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Change in histone H3 phosphorylation, MAP kinase p38, SIR 2 and p53 expression by resveratrol in preventing streptozotocin induced type I diabetic nephropathy

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Abstract
Resveratrol has been reported to have a wide variety of biological effects. However, little is known regarding its role on phosphorylation of histone H3, MAP kinase p38, SIR2 and p53 in type I diabetic nephropathy (DN). Hence, the present study was undertaken to examine changes in the above said parameters by resveratrol treatment. Male Sprague-Dawley rats were rendered diabetic using a single dose of streptozotocin (55 mg/kg, *i.p.*). DN was assessed by measurements of blood urea nitrogen and creatinine levels. Phosphorylation of histone H3, SIR2, p53 and MAP kinase p38 expression were examined by western blotting. This study reports that treatment of resveratrol prevents the decrease in the expression of SIR2 in diabetic kidney. It also prevents increase in p38, p53 expression and dephosphorylation of histone H3 in diabetic kidney. This is the first report which suggests that protection against development of diabetic nephropathy by resveratrol treatment involves change in phosphorylation of histone H3, expression of Sir-2, p53 and p38 in diabetic kidney.

Keywords: *Resveratrol, diabetic nephropathy, SIR2, oxidative stress, p38, H3 phosphorylation, p53*

Abbreviations: *STZ, streptozotocin; SIR2, silent information regulator 2; MAP, mitogen activated protein; TBARS, thiobarbituric acid reactive substances; ROS, reactive oxygen species; SOD, superoxide dismutase; BUN, blood urea nitrogen.*

Introduction

Diabetic nephropathy (DN) is the leading cause of end stage renal disease worldwide and is associated with increased cardiovascular risk. It can be defined as a progressive rise in urine albumin excretion, coupled with increasing blood pressure, leading to declining glomerular filtration and eventually end stage renal failure [1]. Current therapeutic approach or treatment can slow down the development of disease but not stop the progression of end stage renal failure, i.e. glycaemic and blood pressure control [2].

Increased oxidative stress in diabetes is shown to play a pivotal role in the pathogenesis of diabetic nephropathy and inhibition of oxidative stress ameliorates the manifestations associated with diabetic nephropathy. Reactive oxygen species (ROS) can damage cellular macromolecules and act as proapoptotic agents [3]. Persistent hyperglycemia generates a hyperosmotic shock environment. Osmotic stress

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triggers various cell responses, including a significant increase in apoptosis, involving tubular and interstitial cells of cortex and medulla. Apoptosis, widely observed in different cells of various organisms, shows a unique morphological pattern of cell death characterized by chromatin condensation, membrane blebbing and cell fragmentation. Inter-nucleosomal DNA cleavage is the most prominent event in apoptosis and thus widely used as a marker of cell death process. It has been demonstrated that hyper osmotic shock-induced cell apoptosis and the accompanying biochemical changes, i.e. activation of caspase-3 and DNA fragmentation in the cells. Antioxidants, such as ascorbic acid and α-tocopherol block this cell induced apoptosis [4].

There is significant increase in apoptosis in the tubular and interstitial cells during the course of progression of diabetic nephropathy [5,6]. Stress-activated signalling pathways such as NF-κB and p38 MAPK underlie the development of diabetic complications [7]. Previously, it has also been reported that hyperglycemia can activate the p38 MAPK pathway in many cell types, including renal cells [8,9]. Activation of p38 is known to be involved in histone H3 phosphorylation that results in modifying chromatin structure [10]. Phosphorylation of histone H3 at Ser10 facilitates the transcription of immediate early genes [11,12]. However, the exact link between the activation and p38 MAPK and histone H3 phosphorylation at Ser 10 and transcription of genes under type I diabetic condition is not well established.

