Intermittent fasting prevents the progression of type I diabetic nephropathy in rats and changes the expression of Sir2 and p53

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Abstract Diabetic nephropathy (DN) is one of the main causes of end stage renal disease (ESRD) and a leading cause of diabetes mellitus related morbidity and mortality. Recently, sirtuins are reported to have emerging pathogenetic roles in cancer, muscle differentiation, heart failure, neurodegeneration, diabetes and aging. The aim of the present study was to study the role of intermittent fasting (IF) on DN and studying the expression of Sir2 and p53. At biochemical level, we found that IF causes significant improvement in blood urea nitrogen (BUN), creatinine, albumin and HDL cholesterol, parameters that are associated with the development of DN. Diabetic rats on IF also show significant improvement in onset of hypertension. Interestingly, the expression of Sir2, a NAD dependent histone deacetylase, decreases in diabetic rat kidney and this decrease is overcome by IF. Moreover, we provide evidence for involvement of mitogen activated protein kinases (MAPK) cascade in mediating the effects of IF as there is reduction in the expression of p38 which gets induced under diabetic condition. This was further accompanied by the concomitant decrease in cleavage of caspase3 and p53 expression. These findings suggest that IF significantly improves biochemical parameters associated with development of DN and changes the expression of Sir2 and p53. © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

Dietary restriction (DR) can reduce body weight and normalize blood glucose, insulin, and leptin levels in obese animals and humans [1]. It also increases both the mean and maximum lifespan of rodents, and this antiaging effect is associated with enhanced insulin sensitivity [2]. To increase life span two different paradigms of DR have been widely employed in rats and mice. In one paradigm, the animals receive food daily but are limited to a specific amount, which is typically 30–40% less than the ad libitum (AL) consumption of the control group. The second paradigm involves intermittent fasting (IF) in which the animals are deprived of food for a full day, every other day, and are fed AL on the intervening days. There are several possible molecular mechanisms that might explain the beneficial effects of DR on aging and disease including reduction in mitochondrial oxyradical production, induction of a cytoprotective cellular stress response, and stimulation of the production of growth factors [2–4].

DR reduces tumor formation, kidney diseases and increases the resistance of neurons to dysfunction and degeneration in experimental models of Alzheimer’s and Parkinson’s diseases as well as in stroke. It is known that IF without an overall reduction in calorie intake exerts several beneficial effects such as lifespan extension, improved glucose regulation and neuroprotection [5]. IF and calorie restriction (CR) increases insulin sensitivity that results in reduced plasma glucose and improved glucose tolerance [6]. It is not only known to reduce the oxidative stress [2] but also increases cellular resistance to various type of stress and enhances immune functions [3].

Diabetic nephropathy (DN) is the leading cause of ESRD resulting in diabetes related morbidity and mortality [7]. It is associated with protein that eventually spills into the urine (proteinurea) because of damaged or destroyed filters, and when the entire filtration system breaks down, the kidneys fail to function. DN also refers to the glomerular lesions, glomerular basement membrane thickening, diffuse mesangial matrix increase, nodular mesangial matrix increase, mesangial, endothelial cell proliferation, arteriolar lesions, and tubular cell problems [8,9]. There is significant increase in apoptosis in the tubular and interstitial cells during the course of progression of diabetic nephropathy [10,11]. Stress-activated signaling pathways such as nuclear factor-xB, p38 mitogen activated protein kinase (MAPK), and Jun kinases underlie the development of diabetic complications [12]. It has also been reported previously that high glucose levels can activate the p38 MAPK pathway in many cell types, including renal cells [13,14].

Recently, sirtuins are reported to have pathogenetic role in cancer, muscle differentiation, heart failure, neurodegeneration, diabetes and aging. Silent information regulator 2 (Sir2)-family proteins (sirtuins) are Class III protein deacetylases conserved from prokaryotes to mammals. Sir2 orthologs also have been shown to promote longevity in yeast, worm and...
flies, supporting the hypothesis that sirtuins may act as evolutionarily conserved regulators of aging [15]. SIRT1 is a mammalian homolog of the Saccharomyces cerevisiae chromatin silencing factor Sir2. Sirtuins have also been implicated in several important cellular processes, including transcriptional silencing, genomic stability, DNA repair, adipogenesis and p53-mediated apoptosis [16]. Complex role of p53 in SIRT1 regulation has been suggested. Higher basal expression of SIRT1 in adipose tissue was observed in p53−/− mice than in wild type control. These in vivo results support role for p53 in SIRT1 regulation [17]. Moreover not much is known that whether IF can protect or delay the development of diabetic nephropathy. In the present study, we have tested the hypothesis that IF can improve physiological and biochemical parameters associated with diabetic nephropathy.

