Activation of SIRT1, a class III histone deacetylase, contributes to fructose feeding mediated induction of the α-myosin heavy chain expression.

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Abstract

Fructose feeding has been shown to induce the cardiac α-MHC (myosin heavy chain) expression, and protect the heart from ischemia-reperfusion mediated cell-injury. This study was designed to investigate the mechanism involved in the effect of this sugar on MHC gene expression and cardiac protection. Adult mice were fed with a PTU (6-propyl-2-thiouracil) diet or PTU combined with a fructose-rich diet. PTU treatment made animals hypothyroid and that resulted into total replacement of cardiac α-MHC with the β-MHC isoform. Addition of fructose into PTU diet led to re-expression of α-MHC isoform to a significant level. Similar induction of α-MHC expression was also seen when PTU diet was combined with resveratrol, an agonist of SIRT1 deacetylase. Analysis of heart lysate of these animals indicated that fructose feeding augmented the NAD/NADH ratio and the cardiac SIRT1 levels, thus suggesting a role of SIRT1 in fructose-mediated activation of α-MHC isoform. To analyze a direct effect of SIRT1 on MHC isoform expression, we generated transgenic mice expressing SIRT1 in the heart. Treating these transgenic mice with PTU diet did not let disappearance of α-MHC, as it did in the non transgenic animals. SIRT1 over expression also activated the α-MHC gene promoter in transient transfection assays, thus confirming a role of SIRT1 in the induction of α-MHC expression. Fructose feeding also attenuated the MHC isoform shift and blocked the cardiac hypertrophy response associated with pressure overload, which was again associated with the induction of cardiac SIRT1 levels. These results demonstrate that fructose feeding protects the heart by induction of the SIRT1 deacetylase and highlight its role in the induction of α-MHC gene expression.
Introduction

In response to sustained hemodynamic overload the heart initially compensates with an adaptive hypertrophic growth of the myocardium. Under prolonged stress conditions the heart eventually develops irreversible changes which results in ventricular dilation and heart failure. At the cellular level stress-induced cardiac hypertrophy is associated with increase of myocyte cell-size and activation of fetal genes encoding proteins involved in contractility, Ca$^{2+}$ transport and cell energetics (reviewed in [16]).

One of the major change occurring in myofilaments of hypertrophied hearts is the shift in myosin isoform from $\alpha$- to $\beta$-MHC, which is considered a critical determinant of the diminished myofibrillar ATPase activity as well as the reduced contractile activity of the failing heart [28]. The myosin molecule of a mammalian heart is composed of two MHCs, $\alpha$- and $\beta$- (220 kDa each) and four light chains. The two MHC isoforms are regulated developmentally, as well as in response to various physiologic and pathophysiologic stimuli. The $\alpha$-MHC with high ATPase activity accounts for faster shortening velocity of cardiac myofibers, while the $\beta$-MHC having low ATPase activity leads to greater economy of force generation. In rodents, $\beta$-MHC isoform is the major isoform in the fetal heart, whereas the $\alpha$-MHC predominates in the adult heart [19]. Unlike rodents, in the adult human heart nearly 10% MHC expressed is in the $\alpha$-isoform, and this is reduced to non-detectable levels in failing hearts [30]. Previous studies conducted with transgenic mice and rabbits have indicated that $\alpha$-MHC hearts are at advantage in stress conditions, as opposed to hearts expressing mostly $\beta$-MHC isoform [23, 25]. These studies have indicated that induction of $\alpha$-MHC expression in the failing heart may be beneficial in terms of increasing the myocardial contractility of a hemodynamically challenged heart.

The expression of MHC genes is regulated both by genetic and epigenetic factors. Thyroid hormone which up regulates $\alpha$-MHC and down regulates $\beta$-MHC expression is the most potent stimuli [31]. A great deal of evidence has also demonstrated that changes in the metabolic status of the heart influence myosin isoform expression. In general, decreased activity of the glycolytic pathway with a corresponding increased energy production by free-fatty acid oxidation, as occurs in cases of hypertrophy and diabetes, favors the synthesis of $\beta$-MHC expression [19]. Feeding diabetic animals with a high-fructose diet or free-fatty acid transport inhibitor, which results in increased rate of glycolytic flux has been shown to favor the synthesis of the $\alpha$-MHC isoform [11, 13]. Fructose feeding has been also shown to induce $\alpha$-MHC expression in hypothyroid and
hypophysectomized rats, where β-MHC is the predominant isoform [14, 41]. Additional reports have shown that the hearts of fructose fed rats are protected against ischemia-reperfusion injury [24]. However, the mechanism by which fructose-feeding mediates induction of α-MHC expression and cardiac protection is virtually unknown.

SIRT1 is a member of class-III group of histone deacetylases (HDACs), collectively called sirtuins (SIRTs). They are homologous to the yeast Sir2 gene, which has been implicated in chromatin silencing, cell-survival and aging [32]. Unlike the class-I and II HDACs, the deacetylase activity of SIRTs is dependent upon the NAD/NADH ratio of the cell [21]. SIRT family (SIRTs) members are considered nuclear sensors of redox-signaling. In stress conditions a change in metabolic state of the cell (NAD/NADH ratio) alters the deacetylase activity of SIRTs, and that has been shown to influence the cell fate [18]. Increased cellular NAD content elevates the deacetylase activity of SIRTs, whereas high NADH and nicotinamide levels act as inhibitors of this family [5]. Recent studies have documented a role of SIRTs in genetic control of aging (reviewed in [29]). In yeast, the life span of the organism was shown to be shortened by a null mutation in the Sir2 gene, and it was extended by the presence of an extra copy. Likewise, over-expression of SIRT orthologues increased life span (>50%) of other species including, C. elegans and Drosophila, indicating a role of SIRT family members in controlling longevity of the organism [45]. In the brain, over activation of SIRT1 has been shown to prevent axonopathy and neuronal degeneration [3]. We have recently demonstrated that the SIRT1 protein levels are decreased in end-stage failing hearts, and the over expression of SIRT1 protects cardiomyocytes from oxidative-stress mediated cell damage [38].

