Resveratrol, a Polyphenolic Phytoalexin, Attenuates Diabetic Nephropathy in Rats

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Key Words
Diabetic nephropathy · Lipid peroxidation · Oxidative stress · Resveratrol · Streptozotocin

Abstract
Diabetic nephropathy is a serious microvascular complication and one of the main causes of end-stage renal disease. Various studies have revealed that increased oxidative stress is a major pathophysiological mechanism which is involved in the etiology of diabetic nephropathy. Resveratrol, a polyphenolic phytoalexin present in red wine, is known to possess potent antioxidant properties and thus we aimed to examine its effect on renal function and oxidative stress in streptozotocin (STZ)-induced diabetic rats. Diabetes was induced by a single intraperitoneal injection of STZ (65 mg/kg) in rats. After 4 weeks of STZ injection, rats were divided into four groups: the control rats, diabetic rats and diabetic rats treated with resveratrol (5 and 10 mg/kg, orally) respectively from week 4 up till week 6. At the termination of the experiments, urine albumin excretion, urine output, serum creatinine, blood urea nitrogen, creatinine and urea clearance were measured. The levels of the renal oxidative stress markers malonaldehyde and glutathione and the antioxidant enzymes superoxide dismutase and catalase were measured in kidney homogenate. STZ-injected rats showed significant increases in blood glucose, polyuria, proteinuria and a decrease in body weight compared with age-matched control rats. After 6 weeks, diabetic rats exhibited renal dysfunction, as evidenced by reduced creatinine and urea clearance, and proteinuria along with a marked increase in oxidative stress, as determined by lipid peroxidation and activities of key antioxidant enzymes. Treatment with resveratrol significantly attenuated renal dysfunction and oxidative stress in diabetic rats. The present study reinforces the important role of oxidative stress in diabetic kidney and points towards the possible antioxidative mechanism being responsible for the renoprotective action of resveratrol.

Introduction
Diabetic nephropathy is a microvascular complication of diabetes mellitus morphologically characterized with the thickening of the glomerular basement membrane and by expansion of the mesangial matrix which correlates with glomerular filtration function. Recent studies indicate that reactive oxygen species (ROS) play a key intermediate role in the pathophysiology of diabetic nephropathy [1, 2]. Chronic hyperglycemia, the main determinant of initiation and progression of diabetic nephropathy, not only generates more reactive oxygen metabolites but also attenuates an antioxidative mechanism through non-enzymatic glycosylation of the antioxidant
enzymes [3, 4]. In vitro studies with cultured mesangial cells also reveal that elevated glucose concentrations increase collagen synthesis similar to the in vivo situation [5, 6]. These studies show that hyperglycemia may be toxic either by non-enzymatic reaction of glucose with proteins and subsequent formation of advanced glycosylation end products or by increased metabolism leading to increased oxidative stress and activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) resulting in increased production of cytokines [7].

Resveratrol, a naturally occurring phytoalexin, is present in dietary sources including red wine [8, 9]. Phytoalexins are chemical substances produced by plants as a defense against infection by pathogenic microorganisms such as fungi. Resveratrol (3,4',5-trihydroxystilbene) is reported to have many beneficial effects on human health against atherosclerosis and cardiovascular diseases, inhibition of proliferation of a number of cell lines and antiplatelet activity [9]. Resveratrol has also been found to exert a strong inhibitory effect on superoxide anion and hydrogen peroxide production by macrophages stimulated by lipopolysaccharide or phorbol esters. It has hydroxyl-radical scavenging activity and has recently also been found to possess glutathione-sparing activity [10]. Due to the strong implication of oxidative damage in diabetic nephropathy, the present study was designed to evaluate the effect of resveratrol on renal function, lipid peroxidation and activities of antioxidant enzymes in the kidneys of streptozotocin (STZ)-induced diabetic rats.

