Involvement of Endothelial Nitric Oxide Synthase, Caveolin and Haemoxygenase in Development of Experimental Diabetic Nephropathy in Rats

Taruna Katyal, Arun Garg, R.D. Budhiraja
a. Dept. of Pharmacology, I.S.F. College of Pharmacy, Ghal Kalan, Moga 142001, Punjab, India.

b. PDM college of Pharmacy, Sarai Aurangabad, Bahadurgarh, 124567, Haryana, India

Abstract - Endothelin nitric oxide synthase plays an important role in diabetic nephropathy. Caveolae are the morphological distinct invaginations on the suface of the plasma membrane and are of 50-100nm in diameter. Caveolin decreases nitric oxide by increasing binding of eNOS with caveolin. Hemeoxygenase-1 (OH-1) is a enzyme involve in the catabolism of heme and leads to the formation of NO. Hemeoxygenase level was found to be decreased in diabetes. It has been documented that OH-1 decreases the interaction of eNOS with caveolin by increasing the availibility of NO. This study was designed to determine the role of caveolin & haemoxygenase in eNOS-mediated NO synthesis & and release in DN. Diabetic nephropathy was induced by administering single dose of streptozotocin in wistar rats. DN was clinically assessed by estimation of various biochemical parameters and histopathological studies of renal tissue. DN was assessed by measuring serum creatinine, blood urea nitrogen, proteinuria, lipid profile, serum nitrite/nitrate ratio. The combination of daidzein(caveolin inhibitor) and Hemin (Hemoxygenase activator) showed significant improvement in (BUN, serum creatinine, proteinuria, urinary output, serum nitrite/ nitrate level) renal parameters studied for DN in comparison with single drug administration. Moreover, NG-nitro-L-arginine methyl ester (L-NAME), a selective eNOS inhibitor abolised the ameliorative effect of combination of daidzein and hemin in DN in rats. The finding of this study suggest that haemoxigenase and caveolin plays a important role in the development of DN.

Keywords: Diabetes nephropathy. Nitric oxide. Caveolin. Haemoxygenase

I. INTRODUCTION

Diabetes mellitus is a chronic disease characterized by hyperglycemia, polyuria, polydipsia and weight loss in spite of polyphagia, glycosuria, ketosis and acidosis [1]. DM includes various complications such as retinopathy, neuropathy, cardiomyopathy, coronary artery disease, peripheral vascular disease and nephropathy [2]. DN is considered to be one of the major complications of diabetes mellitus. DN is characterized by thickening of glomerular basement membranes, mesangial expansion, accumulation of extracellular matrix proteins, mesangial expansion with progression into glomerulosclerosis, tubulointerstitial fibrosis [3].

Renin angiotensin system play a pivotal role in the pathogenesis of DN Ang-II levels have been found to be increased in DN [4]. Ang-II increases intraglomerular pressure, infiltration of macrophages and lymphocytes in glomeruli and tubules. Further, ang-II also stimulates superoxide anion production, reduces the bioavailability of NO [5], enhances the synthesis of growth factors such as TGF-β [6, 7] and activates PKC and P38 MAPK pathways, which all collectively deteriorate the diabetic kidney.

Caveolae’s are invaginations of 50–100 nm in size form stable membrane domain [8]. Caveolins are the protein present on the caveolae [9]. Caveolins have three mammalian isoforms known as caveolin-1, caveolin-2, and caveolin-3 [10]. Caveolin expression has been found to be increased in diabetic rats [11]. Caveolin play a important role in the negative regulation of eNOS, means it binds with the eNOS and decreasing the availability of NO [11, 12]. Moreover, caveolin- knockout mice was shown to increased eNOS activity [13].

Hemeoxygenase-1 (OH-1) is a enzyme involve in the catabolism of heme and leads to the formation of bilirubin [14]. It has been documented that OH-1 decreases the interaction of eNOS with caveolin by increasing the availibility of NO [15]. However, it has been reported that the metabolite of OH-1 i.e. bilirubin, increase the bioavailibility of nitric oxide [16]. Moreover, the expression of heme oxygenase is decreases in diabetic rats [17]. Further, pretreatment with hemin shown to improve serum creatinine, BUN in mercuric chloride induced nephropathy in rats [18-20]. Furthermore,
inducers of hemeoxygenase, is reported to increases the release of NO in diabetic rats [21]. In addition, inhibitors of haemeoxgenase i.e. Tin protoporphyrin have been noted to aggravate nephropathy [22].

