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Troxerutin abrogates mitochondrial oxidative stress and myocardial apoptosis in mice fed calorie-rich diet

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ABSTRACT

Mitochondrial oxidative stress plays a major role in the pathogenesis of myocardial apoptosis in metabolic syndrome (MS) patients. In this study, we investigated the effect of troxerutin (TX), an antioxidant on mitochondrial oxidative stress and apoptotic markers in heart of mice fed fat and fructose-rich diet. Adult male *Mus musculus* mice were fed either control diet or high fat, high fructose diet (HFFD) for 60 days to induce MS. Mice from each dietary group were divided into two on the 16th day and were either treated or untreated with TX (150 mg/kg bw, p.o) for the next 45 days. At the end of the study, mitochondrial reactive oxygen species (ROS) generation, oxidative stress markers, levels of intracellular calcium, cardiolipin content, cytochrome *c* release and apoptotic markers were examined in the myocardium. HFFD-feeding resulted in diminution of antioxidants and increased ROS production, lipid peroxidation and oxidatively modified adducts of 8-OHG, 4-HNE and 3-NT. Further increase in Ca^{2+} levels, low levels of calcium transporters and decrease in cardiolipin content were noted. Changes in the mitochondrial structure were observed by electron microscopy. Furthermore, cytochrome *c* release, increase in proapoptotic proteins (APAF-1, BAX, caspases-9 and-3) and decrease in antiapoptotic protein (BCL-2) in HFFD-fed mice suggest myocardial apoptosis. These changes were significantly restored by TX supplementation. TX administration effectively attenuated cardiac apoptosis and exerted a protective role by increasing antioxidant potential and by improving mitochondrial function. Thus, TX could be a promising therapeutic candidate for treating cardiac disease in MS patients.

1. Introduction

Metabolic syndrome (MS) is a cluster of metabolic abnormalities that predisposes the development of type 2 diabetes (T2D) and cardiovascular disease [1]. Modifications in life style mainly excess calorie intake and sedentary habits leads to remarkable increase in the incidence of MS worldwide. The main pathophysiologic hallmark of MS is insulin resistance, a state refers to decreased ability of insulin to promote glucose uptake in targets tissues. Consumption of high calorie diet-induced insulin resistance has been reported to be caused by alterations in energy metabolism [2]. Feeding high fructose, high fat diet (HFFD) for 2–4 months to animals resulted in the development of characteristic features of MS, as observed in human MS [3,4].

Chronic intake of HFFD caused cardiac abnormalities like cardiac hypertrophy, contractile dysfunction, vascular remodeling, oxidative stress and myocardial apoptosis in animal models of MS [4,5]. Further continual excess fuel supply to the heart results in deleterious consequences due to formation of ROS and decreased antioxidant protection [5]. Mitochondria are the primary sites for ROS production, calcium homeostasis and initiation of apoptosis. Mitochondrial ROS production can cause oxidative damage to cellular macromolecules and is considered to be a major event that induces functional and structural damage to cardiac myocytes and promotes cell death [6–8]. Furthermore, excess mitochondrial ROS production has been attributed to the impairment of calcium homeostasis which results in increased calcium influx. Changes in intracellular calcium trigger cytochrome *c* release and caspases mediated apoptosis via opening of the mitochondrial permeability

Abbreviations: ROS, Reactive oxygen species; MPT, mitochondrial permeability transition pore; TBARS, Thiobarbituric acid reactive substances; LHP, lipid hydroperoxides; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; 8-OHG, 8-hydroxyguanosine; 4-HNE, 4-Hydroxy-2,3-nonenal; 3-NT, 3- Nitrotyrosine; SERCA2a, sarcoendoplasmic reticulum calcium ATPase; NCX, Na+/Ca2+ exchanger; APAF-1, Apoptotic protease factor-1; BCL-2, B-cell lymphoma 2; BAX, BCL2-associated X protein.

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transition pore (MPT) [9–11].

Cardiolipin is an anionic phospholipid of the inner mitochondrial membrane, involved in the regulation of the mitochondrial phase of apoptotic process. Cardiolipin is usually found to be bound with cytochrome *c* and changes in cardiolipin content upon oxidative damage to the acyl chains of cardiolipin might result in the dissociation of cardiolipin from cytochrome *c*. Thus, cytochrome *c* is released from the inner mitochondrial membrane which initiates a series of events leading to caspase-mediated apoptosis.

Numerous studies have shown that treatment with antioxidants is useful to protect the heart against oxidative stress [12–14]. Thus attenuation of mitochondrial oxidative stress could be an effective strategy in preventing the myocardial apoptosis in MS-associated heart disease.

Considering the evidence that oxidative stress induced myocardial apoptosis is a key element in the pathogenesis and progression of cardiac diseases, it is mandatory to provide antioxidant therapy. Troxerutin (TX) is a semi-synthetic derivative of the natural bioflavonoid rutin, which has been documented to have strong anti-oxidant and anti-inflammatory activities [15]. In our previous studies, we showed that TX attenuates lipid abnormalities, cardiac hypertrophy and mitochondrial dysfunction [16–18]. However, the effect of TX against myocardial apoptosis during HFFD challenge is unexplored. Therefore, we have investigated mitochondrial ROS production, oxidative stress markers, levels of intracellular calcium, calcium transporters, mitochondrial swelling, cardiolipin content, cytochrome *c* release and apoptotic markers in HFFD-fed mice treated or untreated with TX.

