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Research Article

EFFECT OF TROXERUTIN ON CARDIAC INSULIN SIGNALING IN HIGH FAT, HIGH FRUCTOSE DIET - INDUCED INSULIN RESISTANT MICE

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ABSTRACT
The present study investigates the effects of troxerutin (TX) on insulin signaling in the heart of high fat, high fructose diet (HFFD)-induced insulin resistant mice. Adult male *Mus musculus* mice of body weight 25–30 g were used for the study. HFFD was fed to animals to induce insulin resistance. On the 16th day a group of HFFD-fed mice were supplemented with TX (150 mg/Kg bw, p.o) till the end of the experimental period. Control mice fed control diet and TX-unsupplemented HFFD-fed mice were also maintained. Analyses were performed after 60 days in plasma and heart tissue of mice. HFFD-fed mice displayed higher levels of glucose and insulin, decreased tyrosine phosphorylation of insulin receptor-β (IR-β) and insulin receptor substrate-1 (IRS-1). The association of p85 subunit of phosphotidyl inositol 3 kinase (PI3K) with IRS-1, subsequent Akt phosphorylation and GLUT-4 translocation were reduced. TX treatment to HFFD-fed mice reduced hyperglycemia, hyperinsulinemia and enhanced tyrosine phosphorylation of IR-β, IRS-1, IRS-1-PI3K association, Akt and GLUT-4 activation. These findings suggest that TX activates IRS–PI3K–Akt pathway of insulin signaling and thereby promotes insulin action in the heart. Thus, TX could be a promising agent for the management of insulin resistance.
Key words: High-fat-high-fructose diet, Insulin signaling, Insulin resistance, Troxerutin

INTRODUCTION

Insulin is a potent anabolic hormone that plays an important role in whole body glucose homeostasis. Failure of the target cells to respond to the circulating levels of insulin is termed as insulin resistance which is often manifested clinically through the features of the metabolic syndrome (MS) or type 2 diabetes (T2D). The prevalence of insulin resistance has increased dramatically and now reached epidemic levels throughout the world.[1-2] The pathophysiological consequences of insulin resistance include hyperinsulinemia, impaired glucose tolerance and disturbances in glucose and lipid metabolism which can potentially worsen metabolic efficiency of tissues.[3]

Insulin has profound effects on the myocardium and lack of insulin response in cardiac tissue can lead to alterations in myocardial structure, metabolic pathways and vascular function. Insulin induces glucose uptake in the cardiomyocytes upon binding to the cell surface insulin receptor (IR). Binding of insulin to its receptor activates tyrosine kinase activity of the receptor's β-subunit. This leads to autophosphorylation as well as tyrosine phosphorylation of insulin receptor substrates (IRS) like IRS-1 and -4. These substrates, in turn, interact with phosphatidylinositol 3-kinase (PI3K) and stimulate protein kinase B (Akt), a downstream ser/thr kinase. Insulin is a very potent PKB/Akt activator in the heart.[4] Acitvated Akt favours glucose uptake through glucose transporter-4 (GLUT-4) translocation.

Defects in insulin receptor and IRS-1-4 tyrosine phosphorylation results in insulin resistance. Serine phosphorylation of IRS-1 blocks tyrosine phosphorylation by causing either dissociation of IR and IRS1, degradation of IRS proteins or dissociation of the p85 subunit of PI3K. Increased activity of phosphatases (phospho-tyrosine phosphatase 1B) can decrease the activation of downstream signaling molecules like Akt and protein kinase C.

Troxerutin (TX, $C_{33}H_{42}O_{19}$), a trihydroxyethylated derivative of the natural bio-flavonoid rutin exhibits strong vasoprotective, antioxidative, anti-inflammatory and anti-hyperlipidemic activities.[5-6] Our recent study demonstrated that TX improves whole-body insulin sensitivity, lowers lipid content and oxidative stress and also regulates lipid metabolism in the heart.[6] However, the mechanisms underlying the insulin sensitivity effects of TX have not been investigated so far. Consumption of energy rich foods containing high quantities of fat and fructose induce insulin resistance in rodents and humans.[3] In the present work, we employed the high fat, high fructose diet (HFFD)–fed insulin resistant mice to investigate whether TX improves insulin response in heart tissue. To achieve this, plasma glucose and insulin levels, insulin signal proteins in heart tissue of TX-supplemented and TX-un-supplemented HFFD-fed mice were studied.

MATERIALS AND METHODS
Kits, antibodies and chemicals
Assay kits for glucose and insulin were obtained from Agappe Diagnostics Pvt. Ltd., Kerala and Monobind Microwells Inc., CA, USA respectively. Human recombinant insulin (Humulin R) was procured from Eli Lilly, Indianapolis, USA. IR-β, IRS-1, phosphotyrosine (100), phospho Akt (Threonine 308) and Akt and GLUT-4 antibodies were purchased from Cell Signaling Technology, MA, USA and PI3K antibody from Santa Cruz Biotechnology CA, USA. Enhanced chemiluminescence (ECL, Super signal west pico chemiluminescent substrate) assay kit was obtained from Thermo Scientific, PA, USA. Chemicals and solvents used were purchased from Himedia Laboratories Pvt., Ltd., Mumbai, India.

