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Silymarin ameliorates fructose induced insulin resistance syndrome by reducing de novo hepatic lipogenesis in the rat

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ABSTRACT

High dietary fructose causes insulin resistance syndrome (IRS), primarily due to simultaneous induction of genes involved in glucose, lipid and mitochondrial oxidative metabolism. The present study evaluates effect of a hepatoprotective agent, silymarin (SYM) on fructose-induced metabolic abnormalities in the rat and also assessed the associated thrombotic complications. Wistar rats were kept on high fructose (HFr) diet throughout the 12-week study duration (9 weeks of HFr feeding and subsequently 3 weeks of HFr plus SYM oral administration [once daily]). SYM treatment significantly reduced the HFr diet-induced increase expression of peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α/β, peroxisome proliferator-activated receptor (PPAR)-α, forkhead box protein O1 (FOXO1), sterol regulatory element binding protein (SREBP)-1c, liver X receptor (LXR)-β, fatty acid synthase (FAS) and PPARγ genes in rat liver. SYM also reduced HFr diet mediated increase in plasma triglycerides (TG), non-esterified fatty acids (NEFA), uric acid, malondialdehyde (MDA), total nitrite and pro-inflammatory cytokines (C-reactive protein [CRP], interleukin-6 [IL-6], interferon-gamma [IFN-γ] and tumor necrosis factor [TNF]) levels. Moreover, SYM ameliorated HFr diet induced reduction in glucose utilization and endothelial dysfunction. Additionally, SYM significantly reduced platelet activation (adhesion and aggregation), prolonged ferric chloride-induced blood vessel occlusion time and protected against exacerbated myocardial ischemia reperfusion (MI-RP) injury. SYM treatment prevented HFr induced mRNA expression of hepatic PGC-1α/β and also its target transcription factors which was accompanied with recovery in insulin sensitivity and reduced propensity towards thrombotic complications and aggravated MI-RP injury.

1. Introduction

Insulin resistance syndrome (IRS), a group of metabolic diseases that occur concomitantly leading to the development of type 2 diabetes or cardiovascular diseases (Grundy et al., 2005). The risk factors include hyperinsulinemia, impaired glucose tolerance, abnormalities in circulating lipid levels, endothelial dysfunction, oxidative stress and systemic inflammation (Franchini et al., 2008). Preclinical and clinical studies demonstrate that high fructose (HFr) diet stimulates hepatic de novo lipogenesis and cause hepatic steatosis (Nomura and Yamanouchi, 2012; Tappy and Le, 2012). Use of fructose-sweetened beverages by overweight and obese human subjects for 10 weeks led to significant increase in the hepatic de novo lipogenesis, postprandial plasma triglyceride (TG) levels, visceral adipose volume, dyslipidemia, hyperglycemia and insulin resistance (Stanhope et al., 2009). Fructose feeding significantly augmented expression of sterol regulatory element binding protein (SREBP)-1c in the rats, which was attenuated by peroxisome proliferator-activated receptor gamma coactivator (PGC)-1β antisense oligonucleotides (ASO) (Nagai et al., 2009). ASO treatment also reduced accumulation of TG and offered protection against IRS.

Silymarin (SYM), the flavonolignans extract of Silybum marianum (milk thistle), has emerged as a potential candidate for the treatment of liver diseases. SYM protects liver cells from various toxins, such as carbon tetrachloride, ethanol, acetaminophen, and D-galactosamine (Chrungoo et al., 1997; Halim et al., 1997). It also exhibits infarct sparing potential in liver cells against ischemic injury, iron toxicity, viral hepatitis and radiation (Kropacova et al., 1998; Pietrangelo et al., 1995; Wu et al., 1993). SYM has been proposed to offer hepatoprotection by various mechanisms, which includes protection against glutathione depletion, enhanced detoxification and reduction in oxidative stress (Baer-Dubowska et al., 1998; Basaga et al., 1997; Bosisio et al., 1992; Campos et al., 1989). SYM also exhibits anti-fibrotic, mast cells stabilizing, immunomodulatory and anti-tumor activities (Green-McKenzie and Hudes, 2005; Sonnenbichler et al., 1986). Moreover, SYM treatment reduces circulating levels of pro-inflammatory markers,

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improved metabolic parameters (Federico et al., 2008; Federico et al., 2006; Loguercio et al., 2007) and prevents mitochondrial dysfunction in the rodent model of non-alcoholic steatohepatitis (Serviddio et al., 2010). It has shown to ameliorate hepatic pathology in the patients of non-alcoholic fatty liver disease (NAFLD) (Loguercio et al., 2007). In addition, SYM significantly reduces platelet adhesion and aggregation in the rat (Rui, 1991). The present study was therefore undertaken to explore the effect of SYM against high fructose diet induced IRS and associated cardiovascular complications in the rat.

2. Materials and methods

2.1. Ethics statement

All the experiments were performed with prior approval and according to the guidelines of Institutional Animal Ethics Committee (IAEC) of CSIR-Central Drug Research Institute (CDRI), Lucknow, India (Approval no. 69/08/Pharmacol/IAEC). All procedures were performed under ketamine and xylazine anesthesia and all efforts were made to minimize animal suffering.