Materials and Methods

Animals

Male Sprague-Dawley rats (200–250 g) bred in the Central Animal House facilities of Panjab University were used. Animals were housed under optimal conditions with food and water ad libitum. All experimental protocols were approved by the Institutional Animal Ethics Committee and were conducted according to the Institutional Animal Ethics Committee and the Indian National Science Academy guidelines for the use and care of the animals [11].

Induction and Assessment of Diabetes

STZ (Sigma, St. Louis, Mo., USA) prepared in 0.1 mol/l citrate buffer, pH 4.4, was injected in a single dose of 65 mg/kg i.p. to rats [12]. The age-matched control rats received citrate buffer and were used along with diabetic animals. Diabetes was confirmed after 48 h of STZ injection, the blood samples were collected through the tail vein and plasma glucose levels were estimated by enzymatic GOD-PAP (glucose oxidase peroxidase) diagnostic kit method [13] (Span Diagnostic Chemicals, India). Rats having plasma glucose levels >250 mg/dl were included in the present study [14].

Treatment Schedule

Four weeks after the STZ injection, control and diabetic rats were randomly selected and divided into four groups of 6–7 animals each, i.e. control, diabetic control, diabetic group treated with resveratrol 5 mg/kg and diabetic group treated with resveratrol 10 mg/kg orally. Starting from week 4 till week 6, the control and diabetic control groups received vehicle of resveratrol and treated diabetic groups received a suspension of resveratrol (5 and 10 mg/kg b.w./day) orally respectively. The resveratrol (Sigma) suspension was prepared in 0.5% carboxymethylcellulose solution. All these drugs were administered in a constant volume of 0.5 ml/100 g b.w. of rat.

Blood and Urine Chemistries

At the end of the 6th week, rats were kept individually in metabolic cages for 24 h to collect urine for the measurement of urine output and renal function. Renal function was assessed by measuring plasma and urine levels of creatinine, urea and urine albumin excretion using a semi-auto-analyser (Erba Chem-5 plus; Transasia, Mumbai, India). Creatinine and urea clearance were measured as an index of glomerular filtration rate (GFR). Plasma glucose levels were also measured at 4 weeks and at the end of the experiment to investigate the effect of resveratrol on glucose levels.

Assessment of Renal Oxidative Stress

Preparation of Renal Homogenate.

Assessment of Renal Oxidative Stress

Superoxide Dismutase Activity.

Preparation of Renal Homogenate. MDA, an index of lipid peroxidation, was estimated according to the method of Wills [15]. The amount of MDA formed was measured by acid heating reaction with thiobarbituric acid at 532 nm using an Erba Chem-5 plus semi-auto-analyser (Transasia). The results are expressed as nmol of MDA per mg protein using the molar extinction coefficient of chromophore (1.56 × 10^5 mol⁻¹ cm⁻¹).

Estimation of Reduced Glutathione. Reduced GSH in the kidney was estimated according to the method of Ellman [16]. The homogenate (0.75 ml) was precipitated with 0.75 ml of 4% sulfosalicylic acid. Samples were centrifuged at 1,200 g for 15 min at 4°C. The assay mixture contained 0.5 ml supernatant and 4.5 ml of 0.001 mol/l (in 0.1 mol/l phosphate buffer, pH 8.0) 5,5'-dithiobis-(2-nitrobenzoic acid). The yellow color that developed was read immediately at 412 nm using an Erba Chem-5 plus semi-auto-analyser (Transasia). Results are expressed as nmol GSH/mg protein.

Superoxide Dismutase Activity. SOD activity was assayed according to the method of Kono [17], wherein the reduction of nitroblue tetrazolium inhibited by SOD is measured at 560 nm using an Erba Chem-5 plus semi-auto-analyser. Briefly, the reaction was initiated by the addition of hydroxylamine hydrochloride to the reaction mixture containing nitroblue tetrazolium and post-mitochondrial fraction of the kidney homogenate. Results are expressed as units/mg protein, where 1 unit of SOD enzyme is defined as the amount of enzyme inhibiting the rate of dismutation reaction by 100%.