In this study we examined the participation of SIRT1 deacetylase in cardio-protective effects of fructose feeding. We found that fructose feeding elevates SIRT1 deacetylase levels in the heart. Fructose feeding mediated induction of α-MHC expression, and this effect could be reproduced by over-expression of SIRT1. We also demonstrate that the fructose feeding prevents cardiac hypertrophy in response to pressure overload by activating SIRT1. These results demonstrate for the first time that the SIRT1 deacetylase activity has the potential to regulate myosin isoform expression, and that this effect could be achieved by a simple manipulation of the diet.
Methods

Animal Care: Male mice (20-30 g) of CD1 strain were treated and housed in accordance with the Animal Care and Use Committee guidelines of the University of Chicago. Animal diets of different compositions were purchased from Harlan Teklad, Madison, WI. Regular diet contained (g/kg) corn (774.5g), wheat gluten (194g), L-Lysine (1.5g), calcium carbonate (10g), sodium chloride (10g) and vitamin mix (Teklad-40060) (10g). The iodine-deficient diet contained 0.15% polythiouracil (PTU). Fructose and resveratrol diets contained 40% and 0.067% of these compounds, respectively. All these compounds were added in the diet by replacing same amount of corn. Nutrient information of different diets is given in table 1. Animals fed with this PTU diet have been reported previously to develop typical signs of hypothyroidism without loss of body weight [35, 40]. The circulating T3 (37.78 ng/dl) and T4 (1.246 μg/dl) levels were significantly lower in PTU treated mice than in euthyroid mice (T3 55.7ng/dl; T4 3.78 μg/dl), and they remained unchanged by including fructose or resveratrol in the diet. Plasma insulin (60.5μU/ml) and glucose (100-150mg/dl) levels also remained within control values in mice fed with fructose or resveratrol-rich diet. At the end of experiment animals were anesthetized by intra-peritoneal injection of pentobarbital sodium (200 mg/kg), and hearts were rapidly excised, quickly frozen and stored at -80°C until further analysis. Body and heart weights were obtained at the time of sacrifice.

Aortic banding: The aortic banding was carried out in adult mice to produce pressure over-load hypertrophy as previously described [39]. Adult male CD-1 mice weighing ~30 grams were anesthetized with ketamine (60mg/kg, IP) and xylazine (10 mg/kg, IP) and ventilated with a small rodent ventilator (CWE, Inc., Ardmore, PA). The chest was opened through by performing a mini-sternotomy and the aorta was identified between the innominate and left common carotid arteries and dissected free from surrounding fatty tissue. A 4-0 ticron suture was tied around the aorta over a 27 gauge needle between the origins of the innominate and left common carotid arteries. The needle was subsequently removed. Adequacy of aortic constriction was demonstrated visually at this point by the difference between the bounding pulsations observed in the right common carotid artery (which arises from the innominate artery) and the near absence of visible pulsation in the left common carotid artery. Animals with sham surgery underwent identical procedure with exception of band placement. At the time of sacrifice the presence of band in the
aorta was visually verified and only those animals with intact band in place were included in further study. Animals were sacrificed at different time periods post surgery, their hearts removed and analyzed for the development of cardiac hypertrophy. In 12 wks banded mice, heart weight to tibia length ratio increased by 60-70%, compared to controls, and they had clear signs of ventricular dilation.

**MHC isoform separation:** Cardiac α- and β-MHC isoforms were electrophoretically separated using the method described by Rundell et al [40]. Briefly, heart tissue was pulverized in liquid nitrogen and 50-100 mg of tissue powder was extracted with the buffer containing 50 mM Tris.HCl, pH 6.8, 2.5% SDS, 10% glycerol, 1 mM DTT (dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride) and mammalian protease inhibitors (Sigma). Samples were processed by passing through four cycles of 5 min boiling followed by 30 minutes sonication under water at 4°C. After centrifugation, cleared supernatants were estimated for protein concentration by BSA reagent (Pierce). Twenty microgram of protein samples were resolved by 6.5% SDS-polyacrylamide gel electrophoresis in Laemmli® buffer [26]. The upper tank buffer contained 6.23 mM 2-mercaptoethanol. Gels were electrophoresed at 4°C, initially for 20 min with 25 mA current per gel followed by 45 mA till 150 kDa molecular weight protein marker band reached almost 1 cm above bottom of the gel. On the completion of electrophoresis, gels were removed, fixed in 12.5% TCA for 10 min and stained overnight with 0.03% Coomassie blue according to manufacturer's instructions (RPI). Gels were destained in 5% acetic acid and 7.5% methanol solution and scanned with a Bio-Rad GS 710 flatbed scanner and analyzed with Kodak 1D software.