2. Materials and methods

2.1. Chemical

All the chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise mentioned. Plasma glucose level, blood urea nitrogen (BUN), plasma albumin and plasma HDL-cholesterol were estimated by commercially available kits.

2.2. Animal treatment and development of diabetic nephropathy

All the experiments were approved by the Institutional Animal Ethics Committee (IAEC) and complied with the NIH guidelines on handling of experimental animals. Experiments were performed on male Sprague–Dawley rats in the weight range of 240–260 g which were procured from the central animal facility of the institute, kept at controlled environmental conditions with room temperature 22 ± 2 °C and 12 h light/dark cycles. After one week of acclimatization, animals were divided into four different groups namely: control ad libitum (CAL), control IF (CIF), provided access to food every other day, diabetic ad libitum (DAL) and diabetic IF (DIF), provided access to food every other day. Animals were kept in the mesh bottom cages to prevent the caphrophagy and for accurate estimation of spillage [6].

Diabetes was induced by injecting streptozotocin (STZ) (55 mg kg−1, i.p. dissolved in ice cold sodium citrate buffer, 0.01 M, pH 4.4). Animals with plasma glucose level >250 mg/dl after 48 h post induction of diabetes were included in the study as diabetic animals [18]. Age matched control rat received sodium citrate buffer. The animals of group CIF and DIF were kept on IF, i.e. alternate day fasting and full access of food was provided to CAL and DAL for 8 weeks. DN was evaluated by checking biochemical parameters like plasma creatinine, plasma albumin and BUN.

2.3. Measurement of body weight and food intake

Body weight was measured each week while food intake was measured daily. To keep track of spillage white bedding was used to allow easy separation of unconsumed food and powder from waste and bedding; spilled food was allowed to dry fully to avoid measurement of wet weight added by urine [6].

2.4. Blood pressure recording

Blood pressure (systolic, mean and diastolic) and heart rate were recorded at 8th week post-STZ administration, using a tail cuff blood pressure recorder (IHTC INC, Life Science Instruments, Model no. 29,229; CA, USA). Rats were acclimatized up to one week in heating chamber (24–26 °C) for 20 min before recording the blood pressure (between 9 AM and 11 AM). Blood pressure was measured for each rat and the average was calculated [18].

2.5. Measurement of biochemical parameters

Blood samples were collected from the retro orbital plexus of rats under light ether anesthesia in heparinized centrifuge tubes and immediately centrifuged at 2300g for the separation of plasma. Plasma was stored at −80 °C until assayed. The plasma was used for the estimation of glucose, HDL cholesterol, albumin and BUN. Estimations were carried out as per manufacturer’s instruction provided with commercially available kits. Plasma creatinine concentration was measured by the picric acid colorimetric method [19].

2.6. Measurement of lipid peroxidation and superoxide dismutase (SOD) activity

The lipid peroxide level in animal tissues was measured according to method described by Ohkawa et al. [20]. SOD activity was estimated according to method described by Paoletti et al. [21].

2.7. Histopathology of kidney

Rats were anesthetized under light ether anesthesia, after surgery circulating blood was removed by cardiac perfusion with 0.1 M PBS (pH 7.4, 20–50 ml). After clearance of circulating blood, 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) was perfused for another 5 min (100–200 ml of fixative) to fix the tissues. Kidneys were removed from the animal, decapsulated, sliced transversely, and paraffin-embedded for light microscopic evaluation. Histopathological changes in kidney structure were assessed in at least 25 randomly selected tissue sections from each group studied. Sections were stained with Mayer’s hematoxylin and eosin to examine cell structure.

