Esculetin induced changes in Mmp13 and Bmp6 gene expression and histone H3 modifications attenuate development of glomerulosclerosis in diabetic rats

Vivek Madhukar Surse, Jeena Gupta and Kulbhushan Tikoo

Laboratory of Chromatin Biology, Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S. Nagar, Mohali, Punjab 160 062, India

(Correspondence should be addressed to K Tikoo; Email: tikoo.k@gmail.com)

Abstract

Esculetin, an antioxidant, has been used in the treatment of a variety of diseases. This study aimed to investigate the protective effect of esculetin in attenuating streptozotocin (STZ)-induced type I diabetic nephropathy and to understand the molecular mechanism involved in it. Sprague–Dawley rats were rendered diabetic using a single dose of STZ (55 mg/kg, i.p.). Protein expression of PPARγ and transforming growth factor-β1 (TGF-β1) was detected by immunoblotting and immunohistochemistry respectively. RNA expression levels of Mmp13 and Bmp6 were detected by RT-PCR analysis. In diabetic rats, esculetin treatment resulted in a significant decrease in blood glucose, blood urea nitrogen, and plasma creatinine and increase in plasma albumin levels. Esculetin treatment attenuates the downregulation of PPARγ in diabetic kidney, which in turn blocks the TGF-β1-mediated fibronectin expression. In addition, it attenuates the decrease in mono-methylation (K4) and acetylation of histone H3 in diabetic kidney. RT-PCR analysis revealed that esculetin treatment provides protection by decreasing antifibrotic Bmp6 and increasing fibrogenic Mmp13 mRNA expression in diabetic kidney. This is the first report to show that protection observed by esculetin treatment involves alteration in mRNA expression of Mmp13 and Bmp6 genes either directly via altered histone H3 modifications or indirectly by inhibiting the PPARγ/TGF-β1 pathway.

Journal of Molecular Endocrinology (2011) 46, 245–254

Introduction

A number of natural products with a coumarinic moiety have been reported to have multiple biological activities (Wu et al. 2007). In a similar way to isomeric flavonoids, coumarins might affect the formation and scavenging of reactive oxygen species (ROS) and influence processes involving free radical-mediated injury (Chang et al. 1996, Fylaktakidou et al. 2004). Esculetin is a coumarin derivative contained in many plants, such as Artemisia capillaris (Compositae), the leaves of Citrus limonia (Rutaceae; Chang et al. 1996), and Ceratostigma willmottianum (Yue et al. 1997) that are used as folk medicines. It has been shown to have multiple biological activities including the inhibition of xanthine oxidase activity (Egan et al. 1990), platelet aggregation (Okada et al. 1995), and protection against N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats (Matsunaga et al. 2005). It also decreases the activity of ferric soybean lipoxygenase 1 (Kemal et al. 1987) and 5-lipoxygenase (Neichi et al. 1983). In addition, esculetin shows antioxidant activity (Payá et al. 1992), an inhibitory effect on the growth of human breast cancer cells (Noguchi et al. 1995), and a synergistic effect with retinoic acid on the differentiation of human leukemia cells (Hofmanová et al. 1996). However, to date no report is available regarding its role in preventing the progression of diabetic nephropathy.

Diabetic nephropathy is one of the most common cause of end-stage renal failure (Zimmet et al. 2001). It is characterized by specific renal morphological and functional alterations associated with diabetes. Features of early diabetic renal changes are glomerular hyperfiltration, glomerular and renal hypertrophy, increased urinary albumin excretion, increased basement membrane thickness, and mesangial expansion with the accumulation of extracellular matrix (ECM) proteins (van Dijk & Berl 2004). Hyperglycemia-induced oxidative stress has been reported to be responsible for the development and progression of diabetic vascular complications including nephropathy (Prabhakar et al. 2007) by upregulating the transforming growth factor-β1 (TGF-β1) and the ECM protein expression in the glomerular mesangial cells (Sharma et al. 2005). ROS are also known to induce TGF-β1 expression, and this further activates stress-activated signaling pathways in diabetic kidney (Onozato et al. 2002). In addition, the interaction of TGF-β1 with its
receptors induces the phosphorylation and nuclear translocation of the receptor-regulated Smad2 and Smad3 transcription factors. Mesangial cells express Smad2/3, Smad4, and Smad7 (inhibitory Smad), which mediates TGF-β1-induced gene expression, including ECM genes such as plasminogen activator-1 and type I collagen and fibronectin (Bottinger & Bitzer 2002, Runyan et al. 2004).