**Western analysis:** Heart tissue was powdered in liquid nitrogen and homogenate prepared in RIPA buffer (1x PBS, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 10mg/ml PMSF, 100mM sodium orthovanadate, and protease inhibitors). Protein concentration was measured using BioRad protein assay reagent. Typically, 75-100µg of protein sample (unless otherwise specified) was heated at 95°C for 5-10 minutes and resolved by 8% SDS-polyacrylamide gel electrophoresis. Western blot analysis was performed according to the standard procedures. The primary antibodies used for the analysis were as follows: rabbit anti-SIRT1 antibody from Upstate (07-131), ANF (Peninsula, T4-14) and actin (SantaCruz, sc1616). Primary antibodies were typically used at 1:1000 dilution and secondary (HRP-conjugated) anti-rabbit or anti-mouse antibodies were used at 1:3000 dilution.
RNA analysis: Total RNA was extracted from heart using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Northern blot analysis was performed as described earlier with oligonucleotide probe complementary to the unique 3'-untranslated sequences of the mouse α-MHC mRNA and GAPDH cDNA probe [39].

Cell culture and transfection: Primary cultures of cardiac myocytes were prepared from 2 day old neonatal rat hearts as described previously [38]. Myocytes were initially grown in Dulbecco’s Modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum and 5 mg/ml each of penicillin and streptomycin (Invitrogen) for 48 hours. Subsequently, cells were maintained in serum-free medium. For promoter/reporter gene analysis, 48h after plating, typically 1x10⁵ myocytes per well were co-transfected with plasmids using lipofectamine reagent (Invitrogen). After 48 hours of transfection cells were harvested, cell lysates prepared and assayed for luciferase, β-galactosidase activities and protein content. The following plasmids were used for transfection analysis: α-MHC/luciferase reporter plasmid containing -612bp of rat α-MHC gene promoter, β-MHC/luciferase reporter plasmid containing -450bps of rat β-MHC gene promoter, and the expression plasmids encoding wild-type SIRT1 or H355A SIRT1 mutant.

Generation of α-MHC-SIRT1 transgenic mouse: The SIRT1 cDNA was released from pcDNASIRT1 plasmid with SalI digestion and cloned into the blunted-SalI site of a pBluescript II KS(+) plasmid containing the α-MHC promoter (a generous gift from Dr. J. Robbins, The Children’s Hospital and Research Foundation, Cincinnati, OH). The orientation of transgenic construct was confirmed by sequencing. Constructs were linearized and PBS backbone was digested out of the plasmid. The purified DNA construct was injected into the pronuclear stage zygotes of CD1 mouse strain according to the standard transgenic procedure of University of Chicago transgenic facility. At 2-3 wk of age, tail DNA was analyzed to confirm transgenic mice. The SIRT1 (+/-) knockout (KO) mice (CD1 strain) were kindly provided by Dr. M. McBurney, University of Ottawa, Canada. As the fertility rate of SIRT (+/-) KO mice was very low, both sexes of these mice were utilized in the study.

Cell size analysis: The heart sections were fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton-X100 and then stained with wheat germ agglutinin (WGA-100µg/ml) coupled to tetramethylrhodamine isothiocyanate (TRITC) (Sigma) for 30 minutes. The sections were washed three times with PBS and mounted. The average cell size of
myocytes was calculated after measuring area of 100 cells at 10 different places from each section. Images were captured using a Zeiss Axioscope microscope equipped with 40x acroplan objective lens with an Axiocam color camera and the ocular power of 10x. The area of the images captured was measured using the ImageJ program (http://rsb.info.nih.gov).

**Detection of fibrosis:** Mouse heart sections were washed in PBS and fixed in 3.7% formaldehyde in PBS for 15 minutes. After washing with PBS, sections were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, blocked with 5% goat serum and/or 1 h at room temperature, and then incubated with the anti-vimentin (V6630- Sigma) or anti-myomesin (sc-50435, SantaCruz) antibodies. After several washes sections were incubated with secondary antibodies, anti-mouse IgG Marina Blue (M-10991-Invitrogen), or anti rabbit-IgG.FITC (sc 2090). Subsequently, sections were extensively washed and mounted using Vectashield mounting medium containing propidium iodide (H-1300). The blue stained area depicting fibrosis in the heart section was measured using the ImageJ program (http://rsb.info.nih.gov).

**Estimation of NAD and NADH levels:** The NAD levels were measured according to the method described by Jacobson and Jacobson [22] with slight modification. Fifty milligram of frozen crushed tissue was suspended in 200µL of 0.5M perchloric acid. The heart extract was neutralized with equal volume of 1M KOH and 0.33M KH₂PO₄/K₂HPO₄ (pH7.5), centrifuged to collect the supernatant and to remove the KClO₄ precipitate. The supernatant (50µl) (or NAD standard) was added to 200µL of NAD reaction mixture (600mM ethanol, 0.5mM 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide [MTT], 2mM phenazine ethosulfate, 5mM EDTA, 1mg/mL BSA, 120mM bicine at pH 7.8) and incubated for 5min at 37°C. The reaction was initiated by adding 25µl of alcohol dehydrogenase (0.5mg/ml in 100mM bicine, pH 7.8) and incubating for 20min at 37°C. It was stopped by adding 250µl of 12mM iodoacetate. The O.D. of the reaction mix was read at 570nm wavelength. The NAD content was measured from the standard curve and normalized to the protein content of the sample. NADH levels were measured using NAD/NADH quantification kit from Biovision (K337, Bio vision research products, Mountain View CA).
**Scanning densitometry and statistical analysis:** Autoradiograms were scanned using Scion Image for Windows analysis software, based on NIH image for Macintosh by Wayne Rasband (National Institute of Health, Bethesda, MD). Signal intensity was adjusted for background density of the blot. Data are presented as mean±SE. Statistical difference among groups was determined using either Student t-test, for two groups, or one way ANOVA, for more than two groups.
Results