Since Caveolin inhibitor and hemin produce their renoprotective action by different mechanisms, it is likely that their combined use would result in an additive renoprotective effect. Moreover, interaction of these pharmacological agents shall be employed the investigate the role of caveolin, haemeoxgenase and NO in diabetes induced nephropathy in rats. The aim of the study to investigate this hypothesis.

II. MATERIALS AND METHODS

A. Animals

Wistar rats (230–260 g) were used in these studies. Protocol was approved by Institutional Animal Ethical Committee (IAEC). Rats were given chow diet and water ad libitum. DM was induced in rats by administration of streptozotocin (STZ) (50mg/kg/i.p once). Diabetic rats develop nephropathy after 42 days of STZ administration [23].

B. Induction and Estimation of Blood glucose

Collection of the blood sample was done from retro orbital sinus and blood glucose levels was estimated by glucose oxidase–peroxidase (GOD–POD) method [24] using kit (Coral clinical system, Goa, India).

C. Assessment of diabetic nephropathy

Blood urea nitrogen (BUN) was estimated by Berthelot method [25].

Protein in urine was estimated by Pyragallol red method [26].

Serum creatinine was assessed by alkaline picrate method by using standard diagnostic kits [27] (Cresent biosystems, Goa, India).

D. Measurement of serum nitrite and nitrate levels

Nitrite and nitrate are the primary oxidation products of NO subsequent to reaction with oxygen and, therefore, the nitrite/nitrate concentration in serum was used as an indirect measure of NO synthesis. Quantitation of nitrate and nitrite was based on the Griess reaction, which involve a reaction formation of strong chromophore due to reaction between naphthylethylenediamine and sulfanilamide. Total nitrite/ nitrate concentration was calculated by using standard sodium nitrate. Results were expressed as micromoles per liter [28].

E. Assessment of Lipid Profile

The total cholesterol & triglycerides were done by cholesterol oxidase peroxidase (CHOD-PAP) & glycerophosphate oxidase peroxidase (GOD-PAP) methods [29-31] using commercially available kits (Crest Biosystems, Goa, India).

F. Drugs and chemicals

Streptozotocin, Daidzein, L-NAME were purchased from Sigma-Aldrich Ltd., St. Louis, USA. Hemin and L-arginine were obtained from Tocris bioscience boston biochem. Park Ellisville, Missouri, USA. All other chemical were used in the study as analytical grade.

III. RESULTS AND STATISTICAL ANALYSIS

All values are expressed as mean± SD. Data obtained from different groups were statistical analyzed using two-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests. The P<0.05 was considered to be statistically significant.

A. Effect of pharmacological agents on serum glucose

After administration of STZ, the rats those showed blood glucose greater than 250mg/dl were selected and grouped as diabetic rats. Treatment with daidzein caveolin inhibitor (0.2 mg/kg, sc), hemoxgenase activator, Hemin (15mg/kg/i.p.), and L-arginine nitric oxide precursor (2 g/l) did not produce significant change in the glucose level in diabetic rats (Table 1).

B. Effects of pharmacological agents on Blood urea nitrogen, total protein and urinary output

The marked increased in the level of BUN, total protein, and volume of urinary output were noted in diabetic rats as compared to normal rats. The treatment of hemin in combination with L-arginine partially reduced the level BUN, total protein, and urinary output.

However, co-administration of hemin and daidzein markedly reduced BUN, total protein, and urinary output when compared with diabetic rats. Moreover, 14 days treatment of L-NAME (10mg/kg, i.p.) abolished the ameliorative effect of all combinations mentioned above (Figs. 1, 3).

C. Effect of pharmacological agents on serum creatinine

There is increase in concentration of creatinine was noted in diabetic rats as compared to normal rats. Treatment with daidzein slightly reduced serum creatinine level when compared with diabetic rats. However, concurrent administration of hemin and daidzein significantly reduced the serum creatinine levels when compared with diabetic rats. Moreover, 14 days treatment of L-NAME (10mg/kg, i.p.) abolished the ameliorative effect of all combinations mentioned above (Fig. 2).

