomyocytes. To evaluate cardiomyocyte capacity for fructose uptake, expression of GLUT5 (Slc2a5) in adult mouse cardiomyocytes was determined. Real-time RT-PCR analysis demonstrated GLUT5 expression in mouse cardiomyocytes. Initial fluorescence for amplified GLUT5 PCR product was ~18–20 cycles for mouse cardiomyocytes and 22–24 cycles for mouse heart homogenate (Fig. 6A), indicating that cardiomyocytes are the primary cell type expressing GLUT5 in cardiac tissue. Initial fluorescence for GLUT5 in mouse small intestine tissue was detected at ~12–14 cycles (Fig. 6A). Reverse transcription “negative” and “no template control” samples did not amplify in PCR reactions. The melt curve, generated by progressive temperature increase to 95°C post-PCR amplification, revealed a single peak for GLUT5 samples, indicating high PCR product purity (data not shown). Figure 6B presents the conventional RT-PCR gel image of the GLUT5 PCR product band at 492 bp in mouse cardiomyocyte samples. Mouse small intestine represents a positive control. No evidence of the 492-bp product was observed in the negative control. Given previous reports of substrate regulation of GLUT5 in noncardiac tissues (11), the gene expression of GLUT5 in response to a 12-wk high fructose diet was examined. Real-time RT-PCR demonstrated that GLUT5 gene expression was similar in fructose- and control-fed mouse hearts (Fig. 6C).

**DISCUSSION**

This study provides the first evidence that high fructose intake profoundly alters cardiomyocyte Ca\textsuperscript{2+} handling and...
Ca\textsuperscript{2+} responsiveness. Ventricular myocytes isolated from mice subjected to a limited-duration 12-wk fructose feeding intervention exhibited markedly reduced Ca\textsuperscript{2+} transient amplitude and lower diastolic Ca\textsuperscript{2+}. Examining the underlying mechanisms involved, we have shown that suppressed Ca\textsuperscript{2+} transient amplitude is linked with a reduced level of SERCA2a. Decreased phosphorylation of the pump regulator PLB at both PKA and CaMKII target sites was demonstrated, combined with increased dephosphorylation capability of phosphatase PP2A, which targets PLB. Paradoxically, these Ca\textsuperscript{2+} perturbations were observed in the absence of discernible alterations in the myocyte twitch characteristics. By quantifying the dynamic relationship between myocyte Ca\textsuperscript{2+} levels and shortening, it was possible to identify a compensatory increase of Ca\textsuperscript{2+} responsiveness as the likely outcome of an α- to β-MHC isoform expression shift. Drawing on our previous work with this model, we have strong evidence to make the case that these molecular mechanisms can be attributed to increased myocardial ROS production and Akt signaling downregulation occurring as a consequence of myocardial insulin resistance.

The compelling finding is that, despite marked reduction in cardiomyocyte systolic Ca\textsuperscript{2+} availability induced by fructose feeding, a major adaptive increase in Ca\textsuperscript{2+} responsiveness allows myocytes to preserve peak shortening, at least for this relatively short duration of dietary treatment. With ongoing dietary exposure (or with additional coincident morbidity factors, i.e., hypertension), this adaptive capacity would be expected to reach a limit, precipitating cardiac functional demise. The biological and potential clinical importance of these findings is that significant underlying cellular excitation-contraction coupling disturbance may occur before cardiac functional impact is observable in vivo (or ex vivo) with high fructose intake. The capacity for direct fructose involvement in potentially mediating some of these actions was identified in our study by demonstration of fructose-specific transporter GLUT5 (Slc2a5) expression in murine cardiomyocytes.

