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Chrysin, an anti-inflammatory molecule, abrogates renal dysfunction in type 2 diabetic rats

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ABSTRACT

Diabetic nepropathy (DN) is considered as the leading cause of end-stage renal disease (ESRD) worldwide, but the current available treatments are limited. Recent experimental evidences support the role of chronic microinflammation in the development of DN. Therefore, the tumor necrosis factor-alpha (TNF-α) pathway has emerged as a new therapeutic target for the treatment of DN. We investigated the nephroprotective effects of chrysin (5, 7-dihydroxyflavone) in a high fat diet/streptozotocin (HFD/STZ)-induced type 2 diabetic Wistar albino rat model. Chrysin is a potent anti-inflammatory compound that is abundantly found in plant extracts, honey and bee propolis. The treatment with chrysin for 16 weeks post induction of diabetes significantly abrogated renal dysfunction and oxidative stress. Chrysin treatment considerably reduced renal TNF-α expression and inhibited the nuclear transcription factor-kappa B (NF-κB) activation. Furthermore, chrysin treatment improved renal pathology and suppressed transforming growth factor-beta (TGF-β), fibronectin and collagen-IV protein expressions in renal tissues. Chrysin also significantly reduced the serum levels of pro-inflammatory cytokines, interleukin-1beta (IL-1β) and IL-6. Moreover, there were no appreciable differences in fasting blood glucose and serum insulin levels between the chrysin treated groups compared to the HFD/STZ-treated group. Hence, our results suggest that chrysin prevents the development of DN in HFD/STZ-induced type 2 diabetic rats through anti-inflammatory effects in the kidney by specifically targeting the TNF-α pathway.

Introduction

End stage renal disease (ESRD) is a global health issue that has reached epidemic proportions across the world. Diabetic nephropathy (DN), a major microvascular complication of diabetes mellitus (DM), especially of type 2 diabetes, is considered as the most important risk factor for ESRD (Ritz et al., 1999). Although the role of hyperglycemia, insulin resistance, hyperlipidemia and oxidative stress in the pathogenesis of DN is well established, however, the intrinsic mechanisms responsible for the development of renal injury are still unknown. Recent experimental evidences have supported the role of inflammatory molecules in the initiation and progression of DN (Bruno et al., 2003; Navarro, 2003). In this context, tumor necrosis factor-alpha (TNF-α) has received much attention (Mora and Navarro, 2004; Navarro, 2003). TNF-α is a pro-inflammatory cytokine synthesized by monocytes, macrophages and T lymphocytes, and has an important function in lipid metabolism, insulin resistance and inflammatory responses. However, it is also secreted by resident renal cells where it induces a variety of effects on renal structure and function (Dong et al., 2007; Mariano et al., 1997). In diabetic kidney, TNF-α is implicated in the promotion of local oxidative stress (Koike et al., 2007; Mensah-Brown et al., 2005), increased protein permeability (McCarthy et al., 1998), and the induction of renal cell loss (Boyle et al., 2003). TNF-α is also responsible for the development of various hemodynamic and metabolic changes in diabetic kidney, including infiltration of immune cells, reduced glomerular filtration rate (GFR) as well as altered endothelial function (Baud and Ardaillou, 1995). Hence, the targeted inhibition of TNF-α expression is a promising approach to inhibit, both, renal chronic microinflammation as well as the development of DN.

The current available treatment strategies for DN are not optimal, both in the prevention and treatment of DN. Moreover, conventional therapeutic drugs are found to produce numerous side-effects. So, it is desirable to find therapeutic agents that could prevent, both, initiation as well as progression of DN, and unlike other drugs, also do not produce side effects. Foods rich in antioxidants, such as polyphenolics, terpenoids, and coumarins, have preventive effects against cancer, inflammatory disorders and neurological degeneration (Jain et al., 1999). Flavonoids are a large family of polyphenolic compounds synthesized by plants, and are divided into various classes such as flavonoids, flavones, anthocyanidins and isoflavonoids. Flavonoids possess a wide range of pharmacological properties with minimum side-effects (Cook and Samman, 1996), and the study of their mechanism of action has

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been the subject of considerable interest in recent years. In this context, chrysin (5,7-dihydroxyflavone), found abundantly in plant extracts, honey and bee propolis (Harminder and Chaudhary, 2011; Pichichero et al., 2010), has received attention because of its potent antioxidant and anti-inflammatory properties (Cho et al., 2004; Lapidot et al., 2002).

In the present study, we have investigated the effects of chrysin on DN in the high fat diet/streptozotocin (HFD/STZ) induced type 2 diabetic Wistar albino rat model. To evaluate the mechanism of action, we hypothesized that chrysin may improve the renal functions in HFD/STZ type 2 diabetic rat model through the inhibition of TNF-α mediated chronic inflammatory processes.

Materials and methods

Chemicals and reagents. Chrysin, streptozotocin (STZ), oxidized glutathione (GSSG), reduced glutathione (GSH), glutathione reductase (GR), reduced nicotinamide adenine dinucleotide phosphate (NADPH), bovine serum albumin (BSA) and dimethyl sulphoxide (DMSO) were purchased from Sigma Aldrich Chemicals Pvt. Ltd. (New Delhi, India). Hydrogen peroxide, Tris-buffer, thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from SD-Fine Pvt. Ltd. (Mumbai, India). All other chemicals used were of analytical grade.

Animal model. Adult male Wistar albino rats (150–180 g) used for the present study were housed in the animal house facility of Jamia Hamdard, New Delhi and were kept in a controlled environment under standard conditions of temperature and humidity with an alternating 12 hr light and dark cycle. The animals had free access to food and water *ad libitum*. The animals were maintained in accordance with guidelines prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the study was approved by the animal ethics committee of Jamia Hamdard, New Delhi, India (Registration no. 173/CPCSEA, project No. 721).