Animals
Adult male *Mus musculus* albino mice of Swiss strain weighing 25-30 g were used for the study. Animals were obtained and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital (RMMC and H),

Annamalai Nagar, Tamil Nadu, India. The animals were housed under hygienic conditions (22–24°C) in polypropylene cages and maintained on a 12h light/12 h dark cycle. Animals had free access to water and standard pellet diet. The animals were cared according to the guidelines of the Institutional Animal Ethical Committee (IAEC) in accordance with the Indian National Law on Animal Care and Use and this study protocol was approved by the IAEC, RMMC and H (Reg. No. 160/1999/CPCSEA-888).

Diet preparation
The high fat-high fructose diet (HFFD) prepared in the laboratory had the following ingredients (g/100g): fructose 45.0, groundnut oil 10.0, beef tallow 10.0, casein 22.5, DL-methionine 0.3, wheat bran 5.5, vitamin mixture 1.2 and mineral mixture 5.5. The standard diet, commercially obtained from Sai Enterprise, Chennai contained 60% (w/w) starch, 22.08% (w/w) protein and 4.38% (w/w) fat, provided 382.61 cal/100 g while HFFD provided 471.25 cal/100 g.

Study protocol
Mice were randomly divided into 4 experimental groups with 6 mice in each group after acclimatization for a period of one week. The following groups were maintained for the period of 60 days. Group I- CON, Group II- HFFD, Group III- HFFD + TX, and Group IV- CON+TX. TX was administered orally from the 16th day till the end of the experimental period. At the end of the experimental period, six mice from each group were fasted overnight and sacrificed by cervical dislocation. Blood collected from retro-orbital plexus in vials containing EDTA was centrifuged at 371x g for 10 min to obtain plasma. Heart was dissected out and washed in ice-cold saline, weighed and used for further studies.

Biochemical parameters
Insulin sensitivity tests
Plasma glucose and insulin levels were measured using ELISA kit according to manufacturer's instructions. Insulin sensitivity indices like homeostasis model assessment (HOMA)[7] and quantitative insulin check index (QUICKI)[8] were calculated using appropriate formulae.

$$\text{HOMA-IR} = \text{glucose (mmol /L)} \times \text{insulin (}\mu\text{IU/mL) /22.5}$$
$$\text{QUICKI} = 1 / \text{Log insulin (mU/L)} \times \text{log glucose (mg/ dL)}$$

Insulin stimulation
At the end of the experimental period, animals from each group (n=4) were injected intraperitoneally 15 unit/kg human recombinant insulin. Animals were sacrificed after 30 min. The heart tissue was removed, weighed and homogenized in ice-cold homogenization buffer (50 mM Tris, 0.25% SDS, 150 mM NaCl, 1% NP-40, 1 mM EDTA and a protease inhibitor cocktail) and centrifuged at 1500 rpm for 10 min at 4°C. The supernatant obtained was again centrifuged at 12,000 rpm for 20 min at 4°C. The protein concentration was estimated by the method of Lowry et al.[9]

Immunoprecipitation
Tyr phosphorylation of IRβ, tyr phosphorylation of IRS-1 and IRS-1 association with PI3K were assessed by immunoprecipitation and immunoblotting. Heart homogenates containing an equal amount of protein were subjected to immunoprecipitation, at 4°C, using antibodies specific to anti-IRβ and anti-IRS-1. The immune complexes were precipitated by the addition of 50 μL protein-A agarose

slurry to each sample, and then incubated at 4°C for 2 h. Immune complexes were pelleted at $4000 \times g$ for 10 min at 4°C and washed thrice with homogenization buffer. The pellets were suspended in Laemmli sample buffer and boiled for 5 min. Proteins were resolved by 8%-10% SDS - polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto polyvinylidene fluoride membranes. The membranes were then blocked in Tris buffered saline – Tween 20 (TBST) containing 3% bovine serum albumin (pH 7.4) for 2 h at room temperature. Proteins were immunoblotted with anti-phospho tyr antibody (for IRβ and IRS-1), and PI3K antibody for IRS-1. The membranes were washed with TBST and incubated with their respective secondary antibodies for 2 h at room temperature. Blots were subsequently stripped and reprobed with the same antibody used for immunoprecipitation. Protein band detection was performed by enhanced chemiluminescence assay (Thermo Scientific super signal west pico chemiluminescent substrate, Rockford, USA) as per the manufacturer's instructions. The band densities of phosphorylation were normalized with the band density of corresponding total protein. Quantitative comparisons of protein abundance between various groups were performed using Image J software (US National Institutes of Health).