Fructose feeding induces α-MHC expression: The effect of fructose-feeding on MHC isoform expression was determined by feeding adult mice for 6 wks with a PTU diet or PTU diet combined with fructose (40%). Physical parameters of mice fed with different diets are shown in table 2. There was no change in heart weight, body weight or heart weight/body weight ratio between different groups of animals, indicating that diets utilized in this study had no harmful effect on the heart or body weight of the animal, consistent with previous reports [36]. Analysis of MHC isoforms showed that adult mouse heart expressed mostly α-MHC isoform, while neonatal mouse heart expressed both α- and β-MHC isoforms, as expected. In PTU-treated hypothyroid animals the α-MHC isoform was completely replaced by the β-MHC isoform (Fig.1A & 1C). However, in animals, fed with the PTU diet containing fructose, a substantial amount of α-MHC isoform persisted. These data are in agreement with many previous reports where fructose-feeding had been found to augment the levels of α-MHC isoform [12, 11]. To determine the mechanism behind the fructose-mediated induction of α-MHC, we carried out micro-array analysis of the heart mRNA obtained from the two groups. We found that the transcript level of SIRT1 deacetylase was notably elevated (~3 fold), in fructose-fed mice, compared to animals fed with the PTU diet (data not shown). These results were confirmed by the western-blot analysis (Fig. 1B). To investigate whether the induction of SIRT1 deacetylase was sufficient to elevate the α-MHC expression, we fed mice with a PTU diet modified to contain resveratrol (0.067%), a potent inducer of SIRT1. As shown in figure 2A, we found induction of α-MHC expression in resveratrol fed mice as well, which correlated with the increased levels of SIRT1 (110 KDa) in these hearts (Fig. 2B). These results suggested that SIRT1 deacetylase may have a role in the expression of cardiac myosin heavy chain isoforms.

SIRT1 induction contributes to fructose- as well as resveratrol-mediated α-MHC induction: To determine whether SIRT1 was indeed involved in fructose-mediated induction of MHC expression, we fed mice with a PTU diet containing both resveratrol (0.067%) and fructose (40%) for 6 wks. Analysis of MHC isoform of these hearts indicated that the level of α-MHC induction was the same, as it was seen with diets containing either fructose or resveratrol (Fig 2A & 2D). Since no additive effect of fructose and resveratrol was observed, it indicated that both compounds are likely to work through a common
mechanism, that is, induction of SIRT1 deacetylase. To support this finding we analyzed the response of SIRT1 knockout mice to fructose feeding. SIRT1 null (-/-) mice do not survive beyond few days of birth and have been shown to possess cardiac phenotype [8]. The heterozygous SIRT1 (+/-) KO mice survive and mature normally, though, they are less fertile compared to wild type. There is no notable difference in heart weight to body weight ratio between SIRT1 (+/-) KO and wild type mice. We therefore selected to examine the effect of fructose feeding in SIRT1 (+/-) KO mice. As shown in figure 2C, feeding of SIRT1 (+/-) KO mice with PTU diet led to total replacement of α-MHC with β-MHC isoform. When these mice were fed with PTU diet containing fructose, no change in MHC profile was observed, compared to PTU alone treated animals. These results strongly indicated that SIRT1 induction plays a role in fructose-mediated activation of the α-MHC expression.

SIRT1 over expressing transgenic mice are less responsive to PTU mediated reduction of α-MHC levels: To further substantiate a role of SIRT1 in MHC isoform shift, we generated two transgenic mouse lines which express different amounts of SIRT1 under the control of a cardiac-specific promoter. Transgenic line one (SIRT1.tg1) and two (SIRT1.tg2) have 10 and 2.5 fold increase in cardiac specific expression of SIRT1, above the endogenous deacetylase levels, respectively. Both transgenic lines were put on PTU diet for six weeks, then the animals were sacrificed and the heart lysate analyzed for the MHC isoform expression. We found that, while in control non-transgenic mice PTU diet resulted in complete replacement of α-MHC, SIRT1 transgenic animals still expressed a significant amount of α-MHC isoform after 6 wks of PTU treatment (Fig. 3A). The level of α-MHC isoform correlated with the amount of SIRT1 expressed in the hearts. In the SIRT1.tg1 line with 10 fold increase in SIRT1, almost 3 fold more α-MHC expression was detected compared to SIRT1.tg2 line which had a 2 fold increase in expression of the deacetylase (Fig 3A & 3B). We also analyzed MHC mRNA levels in these transgenic mouse hearts. As shown in figure 3C, α-MHC mRNA levels were reduced to nearly non-detectable levels in the PTU fed non-transgenic mice, whereas, a significant amount of α-MHC transcripts was detected in PTU fed SIRT1 transgenic mice. These results indicated that SIRT1 expression elevates the steady state level of the α-MHC mRNA transcripts.

SIRT1 deacetylase activates the α-MHC gene promoter: HDACs are known to regulate gene expression both at transcriptional and post-transcriptional levels. To examine whether the observed effect of SIRT1 on α-MHC expression was due to change in the rate
of MHC gene transcription, we analyzed the effect of the SIRT1 on both the α- and β-MHC gene promoters. Primary cultures of cardiomyocytes were transfected with the α-MHC promoter/reporter construct together with the expression vectors encoding either the wild-type or the mutant SIRT1 deacetylase. We found that over expression of the wild type SIRT1 increased the α-MHC promoter activity in a concentration dependent manner, but not the mutant deacetylase (Fig 3D). We, however, found no direct effect of SIRT1 over expression on the β-MHC promoter (Fig 3E). These results, thus, demonstrated that the SIRT1 deacetylase regulates the MHC isoforms switch in favor of α-MHC expression by altering the rate of gene transcription.