D. Effect of pharmacological agents on serum cholesterol

The serum cholesterol was noted to be increased in diabetic rats when compared with normal rats. None of the pharmacological intervention (hemin, L-arginine or daidzein) either alone or in combination could reduce the elevated serum cholesterol level in diabetic rats (Fig. 4).
E. Effect of pharmacological agents serum triglycerides

Diabetic rats after 42 days of STZ administration showed increase in serum triglycerides level when compared with normal rats. Treatment of hemin or in combination with L-arginine partially decreased the serum triglycerides level when compared with diabetic rats. However, co-administration of daidzein with hemin significantly decreased the serum triglycerides when compared to diabetic rats. Moreover, 14days treatment of L-NNAME (10mg/kg, i.p.) abolished the ameliorative effect of all combinations mentioned above (Fig. 5).

F. Effect of pharmacological agents on serum nitrite/nitrate level

The marked decreased in the level of serum nitrite/nitrate was noted in diabetic rats as compared with normal rats. Combination of daidzein and hemin significantly increased the serum nitrite/nitrate level as compared to diabetic rats. Moreover, 14days treatment of L-NNAME (10mg/kg, i.p.) abolished the ameliorative effect of all combinations mentioned above. (Table. 1).

G. Histopathological studies

The section of 3 µM in thickness were made and stained with hematoxylin and eosin to assess the pathological changes of glomeruli using the light microscopy (400 X). The diabetic rats were noted to develop pathological changes in the glomeruli such as glomerular capillary size reduction and extracellular mesangial expansion as compared to normal rats. The administration of Sodium nitrite, L-Arginine & Daidzein, Hemin markedly reduced the pathological changes in glomeruli by improving the glomerular capillary size and reducing the mesangial expansion (Fig.6).

IV. DISCUSSION

Diabetes mellitus was induced by single administration of streptozotocin (50mg/kg/i.p.) and increase in serum creatinine, BUN and total protein were noted as a marker of renal dysfunction [32-35]. In our study, serum creatinine, blood urea nitrogen and proteinuria has been noted to be increased after STZ administration. Vascular endothelial dysfunction takes place in diabetic nephropathy [36, 37]. The decrease nitrite/nitrate (serum) concentration indicates the dysfunction in vascular endothelium [38, 39]. However, numerous studies suggest that eNOS production has been found to be decreased in diabetic rats [36]. Hyperlipidemia is an independent risk factor for the induction and progression of diabetes induced nephropathy [40, 41]. Therefore total cholesterol, triglycerides has been estimated in present study to assess the effect of dyslipidemia. This contention is supported by the results obtained in the present study that marked increase in triglycerides was noted in diabetic rats with nephropathy.

Renin angiotensin aldosterones play a pivotal role in the pathogenesis of diabetes and get upregulated in diabetic nephropathy [42]. Angiotensin-II increases intraglomerular pressure, infiltration of macrophages and lymphocytes in glomeruli and tubules [43]. Ang-II also stimulates superoxide anion production, reduces the bioavailability of NO [5], and enhances the synthesis of growth factors such as TGF-β [6, 7] and which leads to diabetic nephropathy. Growing evidences suggest that decrease in NO level contribute to diabetic nephropathy [44, 45].

Caveolae’s are invaginations of 50–100 nm in size and form stable membrane domain, present on the caveolin [8, 10]. Further, Caveolin down regulates the level of nitric oxide [46]. Caveolin expressions were shown to be increased in diabetic conditions that decrease the NO level by increasing the interaction of eNOS with caveolin [11]. Numerous researches suggest that daidzein, reduce the expression of caveolin and increase the release of nitric oxide in diabetic rats [28, 47]. To reduce the effect of estrogen, only male were used in the study. However, treatment with daidzein slightly prevent nephropathy by decreasing serum creatinine, proteinuria, BUN, triglycerides and increasing the level of serum nitrite/nitrate in diabetic rats. It may be conclude that treatment with daidzein may enhance the level renal nitric oxide level inhibiting its interaction of eNOS with caveolin [28].