Various antioxidants have been reported to prevent the upregulation of TGF-β1 and fibronectin induced by high-glucose and H2O2 (Ha & Kim 1999). Previous studies both in our laboratory and by other groups have reported that antioxidants such as lipoic acid (Packer et al. 2001), vitamin E (Lonn et al. 2002), curcumin (Tikoo et al. 2008) are able to prevent the progression of diabetic nephropathy. Hence this study was conceived to study the effect of esculetin in the progression of streptozotocin (STZ)-induced type I diabetic nephropathy and to understand the molecular mechanism involved in it.

### Materials and methods

#### Animal studies

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 and kept at controlled environmental conditions at room temperature 22±2°C and 12 h light:12 h darkness 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 (Tikoo et al. 2007b). Briefly, diabetes was induced by injecting a single dose of 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

#### Table 1  Effect of esculetin on blood urea nitrogen, plasma albumin, plasma glucose, and plasma creatinine levels

<table>
<thead>
<tr>
<th>Groups</th>
<th>Blood urea nitrogen (mg/dl)</th>
<th>Plasma albumin (g/dl)</th>
<th>Plasma glucose (mg/dl)</th>
<th>Plasma creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal/control</td>
<td>21±2</td>
<td>3.78±0.06</td>
<td>112±3</td>
<td>1.21±0.02</td>
</tr>
<tr>
<td>Normal/esculetin</td>
<td>16±3</td>
<td>4.08±0.13</td>
<td>118±2</td>
<td>1.24±0.12</td>
</tr>
<tr>
<td>Diabetic/normal</td>
<td>59±3†,a,b</td>
<td>2.23±0.06†,a,b</td>
<td>534±15†,a,b</td>
<td>2.30±0.09†,a,b</td>
</tr>
<tr>
<td>Diabetic/esculetin (50 mg/kg per day, p.o.)</td>
<td>31±2†,a,b,c</td>
<td>2.70±0.12†,a,b,c</td>
<td>447±8†,a,b,c</td>
<td>1.53±0.12†,c</td>
</tr>
<tr>
<td>Diabetic/esculetin (100 mg/kg per day, p.o.)</td>
<td>25±2†,a,b,c</td>
<td>2.93±0.02†,a,b,c</td>
<td>407±5†,a,b,c</td>
<td>1.42±0.14†,c</td>
</tr>
</tbody>
</table>

Blood urea nitrogen, plasma albumin, plasma glucose and plasma creatinine levels were estimated after 8 weeks. Values are represented as mean±S.E.M. (n=8), *P<0.05, †P<0.01, and ‡P<0.001.

*Normal/control.
*aNormal/esculetin.
*bDiabetic/normal.
*cDiabetic/esculetin (50 mg/kg/day, p.o.).

#### Table 2  Effect of esculetin on body weight, kidney weight and kidney/body weight ratio. Body weight, average kidney weight and average kidney/body weight ratio were estimated after 8 weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Average kidney weight (g)</th>
<th>Average kidney/body weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal/control</td>
<td>373±10</td>
<td>0.89±0.005</td>
<td>0.27±0.007</td>
</tr>
<tr>
<td>Normal/esculetin</td>
<td>374±15</td>
<td>0.84±0.031</td>
<td>0.23±0.013</td>
</tr>
<tr>
<td>Diabetic/normal</td>
<td>125±8†,a,b</td>
<td>1.06±0.022</td>
<td>0.81±0.051†,a,b</td>
</tr>
<tr>
<td>Diabetic/esculetin (50 mg/kg per day, p.o.)</td>
<td>170±12†,a,b,c</td>
<td>0.94±0.029</td>
<td>0.53±0.034†,a,b,c</td>
</tr>
<tr>
<td>Diabetic/esculetin (100 mg/kg/day, p.o.)</td>
<td>196±4†,a,b,c</td>
<td>0.90±0.037</td>
<td>0.50±0.017†,a,b,c</td>
</tr>
</tbody>
</table>

Values are represented as mean±S.E.M. (n=8), *P<0.05 and †P<0.001.