Fructose feeding attenuates the cardiac hypertrophy response secondary to pressure overload: Induction of the α-MHC isoform has been shown to be associated with physiological hypertrophy, and the agents capable of inducing α-MHC have been shown to block the development of pathological hypertrophy [19]. We therefore asked whether a fructose diet capable of inducing the α-MHC isoform could suppress the cardiac hypertrophy response to pressure overload. Animals were fed with the fructose diet for 4 weeks and then subjected to aortic banding. These animals were maintained on the fructose diet for next 12 weeks and subsequently they were sacrificed and different hypertrophy marker analyzed. Fructose feeding had no noticeable effect on the body weight of the animal compared to standard diet. In these experiments we observed that, as compared to controls, animals fed with fructose-rich diet had significantly higher rate of survivability over the 12 weeks period of aortic banding (data not shown). As expected, control animals receiving regular diet produced a large amount of cardiac hypertrophy (60-70%) following 12 weeks of banding. However, this hypertrophy response to pressure overload was markedly attenuated in fructose fed mice, which produced merely 15% of hypertrophy after 12 wks of banding (Fig 4A & 4B).

To confirm the anti-hypertrophy effect of fructose feeding, we analyzed the expression levels of fetal genes (e.g. MHC and ANF genes) in these two groups of hearts. As shown in figure 4C & 4D, MHC isoforms ratio shifted from 100% α-MHC in sham operated controls to nearly 60:40 α:β-MHC isoforms in mice with aortic banding, as expected. In fructose-fed mice, however, no appreciable change in MHC isoform was noticed following aortic banding. In these hearts, MHC isoforms ratio was comparable to that seen in sham operated controls, suggesting that the induction of β-MHC at the expense of α-MHC, as generally occurs with pressure overload hypertrophy, was
prevented in fructose fed mice. The anti-hypertrophy effect of fructose feeding was also apparent at the expression level of ANF in these two groups of hearts. While there was a marked induction of ANF expression in the banded mice fed with the regular diet, no significant change was observed in mice fed with the fructose rich diet (Fig. 4E & 4F).

To add further weight to these findings we analyzed two other markers of cardiac hypertrophy: myocyte cross-sectional area and interstitial fibrosis. The average cross-sectional area of ventricular myocytes was significantly increased in control banded mice, but not in banded mice fed with fructose diet; again demonstrating impaired hypertrophic response in fructose-fed mice (Fig 5A & 5B). To examine interstitial fibrosis, we stained heart sections of control and fructose-fed mice with anti-vimentin antibody, which reacts specifically with intermediate filaments of cardiac fibroblasts [7]. In control mice, aortic banding led to marked increase of fibroblast proliferation as seen with increased blue color staining in interstitial spaces of the heart sections. However, in the fructose-fed mice no significant change in cardiac fibrosis was observed following aortic banding (Fig 5C & 5D).

In order to determine whether these beneficial effects of fructose diet were related to induction of SIRT1, we measured the deacetylase levels in different groups of mice hearts. As shown in figure 5E, SIRT1 levels were notably decreased in banded mice hearts fed with a regular diet, consistent with a previous report [38]. However, they were maintained to nearly control levels in mice fed with a fructose-rich diet. These data indicated that fructose-feeding blocks cardiac hypertrophy response to pressure overload possibly by induction of SIRT1 deacetylase.

Fructose feeding maintains cardiac NAD levels: Results described above demonstrated that the long term feeding of mice with a fructose containing diet increased cardiac SIRT1 levels, which is a NAD-dependent deacetylase. We therefore reasoned whether fructose feeding can alter the SIRT1 levels by changing the cellular levels of NAD and hence NAD/NADH ratio. To test this possibility, we measured NAD and NADH levels in the heart lysate prepared from the snap frozen samples of control and fructose fed mice. We found that the heart NAD levels were significantly lower in banded mice, compared to sham controls. In fructose-fed mice, however, NAD levels did not decline significantly following aortic banding, and they were maintained at a level comparable to NAD levels found in the sham controls (Fig 6A). The measurement of NADH levels of these hearts gave very different picture of nucleotide regulation. As shown in Fig 6B, no significant decrease in NADH levels was noticed following aortic banding in mice fed with
the regular diet; whereas, it was significantly lower in fructose fed mice. When these values were calculated in terms of NAD/NADH ratio, we found that this ratio was markedly reduced in banded mice on regular diet, but not in fructose fed mice, compared to sham controls (Fig 6C). These results indicated that fructose feeding upholds cellular NAD and NAD/NADH ratio, and that, in part, may contribute to increased levels of SIRT1 in mice subjected to aortic banding. Previous studies from this laboratory, as well as by others have demonstrated a role of SIRT1 in protecting cardiomyocytes from oxidative-stress mediated cell-death [38]. A role of SIRT1 in blocking the agonists mediated hypertrophy response has been also demonstrated [1, 2, 37].
In this study, we show that fructose feeding of mice attenuates the hypothyroid mediated myosin isoform shift and the cardiac hypertrophic response associated with chronic pressure overload. We found that fructose feeding increases the cellular NAD/NADH ratio and the levels of SIRT1 deacetylase. The effect of fructose on myosin isoforms shift was reproduced in transgenic mice having SIRT1 over expressed in the heart, and it was absent in SIRT1 (+/-) KO animals having reduced levels of the deacetylase. A role of SIRT1 in blocking the agonist-mediated cardiomyocyte hypertrophic response and myocyte cell death has been demonstrated previously by us as well as by others [2, 38]. Thus, the beneficial effects of fructose feeding observed in this study are likely to be contributed by the induction of SIRT1 deacetylase. The data presented here support many previous studies where fructose feeding has been shown to inhibit apoptosis induced by oxidative-stress of the tissue [15, 24].