2.2. Materials

Silymarin (Silybon-140 mg) was procured from Micro Labs Ltd (India). Anti-Akt, anti-phospho-Akt (Thr308), anti-eNOS and anti-phospho-eNOS (Serine 1177) antibodies were from Cell Signaling Technology (USA). ELISA kits for C-reactive protein (CRP), interleukin-6 (IL-6), interferon-gamma (IFNγ), and tumor necrosis factor (TNF) were obtained from BD Biosciences (USA) and insulin kit was procured from Millipore (USA). TG ELISA kit was procured from BioVision Inc (USA). RevertAidTM H Minus First Strand cDNA synthesis kit was obtained from Fermentas (USA). AccuSure blood glucose monitoring system kit was from MicroGene Diagnostic Systems Pvt. Ltd. Kits for TG, non-esterified fatty acids (NEFA), uric acid, alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL) and direct bilirubin (conjugated bilirubin; DBIL) were from Randox Laboratories Ltd. (UK). Creatine kinase-MB fraction (CK-MB) and lactate dehydrogenase (LDH) assay kits were procured from Merck Diagnostic (India). Fructose was procured from Sisco Research Laboratories (India) and all the other reagents used in the experiments were from Sigma Chemicals (USA).

2.3. Experimental design and sample preparation

Eight weeks old, male Wistar rats were obtained from National Laboratory Animal Center (NLAC), CSIR-CDRI, Lucknow, India. The animals ($n=3$ per cage) were maintained at 24 ± 0.5 °C, 12 h day/night cycle with ad libitum water and chow pellets in the animal house of CSIR-CDRI. After one week, the animals were randomly distributed in two groups: first group received normal chow diet and another received high fructose (HFr) diet containing 35% fructose, 5% saturated fat (coconut oil), essential vitamins, and minerals along with 25% fructose in drinking water. Normal chow and HFr diet were prepared at CSIR-CDRI diet production facility using various components as described earlier (Prakash et al., 2011a). After 9 weeks of feeding, HFr group was further split into SYM (30, 100 and 300 mg kg^{-1}; once-daily per oral) and vehicle (aqueous carboxymethylcellulose [CMC] suspension) treated groups for 3 additional weeks along with 35% fructose in diet and 25% fructose in drinking water up to 12 weeks. Throughout the study period (9 weeks on HFr diet plus 3 weeks on SYM), rats were completely restricted from fructose free drinking water. Adoption of fructose feeding regimen through diet and water was in accordance to the previously published reports (Panchal et al., 2011; Stranahan et al., 2008). Previous study from our laboratory demonstrated that fructose feeding through diet and water induced IRS, mimics pre-diabetic state in humans. At the end of 12 weeks, animals were anaesthetized and blood was collected through cardiac puncture into a plastic syringe containing 1.9% tri-sodium citrate. To obtain serum, blood was collected separately in dry tubes without the anti-coagulant. Blood was centrifuged at 2500*g* for 15 min at 20 °C to obtain plasma and serum. Left ventricle, liver and aorta were quickly removed, washed thrice with chilled 0.9% NaCl and were immediately snap-frozen in liquid nitrogen. All tissue samples were stored at −80 °C until further analysis. To measure tissue nitrite, snap-frozen left ventricle heart sample was crushed in liquid nitrogen and 10% (w/v) tissue homogenate was prepared in ice cold 0.03 M sodium phosphate buffer by using Ultra-Turrax T25 (USA) homogenizer (Prakash et al., 2011a).

2.4. Intra-peritoneal glucose tolerance test (ipGTT)

ipGTT was carried out after giving intra-peritoneal injection of glucose (2 g kg^{-1}) to overnight fasted rats. Blood samples were collected from the tail vein at 0, 15, 30, 60, and 120 min after glucose loading (Prakash et al., 2011a). Glucose content was estimated by using AccuSure, a blood glucose monitoring kit.

2.5. Analysis of inflammatory markers and biochemical measurements

Plasma concentration of C-reactive protein (CRP), interleukin 6 (IL-6), interferon gamma (IFN-γ) and tumor necrosis factors (TNF) were assessed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. TG, insulin, non-esterified fatty acids (NEFA), uric acid, alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), and direct bilirubin (DBIL; conjugated Bilirubin) were measured using commercial kits as per the manufacturer's protocol.

2.6. Vascular reactivity

Vascular reactivity was assessed as described earlier (Prakash et al., 2011a). In brief, chest was opened and thoracic aorta was excised from the anaesthetized rat and immediately placed in ice-cold Kreb's bicarbonate solution. Transverse 4 mm wide rings were cut and mounted in 10 ml organ bath containing Kreb's solution. Aortic rings were exposed to KCl Kreb's buffer (80 mM) in order to assess the maximum tissue contractility. Concentration dependent relaxant response to acetylcholine (Ach) was then monitored in phenylephrine (PE; 1 μM) precontracted rings. Tissue contractility and viability was assessed by exposing the rings to KCl Kreb's buffer (80 mM) in all the groups (Khanna et al., 2011).

2.7. Quantitative real time qPCR (RT-qPCR)

Liver was aseptically removed, washed thrice with chilled 0.9% NaCl and RNA was isolated using TRIZOL. Further, cDNA was synthesized by using RevertAidTM H Minus First strand cDNA Synthesis Kit as per manufacturer's protocol. The abundance of transcripts was assessed by RT-qPCR on a LightCycler 480 real-time PCR system (Roche Applied Science, Mannheim, Germany) with a SYBR Green detection system. RT-qPCR was performed in 20 μl final volume using the Maxima SYBR Green/ROX qPCR Master Mix (2x) (Fermentas Life Sciences). For each run, samples were analyzed in duplicate for the gene of interest as well as for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Three-step cycling protocol (initial denaturation at 95 °C for 5 min, 40 cycles of 30 s denaturation at 95 °C, 1 min for annealing at 54 °C, and 30 s