*Normal/control.
*aNormal/esculetin.
*bDiabetic/normal.
glucose level >16.7 mmol/l after 48 h of STZ injection were included in the study as diabetic animals. Diabetic animals after 4 weeks (when the rats develop characteristics of diabetic nephropathy; increased blood urea nitrogen (BUN), and creatinine) were divided into three groups, namely diabetic/control (n=8), diabetic/treated with esculetin (50 mg/kg per day, p.o., for 4 weeks, n=8), and diabetic/treated with esculetin (100 mg/kg per day, p.o., for 4 weeks, n=8). Along with these groups, there was one age-matched normal/control group (n=8) and two normal control with esculetin (50 mg/kg per day, p.o., 100 mg/kg per day, p.o. for 4 weeks, n=8) treated groups. At the end of 8 weeks, blood samples were collected and immediately centrifuged at 2300 g for the separation of plasma. The plasma was used for the estimation of glucose, albumin, BUN, and creatinine as described previously (Tikoo et al. 2007a,b).

Assessment of lipid peroxidation (thiobarbituric acid reacting substances) in plasma and kidney of diabetic rats

Renal oxidative stress was measured as described previously (Tikoo et al. 2007b). Briefly, after killing rats, the kidneys were excised and rinsed with normal saline and weighed. After weighing, the 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. (1979).

Histopathological evaluation and immunostaining

From each rat, parts of the kidney tissue were fixed in 10% formalin in PBS and embedded in paraffin. Sections (2 μm) were stained with hematoxylin and eosin. Glomerular damage was assessed using a semi-quantitative score by a blinded observer as follows: 0, no lesion; 1, <25% damage; 2, 25–49% damage; 3, 50–74% damage; 4, 75–100% damage, respectively, as described (Ninichuk et al. 2008). Totally, 15 glomeruli were analyzed per section. All immunohistological studies were performed on paraffin-embedded sections. The rabbit antibody was used as primary antibody: anti-fibronectin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, 1:50) and anti-TGF-β1 (Santa Cruz, 1:50). The intensity of spot was graded from 1 to 4 (1, slight or no color; 2, very low color; 3, moderate brown color; and 4, very intense brown color). The immunohistochemistry score was expressed as mean ± S.E.M. for each experimental group.

Histone extraction and immunoblotting

Kidneys were manually dissected, and histone isolation and western blotting were performed as described previously (Tikoo et al. 2008). Immunoblot analysis was performed by using anti-Ac-Histone H3 (K9/14; rabbit 1:5000, Upstate, Lake Placid, NY, USA), anti-Me-Histone H3 (K4) (rabbit 1:2000), anti-Histone H3 (rabbit 1:5000, Upstate), anti-PPARγ (rabbit 1:500), anti-actin (rabbit 1:2500, Sigma), and HRP-conjugated secondary antibodies (Santa Cruz). Proteins were detected with the enhanced chemiluminescence esculetin

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system and ECL hyperfilm (Amersham Pharmacia Biotech, UK Ltd). Immunoblots were quantitated by densitometric analysis and the exposures were in the linear dynamic range. The densitometry analysis was performed by Image J Software (NIH, Bethesda, MD, USA).

**PCR**

RNA was isolated from kidney using RNA extraction kit (Qiagen). After reverse transcription with Superscript II (Invitrogen), real-time RT-PCR was performed on a Light Cycler 480 (Roche) using Light Cycler Fast Start DNA master plus kit (Roche Diagnostics) and the specific forward and reverse primers (Midland Certified Reagent Company, Inc., Midland, TX, USA) for Bmp6 (left: 3'-GGGAGAGGACTGGAGCCCGG-5', right: 3'-AGGCCGGGAGATGGCTGTT-5') and Mmp13 (left: 3'-TGACCAAACCTTGCGGGGG-5', right: 3'-TTGTAGCCTTTGGAGCTGCTTGTCC-5'). After amplification, a melting curve analysis was performed to verify the specificity of the reaction. 18s gene (left: 3'-GCAATTATTCCCCATGACG-5', right: 3'-AGGGCCTCACTAACCATCC-5') was used as an internal control and results were expressed as fold change over control rats.