2.8. Protein isolation and Western blotting

Total proteins were isolated from kidney tissue homogenates by sonication and nuclei were isolated according to method described by Tikoo et al. [22]. Histones were acid extracted using 0.25 M HCl. Nuclei were suspended in buffer [1% NP-40, 10 mM Tris, 10 mM NaCl, 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride (PMSF)] prior to sonication and its protein concentration was determined by Lowry method [23]. For Western blot analysis, proteins were transferred onto nitrocellulose membrane and immunoblot analysis was performed by using the Anti p38 (rabbit polyclonal 1:500, Santa Cruz), Anti p53 (mouse monoclonal, 10 μg/ml, Calbiochem), Anti Sir2 (rabbit polyclonal 1:500, Sigma), Anti-actin (rabbit 1:2500, Santa Cruz), p-Histone H3 ser-10 (rabbit 1:2000, Santa Cruz), Anti Caspase-3 (rabbit 1:1000, Calbiochem), Anti Histone H3 (rabbit 1:5000, Upstate) and HRP-conjugated secondary antibodies (anti-rabbit, Santa Cruz). Proteins were detected with the enhanced chemiluminescence (ECL) system and ECL Hyperfilm (Amersham Pharmacia Biotech).

2.9. Statistical analysis

Experimental values are expressed as mean ± S.E.M. Comparison of mean values between various groups was performed by one-way-analysis of variance (one-way-ANOVA) followed by multiple comparisons by Tukey test. P value <0.05 is considered to be significant.

3. Results

3.1. Effect of IF on bodyweight and food intake in diabetic animals

Despite of increased food intake in diabetic rats as compared to controls, we did not observe the corresponding gain in weight of diabetic animals. However, diabetic rats kept on IF showed a slight reduction in the body weight in the earlier weeks which may be due to sudden restriction of daily food intake (Fig. 1B). Total food intake was significantly reduced in both CIF as well as DIF groups as compared to respective CAL and DAL which is primarily because of restricting food (Fig. 1A).

3.2. IF inhibits the rise in blood pressure in diabetic animals

STZ induced diabetic condition is accompanied by a significant rise in blood pressure [18]. DR is reported to alleviate the rise in blood pressure in normal conditions [24]. In order to check whether IF plays any role in altering the hemodynamic regulation, we have recorded blood pressure at 8th week in all four groups by tail cuff noninvasive method. DAL group
showed significant increase in systolic, mean and diastolic blood pressure as compared to CAL. IF prevents the rise in blood pressure in both the diabetic as well as control rats on IF, as is evident from Table 2. Since, rise in blood pressure results in a decline in the process of glomerular filtration eventually leading to nephropathic damage. IF results in prevention to glomerular damage by decreasing the blood pressure (see Fig. 3D).

3.3. Effect of IF on plasma glucose, BUN, plasma creatinine, plasma albumin and plasma HDL-cholesterol

After eight week of STZ treatment, we observed a significant increase in BUN in DAL (51 ± 4.99) as compared to CAL (20 ± 1.80) group which indicates development of DN. However, IF significantly decreases BUN in DIF (30 ± 0.78) group thereby preventing the progression of DN. Similarly plasma creatinine another biomarker of DN increases significantly in DAL (2.04 ± 0.17) group as compared to CAL (1.46 ± 0.08), while IF significantly reduces the increase in plasma creatinine in DIF (1.41 ± 0.04) group. Plasma albumin levels are lower in DAL (2.08 ± 0.08) group in comparison to CAL (2.85 ± 0.08). In case of DIF plasma albumin (2.4 ± 0.07) was increased significantly in comparison to DAL. So, IF protects the depletion of albumin through urinary excretion indicating improved functional state of nephron. Moreover, plasma HDL cholesterol was significantly decreased in DAL (21 ± 3.62) groups in comparison to CAL (32 ± 0.89) group while it gets restored to normal level in DIF (31 ± 1.48) group. However, we failed to find any significant difference in plasma glucose levels between DAL and DIF group. Restoration of biochemical parameters like BUN, plasma creatinine, plasma albumin and plasma HDL-cholesterol of diabetic rats towards control rat values by IF suggests its role in providing protection in progression of DN (see Table 1).

3.4. IF decreases lipid peroxidation and increases SOD level

CR is known to increase the resistance to oxidative stress [25] and oxidative stress is one of the known culprits in the development of nephropathy [26]. We sought to assess the levels of oxidative stress markers like malondialdehyde (MDA) and SOD. IF lowers the oxidative stress because it decreases the lipid peroxidation and increases the SOD level. Lipid peroxidation significantly increases in DAL group (382.2 ± 29.2) as compared to CAL group (236.6 ± 9.76) while it was significantly lower in DIF group (255.83 ± 13.7). On the other side, MDA level in DIF group was closer to CAL and CIF groups suggesting that IF protects from oxidative stress [27] (Fig. 2A). SOD level was significantly decreased (9/C2450%) in DAL group as compared to CAL which was almost reversed by IF (Fig. 2B).
3.5. IF protects glomerular damage

Glomerular hypertrophy and glomerular injury is a prominent feature of diabetic rats kidney. Fig. 3 represents Mayers hematoxylin and eosin stained sections from CAL, CIF, DAL and DIF group. Kidney section of DAL rats showed marked microscopic changes like glomerular hypertrophy and vacuolations compared to CAL rats (see Fig. 3 A&C). The incidence and intensity of tubular vacuolations and glomerular hypertrophy as well as other degenerative structures were much lower in DIF (see Fig. 3 D).