The fructose-rich diet (40%) utilized in this study did not alter body weight of the animals even after 12 wks of feeding, indicating that these animals did not receive excessive caloric intake. In a previous study, Jordan et al reported that rats fed with a fructose rich diet were protected against ischemia reperfusion mediated cardiac injury [24]. In this study authors examined the effect of different time periods of fructose feeding, starting from 3 days to 4 weeks, and found the same amount of protection in terms of reduced infarct size. The study interpreted that the protection offered by fructose diet is specific to dietary ingestion of fructose and not secondary to metabolic abnormalities (insulin resistance) associated with it. Authors concluded that the fructose feeding induced cardio-protection is a “chemical preconditioning” response of the heart. Similarly, in an earlier study examining the effect of fructose on MHC isoform expression, Dillmann et al reported that a diet containing 60% fructose could prevent α-MHC decline in hypothyroid rats [14]. These authors also examined the circulating thyroid and insulin levels in fructose fed animals and found no change related to sugar ingestion. They inferred that the induction of α-MHC isoform by fructose feeding is a “fructose effect” and not related to hormonal status of the animal. Cytoprotective effects of fructose had been also observed in other tissues including hepatocytes. Frenzel et al have reported that the anti-apoptotic effect of fructose administration was independent of cell ATP content. Rather, it was related to reduced synthesis of reactive oxygen species [15]. All these
protective effects of fructose administration can be explained by our observation that this sugar is capable of elevating the cellular SIRT1 deacetylase levels.

SIRT1 is considered a longevity factor, as the over expression of this deacetylase has been found to increase life span of the organism. SIRT1 activation suppresses gene transcription and promotes cell survival by deacetylating targets such as histones, NFkB, MEF2, p53, Ku70 and FOXO group of factors [6, 43, 46]. Some of these targets have been also implicated in development of cardiac hypertrophy [20]. Thus, SIRT1 can block the cardiac hypertrophic response by deacetylating one or more of these targets.

Another important finding reported in this study is that SIRT1 activation prevented the MHC isoform shift associated with hypothyroidism or hypertrophy of the heart. Animals fed with resveratrol rich diet and the transgenic animals expressing SIRT1 responded similarly to PTU treatment. In both groups a significant amount of α-MHC expression was noticed even when animals were made hypothyroid for several weeks, indicating that the deacetylase activation has a direct effect on the α-MHC gene expression. This is also supported by findings of transient transfection assays, where SIRT1 over-expression was found to stimulate the α-MHC gene promoter activity. In this study, however, we did not find a direct effect of SIRT1 on the β-MHC gene promoter. In contrast, Fulco et al have shown that over expression of SIRT1 in skeletal muscle cells suppressed the expression of endogenous β-MHC genes [17]. This discrepancy could be due to different cell background i.e. skeletal versus cardiac, or the endogenous gene versus shorter promoter fragment, examined in the two studies. Results obtained from PTU treated SIRT1 transgenic animals have shown that the deacetylase expression prevents the α-MHC decline, but not the induction of β-MHC isoform associated with hypothyroidism. Thus, these results indicate that SIRT1 activation has a profound (if not exclusive) effect on the cardiac α-MHC gene expression. To the best of our knowledge this is the first report demonstrating that stimulation of a histone deacetylase up-regulates the α-MHC gene expression. Previous studies carried out with a class-I & II HDAC inhibitor, TSA (trichostatin A), have shown that blocking (rather than activation) of HDAC activity up-regulates the expression of this transcript [10]. Thus, our data presented here reveals that different classes of HDACs have different effects on the MHC isoform expression, which could be related to different substrates targeted by different class of HDACs. The underlying mechanism of α-MHC induction by SIRT1 activation is not known at present. One explanation can be deduced from the ability of SIRT1 to antagonize the effects PARP1 and Ku70. Both of these molecules have been shown to suppress the
activity of α-MHC gene promoter, and their expression level has been shown to be up-regulated in failing hearts where α-MHC levels are suppressed [38, 39, 44]. In the present study, lack of staining of heart section for vimentin suggests inhibition of fibroblast proliferation in fructose fed mice. This effect of fructose can be also attributed to SIRT1 activation, as resveratrol has been recently shown to inhibit angiotensin-II and TGFβ mediated cardiac fibroblast proliferation and differentiation [33].