![Figure 2](http://dx.doi.org/10.1530/JME-10-0154) Esculetin decreases the renal fibrosis in diabetic kidney. (A) Images show representative TGF-β1 immunostaining in renal sections (original magnification 100×): (a) normal/control, (b) diabetic/normal, (c) diabetic/esculetin (50 mg/kg), (d) normal/esculetin (50 mg/kg). Arrows indicate increased TGF-β1 staining in renal sections from diabetic animals compared with control rats. TGF-β1 was identified by immunostaining on renal sections from all groups and the graph illustrates the mean percentage of immunohistochemical score ± S.E.M. from all rats in each group (n=8). Data represent mean ± S.E.M. from 15 cortical glomerular cross sections. **P<0.01 considered significantly different from normal/control rats and *P<0.05 considered significantly different from diabetic/normal rats. Increase in TGF-β1 was observed in STZ-induced type I diabetes indicating the development of renal fibrosis, and esculetin (50 mg/kg) decreases the expression of TGF-β1. (B) Images show representative fibronectin immunostaining in renal sections (original magnification 100×): (a) normal/control, (b) diabetic/normal, (c) diabetic/esculetin (50 mg/kg), (d) normal/esculetin (50 mg/kg). Arrows indicate increased fibronectin staining in renal sections from diabetic animals compared with control rats. Fibronectin was identified by immunostaining on renal sections from all groups and the graph illustrates the mean percentage of immunohistochemical score ± S.E.M. from all rats in each group (n=8). Data represent mean ± S.E.M. from 15 cortical glomerular cross sections. ***P<0.001 considered significantly different from normal/control rats and #P<0.05 considered significantly different from diabetic/normal rats. Increase in expression of fibronectin was observed in diabetic kidney indicating the development of renal fibrosis, and esculetin treatment prevents the accumulation of fibronectin. Full color version of this figure is available via http://dx.doi.org/10.1530/JME-10-0154.
Statistical analysis

Experimental values are expressed as mean ± S.E.M. Comparison of mean values between various groups was performed by one-way ANOVA followed by Tukey’s test. \( P \) value < 0.05 is considered to be significant. Histological damage was analyzed by one-way Kruskal–Wallis test.

Results

Esculetin treatment attenuates the development of STZ-induced type I diabetic nephropathy

We observed a significantly higher plasma glucose level in diabetic rats after 8 weeks of STZ treatment compared with the control group. However, treatment with esculetin (50 and 100 mg/kg per day) significantly reduced the plasma glucose level in diabetic rats (see Table 1). The increased BUN and plasma creatinine indicates the development of diabetic nephropathy in rats (Yokoi et al. 2001, Breyer et al. 2005). Esculetin (50 and 100 mg/kg per day) treatment significantly reduced the elevated BUN and plasma creatinine level in diabetic rats (see Table 1). Also, plasma albumin level was significantly decreased in diabetic animals compared with age-matched control rats and this decrease was attenuated by esculetin treatment. Maintenance of these biochemical parameters closer to control rat level by esculetin treatment suggests its role, either directly or indirectly, in providing protection against the development of diabetic nephropathy or delaying its progression.