Western blotting analysis
Protein extracts of heart tissue were obtained by ice-cold lysis buffer supplemented with a protease inhibitor cocktail. Protein content was determined by Lowry et al[9] and 50-100 μg of protein was electrophoresed using SDS-PAGE under reducing conditions. The separated proteins were transferred to a polyvinylidene difluoride membrane. Proteins of interest were revealed with antibodies specific to IRβ, IRS-1, PI3K, AKT and GLUT4-. Then the bound antigen-antibody complexes were detected using horseradish peroxidase-conjugated secondary antibodies by incubation for 1h at room temperature. Protein bands were revealed using Immobilon Western Chemiluminscent HRP Substrate-ECL detection system and quantitated by densitometry using Image J software (National Institute of Health Bethesda, MD, USA).

Statistical analysis
Values are expressed as means ± SD for 6 mice in each group for biochemical analysis, and n=4 for western blotting. Significant differences between groups were examined by one–way analysis of variance (ANOVA) followed by Tukey's test. P values < 0.05 were considered statistically significant.

RESULTS
Plasma biochemical analysis
Plasma glucose concentrations were significantly higher in HFFD-fed mice at 0 min and 120 min after glucose challenge as compared to other groups suggesting impaired glucose tolerance. TX-treated HFFD group showed significant reduction in glucose load after 2 hours compared to HFFD-fed group. Significantly higher values of insulin levels in plasma and insulin/glucose ratio, and lower $ISI_{0,120}$ values were observed in HFFD fed- mice. Higher values of HOMA and lower values of QUICKI were also noted in HFFD-fed mice as compared to control mice suggesting the inefficient insulin action and the development of peripheral insulin resistance. TX administration significantly reversed the status in HFFD-fed mice. No significant differences in plasma

glucose and insulin levels were observed between CON+TX and CON groups (Table 1).

Insulin signaling proteins in heart
Protein levels of IR-β
Western blot analysis of IR-β in heart of experimental groups is shown in Figure1. Insulin-stimulated tyrosine phosphorylation of IR-β was reduced by 42% in HFFD-fed mice as compared to CON mice. TX treatment significantly increased tyrosine phosphorylation of IR-β levels to 83% in HFFD-fed mice. There were no significant alterations in IR-β protein levels between CON and CON+TX group.

Protein abundance of IRS-1
HFFD feeding resulted in reduced insulin-stimulated tyrosine phosphorylation of IRS-1 (by 48%) as compared to CON mice (Figure.2). TX treatment significantly improved the tyrosine phosphorylation status. Phosphorylation status of IRS-1 in TX-treated mice was lower only by 19% with respect to control. The protein levels of IRS-1 did not vary between CON group and the CON+TX group.

Abundance of IRS-1-PI3K association
IRS-1-PI3K association was found to be decreased by 53% in HFFD-fed mice as compared to CON mice in insulin stimulated condition. After TX-treatment, significant increase in PI3K association with IRS-1 was observed in HFFD-fed mice (Figure.3). No significant differences were observed in protein levels of IRS-1 between CON and CON+TX groups.

Downstream signaling molecules
Phosphorylation status of Akt
Insulin–mediated threonine phosphorylation of Akt was measured in the hearts of HFFD-fed mice to determine whether decreased cardiac insulin signaling was responsible (insulin resistance) for impaired GLUT-4 membrane translocation. Insulin stimulation significantly blocked Akt phosphorylation (by 45%) in HFFD-fed mice as compared to CON mice. TX administration significantly increased the phosphorylation status and it was improved to 80%. TX-treated and untreated CON mice showed no significant changes in Akt protein level (Figure.4)

GLUT-4 translocation
GLUT-4 protein was examined in the membrane fraction of heart using immunoblotting is shown in Fig.5. HFFD-fed mice had a marked reduction of membranous GLUT-4 protein (by 46%) as compared to CON mice (Figure.5). Interestingly, administration of TX restored these levels in

HFFD-fed mice. With respect to control, GLUT-4 abundance was lower only by 18% in TX-treated mice. There were no significant differences between the CON and CON+TX groups.

DISCUSSION
For the first time, the study found that the supplementation of TX to insulin resistant mice improves cardiac insulin sensitivity by activating IRS–PI3K–Akt pathway and GLUT-4 translocation. Multitude of studies in human and animals have demonstrated that the macronutrient composition of a diet is an important environmental determinant of the quality of insulin action.[10-11] High fat and high fructose intake contributes to obesity, hyperlipidemia, glucose intolerance and insulin resistance.[12] Feeding high calorie diet led to hyperglycemia and hyperinsulinemia which has been already documented in our earlier studies[12,13] HOMA-IR and QUICKI values serve as markers for insulin resistance which is substantiated by high blood glucose even during hyperinsulinemia. Administration of TX to HFFD mice resulted in significant reduction in plasma levels of glucose, insulin probably by improving insulin sensitivity. Studies reported that high calorie diet causes defects in insulin signaling and blunts Akt-mediated GLUT-4 translocation in heart.[3,14] Since TX improved glucose and insulin levels in heart tissue we next studied whether TX can mitigate the alterations in insulin signaling caused by HFFD.