Establishing cardiac- and cardiomyocyte-direct actions of high fructose intake. In other rodent type 2 diabetic models, cardiomyopathy has been observed, generally coincident with pressure or volume loading from hypertension or obesity (5). With our model, we are positioned to evaluate cardiac functional effects without confounding load influence. Our evidence indicates that high dietary fructose intake is primarily exerting a direct effect on the myocardium and cardiomyocytes (at least in the early stages of the cardiopathy). The marked myocardial structural and Ca\textsuperscript{2+}-handling disturbances are observed in the context of rather moderate systemic perturbation, i.e., no hyperinsulinemia and no hemodynamic loading. The finding that cardiomyocytes express the GLUT5 fructose transporter supports the contention that myocyte fructose entry may have a role in directly modifying metabolic signaling pathways and fueling excitation-contraction coupling. We have previously reported that insulin resistance is evidenced in this model at a myocardial level by downregulation of the insulin-responsive PI3K/Akt pathway (35). The cardiac-specific signaling alterations identified with this dietary intervention may potentially contribute to excitation-contraction coupling modulation, and we now also identify the possibility of cardiac impact contingent on tissue fructose uptake. Further work is required to fully dissect the direct and indirect myocardial effects of dietary fructose.

Murine cardiomyocytes have fructose transport capacity. The present study is the first to demonstrate the presence of the fructose-specific transporter GLUT5 in murine cardiomyocytes. We have recently demonstrated that isolated rat cardiomyocytes have the capacity to utilize exogenously supplied fructose (34). In vitro fructose supplementation is able to abrogate the functional deficits induced by cardiomyocyte treatment with the glucose metabolic inhibitor 2-deoxyglucose. Together these studies indicate the presence of an operational cardiomyocyte fructose GLUT5 transporter.

Plasma fructose concentrations are elevated in response to high dietary fructose intake (2, 25), and increased fructose availability has been shown to induce GLUT5 expression in gut epithelial cell types (11). In contrast, we found no evidence of fructose feeding-induced cardiac GLUT5 expression upregulation in cardiac tissue. It is probable that, with elevated extracellular fructose levels, GLUT5 activity is increased, and this possibility requires investigation. Our previous work identified elevated myocardial ROS production associated with fructose feeding, consistent with dysregulated phosphofructokinase-bypass fructose metabolism (31, 35). These findings provide the impetus for further work to characterize the role of cardiac fructose uptake and metabolism in dietary fructose-induced cardiac pathology.

Fig. 6. GLUT5 gene expression in cardiomyocytes. A: real-time PCR fluorescence depicting GLUT5 gene expression relative to 18S in adult mouse cardiomyocytes, heart homogenate, and small intestine (positive control). B: DNA gel image from conventional RT-PCR of GLUT5 in adult mouse cardiomyocytes and heart homogenate samples. GLUT5 primers were designed to obtain a 492-bp PCR product. Small intestine (“int”) tissue was used as a positive control. Negative control (“neg”) was obtained by RNA that was not reverse transcribed to cDNA. C: GLUT5 gene expression in ventricular tissue from control- and fructose-fed mice. Data are presented as means ± SE, n = 6.
Ca$^{2+}$-handling abnormalities in myocytes of fructose-fed mice. There has been no previous evaluation of the impact of a high fructose diet on cardiomyocyte functional status. Our investigation reveals a marked disturbance of Ca$^{2+}$ handling, apparent after a limited-duration feeding intervention of 12 wk. Ca$^{2+}$ reuptake into the sarcoplasmic reticulum (SR) was impaired, as evidenced by prolonged decay of cytosolic Ca$^{2+}$ during the relaxation phase. Ca$^{2+}$ decay during relaxation is primarily mediated by the ATP-dependent SERCA2 pump, regulated by PLB. Reduced SERCA2a protein expression coincident with increased inhibition (due to suppressed PLB phosphorylation at both Ser$^{16}$ and Thr$^{17}$ sites) was demonstrated in fructose-fed mouse hearts and would be expected to induce Ca$^{2+}$ transient prolongation. The observed increase in PP2A protein expression (the structural and catalytic subunits) is also consistent with decreased phosphorylation of PLB, and further work utilizing a coimmunoprecipitation approach is warranted. CaMKII targets the PLB Thr$^{17}$ site, and a recent work utilizing a coimmunoprecipitation approach is also consistent with decreased phosphorylation of PLB, and would minimize diastolic Ca$^{2+}$ levels. Decreased Ca$^{2+}$ influx via the L-type Ca$^{2+}$ channel is probable, as has been recently demonstrated in myocytes from a different model of insulin resistance (13), but this has not been measured in the present study. Reduced diastolic Ca$^{2+}$ levels could be partially ascribed to lower SR diastolic Ca$^{2+}$ leak, and it is also feasible that fructose may facilitate subcellular local glycolytic support of Na$^{+}$-K$^{+}$-ATPase-coupled Na$^{+}$/Ca$^{2+}$ exchange, which would minimize diastolic Ca$^{2+}$ levels (38, 47). Measurement of SR load level, and electrophysiological interrogation of Ca$^{2+}$ fluxes modified by high fructose exposure, is now required.