In addition, after 8 weeks of STZ treatment, diabetic animal showed a significant decrease in body weight and increase in kidney weight compared with normal rats. Moreover, kidney/body weight ratio was doubled compared with normal rats. Treatment of esculetin for 4 weeks (50 and 100 mg/kg per day) provides protection against body weight loss in diabetic rats compared with untreated diabetic rats. In addition, it also attenuates the increase in kidney weight in diabetic rats. The increased kidney weight/body weight ratio in diabetic rats is a marker for the development of diabetic nephropathy and our results show a significant decrease in kidney weight/body weight ratio in diabetic rats after treatment of esculetin (see Table 2)

As we did not observe any significant difference between the 50 and 100 mg/kg dose, for further mechanistic studies, we only incorporated the esculetin 50 mg/kg dose. On tissue level, we observed a glomerular damage in diabetic kidney compared with normal control rats by histopathological analysis (Fig. 1). In addition, nephropathy was further confirmed by the expression of fibronectin and TGF-β1 in the kidney of these animals, suggesting the development of renal fibrosis in diabetic animals (Fig. 2). Esculetin treatment is able to reduce the glomerular damage and accumulation of fibronectin in diabetic kidney (Figs 1 and 2). This finding further confirms our biochemical observations that esculetin treatment provides protection against the progression of STZ-induced diabetic nephropathy.

Esculetin decreases the level of thiobarbituric acid reacting substances in plasma as well as in kidney of diabetic animals

STZ-treated diabetic rats show higher levels of thiobarbituric acid reacting substances (TBARS) in both plasma and kidney compared with control rats (Fig. 3), suggesting the presence of oxidative stress.
Treatment with esculetin significantly reduced the levels of TBARS in both plasma and kidney of diabetic rat, suggesting that esculetin treatment attenuates the increase in oxidative stress in plasma as well as in kidney of diabetic animals.

**Esculetin treatment attenuates change in PPARγ/TGF-β1 expression in diabetic kidney**

Further, we sought to check the effect of esculetin treatment on expression of the PPARγ/TGF-β1 pathway in STZ-induced diabetic rat kidney. Our data suggest that there was a significant decrease in the expression of PPARγ in diabetic rat kidney compared with control rat and treatment of esculetin attenuates the same (Fig. 4). In addition, we observed increased expression of TGF-β1 and accumulation of the ECM protein, fibronectin, in renal sections of diabetic animals (Fig. 2). Esculetin treatment provides protection against the increase in expression of TGF-β1 and fibronectin in diabetic rat kidney and hence shows efficacy in attenuating glomerulosclerosis (Fig. 2). These results indicate that esculetin provides protection against the development of diabetic nephropathy by altering the PPARγ/TGF-β1 pathway.

**Esculetin attenuates the decrease in histone H3 acetylation (K9/14) and mono-methylation (K4) in diabetic kidney**

To further understand the downstream mechanism responsible for providing protection against diabetic nephropathy by esculetin, we studied the covalent modifications of histone H3. Recently, we have reported that in a diabetic kidney, there is decrease in acetylation and phosphorylation of histone H3 and treatment with curcumin reverses these changes in histone H3 modifications (Tikoo et al. 2008). Hence, we checked the effect of esculetin treatment on the level of acetylation (K9/14) and mono-methylation (K4) of histone H3 in diabetic kidney. Our results show that esculetin treatment attenuates the decrease in acetylation (K9/14) and mono-methylation (K4) of histone H3 in STZ-induced diabetic rat kidney (Fig. 5A and B). In addition, esculetin treatment alone in control rats induces acetylation of histone H3 compared with control untreated rats (Fig. 5A, *P<0.05). These results suggest that esculetin treatment reverses the changes in histone H3 modifications, thereby regulating the expression of genes that are responsible for preventing the development of diabetic nephropathy.

**Treatment of esculetin averts the changes in mRNA expression of Mmp13 (fibrogenic) and Bmp-6 (antifibrotic) genes in diabetic kidney**

As evident from the previous results, esculetin treatment attenuates the changes in histone H3 modifications and thus alters the chromatin organization and alters expression of genes responsible for development of diabetic nephropathy. To further see the effect of these alterations in chromatin organization on the regulation of renal fibrosis-related gene expressions, we checked the mRNA expression of \( \text{Mmp13} \) (fibrotic) and \( \text{Bmp6} \) (antifibrotic) in diabetic kidney by RT-PCR analysis. Our results show for the first time that there is an increase in mRNA level of \( \text{Mmp13} \) and a decrease in mRNA expression of \( \text{Bmp6} \) in diabetic rat kidney. Esculetin treatment further averts these changes in gene expression in diabetic kidney (Fig. 6). These results suggest that esculetin treatment both directly or indirectly alters the expression of \( \text{Mmp13} \) and \( \text{Bmp6} \) and thus provides protection against the development of renal fibrosis.
Discussion and conclusion