Resveratrol (trans-3,4',5-tri-hydroxystilbene), is a natural phytoalexin known to extend the life span of evolutionary distant species including *S. cervisiae*, *C. elegans* and *D. melanogaster* in a SIR2 dependent manner [13–16]. Howitz et al. [13] have reported that resveratrol, a polyphenol found in red wine, is the potent activator of SIRT1 for both the acetylated substrate and NAD(+) and increases cell survival by stimulating SIRT1-dependent deacetylation of p53. They have also reported that it mimics effects of calorie restriction by stimulating SIR2, in yeast. Resveratrol has also been shown to shift the physiology of middle aged mice on a high calorie diet towards that of mice on a standard diet [17]. Moreover, resveratrol is also reported to have a wide variety of biological effects [18] as an antioxidant [19], as an anti-cancer agent [20] and also as anti-inflammatory [21]. However, little is known regarding its effect on post-translational modification of histone H3, p38, SIR2 and p53 expression in type I diabetic kidney. Therefore, the present study was undertaken to examine changes in histone H3 phosphorylation, SIR2, p53 and MAP kinase p38 expression by resveratrol in Streptozotocin (STZ) induced type I diabetic nephropathy.

Materials and methods

Chemicals

All the chemicals were purchased from Sigma (St. Louis, MO), unless otherwise mentioned.

Animal treatment

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 (SD) 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 $22 \pm 2^{\circ}$C and 12 h light/dark cycles. After 1 week of acclimatization, animals were randomly divided into two groups at the start of the experiment. In the first group, type I diabetes was induced as described previously [22]. Briefly, diabetes was induced by injecting a single dose of streptozotocin (STZ) (55 mg/kg, *i.p.* dissolved in ice cold sodium citrate buffer, 0.01 M, pH 4.4). Age matched control rats received sodium citrate buffer. Animals with plasma glucose level > 250 mg/dl after 48 h post-induction of diabetes were included in the study as diabetic animals. Diabetic animals after 2 weeks were divided into two groups, namely diabetic/control ($n = 6$) and diabetic/treated with resveratrol (10 mg/kg/day, *ip*, for 4 weeks, $n = 6$). Along with these groups, there was one age matched normal/control group ($n = 6$) and one normal/with resveratrol treated group ($n = 6$). Treatment of resveratrol was started from the 5th week and continued until the end of week 8 (4 weeks treatment). Each animal in the control group received 0.25 ml/day of vehicle, *ip*.

Estimation of plasma glucose, blood urea nitrogen and creatinine

Blood samples were collected from rat tail vein under light ether anaesthesia in heparinized centrifuge tubes and immediately centrifuged at 2300 g for the separation of plasma. Plasma was stored at -80°C until assayed. The plasma was used for the estimation of glucose, blood urea nitrogen (BUN) and creatinine as described previously [22,23].

Blood pressure recording

Blood pressure (Systolic, mean and diastolic) was recorded at the 8th week post-STZ administration, using a tail cuff blood pressure recorder (IITC INC, Life Science Instruments, CA). Blood pressure was measured three times for each rat and the average was calculated, as described previously [22].

Table I. Effect of resveratrol on body weight (g), kidney weight (g) and kidney weight/body weight ratio in diabetic animals. Body weight and kidney weight were taken after 8 weeks.

Groups	Body weight (BW) in g	Kidney weight (KW) in g	KW/BW ratio*1000
Normal/control	391 ± 18	0.76 ± 0.03	1.9 ± 0.05
Normal/resveratrol treated	390 ± 11	0.71 ± 0.05	1.8 ± 0.06
Diabetic/control	181 ± 14***[a]	1.23 ± 0.13*[a]	6.8 ± 0.07***[a]
Diabetic/resveratrol treated	227 ± 8	0.85 ± 0.02*[b]	3.7 ± 0.18***[b]

All the values were represented as Mean ± SEM ($n = 6$), *** $p < 0.001$, * $p < 0.05$.
[a] significantly different from normal/control; [b] significantly different from diabetic/control.