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**Catalase Activity.** Catalase activity was assayed according to the method of Luck [18] in which the breakdown of hydrogen peroxide (H$_2$O$_2$) is measured at 240 nm. Briefly, the assay mixture consisted of 3 ml H$_2$O$_2$ phosphate buffer (1.25 $\times$ 10$^{-2}$ H$_2$O$_2$ mol/l) and 0.05 ml supernatant of the kidney homogenate (10%) and the change in absorbance was recorded at 240 nm using Erba Chem-5 plus semi-auto-analyser. Enzyme activity was calculated using the millimolar extinction coefficient of H$_2$O$_2$ (0.07). Results are expressed as mmol H$_2$O$_2$ decomposed/min/mg protein.

**Protein Estimation.** The protein content of the renal homogenate was measured according to the method of Lowry et al. [19] using bovine serum albumin as the standard.

**Renal Histology**

The kidneys were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Renal sections (5 $\mu$m) were cut, deparaffinized, hydrated and stained with haematoxylin and eosin. The renal sections were examined in a blind fashion for apical blebbing, interstitial fibrosis, hyaline casts, glomerular changes and arteriolopathy in all the treatments (see fig. 5). A minimum of 10 fields for each kidney slide were examined and assigned for severity of changes using scores on a scale of none (−), mild (+), moderate (++), and severe (+++) damage.

**Statistical Analysis**

Results were expressed as mean ± SEM. The significance of differences was analyzed using ANOVA followed by Dunnett’s t test. $p < 0.05$ was considered significant. Unpaired Student’s t test was used to compare differences between two groups.

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**Table 1.** Effect of 2 weeks of treatment with resveratrol (5 and 10 mg/kg, p.o.) on control and STZ-induced diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>Control</th>
<th>STZ</th>
<th>STZ + resveratrol (5)</th>
<th>STZ + resveratrol (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose level, mg/dl</td>
<td>113.30 ± 1.943</td>
<td>382.5 ± 16.52*</td>
<td>333.75 ± 7.181**</td>
<td>314.5 ± 9.432**</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>236.25 ± 3.75</td>
<td>168 ± 2.71*</td>
<td>175.46 ± 6.45</td>
<td>197.5 ± 4.78**</td>
<td></td>
</tr>
<tr>
<td>Food intake, g</td>
<td>28.25 ± 1.652</td>
<td>58.75 ± 2.689*</td>
<td>47.25 ± 1.11**</td>
<td>32.5 ± 1.71**</td>
<td></td>
</tr>
<tr>
<td>Water intake, ml</td>
<td>35.26 ± 1.23</td>
<td>108.25 ± 1.65*</td>
<td>85.5 ± 1.1***</td>
<td>48.26 ± 2.27**</td>
<td></td>
</tr>
<tr>
<td>Urine output, ml</td>
<td>9.37 ± 0.63</td>
<td>42.5 ± 0.816*</td>
<td>35.46 ± 1.08**</td>
<td>16.69 ± 0.47**</td>
<td></td>
</tr>
<tr>
<td>Urine albumin excretion, mg/dl</td>
<td>3.83 ± 0.497</td>
<td>11.25 ± 0.854*</td>
<td>8.46 ± 0.312**</td>
<td>6.75 ± 0.75**</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.31 ± 0.0261</td>
<td>1.42 ± 0.131*</td>
<td>0.89 ± 0.0389**</td>
<td>0.81 ± 0.0239**</td>
<td></td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>24.5 ± 0.645</td>
<td>58.25 ± 1.75*</td>
<td>50.5 ± 2.21**</td>
<td>43.52 ± 1.02**</td>
<td></td>
</tr>
<tr>
<td>Creatinine clearance, ml/24 h</td>
<td>0.71 ± 0.01</td>
<td>0.25 ± 0.02*</td>
<td>0.43 ± 0.06**</td>
<td>0.65 ± 0.02**</td>
<td></td>
</tr>
<tr>
<td>Urea clearance, ml/24 h</td>
<td>0.8 ± 0.06</td>
<td>0.31 ± 0.06*</td>
<td>0.47 ± 0.08**</td>
<td>0.7 ± 0.03**</td>
<td></td>
</tr>
<tr>
<td>BP, mm Hg</td>
<td>112.5 ± 3.27</td>
<td>205.14 ± 11.18*</td>
<td>178.86 ± 8.64</td>
<td>140.05 ± 10.69**</td>
<td></td>
</tr>
</tbody>
</table>