In this study, we provide evidence that treatment with esculetin protects the progression of diabetic nephropathy involving changes in the PPARγ/TGF-β1 pathway, covalent modifications of histone H3, and Mmp13 and Bmp6 gene expression. In addition, our results also demonstrate that esculetin treatment significantly lowers BUN, plasma creatinine, and attenuates the decrease in plasma albumin levels associated with diabetic nephropathy. Moreover, a recent report suggests the potential of esculetin in lowering the blood glucose levels due to inhibition of glycogen phosphorylase enzyme (Atsushi et al. 2008). We also demonstrate that esculetin reduces the plasma glucose levels in diabetic animals compared with normal control animals.

Esculetin is recognized as an inhibitor not only of the lipoxygenase and cyclooxygenase enzymatic systems but also for the neutrophil-dependent superoxide anion generation system (Fylaktakidou et al. 2004). It has been shown to exhibit 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, hydroxyl radical scavenging, and intracellular ROS scavenging activities (Kim et al. 2008). The radical scavenging activity of esculetin resulted in the protection of cells from lipid peroxidation, protein carbonyl, and DNA damage induced by H2O2. The protective effects of esculetin against the increase in 8-oxodG and TBARS levels were determined after 1,1-dimethylhydrazine (DMH) administration (Kaneko et al. 2003, 2007). Elevated malondialdehyde (MDA) level and decrease in superoxide dismutase (SOD) level in diabetic kidney is an indicator of increased oxidative stress (Chang et al. 2005). We also observed the increased levels of TBARS in this study, in both plasma and kidney of diabetic rats, and esculetin treatment reduces these elevated levels, indicating that the radical scavenging property of esculetin may be responsible for reduction in lipid peroxidation and protects the progression of diabetic nephropathy.

Increasing evidence shows the ability of PPARγ in improving insulin sensitivity and its anti-fibrotic potential (Li et al. 2006). Studies have shown that PPARγ agonists not only ameliorate diabetic nephropathy but also exert beneficial actions in the attenuation of renal fibrosis associated with non-diabetic chronic kidney disease like non-diabetic glomerulosclerosis (Ma et al. 2001) and experimental crescentic glomerulonephritis (Haraguchi et al. 2003). In rat remnant kidney model of renal fibrosis, administration of the PPARγ agonist, troglitazone, is associated with a reduction of proteinuria, serum creatinine, and glomerulosclerosis. PPARγ activation is also known to decrease glomerular cell proliferation and suppress TGF-β1 expression (Guo et al. 2004, Kawai et al. 2008). In vitro investigations have also revealed that PPARγ activators are capable of inhibiting cell proliferation.
and suppressing the expression of ECM components such as type I collagen and fibronectin (Zheng et al. 2002, Panchapakesan et al. 2005). Activation of PPARγ by its ligands is shown to have a direct effect on the processes of renal fibrogenesis (Nicholas et al. 2001). Our results also demonstrate that treatment with esculetin attenuates downregulation of the PPARγ in diabetic rat kidney, thus indicating its antifibrotic effect. In addition, decrease in expression of TGF-β1 and fibronectin in diabetic kidney by esculetin treatment clearly suggests that esculetin protects the development of renal fibrosis associated with diabetic nephropathy.