Assessment of renal oxidative stress markers

Oxidative stress markers were measured as described previously [22]. Briefly, after sacrificing rat, the kidneys were excised and rinsed with normal saline and weighed. After weighing, kidney tissue was minced properly and the homogenate was prepared in cold phosphate-buffered saline (pH 7.4) and centrifuged at 700 g. Supernatant was collected and used for estimations. The lipid peroxide level in animal tissues was measured according to the method described by Ohkawa et al. [24]. SOD activity was estimated according to the method described by Paoletti and Mocali [25].

Protein isolation and western blotting

Nuclei, histone isolation and western blotting were performed in kidney tissues as described previously [22,23,26]. Immunoblot analysis was performed by using Anti p-Histone H3 ser-10 (rabbit 1:2000, Santa Cruz, CA), Anti Histone H3 (rabbit 1:5000, Upstate, Lake Placid, NY), Anti p53 (mouse monoclonal, 10 μg/ml, Calbiochem), Anti Sir 2 (rabbit 1:500, Sigma, St. Louis, MO), Anti p38 (rabbit 1:500, Santa Cruz, CA), Anti-actin (rabbit 1:2500, Sigma, St. Louis, MO) and HRP-conjugated secondary antibodies (anti-rabbit) from Santa Cruz (CA). Proteins were detected with the enhanced chemiluminescence (ECL) system and ECL Hyperfilm (Amersham Pharmacia Biotech, UK Ltd, Little Chalfont, Buckinghamshire, UK).

Table II. Effect of resveratrol treatment on plasma glucose (PGL), blood urea nitrogen (BUN) and plasma creatinine (PCR) in diabetic rats. Biochemical parameters were estimated after 8 weeks.

Groups	PGL (mg/dl)	BUN (mg/dl)	PCR (g/dl)
Normal/control	107 ± 2	24 ± 1	1.2 ± 0.08
Normal/resveratrol treated	119 ± 3	21 ± 2	1.3 ± 0.05
Diabetic/control	452 ± 30***[a]	56 ± 4***[a]	1.8 ± 0.11***[a]
Diabetic/resveratrol treated	418 ± 13	37 ± 2**[b]	1.0 ± 0.1**[b]

All the values were represented as Mean ± SEM ($n = 6$), *** $p < 0.001$, ** $p < 0.01$.
[a] significantly different from normal/control; [b] significantly different from diabetic/control.

Statistical analysis

Experimental values are expressed as mean ± SEM. 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.

Results

Change in body weight and kidney weight/body weight ratio by resveratrol treatment

Diabetic animals showed significant decrease in body weights and increase in kidney weight. Moreover, kidney weight/body weight ratio doubled as compared to normal/control rats. Treatment with resveratrol prevents weight loss in diabetic rats up to a certain extent. However, resveratrol treatment leads to a significant reduction in the kidney weight/body weight ratio as compared to diabetic/control rats (Table I).

Effect of resveratrol treatment on plasma glucose, plasma creatinine and BUN

A plasma glucose level of diabetic rats was significantly higher than the normal/control group. Diabetic rats treated with resveratrol did not show any significant decrease in glucose level. An increase in plasma creatinine level was observed in diabetic/control rats as compared to normal/control group. However, treatment with resveratrol for 4 weeks significantly decreased plasma creatinine level in diabetic rats (Table II). Similarly, BUN of diabetic/control rats was significantly higher than in the normal/control group and there was a significant drop in its level on treatment with resveratrol (Table II). Increase in plasma creatinine level and BUN level indicates the development of diabetic nephropathy in rats [27,28].

Effect of resveratrol treatment on haemodynamic parameters

In diabetic/control rats systolic blood pressure (SBP), mean blood pressure (MBP) and diastolic blood pressure (DBP) were significantly higher as compared

Table III. Effect of resveratrol treatment on systolic blood pressure (SBP), mean arterial blood pressure and diastolic blood pressure (DBP) of diabetic rats. Blood pressure was measured after 8 weeks.