3.6. Change in expression of p38 and caspase-3 by IF in diabetic rat kidney

It has been reported that hyperglycemic condition results in activation of p38 [28]. It is a proapoptotic protein that plays a pathological role in diabetic condition [13]. Our data indicates a significant increase in expression of p38 in DAL kidney (Fig. 4A). However, this increase in p38 level was prevented by IF, suggesting decrease in progression of DN. In case of diabetic rat kidney, there is an increase in cleavage of caspase-3 indicated by the appearance of 20 kDa cleaved fragment (Fig. 4B) as compared to the control rat kidney. Significant reduction in 20 kDa cleaved caspase-3 fragment was observed in diabetic rat kidney on IF (see Fig. 4B).

3.7. IF decreases expression of p53 in diabetic rat kidney

It has been reported that under hyperglycemic conditions expression of p53 significantly increases in the mouse blastocyst [29], and in myocytes [30]. We also observed increase in p53 expression in diabetic kidney (Fig. 4C). Moreover, our data shows that IF results in a marked reduction in the expression of p53 in diabetic kidney (Fig. 4C).

3.8. IF in diabetic rats increases Sir2 activity

The yeast Sir2 gene appears to promote survival in a wide range of organism and the mammalian ortholog of Sir2, sirtuins, represses the activity of p53 and therefore inhibits apoptosis. It has been shown that p53 is deacetylated and its expression is downregulated by Sir2. Thus, Sir2 negatively regulates p53 dependent apoptosis in response to cellular damage [31–33]. Fig. 4C shows Sir2 expression by Western blot analysis of IF diabetic rat kidney. Our result clearly shows that there is decrease in expression of Sir2 in DAL rat kidney (Fig. 4C, lane c). IF significantly increases its expression in DIF.
Thus, we provide indirect evidence that IF is working at two levels, on one hand it is reducing p53 expression and on the other hand it is also preventing its activation by activating deacetylating enzyme Sir2.

3.9. Change in histone H3 phosphorylation by IF in diabetic rat kidney

Activation of p38 under diabetic condition suggested us to look for downstream signaling molecules that result into modifying chromatin structure. Phosphorylation of histone H3 at serine 10 occurs usually when cells enters into mitosis [34]. Several toxicants have also been shown to induce histone H3 phosphorylation, which results into premature chromatin condensation and cell death [35]. Fig. 4D shows dephosphorylation of histone H3 under diabetic condition. However, IF to diabetic animals prevents histone H3 dephosphorylation. This change in histone H3 phosphorylation can only be explained if we assume that IF either directly or indirectly prevents cells to undergo mitotic arrest.

4. Discussion

Neuroprotective effect of IF studies have reported that IGF-1 signaling is involved in experimental models of neurodegenerative disorders [36,37]. However, there are no reports, till date, pertaining to the role of IF in DN and the mechanisms involved therein. IF results in a significant decrease in BUN and plasma creatinine levels after the 8 weeks of induction of diabetes. Rats on IF also show increased plasma albumin as well as HDL-cholesterol.

Chronic hyperglycemia increases oxidative stress and considerably modifies the structure and function of proteins and lipids, due to glycoxidation and peroxidation [38,39]. These
modified products could contribute to the morphological and functional abnormalities to the blood vessels as well as kidney which may lead to increase in blood pressure. If this persists for a longer time, it may lead to kidney failure. We observed increase in blood pressure in STZ induced diabetic rat after eight weeks of induction of diabetes. These DAL rats showed significant increase in mean, systolic and diastolic blood pressure as compared to CAL. Moreover, eight weeks of IF results in a significant decrease in mean systolic and diastolic blood pressure.