Molecular mechanisms for maintaining cardiomyocyte contractile performance despite Ca$^{2+}$-handling abnormalities. Cardiac functional effect of dietary fructose has been evaluated previously using in vivo echocardiography in rodents. Inconsistent findings have been reported, both lack of functional effect [8-wk treatment (10, 43)] and performance decrement [2-wk treatment (9)]. These discrepancies may reflect differential levels of coincident hypertension and fibrotic pathology and the extent to which myocyte compensatory responses allow for maintenance of cardiac function, even when there is progressive underlying abnormality of excitation-contraction coupling. In vitro fructose manipulation in a cell culture setting has demonstrated an effect of 24-h fructose exposure on cardiomyocyte function (41). No previous study has investigated the effect of in vivo chronic high fructose on cardiomyocytes isolated from fructose-fed animals. In this study, a significant alteration in Ca$^{2+}$ transients was observed without coincident effect on cardiomyocyte twitch function. These findings contrast with previous reports of cardiomyocyte contractile dysfunction in sucrose-fed mice (12, 49) and may reflect the different systemic and cardiovascular phenotype induced by sucrose and fructose diets. High sucrose diets have been reported to induce hyperinsulinemia and hypertension with normal glucose levels (12, 15, 49), whereas the fructose-fed mouse model employed in the present study exhibits normal insulin levels, hyperglycemia, and no change in blood pressure (35).

Given the major reduction in myocyte Ca$^{2+}$ transient amplitude induced by high fructose intake, the observed lack of effect on myocyte contractile performance was conspicuous. Based on the shift in the Ca$^{2+}$-shortening phase-loop plot, our findings indicate that increased myocyte Ca$^{2+}$ responsiveness compensates for Ca$^{2+}$ signal reduction to maintain contractile function. At a molecular level, at least part of the underlying mechanism involves an increase in the α-MHC-to-β-MHC expression ratio. The α-MHC isoform is associated with higher ATPase activity, increased cross-bridge kinetic, and enhanced Ca$^{2+}$ sensitivity (3, 16, 36). An expression shift to increase the relative level of α-MHC seems paradoxical in a setting of tissue insulin resistance, potentially limiting glucose supply. It is generally considered that a shift to increased β-MHC expression is an “economical” adaptation, given the ATP requirements of the two isoforms. However, data from transgenic animals have shown that moderate α-MHC isoform expression can be beneficial in stress states, suggesting that a relative increase in the α-MHC-to-β-MHC expression ratio may represent a strategic outcome that compensates for the higher energy utilization of this isoform (19). In the present study, cardiomyocyte contractility was maintained in fructose-fed mice despite dramatic reductions in Ca$^{2+}$ transient amplitude. It is well established that the α-MHC-to-β-MHC expression ratio is linearly correlated with power output in isolated myocytes and intact hearts (1, 23). Thus the observed increase in α-MHC/β-MHC expression may allow for contractility maintenance by offsetting the reduction in activator Ca$^{2+}$ available to the myofilaments. In this study, the MHC isoform shift was characterized at the mRNA level. This approach has been previously validated in a rodent model of hypertensive cardiopathy (43). A more detailed dissection of the contribution of MHC isoform switch to myofilament Ca$^{2+}$ responsiveness, including analysis of MHC protein content and activity, is now warranted.