Groups	SBP (mmHg)	MAP (mmHg)	DBP (mmHg)
Normal/control	113±0.7	91±0.3	81±0.4
Normal/resveratrol treated	116±1.6	89±0.7	79±1.4
Diabetic/control	144±2.1***[a]	120±1.7***[a]	109±1.3***[a]
Diabetic/resveratrol treated	120±6.4**[b]	99±4.9***[b]	88±4.4***[b]

All the values were represented as Mean±SEM ($n=6$), *** $p<0.001$, ** $p<0.01$.
[a] significantly different from normal/control; [b] significantly different from diabetic/control.

to the control group indicating the development of hypertension. However, treatment with resveratrol significantly improved hypertensive condition of diabetic rats (Table III).

Effect of resveratrol treatment on oxidative stress markers

Treatment of resveratrol showed significant change in thiobarbituric acid reacting substances (TBARS) and superoxide dismutase (SOD) levels in diabetic rats. Diabetic/control rats show higher levels of TBARS as compared to normal/control rats (see Figure 1A). Treatment with resveratrol significantly reduces the levels of TBARS in diabetic rat kidney. Diabetic/control animals also show significant decrease in SOD level as compared to normal/control animals (see Figure 1B). Treatment with resveratrol leads to an increase in the SOD activity in diabetic animals. However, resveratrol *per se* did not show any significant effect on oxidative stress markers (see Figures 1A and B).

Resveratrol treatment decreases the expression of p38 in diabetic rat kidney

It has been reported that hyperglycemic conditions also result in p38 activation [29]. Proapoptotic protein p38 plays a pathological role in diabetic condition [8]. Level of p38 expression was checked on resveratrol treatment in diabetic rats. Our data

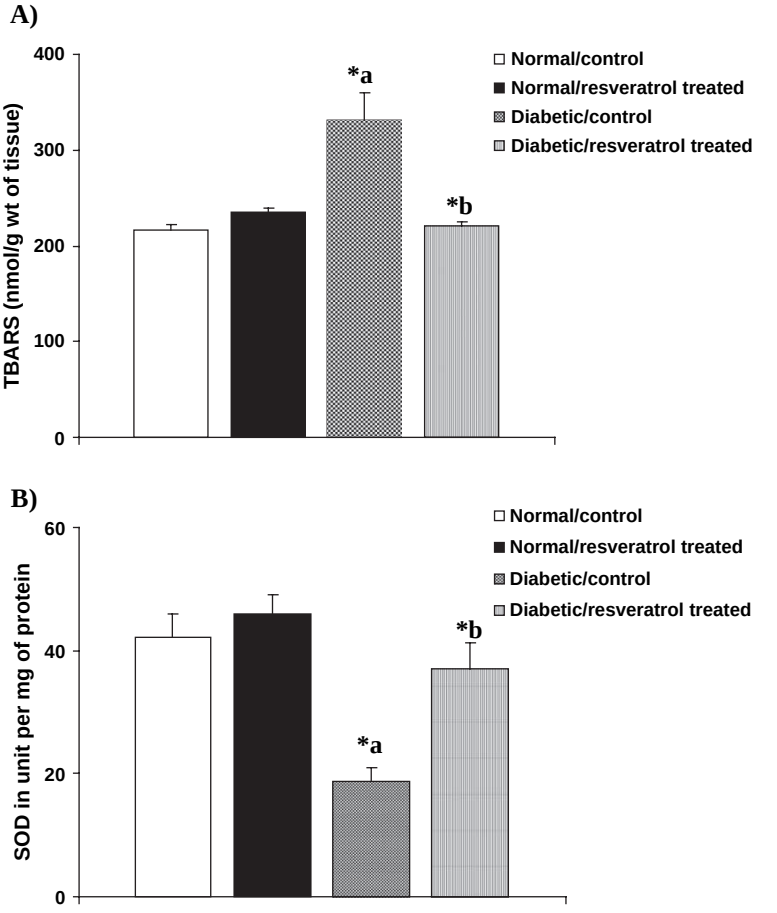

Figure 1. Effect of resveratrol treatment on TBARS and SOD levels in diabetic rat kidney: (A) TBARS and (B) SOD. All the values were represented as Mean±SEM ($n=6$), *$p<0.05$; Where, a vs normal/control group and b vs diabetic/control.