Oxidative stress has been suggested extensively as a potential mechanism for diabetic kidney disease because it promotes the formation lipid peroxidation products and decreases the antioxidant defense by decreasing the level of antioxidant enzymes like SOD, catalase and glutathione. The importance of oxidative stress in pathology is dependent on both its sources and its targets. In diabetes, hyperglycemia has been considered as the major culprit of oxidative stress induced damage inflicted on the various tissues including kidney leading to the development of diabetic nephropathy [26]. Remarkably, IF resulted in a significant increase in the SOD levels in the kidneys of diabetic rats. The MDA levels are also found to be reduced significantly in the diabetic rats on IF.

Recently, Sir2 gene was reported to be downregulated in the skeletal muscles of conditional knockout muscle insulin receptor knockout (MIRKO) mice [40]. We found decrease in level of Sir2 in diabetic kidney and IF prevents this decrease. These results further implicate this enzyme as a putative molecular marker of nephropathic condition. Although, the mechanisms of its down regulation in diabetic conditions are not till yet clear.

Sirtuins target many proteins that are not histones they have been demonstrated to bind and deacetylate p53 in vitro and in vivo [32,33,41]. p53 is transcriptional activator, and its activation results in cell cycle arrest, senescence or the initiation of programmed cell death. Over expression of sirtuins has been shown to inhibit p53-dependent apoptosis in response to DNA damage and oxidative stress [33]. Recently, it has been reported that increased expression of p53 gene under diabetic condition is associated with renal apoptosis [42]. We found increased expression of p53 protein in the kidneys of diabetic animals as compared to their respective controls. However, the expression of p53 is reduced significantly in the kidneys of diabetic rats on IF regimen. The expression as well as activation of p53 is thought to be mediated by Sir2 dependent deacetylation. They both share an inverse relationship as is evident from our results wherein the Sir2 expression is decreased and at the same time p53 is upregulated.

Attenuation of p53 expression in diabetic rats kept on IF regimen indicates the involvement of anti apoptotic cascades. Since caspase activation forms the mainstay of apoptotic events, therefore we evaluated the cleavage of caspase-3 in diabetic kidney. Caspases when activated initiate a cascade of events leading to cleavage of PARP and self autolysis resulting in the appearance of a 20 kDa fragment signifying apoptosis. Recently Susztak et al. [13] have demonstrated that activation of caspases-3 and p38 are involved in podocyte apoptosis during the onset of diabetic nephropathy. Our data also shows that cleaved 20 kDa fragment is markedly reduced in the kidneys of diabetic rats. Although the mechanism by which IF exerts its anti apoptotic effects is yet to be understood in detail but the decrease in caspase-3 cleavage by IF and also suppression and activation of p53 and Sir2, respectively. Suggest a cross talk between Sir2, p53 and caspase-3.

Stress activated protein kinase p38 is also known to be activated under diabetic conditions [28,43] and the role of p38 is well documented as an upstream mediator of apoptotic cell death. Interestingly, the expression of p38 is increased in the diabetic rat kidney which got deceased by IF. Activation of p38 MAPK pathway is known to induce phosphorylation of histone H3 [44]. However, we observed decrease in histone H3 phosphorylation (Ser-10) in the diabetic rat kidney. Interestingly, this decrease in phosphorylation of histone H3 is prevented in the kidneys of rat on IF. Phosphorylation of histone H3 at Ser10 facilitates the transcription of immediate early genes [45–49], whereas during mitosis such phosphorylation...
facilitates chromosome remodeling and condensation [50–52]. Hendzel et al. [53] have shown that, phosphorylation of H3 and apoptotic chromosome condensation are unrelated events and chromosome condensation can occur without phosphorylation of Ser-10. Thus, decrease in histone H3 phosphorylation under diabetic condition can not suggest that cells are getting prevented against apoptotic cell death. However, these results signify the regulatory role of histone phosphatases and histone kinases in diabetic conditions. The increase in phosphorylation of histone H3 in IF might be due to specific inhibition of histone phosphatases or activation of histone kinases. Our data provides indirect evidence that IF increases phosphorylation of histone H3 which is not histone kinase dependent rather it is histone phosphatase dependent. This can be explained if we assume histone kinases are activated by upstream kinase p38. However during IF, expression of p38 is downregulated (see Fig. 5).

In conclusion, we demonstrate the nephroprotective effect of IF in diabetes. Although the precise mechanisms leading to activation of Sir2 and downregulation of proapoptotic p53 in IF are yet to be understood in detail, but these results definitely underscore the role of dietary regimens in pathophysiological condition.

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