* $p < 0.05$ as compared to control group. ** $p < 0.05$ as compared to the STZ-treated group.

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**Results**

**Effect of Resveratrol on Blood Glucose, Body Weight, Water Intake, Food Intake, Blood Pressure and Renal Function of Diabetic Rats**

Four weeks after STZ injection, diabetic animals exhibited increased blood glucose levels (113.30 ± 1.943 and 382.5 ± 16.52 mg/dl) for control and diabetic rats, respectively; $p > 0.05$ and decreased body weight (236.25 ± 3.75 and 168 ± 2.71 g for control and diabetic rats, respectively; $p < 0.05$) compared with control rats. At the end of the 6 weeks, diabetic rats exhibited significantly increased plasma glucose levels and decreased body weights compared with control rats. Chronic treatment with resveratrol (5 and 10 mg/kg/day) in diabetic rats from the 4th to 6th week altered plasma glucose levels as compared with vehicle-treated diabetic rats. Treatment with a higher dose of resveratrol attenuated the decrease in body weight in diabetic rats but this effect was not significant as compared with vehicle-treated diabetic rats (table 1). Further, a significant increase in food intake, water intake and systolic blood pressure was observed in STZ-injected rats.

Diabetic rats exhibited marked polyuria, increased urinary albumin excretion, high serum creatinine as well as blood urea nitrogen. Chronic treatment with resveratrol at a higher dose (10 mg/kg) effectively reduced diabetic proteinuria, polyuria and increased serum creatinine and blood urea nitrogen. Creatinine and urea clearance were also significantly improved following the administration of resveratrol (5 and 10 mg/kg) to diabetic rats compared with untreated diabetic rats (table 1).
Effect of Resveratrol on Oxidative Stress Markers in Control and Diabetic Rats

Effect of Resveratrol on Renal MDA and GSH Levels. At the end of the experiment, diabetic rats showed a significant increase in lipid peroxidation as indicated by a marked increase in renal MDA and decrease in GSH levels as compared with vehicle-treated control rats. Chronic treatment of a higher dose of resveratrol (10 mg/kg/day) in diabetic rats significantly reversed the increased lipid peroxidation and decreased GSH as compared with vehicle-treated diabetic rats (fig. 1, 2).

Effect of Resveratrol on Renal SOD and Catalase Activities. At the end of the experiment, diabetic rats showed a significant decrease in the kidney SOD and catalase activities. Chronic administration of resveratrol (10 mg/kg/day) in diabetic rats significantly reversed the decrease in the kidney SOD and catalase activities as compared with vehicle-treated diabetic rats (fig. 3, 4).

Effect of Resveratrol on STZ-Induced Diabetic Renal Morphological Changes. The renal morphological changes observed were scored and summarized in table 2. The light microscopic findings of kidney of control rats showed normal glomeruli and efferent arterioles (fig. 5a). The kidneys of diabetic rats showed marked histological changes in the cortex and outer medulla as hyaline casts, glomerular thickening and moderate interstitial fibrosis and arteriolopathy (fig. 5b). The interstitial and glomerular alterations were attenuated by resveratrol treatment (5 and 10 mg/kg/day) in diabetic rats (fig. 5c, d).
Table 2. Effect of resveratrol (5 and 10 mg/kg) treatment on morphological changes as assessed by histopathological examination of kidney in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Group</th>
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<th>STZ + resveratrol (5)</th>
<th>STZ + resveratrol (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epical blebbing</td>
<td>–</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Glomerular thickening</td>
<td>–</td>
<td>+++</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>–</td>
<td>+++</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>Arteriolopathy</td>
<td>–</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Hyaline cast</td>
<td>–</td>
<td>++</td>
<td>++</td>
<td>–</td>
</tr>
</tbody>
</table>

– = None, + = mild, ++ = moderate, +++ = severe.