indicate significant increase in expression of p38 in diabetic kidney (Figure 2A, lane b). However, this increase was prevented by resveratrol treatment (Figure 2A, lane c).

Treatment of resveratrol changes the expression of p53 and SIR2 in diabetic rat kidney

Under hyperglycemic conditions expression of p53 significantly increases in the mouse blastocyst [30] and in myocytes [31]. We also observe increase in p53 expression in diabetic kidney (Figure 2B, lane b). Moreover, our data shows that treatment of resveratrol shows a decrease in expression of p53 in diabetic kidney (Figure 2B, lane c). p53 is deacetylated and its expression is down-regulated by SIR2 [32]. Thus, SIR2 negatively regulates p53-dependent apoptosis in response to cellular damage [33]. Our results show that there is a decrease in expression of SIR2 in

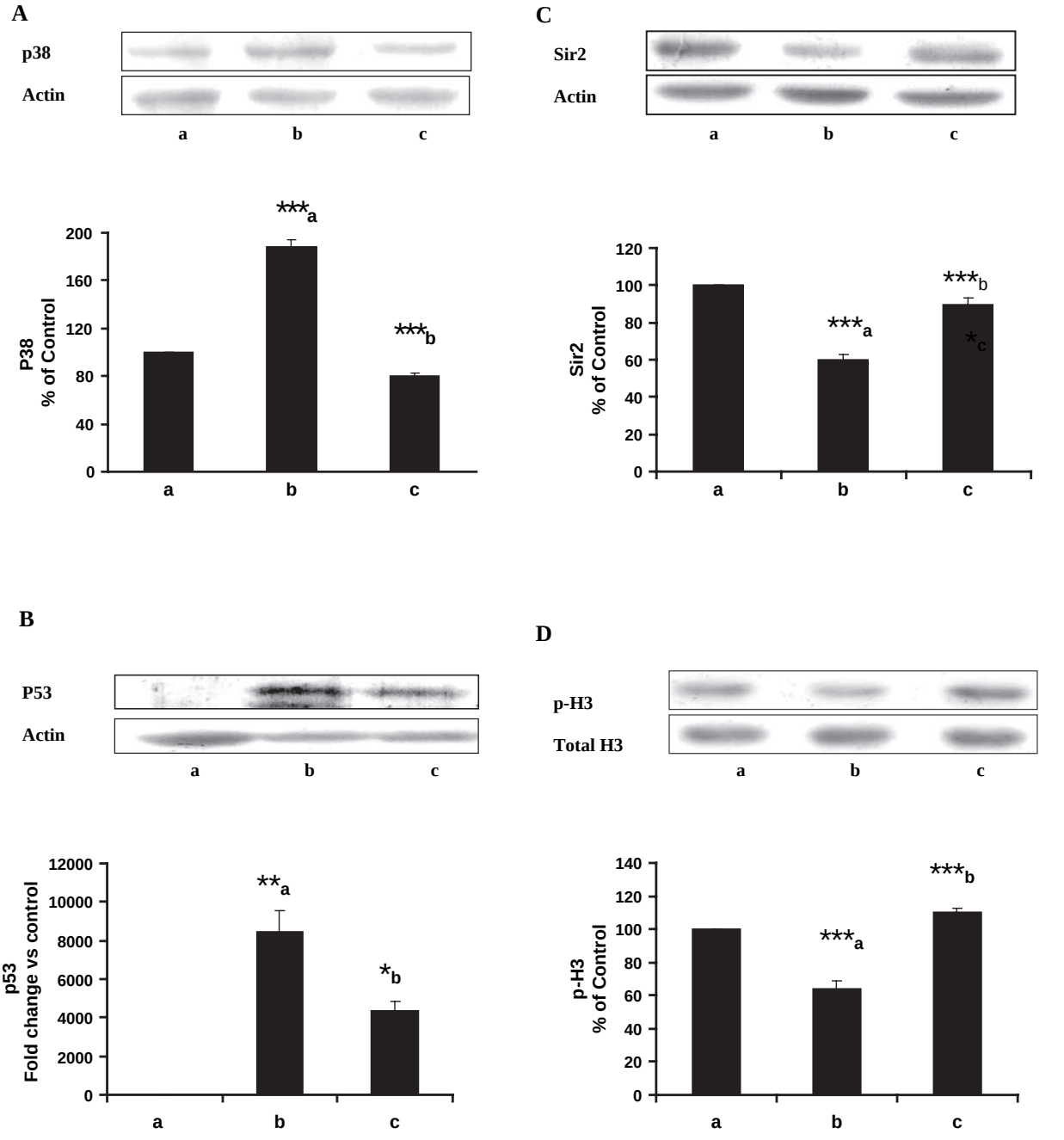

Figure 2. Western blot of p38, p53, SIR2 and p-Histone H3 in rat kidney after 4 weeks treatment of resveratrol: Western blot of (A) p38, (B) p53, (C) SIR2 and (D) p-H3 (Ser-10). Where, a (normal/control), b (diabetic/control) and c (diabetic/resveratrol treated). Similar results were obtained in three independent experiments. All the values were represented as Mean ± SEM, ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; Where, a vs normal/control and b vs diabetic/control.

diabetic rat kidney (Figure 2C, lane b). However, treatment of resveratrol significantly increased SIR2 expression in diabetic condition (Figure 2C, lane c). This can only be explained if we assume that resveratrol directly or indirectly protects cells from undergoing apoptosis.

Resveratrol prevents dephosphorylation of histone H3 in diabetic kidney