Elevated myocardial PP2A levels have also been shown to mediate an increase in myofilament Ca$^{2+}$ sensitivity. Dephosphorylation of cardiac troponin I by PP2A relieves inhibition of myosin ATPase activity and slows Ca$^{2+}$ dissociation from troponin C (21, 42). Fructose-induced upregulation of PP2A subunits A and C in the present study may play a role in maintaining cardiomyocyte contractile performance despite marked suppression of Ca$^{2+}$ cycling. PP2A activation may involve p21-activated kinase 1 regulated by Ras-related small G proteins (21, 22), but this possibility has not been explored in this study. NHE-mediated modulation of myofilament Ca$^{2+}$ sensitivity (20) might also be involved and was investigated, since this transporter has been shown to be upregulated in rodents with genetically induced insulin resistance (13). No change in NHE-1 expression was detected. This finding does not preclude a role for pH modulation, since even without NHE1 expression change there may be altered Na$^{+}$/H$^{+}$ exchange activity. Expression and/or activity shifts in other pH regulatory transporters could be considered.

The low Ca$^{2+}$ “operational state,” an adaptive phenotype vulnerable to challenge? The capacity of myocytes from fructose-fed mice to maintain contractile performance in the con-
text of significant reduction in operational (systolic and diastolic) Ca$^{2+}$ levels is striking. Because net contractility is maintained (equivalent twitch amplitude and maximum rate of shortening), the increase in Ca$^{2+}$ responsiveness appears to offset the decreased Ca$^{2+}$ availability. This finding is suggestive of a subcellular compartmentation process that may selectively direct energy intermediates to support myofilament ATPase activity in preference to SERCA2 activity. In the insulin-resistant state, while glucose uptake would be expected to be diminished, a partially augmented fructose supply may be targeted to service myofilament energy demand. Elevated ROS levels in conjunction with increased Ca$^{2+}$ levels are known promoters of programmed cell death (4, 6); thus, it could be speculated that this low Ca$^{2+}$ adaptive strategy protects myocyte viability in a high-stress state. We have previously shown in this model of fructose feeding that autophagy induction is upregulated (35) and identified this mode of cell death as a potential mechanism responsible for myocyte attrition and fibrosis infiltration. The longevity and robustness of such an adaptive strategy may be limited. As a relatively short-term contingency, the low Ca$^{2+}$ operational state may be effective, but over the longer term and when additional loading or an inotropic challenge presents, contractile reserve may be inadequate. Indeed, a previously reported finding that fructose-fed mice exhibit more significant deterioration of cardiac function in response to transthoracic aortic constriction is consistent with this proposition (10). The adaptive strategy of increasing responsiveness to Ca$^{2+}$ may be an effective short-term expediency with long-term detriment.

In conclusion, this investigation provides the first evidence that high dietary fructose induces profound disturbance of cardiomycyte Ca$^{2+}$ handling and responsiveness. We provide the first evidence that the fructose-specific GLUT5 transporter is expressed in murine cardiomyocytes. The findings from this study provide important mechanistic insight into the pathogenesis of insulin-resistant cardiomypathy and suggest that myocyte Ca$^{2+}$-handling abnormalities occur at an early disease stage, with recruitment of compensatory molecular shifts to preserve contractile function. The progression of this early adaptive short-term response to longer-term functional maladaptation requires further investigation, and extended longitudinal studies to map the time course of transition beyond adaptation/compensation are necessary. The potential clinical importance of these findings is that significant underlying cellular excitation-contraction coupling disturbance may occur before cardiac functional impact is observable in vivo in the insulin-resistant state associated with high fructose intake. This work identifies substrate for development of treatment strategies directed to molecular targets for effective intervention before disease progression to advanced diabetic cardiomyopathy.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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