Several studies point out that insulin resistance is an early event that precedes both functional and pathological changes in the diabetic myocardium.[15,16] IRS proteins contain numerous tyrosine, serine and threonine phosphorylation sites which favours communication between insulin and signaling pathways.[17] In this study, the abundance of tyrosine phosphorylation of IRβ, IRS-1, IRS1-PI3K association and Akt (Threonine phosphorylation) in HFFD-fed mice were significantly reduced when compared with control mice. Reduction in tyrosine phosphorylation of IRS family members has been observed in insulin-resistant animal models and human subjects, including those who are obese or made insulin resistant by lipid infusion.[14,18] Phosphorylation of IRS proteins at particular serine residues inhibits the interaction of IRS proteins with the insulin receptor which in turn leads to reduction in tyrosine phosphorylation of IRS and subsequently decreases activation of PI3K.[17] Interestingly, high-fat diet–induced insulin resistance is ameliorated when specific serine/threonine kinases are genetically ablated or pharmacologically inhibited.[19,20]

Table1: Effect of TX on glucose and insulin levels and insulin sensitivity indices of experimental mice

Parameters	CON	HFFD	HFFD+TX	CON+TX
Fasting glucose (mg/dL)	75±3.1	125.2±9.6[a]	90.3±5.3[b]	75.5±3.4
Glucose at120 min (mg/dl)	88±4.3	170±14.2[a]	102±8.2[b]	90±4.5
Insulin (μU/mL)	20.3±1.0	30.6±2.6[a]	24.01±1.6[a,b]	20.7±1.1
Insulin/glucose ratio	0.27±0.01	0.24±0.02[a]	0.26±0.01[a,b]	0.30±0.01
ISI $_{0,120}$	3.93±0.18	0.96±0.07[a]	2.36±0.14[a,b]	3.8±0.16
HOMA-IR	0.39±0.01	0.98±0.06[a]	0.53±0.04[b]	0.38±0.01
QUICKI	0.30±0.01	0.25±0.01[a]	0.28±0.02[a,b]	0.30±0.01