Phosphorylation of histone H3 at serine 10 occurs usually when cells enter into mitosis [34]. Several toxicants have also been shown to induce histone H3 phosphorylation, which results into premature chromatin condensation and cell death [10,35]. Figure 2D, lane b shows dephosphorylation of histone H3 under diabetic condition. However, treatment of resveratrol prevents histone H3 dephosphorylation (Figure 2D, lane c). Change in histone H3 phosphorylation suggests that resveratrol is preventing cells undergoing mitotic arrest and eventually the process of cell death.

Discussion

In the present study, we provide evidence that resveratrol treatment shows protection against development of diabetic nephropathy. In rats under STZ-induced hyperglycemia, chronic administration of resveratrol protects against kidney damage and concurrently attenuates oxidative stress in these tissues. Apart from showing protection in biochemical and oxidative stress parameters by resveratrol treatment, our data also shows the anti-apoptotic role of resveratrol in protecting animals from developing diabetic nephropathy. Besides anti-cancer activities [36], resveratrol also exhibits pronounced antioxidant properties by its ability to inhibit hydrogen peroxide- or lipid hydroperoxide dependent lipid peroxidation of cellular membrane lipids [37]. In addition to its free radical scavenging and anti-apoptotic properties, resveratrol also exhibits an anti-inflammatory activity via down-regulation of COX-2 [38], nitric oxide synthase through suppression of NF-κB activation [39] and an oestrogenic agonistic activity [40] as its structure resembles a synthetic oestrogen, diethylstilbestrol. This pharmacological action is very useful in preventing mortality due to infection during diabetes, because diabetic mellitus patients suffer from infections more often than normal individuals.