2. Materials and methods

2.1. Chemicals

TX and cardiolipin (Bovine heart) were procured from Sigma Chemical Company, St. Louis, MO, USA. The solvents and other chemicals used of analytical grade were purchased from Himedia Laboratories Pvt., Ltd., Mumbai, India.

2.2. Animals maintenance and study design

Adult male *Mus musculus* mice weighing 25–30 g were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital (RMMCH), Annamalai Nagar, Tamil Nadu, India. The animals were housed under temperature-controlled room with a standard light/dark cycle. The animals were fed either control diet or HFFD for 60 days. The control diet (Sai Enterprises, Chennai, India) had 4.38% fat and 60% starch and 22.08% protein and provided 382.61 cal/100 g whereas HFFD had 20% fat (10% beef tallow; 10% groundnut oil), 22.5% protein and 45% fructose and provided 471.25 cal/100 g. HFFD was prepared fresh every day in the laboratory.

Mice were randomly divided into 4 experimental groups. Group I - Control (CON), Group II (HFFD); Group III (HFFD + TX); Group IV (CON + TX). Diet and water were provided *ad libitum* to the animals. 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. The experimental protocols were approved by the IAEC, RMMCH, Annamalai University (Reg. No. 160/1999/CPCSEA-888).

At the end of 15 days, the development of insulin resistance was investigated by measuring plasma glucose and insulin levels in plasma of experimental animals. Insulin sensitivity was assessed by calculating homeostatic model assessment [HOMA] [19] and quantitative insulin sensitivity check index [QUICKI] [20]. After confirming the development of insulin resistance in HFFD-fed animals, TX (150 mg/kg bw) was administered through intragastric intubation from 16th day for the next 45 days. At the end of the study period six mice from each group were fasted overnight and sacrificed by cervical dislocation. The heart was dissected out and washed in ice-cold saline, weighed and used for further analysis.

2.3. Isolation and preparation of mice heart mitochondria

The mitochondrial fraction from heart tissue of experimental animals was isolated by differential centrifugation according to the method of Uysal et al., 1989 [21]. A 10% heart homogenate was prepared in ice-cold isolation buffer containing 10 mmol/L Tris, 0.25 mol/L sucrose and 0.5 mmol/L EDTA, at a pH of 7.4. The homogenate was centrifuged twice at 600 g for 10 min and then the supernatants obtained were centrifuged twice at 10 000 g for 20 min at 4 °C. The supernatant of this spin was the cytosolic fraction. The pellet was washed with ice-cold KCl (0.15 mol/L) and then resuspended in the Tris/sucrose buffer for use as the mitochondrial fraction. Protein content was determined by the method of Lowry et al., 1951 [22] The purity of mitochondrial fraction was confirmed by assaying succinate dehydrogenase activity [23].

2.4. Biochemical analysis

Thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LHP), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the content of reduced glutathione (GSH) were assayed in heart mitochondrial fraction by the standard methods elsewhere [24]. Intracellular ROS generation was determined in the heart mitochondrial fraction using dichlorofluorescin-diacetate (DCFH-DA) as mentioned elsewhere [25].

2.5. Measurement of mitochondrial swelling

Mitochondrial preparation (1 mg protein/ml) was used for swelling study. The medium contained 125 mM sucrose, 10 mM HEPES buffer, pH 7.2 and 4.0 μM rotenone in a final volume of 1.0 ml. After incubation for 5 min at 37 °C, 2.5 mM succinate (energizer) was added to the suspension followed by the inducer Ca^{2+} (10 nmol/mg protein). The decrease in absorbance at 540 nm was recorded. Decreasing values of absorbance indicate mitochondrial swelling [26].

2.6. Calcium estimation

Calcium was extracted from heart mitochondria by tri-acid digestion technique. Mitochondrial pellet was digested with 70% perchloric acid, conc. nitric acid and sulphuric acid (2:1:2) and heated until becoming colorless. The samples were then neutralized with 20% sodium hydroxide, filtered and subjected to calcium estimation by the method of Bauer et al., 1981 using a reagent kit (RFCL limited, Uttarkand, India) [27].

2.7. Detection of cardiolipin

Lipids were extracted from heart tissue according to the procedure described by Folch et al., 1957 [28]. Bovine heart cardiolipin was used as an internal standard. Twenty microliters of samples were run through C18- column (150 × 3.2 mm) fixed to HPLC Shimadzu analytic reverse phase system. A solvent gradient was run using acetonitrile-2-propanol (8:2, by volume) to acetonitrile-2-propanol 5:5 in 30 min. The flow rate was 1 ml/min and detection was carried out by fluorescence detector set at an excitation