Values are means ±SD of 6 mice from each group. CON- control mice; HFFD-High fat-high fructose diet-fed mice; HFFD+TX-High fat-high fructose diet-fed mice treated with troxerutin; CON+TX- control mice + troxerutin. ISI-Insulin sensitivity index, HOMA-Homeostasis model assessment, QUICKI- Quantitative insulin check index
[a] Significant as compared to CON(P < 0.05; ANOVA followed by Tukey's test).
[b] Significant as compared to HFFD (P < 0.05; ANOVA followed by Tukey's test).
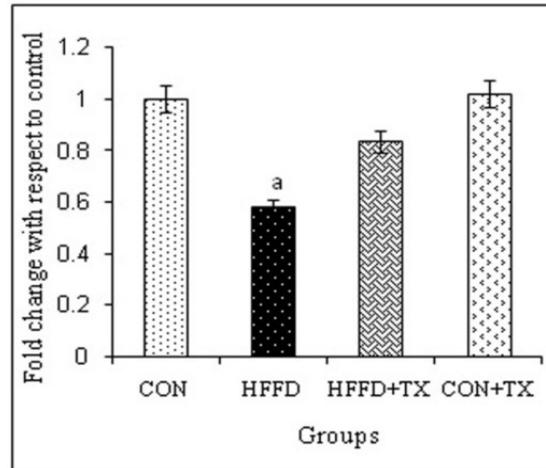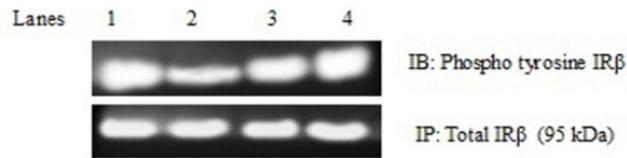
Figure1: Effect of TX on insulin stimulated tyrosine phosphorylation of IRβ in heart of experimental mice. Values shown are mean ± SD of 4 experiments. Lane 1: CON, Lane 2: HFFD, Lane 3: HFFD+TX, and Lane 4: CON+TX. Densitometry data is given after normalization with respective total form. The values are expressed as fold change with respect to control group. [a] Significant as compared to CON(P < 0.05; ANOVA followed by Tukey's test). [b] Significant as compared to HFFD (P < 0.05; ANOVA followed by Tukey's test).
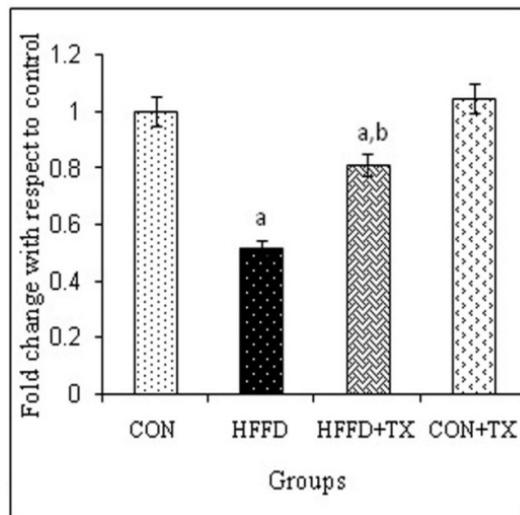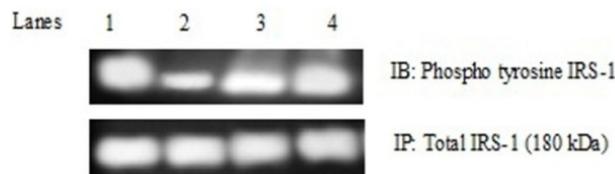
Figure 2: Effect of TX on insulin stimulated tyrosine phosphorylation of IRS-1 in heart of experimental mice. Values shown are mean ± SD of 4 experiments. Lane 1: CON, Lane 2: HFFD, Lane 3: HFFD+TX, and Lane 4: CON+TX. Densitometry data is given after normalization with respective total form. The values are expressed as fold change with respect to control group. [a] Significant as compared to CON(P < 0.05; ANOVA followed by Tukey's test). [b] Significant as compared to HFFD (P < 0.05; ANOVA followed by Tukey's test).
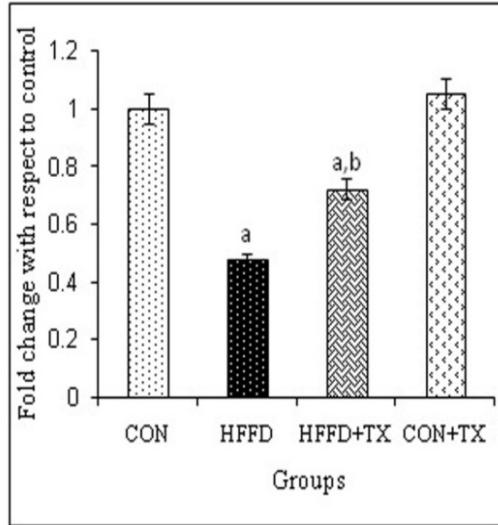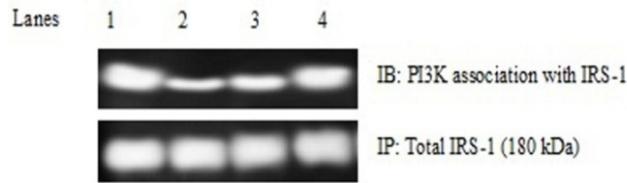
Figure 3: Effect of TX on PI3K with IRS-1 in the heart of experimental animals. Values shown are mean ± SD of 4 experiments. Lane 1: CON, Lane 2: HFFD, Lane 3: HFFD+TX, and Lane 4: CON+TX. Densitometry data is given after normalization with respective total form. The values are expressed as fold change with respect to control group. [a] Significant as compared to CON(P < 0.05; ANOVA followed by Tukey's test). [b] Significant as compared to HFFD (P < 0.05; ANOVA followed by Tukey's test).
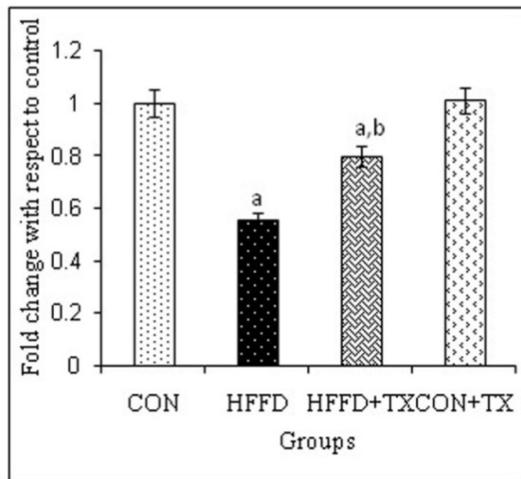
Figure 4: Effect of TX on insulin-stimulated Akt activation in heart of experimental mice. Values shown are mean ± SD of 4 experiments. Lane 1: CON, Lane 2: HFFD, Lane 3: HFFD+TX, and Lane 4: CON+TX. Densitometry data is given after normalization with respective total form. The values are expressed as fold change with respect to control group. [a] Significant as compared to CON(P < 0.05; ANOVA followed by Tukey's test). [b] Significant as compared to HFFD (P < 0.05; ANOVA followed by Tukey's test).
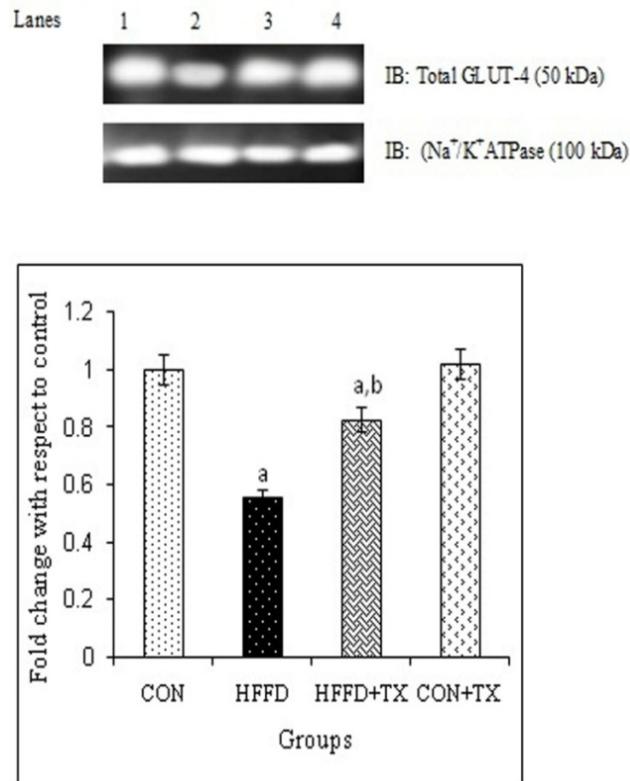
Figure 5: Effect of TX on GLUT-4 activation in heart of experimental animals. Values shown are mean ± SD of 4 experiments. Lane 1: CON, Lane 2: HFFD, Lane 3: HFFD+TX, and Lane 4: CON+TX. Densitometry data is given after normalization with Na⁺/K⁺ATPase. The values are expressed as fold change with respect to control group. ᵃ Significant as compared to CON(P < 0.05; ANOVA followed by Tukey's test). ᵇ Significant as compared to HFFD (P < 0.05; ANOVA followed by Tukey's test).