**Fig. 5.** Hematoxylin- and eosin-stained sections of rat kidneys: a normal glomeruli and arteriole of control rats; b glomerular thickening, interstitial fibrosis and arteriolopathy in diabetic rats; c, d glomeruli and arteriole of resveratrol-treated (5 and 10 mg/kg/day, respectively) diabetic rats that reduced the development of STZ-induced diabetic alterations in kidney.
Discussion

In the present study, STZ-injected rats demonstrated typical characteristics of diabetes mellitus such as hyperglycemia, polyuria, growth retardation and an increase in urinary albumin excretion. With the onset of diabetes mellitus there is a subsequent decrease in creatinine and urea clearance. It has also been observed that increased blood urea nitrogen and serum creatinine in diabetic rats indicates progressive renal damage which is taken as an index of altered GFR in diabetic nephropathy [20]. There have been reports that decreased GFR is associated with the formation of reactive oxygen intermediates [21]. In our results there was a significant correlation between renal dysfunction and renal oxidative stress. Chronic hyperglycemia, a well-recognized pathogenetic factor of long-term complications in diabetes mellitus, is reported to generate not only more ROS but also attenuates antioxidative mechanisms through glycation of the scavenging enzymes. Lipid peroxidation of unsaturated fatty acids, one of the major reactions in vivo, has been proven to be an index of increased oxidative stress and the subsequent cytotoxicity. In STZ-induced diabetic rats exhibiting albuminuria, a marker of glomerular injury in diabetes, there were significantly higher levels of lipid peroxides in renal homogenates suggesting increased oxidative stress in diabetic kidneys. A marked improvement in renal function by resveratrol in diabetic rats may involve its inhibitory effect on ROS, lipid peroxidation and subsequent formation of vasoactive mediators. Resveratrol has been found to exert a strong inhibitory effect on superoxide anion and H$_2$O$_2$ production by macrophages stimulated by lipopolysaccharides or phorbol esters [10]. It has hydroxyl-radical scavenging activity and has recently been found to possess GSH-sparing activity [22]. Moreover, resveratrol was found to strongly inhibit NO generation in activated macrophages, as measured by the amount of nitrite released into the culture medium, and resveratrol strongly reduced the amount of cytosolic iNOS protein and steady-state mRNA levels, thereby reducing the formation of peroxynitrite, a potent oxidant. As an antioxidant, resveratrol also reduced oxidative stress in blood platelets [23].

Direct high glucose-induced PKC and MAPK activation also caused increased production of oxidative stress in the diabetic kidney [24]. Hyperglycemia-induced PKC activation is one of the mechanisms responsible for the generation of free radicals through advanced glycosylation end product formation, the polycl pathway and cytokine production in diabetic nephropathy. Glucose-induced oxidative damage and the production of TGF-β are the final common mediators of the principal lesions of renal disease in diabetes mellitus and cause renal/glomerular hypertrophy and extracellular matrix expansion [25]. It has also been reported that resveratrol inhibited PKC activity, and therefore has an effect on associated signaling networks which may, in part, underlie the mechanism(s) by which this agent exerts renoprotective properties [26].

In conclusion, the findings of the present study strongly suggest that oxidative stress has a prime role in the pathophysiology of diabetic nephropathy and that resveratrol is able to attenuate the renal damage in diabetes through its antioxidative action. These results imply that resveratrol could be used as an adjuvant therapy with a conventional hypoglycemic regimen to treat diabetic complications.

Acknowledgement

The Senior Research Fellowship by the Defence Research Development Organisation (DRDO), New Delhi, is gratefully acknowledged.

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