How does fructose feeding stimulate SIRT1 levels? Although, a precise answer to this question is not known at present, it has been shown that a change in cell NAD content regulates the SIRT1 deacetylase synthesis. The data presented here show that hypertrophied hearts had significantly lower NAD/NADH ratio, and that fructose feeding prevents this change. We also found that fructose diet increases NAD/NADH ratio and SIRT1 deacetylase levels in control mice not subjected to aortic banding, suggesting that this sugar may have a direct impact on cellular NAD/NADH ratio. Utilization of fructose by cells requires its phosphorylation by ketohexokinase before it enters into the glycolytic pathway. Ketohexokinase is abundantly present in the liver, but heart muscle has almost no detectable activity of this enzyme [4]. The other enzyme which can phosphorylate fructose is hexokinase. Although the primary function of hexokinase is to phosphorylate glucose, it is relatively non-specific enzyme with a Michaelis-Menton constant for fructose being ~10 fold that of glucose [27]. This indicates that phosphorylation of fructose can occur in the heart only if the intracellular concentration of fructose is high compared to glucose. Once fructose is phosphorylated at carbon 6 position by hexokinase it enters into regular glycolytic pathway. The main difference in glucose and fructose metabolism is then the mode of sugar transport into the cell interior. While fructose can freely enter into cells, glucose is transported actively via an insulin-dependent mechanism. From the analysis of the glycolytic pathway, it seems that the energy generated by utilization of one molecule of fructose or glucose in the heart is same [27]. The only difference between these two sugars would be that the rate of glycolysis would be expected to be faster with fructose utilization compared to glucose. A faster rate of glycolysis has been shown before to be beneficial for ischemic and decompensated hearts [34]. Enhanced rate of glycolysis has been also demonstrated previously to up-regulate the expression levels of α-MHC gene [13]. Whether faster rate of glycolysis also contributes to augmented levels of NAD is, however, not known. Another possibility could be that fructose impacts the NAD salvage pathway, and thus directly modulates the cellular NAD/NADH ratio.
Our results are in disagreement with a recent study where Chess et al [9] reported that high fructose (61%) diet exacerbated the cardiac hypertrophic response to pressure overload, while the diet containing complex carbohydrates (starch) showed beneficial effects. One major difference between the two studies is that we did not find a change in insulin levels in fructose fed mice, while they have reported a significantly elevated plasma insulin levels in mice fed with this sugar. It is also important to note that the diet with complex carbohydrates, which showed protective effects, had no notable effect on the plasma insulin levels, suggesting that perhaps a change in insulin levels regulates the outcome of the diet to cardiac hypertrophy response [9]. Insulin is known to induce cardiac protein synthesis and develop cardiac hypertrophy [42]. Fructose, by itself does not stimulate insulin synthesis, as glucose does. Therefore, a change in insulin levels in their study, and not in ours, suggests that the metabolic profile of animals in two studies was very different, which may in part explain the observed differences in two studies. Another point worth noting is that, contrary to the feeding protocol utilized by Chess et al [9], where animals were assigned to different diets following surgery, in our case we had pretreated animals with fructose diet for 4 weeks before subjecting them to aortic banding. This period of pretreatment was sufficient to induce cardiac SIRT1 levels, which is likely to invoke a pre-conditioning effect, as suggested by Jordan et al [24]. Similar to our results Dillmann et al [11] have also reported that fructose diet that induces α-MHC expression in diabetic rats does not alter the plasma insulin levels.

In summary, data presented in this study demonstrate that fructose feeding up-regulates the α-MHC expression and blunts the cardiac hypertrophy response to pressure overload. These beneficial effects of fructose are likely to be mediated by activation of the cardiac SIRT1 deacetylase levels. In this study we also present evidence that unlike class-I and II HDACs, activation of a member of class-III HDAC, SIRT1 has a direct effect on the myosin isoform shift in favor of α-MHC expression. These studies provide a mechanism for cytoprotective effect of fructose as observed previously in many other tissue and models, and suggest that limited addition of fructose in the diet may provide benefits to the heart challenged with haemodynamic overload.
Acknowledgement

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References


Figure Legends

**Figure 1**: Fructose feeding induces the α-MHC expression. (A) Adult mice were fed with a PTU, fructose (fruc) or PTU plus fructose-rich diet for 6 weeks. MHC isoforms were separated as described in Methods. Expression of α- and β-MHC isoforms in 4 days old neonatal mouse heart is shown for comparison. Note induction of α-MHC isoform in PTU plus fructose fed mice. (B) Western-blot analysis showing expression of SIRT1 deacetylase in different groups of mice. (C) Quantification of α- and β-MHC isoform levels in different groups of mice. (D) Quantitative presentation of SIRT1 levels in different groups of mice. Cont., control, PTU, 6-propyl-2-thiouracil; *p<0.05 vs PTU treated animals.

**Figure 2**: SIRT1 activation contributes to fructose and resveratrol mediated α-MHC induction. (A) Myosin isoforms expression in different groups of adult wild type mice: Group 1, control (cont) mice fed with regular diet; Group 2, mice fed with PTU diet; Group 3 mice fed with PTU plus resveratrol diet; and Group 4, mice fed with PTU plus resveratrol plus fructose diet for 6 weeks. (B) Expression level of SIRT1 in different groups of wild type and SIRT1 (+/-) knockout (KO) mice. (C) Myosin isoforms profile of SIRT1 (+/-) KO mice fed with PTU or PTU plus fructose diet. (D) Quantification of MHC isoform levels in different groups of mice. *p<0.05 same MHC isoform compared with PTU alone treated group. PTU, 6-propyl-2-thiouracil; Res, resveratrol; Fruc, fructose.

**Figure 3**: Over-expression of SIRT1 deacetylase induces the expression of α-MHC isoform. (A) Expression pattern of MHC isoforms in transgenic lines (SIRT1.tg1 and SIRT1.tg2) made hypothyroid with PTU treatment. Note, as opposed to non-transgenic controls, there was significant amount of α-MHC expression in SIRT1 transgenic animals after PTU treatment. (B) Western analysis showing SIRT1 expression in control and the two transgenic lines. (C) Northern analysis showing expression of α-MHC mRNA in the heart tissue from control and transgenic animals with and without PTU treatment. (D and E) The effect of SIRT1 over-expression on α- and β-MHC gene promoter activity as observed in transient transfection analyses. Values are mean ± SE of 5 -7 transfections. *p<0.05 compared to control having no SIRT1 co-transfection.
Figure 4: Fructose feeding blocks cardiac hypertrophic response to pressure overload. Mice fed with a regular or fructose rich diet were subjected to aortic banding for 12 weeks. (A) Heart weight (HW) to body weight (BW) ratio in different groups of animals. (B) Percent of hypertrophy in control and fructose fed mice following 12 wks of banding. (C) A representative gel showing expression pattern of MHC isoforms in different groups of animals. The adult mouse heart (sham) expressed α-MHC predominantly. Following aortic banding (band) there was nearly equal amount of α and β-MHC isoforms expressed in mice on regular diet, but not in animals fed with the fructose-rich diet (fruc + band). (D) Quantification of α- and β-MHC isoform expression in different groups of mice. (E) Western analysis of ANF expression in different groups. (F) Quantitative presentation of ANF expression detected by the western analysis. ANF, atrial natriuretic factor, NS, not significant.