Chromatin is subject to a diverse array of posttranslational modifications that largely impinge on histone amino termini, thereby regulating access to the underlying DNA involving the conversion of compact heterochromatin into transcription factor accessible euchromatin (Jenuwein & Allis 2001, Egger et al. 2004, Berger 2007). Acetylation of histone at a specific lysine residue is known to increase the transcriptional activity, whereas methylation of histone H3 at a different lysine residue is associated with either gene activation or repression (Berger 2007, Kouzarides 2007, Ruthenburg et al. 2007). In context to diabetes, in vivo chromatin remodeling and histone modifications have been reported to alter the transcription of various inflammatory genes (Miao et al. 2004). Previous reports from our lab also suggest the altered chromatin remodeling and histone H3 modifications in diabetic rat kidney by curcumin treatment (Tikoo et al. 2008). Also, in this study, we observed a decrease in acetylation (K9/14) and methylation (K4) of histone H3 in diabetic rat kidney compared with control rats and treatment with esculetin significantly attenuates this decrease in acetylation and methylation of histone H3 in diabetic kidney. These results indicate that esculetin attenuates these aberrant chromatin remodeling events, which may be involved in altering the expression of fibrogenic genes. Hence, to see the antifibrotic activity of esculetin, we studied the change in the expression of one fibrogenic gene Mmp13 (Lenz et al. 2000) and other antifibrotic gene Bmp6 (Yan et al. 2009). To the best of our knowledge, this is the first report that shows the upregulation of Mmp13 and downregulation of Bmp6 gene expression in diabetic rat kidney and esculetin treatment averts these changes in mRNA expression levels of these genes during the development of renal fibrosis. However, it would be interesting to see the status of histone H3 modifications at the promoter of these genes.

Several reports suggest a link between epigenetic modifications related to diabetic memory after hyperglycemic episodes (El-Osta et al. 2008, Villeneuve & Natarajan 2010). These reports show long-lasting epigenetic changes by transient hyperglycemia, which remains persistent after subsequent normal glycemia. Interestingly, in this study, esculetin treatment reduces hyperglycemia on the one hand and also attenuates epigenetic changes that initiate aberrant chromatin remodeling and alteration in expression of genes.

Figure 6 Treatment with esculetin averts the changes in mRNA expression of Mmp13 (fibrogenic) and Bmp6 (antifibrotic) in diabetic kidney. Mmp13 and Bmp6 mRNA expression was analyzed in total kidney from different experimental groups by RT-QPCR using 18s gene as internal control and results were expressed as fold change over normal/control. Similar results were obtained in three independent sets of experiments. All the values are represented as mean ± S.E.M. (n=3). *P<0.05 and ***P<0.001 considered significantly different from normal/control rats and #P<0.05 and ###P<0.001 considered significantly different from diabetic/normal rats.
These results suggest the protective effect of esculetin against pathophysiology of disease.

In conclusion, this is the first report to show that protection in the development of diabetic nephropathy by esculetin treatment involves changes in expression of PPARγ/TGF-β1 pathway-related proteins. In addition, esculetin treatment attenuates alteration in Mmp13 and Bmp6 gene expression by involving change in acetylation and methylation of histone H3. However, further study needs to be conducted to better understand the mechanisms involved in it, as that can have profound clinical significance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was supported by a grant of the National Institute of Pharmaceutical Education and Research (NIPER).

Author contribution statement

V M S planned and performed all the in vitro experiments and has made substantial contributions to analysis and interpretation of data. J G has carried out RT-PCR experiments and their analysis and was also involved in drafting the manuscript. K T planned and supervised the project and has given final approval of the version to be published.

Acknowledgements

We thank Anil Gaikwad, Ujjwal Mahajan, Abhijit Shinde, Pinakin Karpe, Mukta Sane, Chanchal Gupta, and Sachin Chawla for their helpful discussions and technical assistance. We thank Ajit Vikram for helpful discussion in statistical data analysis.

References

Li Y, Wen X, Spathar BC, Hu K, Dai C & Liu Y 2006 Hepatocyte growth factor is a downstream effector that mediates the antifibrotic action.


Nishibe S & Okuyama T 1995 Search for naturally occurring components from Ceratostigma willmottianum that act as inhibitors of peroxisome proliferator-activated receptor-γ (PPARγ) in mesangial cells. Hypertension 37 722–727.


Ranun Ye, Schnaper HW & Poncelet AC 2004 The phosphatidylinositol 3-kinase/Akt pathway enhances Smad 3-stimulated mesangial cell collagen I expression in response to transforming growth factor-β. Journal of Biological Chemistry 279 2632–2639. (doi:10.1074/jbc.M310412200)


Received in final form 28 February 2011
Accepted 30 March 2011
Made available online as an Accepted Preprint 30 March 2011