Mice fed high fat diet suffer from cardiac insulin resistance as evidenced by increase in diacylglycerol, resulting in activation of PKC which in turn increases serine phosphorylation of IRS-1 with decreased insulin-stimulated IRS-1/2 tyrosine phosphorylation and downstream insulin signaling. [21,22] In addition, high fructose diet can activate serine kinases like c-Jun N-terminal kinase-1 (JNK-1), inhibitor of nuclear factor kappa B kinase (IκB) complex and extracellular signal-regulated kinases that inhibit serine307 phosphorylation of IRS-1 contributing to insulin resistance.[23] Genetic [24] and induced [25] rodent models of obesity and insulin resistance also present reduced tyrosine phosphorylation of IRS proteins. TX-treated HFFD-fed mice showed an improved tyrosine phosphorylation of IRβ, IRS-1 and IRS-1-PI3K association. This effect may be due to its anti-inflammatory and antihyperlipidemic activity. A study reported that that TX administration strongly inhibits the activation of JNK1 and IKKβ/NF-κB induced by endoplasmic reticulum stress in the hippocampus of high-cholesterol-fed mice.[26] Further, our previous study also demonstrated the lipid-lowering effects of TX.[6]

Akt/protein kinase B, a serine/threonine kinase is the downstream target of insulin signaling cascade that activates glucose uptake through GLUT-4. A significant decrease in Akt phosphorylation at Thr308 was observed in the present study which is attributed to defects in the phosphorylation of IRβ and IRS-1 in HFFD-fed mice. Supplementation of TX enhanced IRβ and IRS-1 tyrosine phsphorylation, IRS-1-PI3K association thereby improves Akt activation in HFFD-fed mice. Our findings are similar to that of Lu et al.[26]

reported that TX administration significantly improved insulin sensitivity in high-cholesterol-fed mice by increasing the serine phosphorylation of Akt which is due to activation of tyrosine phosphorylation of IR and IRS-1.

Insulin induces the translocation of GLUT-4 from the intracellular storage to the sarcolemmal membrane and so favours glucose entry.[27] Carroll et al.[28] demonstrated that glucose uptake is reduced as a consequence of reduced GLUT-4 protein and impaired insulin signaling in the myocardium of db/db mouse. Lipotoxicity [increased free fatty acid (FFA) and FA metabolites] and reactive oxygen species (ROS) have been shown to attenuate insulin signaling and GLUT-4 translocation in human cardiac muscle biopsies through activation of the IκB kinase (IKK) and other serine kinases.[29] A study also documented decrease in GLUT-4 mRNA level in the myocardium of fructose-fed animals.[30] where as Li et al.[31]observed both a decrease in GLUT-4 mRNA and insulin-stimulated GLUT-4 translocation to the plasma membrane. Consistent with this, we found reduced translocation of GLUT-4 in HFFD-fed mice indicating diminished cardiac glucose uptake. Supplementation of TX restored GLUT-4 thereby facilitates glucose uptake in the myocardium which was again due to lipid-lowering effects of the TX as reported in our earlier study.[6] Based on our previous finding that TX exhibits antihyperlipidemic activity by increasing the AMP-activated protein kinase phosphorylation, which, in turn, facilitates FA oxidation and FA transport in HFFD-fed mice, we strongly suggest that TX, being a potent antioxidant and antihyperlipidemic agent activates IRS-1-PI3K-Akt pathway thereby attenuate insulin

resistance in HFFD-fed mice. TX administration have been shown to effectively improve insulin signaling in the hippocampus of mice –fed a high cholesterol diet [26] and in gastrocnemius muscle of high fat and sucrose-induced type-2 diabetic adult male rat.[32]