Figure 5: Fructose fed mice show impaired cardiomyocyte hypertrophy and fibrosis following aortic banding. (A) Representative heart sections (200x) stained with wheat germ agglutinin coupled with tetramethylrhodamine isothiocyanate to demarcate cell boundaries. (B) Quantification of cardiomyocyte cell-size based on cross-sectional area in sham controls and banded animals fed with the regular or the fructose-rich diet (n = number of animal in each group, with 5-10 sections from each heart). (C) Mouse heart sections stained with anti-myomesin (green) and anti-vimentin (blue) antibodies. Nuclei are stained (red) with propidium iodide. Blue staining with vimentin antibody indicates extent of fibroblasts proliferation. (D) Quantification of fibrosis, n = 5, with 10 sections from each heart. (E) Western analysis of SIRT1 expression in heart lysate of different groups of mice.

Figure 6: Fructose feeding maintains cellular NAD levels following aortic banding. NAD and NADH levels were measured from total heart lysate as described in Methods. (A) NAD levels in heart samples of different groups. (B) NADH levels in the same hearts analyzed in panel A. (C) NAD/NADH ratio calculated from values presented in panels A and B. Values are mean ± SE, n = 5 hearts in each group, NS, not significant.
Table 1: Nutrient information of different diets.

<table>
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<th>PTU diet</th>
<th>Fructose diet</th>
<th>Resveratrol diet</th>
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<tr>
<td>Protein (% by wt)</td>
<td>18.2</td>
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<tr>
<td>Carbohydrate (% by wt)†</td>
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<td>2.9</td>
<td>3.3</td>
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<td>Kcal/g ‡</td>
<td>3.2</td>
<td>3.2</td>
<td>3.5</td>
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† Estimated digestible carbohydrates.
‡ Values are calculated from ingredient analysis (provided by the manufacturer). Wt, weight.
Table 2: Effect of diet on physical parameters of mice:

<table>
<thead>
<tr>
<th>Diet</th>
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<th>BW (g)</th>
<th>HW/BW (g/g)</th>
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<tbody>
<tr>
<td>Regular</td>
<td>0.222 ± 0.02</td>
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<td>Fructose</td>
<td>0.212 ± 0.02</td>
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<td>PTU + resveratrol</td>
<td>0.195 ± 0.01</td>
<td>37.5 ± 2.0</td>
<td>0.0052 ± 0.0003</td>
</tr>
</tbody>
</table>

Animals were fed with different diets for six weeks. Values are mean ± SE of n = 12 animals. PTU, 6-propyl-2-thiouracil. HW, heart weight; BW, body weight.
Figure 1

(A) Actin isoform and α-MHC and β-MHC isoform of Neo. H. and Adult H.

(B) Representative western blots of SIRT1 and actin for Cont, PTU, PTU + Fruc, and Fruc.

(C) Graph showing % MHC isoform α-MHC and β-MHC.

(D) Graph showing Relative SIRT1/actin for Cont, PTU, PTU + Fruc, and Fruc.
Figure 2

(A) Western blot analysis of MHC isoforms in different treatment groups.

(B) Western blot analysis of SIRT1 expression in Wild type and SIRT1 (+/-) KO mice.

(C) Western blot analysis of MHC isoforms in SIRT1 (+/-) KO mice.

(D) Graph showing the percentage of MHC isoforms in different treatment groups. Wild type mice: Cont, PTU, PTU + Res, PTU + Res + Fruc. SIRT1 (+/-) KO mice: PTU + Res, PTU + Res + Fruc. n = 4-6 for each group.

* indicates a statistically significant difference.
Figure 3

(A) Western blot analysis of α-MHC and β-MHC under different conditions.

(B) Western blot analysis showing SIRT1 (110 kDa) and actin levels.

(C) RT-PCR analysis of α-MHC mRNA and GAPDH levels.

(D) Graph showing relative luciferase activity for α-MHC.Luc under different conditions.

(E) Graph showing relative luciferase activity for β-MHC.Luc under different conditions.

* indicates significant difference.
Figure 4
Figure 5

(A) Control, Band, Fruc + band

(B) Myocyte cross-sectional area (μm²)

<table>
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<th>Fruc + band</th>
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<tr>
<td>Control (n=7)</td>
<td>450</td>
<td>550</td>
<td>600</td>
</tr>
<tr>
<td>Band (n=5)</td>
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<td>700</td>
</tr>
<tr>
<td>Fruc + band (n=5)</td>
<td>600</td>
<td>700</td>
<td>800</td>
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</table>

P<0.05

(C) Control, Band, Fruc + band

(D) % increase of fibrosis

<table>
<thead>
<tr>
<th></th>
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<th>Fruc + band</th>
</tr>
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<tbody>
<tr>
<td>Band (n=5)</td>
<td>20%</td>
<td>40%</td>
</tr>
<tr>
<td>Fruc + band (n=5)</td>
<td>40%</td>
<td>60%</td>
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</table>

P<0.05

(E) Cont., Band, Fruc + band

- SIRT1 (110 KDa)
- actin

Figure 5
Figure 6

(A): NAD

(B): NADH

(C) NAD/ NADH ratio

P<0.05
N. S. P<0.05
P<0.05