Induction of type 2 diabetes mellitus (T2DM) and experiment design. To induce T2DM, a well-established method (Danda et al., 2005) was followed. Animals were fed high fat diet (HFD, 60% calories from fat and 70% animal fat) (TD97201; Harlan Teklad, Madison, WI, USA) initially for five weeks followed by a single intraperitoneal (i.p.) injection of a low dose of streptozotocin (STZ, 35 mg/kgbw, in citrate buffer pH 4.5). Seven days later, fasting blood glucose and serum insulin levels were measured. Hyperglycemic and hyperinsulinemic rats were considered to be diabetic and were used in the study after being acclimatized for one week. After acclimatization, 24 rats were randomly divided into four groups with six animals each and subjected to respective treatment: (i) normal control rats (NC) received intragastric (*i.g.*) of vehicle (0.1% DMSO); (ii) diabetic control rats (DC, $n = 6$) received *i.g.* of 0.1% DMSO; (iii) chrysin treated normal rats (Chrysin-N) received *i.g.* of only chrysin (40 mg/kgbw); and (iv) chrysin treated diabetic rats (Chrysin-40) received *i.g.* of chrysin (40 mg/kgbw). Chrysin was dissolved in 0.1% DMSO and administered into the rats by gastric gavage once a day until the end of the study (16 weeks post-induction of diabetes). The dose level of chrysin (40 mg/kgbw) was selected on the basis of preliminary sub-acute toxicity assessment study.

Sample collection and tissue preparation. During the experimental period, the animals were housed individually in metabolic cages at different time periods (0, 4, 8, 12 and 16 weeks) and fasted overnight. The urine samples were collected over a period of 24 h and maintained under mineral oil to avoid evaporation. Post-collection the urinary volume was measured. Blood samples were obtained from the retro-orbital plexus by using micro-hematocrit capillaries and serum was separated from cells by centrifugation for 5 min at 3000 rpm. At the end of the experimental period, the rats were sacrificed by using diethyl ether anaesthesia. The animals were perfused transcardially through ascending aorta with normal saline so as to remove any blood clots from kidneys. The left kidney was rapidly removed for homogenate preparation. Nuclear and cytoplasmic fractions were extracted from renal tissues using a commercial nuclear extract kit (Active Motif, California, USA) following the manufacturer's instructions, and were used for various biochemical and molecular assays. The right kidney was rapidly removed and preserved in 10% buffered formalin solution for histopathological examinations.

Serum biochemical estimations. Fasting blood glucose (FBG) concentration was determined by using a commercially available glucose assay kit (Span diagnostics Pvt Ltd, Surat, India). Serum insulin level was measured by using enzyme linked immunosorbent assay (ELISA) kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions.

Evaluation of renal function. Glomerular filtration rate (GFR) was estimated through creatinine clearance. Creatinine was measured in urine and serum samples by using a commercial assay kit (Span diagnostics Pvt Ltd, Surat, India). Creatinine clearance was calculated by the standard equation (Arreola-Mendoza et al., 2006). Blood urea nitrogen (BUN) levels were measured in serum samples by using commercial assay kit (Span diagnostics Pvt Ltd, Surat, India). Proteinuria was measured by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Assessment of renal markers of oxidative stress. Lipid peroxidation (LPO) was determined in kidney homogenate by measuring the amount of malondialdehyde (MDA), a product formed due to the peroxidation of lipids (Ohkawa et al., 1979). Reduced glutathione (GSH) content was measured by the method of Jollow et al. (1974). Glutathione peroxidase (GPx) activity was determined by the method of Mohandas et al. (1984). Glutathione reductase (GR) activity was determined by the method of Carlberg and Mannervik (1975). Superoxide dismutase (SOD) activity was assayed by the spectrophotometric method (Marklund and Marklund, 1974). Catalase (CAT) activity was measured in renal tissues as described by Claiborne (1985).

Kidney histopathological analysis. For histopathological examination, kidneys from different groups were stained with Jones periodic acid-Schiff (PAS). Quantitative histological damage in the kidney structure was analyzed by taking microphotographs using an Olympus BX50 bright field microscope (Olympus, Japan).

Western blotting of renal TNF-α, NF-κB, TGF-β, fibronectin and collagen-IV. Nuclear and cytoplasmic proteins were extracted from renal tissues using a commercial nuclear extract kit (Active Motif, California, USA) following the manufacturer's instructions. Total protein quantification was performed in both cytoplasmic as well as nuclear fraction using Bradford assay method (Bradford, 1976). NF-κBp65 levels were measured in nuclear fraction, while TNF-α, TGF-β, fibronectin and collagen-IV levels were measured in cytoplasmic fraction. Briefly, samples were reconstituted in sample buffer and denatured by boiling for 5 min. Samples (40 μg protein) were loaded on SDS-PAGE Unit (Bio-Rad, California, USA) containing 5% stacking and 15% resolving polyacrylamide gels and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 μm) (Bio-Rad, California, USA). Non-specific protein binding was blocked by incubation with 3% BSA in Tris-buffered saline (TBS) at 37 °C for 2 h followed by incubation with rat polyclonal antibodies against TNF-α (1:1000, Thermo Fisher Scientific, USA), NF-κBp65 (1:1000, Thermo Fisher Scientific, USA), TGF-β (1:1000, Thermo Fisher Scientific, USA), fibronectin (1:1000, Sigma Aldrich, USA) and collagen-IV (1:1000, Sigma Aldrich, USA) for 16 h at 4 °C. β-actin was used as a loading control for cytoplasmic proteins and histone-H1 was used as a loading control for nuclear protein. Thereafter, horseradish peroxidase-conjugated secondary