In conclusion, the present findings demonstrate that HFFD feeding leads to impaired insulin signaling through reduced IRS-I tyrosine phosphorylation. TX administration improves cardiac insulin signaling by augmenting insulin-stimulated IRS–PI3K–Akt pathway in the heart of HFFD-fed mice. On the whole, this study provides new data on the mechanisms of insulin sensitivity effect of TX in heart of HFFD-fed mice. Since majority of the diabetic patients (90%) are insulin resistant, TX could be recommended as a possible candidate for the prevention and therapy of insulin resistance in type 2 diabetic subjects.

REFERENCES
1. Elliott SS, Keim NL, Stern JS, *et al*. Fructose, weight gain, and the insulin resistance syndrome. The American Journal of Clinical Nutrition 2002;76 (5):911-22. PMID:12399260.
2. De Ferranti SD, Gauvreau K, Ludwig DS, *et al*. Prevalence of the metabolic syndrome in American adolescents: findings from the Third National Health and Nutrition Examination Survey. Circulation 2004; 110 (16):2494-24977. http://dx.doi:10.1161/01.CIR.0000145117.40114.C7. PMID:15477412.
3. Deng JY, Huang JP, Lu LS, Hung LM. Impairment of cardiac insulin signaling and myocardial contractile performance in high cholesterol–fructose fed rats. American Journal of Physiology- Heart and Circulatory Physiology2007;293(2):978–987. http://dx.doi: 10.1152/ajpheart.01002.2006.PMID: 17400720.
4. Deprez J, Bertrand L, Alessi DR, Krause U, Hue L, Rider MH. Partial puri fication and characterization of a wortmannin-sensitive and insulin-stimulated protein kinase that activates heart 6-phosphofructo-2- kinase. Biochemical Journal 2000; 347 (1):305–312. PMID: 10727432.
5. Fan SH, Zhang ZF, Zheng YL, Lu J, Wu DM, Shan Q, *et al*. Troxerutin protects the mouse kidney from D-galactose-caused injury through anti-inflammation and anti-oxidation. International Immunopharmacology 2009; 9(1):91–96. http://dx.doi: 10.1016/j.intimp.2008.10.008. PMID: 19000936.
6. Geetha R, Yogalakshmi B, Sreeja S, Bhavani K, Anuradha CV. Troxerutin suppresses lipid abnormalities in the heart of high-fat–high-fructose diet-fed mice. Molecular and Cellular Biochemistry 2014; 387 (1-2):123-134. http://dx.doi: 10.1007/s11010-013-1877-2. PMID: 24173620.
7. Mathews DR, Hosker JP, Rudenkl AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28 (7):412–419. PMID: 3899825.
8. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, *et al*. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. The Journal of Clinical Endocrinology and Metabolism 2000;85(7):2402–2410. PMID: 10902785.
9. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 1951; 193 (1):265–275. PMID: 14907713.
10. Bessesen DH. The role of carbohydrates in insulin resistance. The Journal of Nutrition 2001; 131(10): S2782–S2786. PMID: 11584106.
11. Axen KV, Dikeakos A, Sclafani A. High dietary fat promotes syndrome X in nonobese rats. The Journal of Nutrition 2003; 133 (7): 2244–2249. PMID: 12840187.
12. Bhuvaneswari S, Arunkumar E, Viswanathan P, Anuradha CV. Astaxanthin restricts weight gain, promotes insulin sensitivity and curtails fatty liver disease in mice fed a obesity-promoting diet. Process Biochemistry2010;45(8):1406–1414. http://dx.doi: 10.1016/j.procbio.2010.05.016.
13. Arunkumar E, Bhuvaneswari **S**, Anuradha CV. An intervention study in obese mice with astaxanthin, a marine carotenoid--effects on insulin signaling and pro-inflammatory cytokines. Food and Function. 2012; 3(2): 120-126. http://dx.doi:10.1039/c/1 fo 10161g. PMID:22089895

14. Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, *et al*. Insulin resistance differentially affects the PI3-kinase- and MAP kinase mediated signaling in human muscle. The Journal of Clinical Investigation 2000;105(3):311–320. http://dx.doi: 10.1172/JCI7535. PMID: 10675357.
15. Taegtmeyer H, Passmore JM. Defective energy metabolism of the heart in diabetes. Lancet 1985; 1 (8421):139–141. http://dx.doi:10.1016/S0140-6736(85)91907-5. PMID: 2857216.
16. Rodrigues B, McNeill JH. The diabetic heart: metabolic causes for the development of a cardiomyopathy. Cardiovascular Research 1992 26 (10):913–922. http://dx.doi.org/10.1093/cvr/26.10.913 PMID: 1486584.
17. Zick Y. Ser/Thr phosphorylation of IRS proteins: a molecular basis for insulin resistance. Science's STKE: signal transduction knowledge and environment 2005 (268):pe4. http://dx.doi: 10.1126/stke.2682005pe4. PMID: 15671481.
18. Goodyear LJ, Giorgino F, Sherman LA, Carey J, Smith RJ, Dohm GL. Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. The Journal of Clinical Investigation1995; 95 (5): 2195–2204. http://dx.doi: 10.1172/JCI117909. PMID: 7537758.
19. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 2001; 293 (5535): 1673–1677. http://dx.doi:10.1126/science.1061620. PMID: 11533494.
20. Kim JK, Fillmore JJ, Sunshine MJ, Albrecht B, Higashimori T, Kim DW, et al. PKC-theta knockout mice are protected from fat-induced insulin resistance. The Journal of Clinical Investigation 2004; 114 (6):823–827. http://dx.doi: 10.1172/JCI200422230. PMID: 15372106.
21. Schenk S, Saberi M, Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. The Journal of Clinical Investigation 2008; 118(9): p.2992-3002. http://dx.doi: 10.1172/JCI34260. PMID: 18769626.
22. Zhang L, *et al*. Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle. Biochimica et Biophysica Acta 2010; 1801(1): p.1-22. http://dx.doi: 10.1016/j.bbalip.2009.09.014. PMID: 19782765.
23. Kim J, Wei Y, Sowers JR. Role of mitochondrial dysfunction in insulin Resistance. Circulatory Research. 2008;102 (4):401-414. http://dxdoi: 10.1161/CIRCRESAHA.107.16547
24. Kerouz NJ, Horsch D, Pons S, Kahn CR. Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS- 2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. The Journal of Clinical Investigation1997; 100:3164–3172. http://dx.doi: 10.1172/JCI119872. PMID: 9399964.
25. Folli F, Saad MJ, Backer JM, Kahn CR. Regulation of phosphatidylinositol 3-kinase activity in liver and muscle of animal models of insulin-resistant and insulin-deficient diabetes mellitus. The Journal of Clinical Investigation 1993; 92 (4):1787–1794. http://dx.doi: 10.1172/JCI116768. PMID: 7691886.
26. Lu J, Wu D, Zheng Z, Zheng Y, Hu B, Zhang Z. Troxerutin protects against high cholesterol-induced cognitive deficits in mice. Brain 2011; 134 (Pt 3):783–797. http://dx.doi: 10.1093/brain/awq376. PMID: 21252113.
27. Zorzano A, Sevilla L, Camps M, Becker C, Meyer J, Kammermeier H, *et al*. Regulation of glucose transport, and glucose transporters expression and trafficking in the heart: studies in cardiac myocytes. The American Journal of Cardiology 1997; 80 (3):65A–76A. PMID: 9293957.
28. Carroll R, Carley AN, Dyck JR, Severson DL. Metabolic effects of insulin on cardiomyocytes from control and diabetic db/db mouse hearts. American Journal of Physiology,Endocrinology Metabolism 2005; 288 (5):E900–E906. http://dx.doi: 10.1152/ajpendo.00491.2004. PMID: 15632103.
29. Nie Y, Ma RC, Chan JC, Xu H, Xu G. Glucose-dependent insulinotropic peptide impairs insulin signaling via inducing adipocyte inflammation in glucose-dependent insulinotropic peptide receptor-overexpressing adipocytes. Federation of American Societies for Experimental Biology Journal 2012; 26(6):2383–2393. http://dx.doi: 10.1096/fj.11-196782. PMID: 22366643.
30. Qin B, Polansky MM, Harry D, Anderson RA. Green tea polyphenols improve cardiac muscle mRNA and protein levels of signal pathways related to insulin and lipid metabolism and inflammation in insulin-resistant rats. Molecular Nutrition and Food Research 2010; 54 (1):S14-23. http://dx.doi: 10.1002/mnfr.200900306. PMID: 20112301.
31. Li P, Koike T, Qin B, Kubota M, Kawata Y, et al. A high-fructose diet impairs Akt and PKCzeta phosphorylation and GLUT4 translocation in rat skeletal muscle. Hormone and Metabolism Research 2008; 40 (8):528–532. http://dx.doi: 10.1055/s-2008-1073162. PMID: 18500676.

32. Sampath S, Karundevi B. Effect of troxerutin on insulin signaling molecules in the gastrocnemius muscle of high fat and sucrose-induced type-2 diabetic adult male rat. Molecular and Cellular Biochemistry 2014; 395(1-2):11–27. http://dx.doi: 10.1007/s11010-014-2107-2. PMID: 24880482.