In the present study, under diabetic nephropathic condition, kidney weight to body weight ratio was increased. Treatment of resveratrol to these animals significantly decreases kidney weight to body weight ratio. However, we failed to observe any significant effect on body weight and hyperglycemia by resveratrol treatment. Moreover, treatment with resveratrol lowers blood urea nitrogen and plasma creatinine in diabetic animals significantly. This can be well explained if we consider increased clearance of blood urea and creatinine by kidney or due to decreases in protein degradation under diabetic condition.

Our results suggest that resveratrol treatment has a substantial effect on lowering blood pressure. It lowers systolic, diastolic and mean arterial blood pressure. This may be due to resveratrol mediated decrease in oxidative stress. Free radicals react with phenolic compounds much faster than with lipids or DNA. Therefore, phenols protect lipids and DNA from oxidative damage. A direct neuroprotective effect of resveratrol against oxidative stress has been demonstrated in PC12 cells [41].

Apart from showing protection in biochemical and oxidative stress parameters, our data also shows the anti-apoptotic role of resveratrol in protecting animals from developing diabetic nephropathy. Our results demonstrate that resveratrol protects the development of diabetic nephropathy by directly or indirectly inhibiting cell death pathways. Several reports show that stress activated kinase p38 is activated during diabetic condition [29,42]. We have also reported an increase in expression of p38 in diabetic kidney and intermittent fasting (IF) prevents this increase [22]. Consistent with our previous reports, treatment of resveratrol also prevents an increase in expression of p38 in diabetic kidney.

Stress activated protein kinase; p38 is known to modify chromatin structure by changing post-translational modifications of histones [10]. Phosphorylation of histone H3 at Ser 10 usually happens only when either cell enter into mitosis or during premature chromatin condensation [43]. Moreover, it is also important for transcriptional regulation in the transcription of immediate early genes [11,12,44]. Phosphorylation of histone H3 Ser 10 has been reported to be linked with initiation of chromosome condensation in G(2) and for proper chromosome segregation at mitosis [44]. Recently, we have shown that there is a decrease in phosphorylation of histone H3 in diabetic kidney and this decrease in phosphorylation was prevented by IF [22]. Dephosphorylation of histone H3 suggests that cells are under mitotic arrest. Treatment of resveratrol prevents decrease in histone H3 phosphorylation in diabetic kidney. It can be easily assumed that resveratrol also prevents cells undergoing mitotic arrest during the progression of diabetic nephropathy.

Phosphorylation of H3 and apoptotic chromosome condensation is unrelated events and chromosome condensation can occur without phosphorylation at Ser-10. Hendzel et al. [45] have also reported chromatin condensation is also not associated with apoptosis. The increase in phosphorylation of histone H3 in resveratrol treated rats might be due to specific inhibition of histone phosphatase or activation

of histone kinases (MSK1 and RSK2). Our data provides indirect evidence that resveratrol increases phosphorylation of histone H3 which is not MAPK p38 dependent rather it is histone phosphatase dependent. It can be explained if we assume histone kinases (MSK1 and RSK2) are activated by upstream kinase p38.

Recently, it has been reported that SIRT1 can prevent oxidative stress induced apoptosis through p53 deacetylation in messengial cells and upregulation of SIRT1 may provide a new strategy for preventing kidney glomerular diseases [32]. Moreover, it has been reported that increased expression of p53 gene under diabetic condition is associated with renal apoptosis [46]. We also provide evidence regarding an inverse relationship between SIR2 and p53 in diabetic rat kidney. Resveratrol is able to prevent the decrease in SIR2 and increase in p53 expression in diabetic kidney, which might be responsible for preventing apoptotic cell death in diabetic kidney.

Hence, keeping in mind the above observations further studies in this direction are to be planned to elucidate the complete mechanism and cross-talk between different pathways involved in anti-diabetic and nephroprotective effect of resveratrol. Understanding the mechanism involved in resveratrol action can have potentially profound clinical implications.

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