Heme oxygenase (HO) is a rate-limiting enzyme that degrades heme, a pro-oxidant into carbon monoxide, iron and bilirubin [14, 48, 49]. It has been documented that OH-1 increasing the availibilty of NO by reducing the interaction of eNOS with caveolin. In addition, it is interesting to note that the metabolite of OH-1 i.e. bilirubin, increase the bioavailabilty of nitric oxide [25]. In our study, treatment of hemin or in combination with L-arginine partially decreased blood urea nitrogen, serum creatinine, total protein, serum triglycerides and increased the level of nitrite/nitrate(serum) level in diabetic rats. Our results are in agreement with previous reports, pretreatment with hemin has also been shown to produce ameliorative effect by improving the level of serum creatinine and blood urea nitrogen in mercuric chloride-induced nephropathy in rats [27]. It has been previously demonstrated that hemin significantly attenuated the proteinurea observed in anti-GBM-antibody-induced nephritis in rats [29].

Administration of daidzein with hemin markedly attenuated the increase in blood urea nitrogen, serum creatinine, total protein, triglycerides, and decreased nitrite/nitrate(serum) ratio, renal reduced glutathione as compare to diabetic control rats. Combination of daidzein and hemin was only found to be statistically significant. This combination (daidzein, hemin) can potentially be studied to toxicological and clinical outcome.

V. CONCLUSION

This is first time reported that concurrent administration of daidzein with hemin is more proficient in the treatment of diabetic nephropathy than any single drug therapy. It may be concluded that treatment of daidzein with hemin may decrease the interaction eNOS with caveolin and hence increasing renal nitric oxide. These
additional protective effects may be due to anti-apoptotic, anti-inflammatory, anti-oxidant effect or other possible additional pathways of hemin. The synergistic ameliorative effect of combination of daidzein with hemin in diabetic nephropathy is a new finding. These finding provide mechanistic insights to explain renoprotective effect of this combined therapy in diabetes.

VI. ACKNOWLEDGEMENT
We are thankful to Mr. Parveen Garg, Chairman Indo soviet Friendship College of Pharmacy, Moga and Mr. Vikash Kumar (Asst. Prof.), PDM College of Pharmacy, Bahadurgarh, motivation and help during the course of this research work.

VII. REFERENCES


Corresponding Author: **Taruna Katyal**
Ph.D Research scholar
I.S.F. College of pharmacy, Moga, Punjab
09953625707, E.ID: tarunakatyal@gmail.com (Taruna Katyal)

**APPENDIX**

Fig. (1). Effect of various pharmacological interventions on blood urea nitrogen. Values are expressed as mean ± S.D. a = p < 0.05 vs Normal control; b = p < 0.05 vs Diabetic control; c = p< 0.05 vs Daidzein in diabetic control; d= p<0.05 vs Hemin in diabetic control; e = p<0.05 vs L-arginine + Hemin in diabetic control. f = p<0.05 vs Daidzein + Hemin in diabetic control.

Fig. (2). Effect of various pharmacological interventions on blood urea nitrogen. Values are expressed as mean ± S.D. a = p < 0.05 vs Normal control; b = p < 0.05 vs Diabetic control; c = p< 0.05 vs Daidzein in diabetic control; d= p<0.05 vs Hemin in diabetic control; e = p<0.05 vs L-arginine + Hemin in diabetic control. f = p<0.05 vs Daidzein + Hemin in diabetic control.

Fig. (3). Effect of various pharmacological interventions on Total Protein. Values are expressed as mean ± S.D. a = p < 0.05 vs Normal control; b = p < 0.05 vs Diabetic control; c = p< 0.05 vs Daidzein in diabetic control; d= p<0.05 vs Hemin in diabetic control; e = p<0.05 vs L-arginine + Hemin in diabetic control. f = p<0.05 vs Daidzein + Hemin in diabetic control.

Fig. (4). Effect of various pharmacological interventions on serum cholesterol level. Values are expressed as mean ± S.D. a = p < 0.05 vs Normal control.

Fig. (5). Effect of various pharmacological interventions serum triglycerides. Values are expressed as mean ± S.D. a = p < 0.05 vs Normal control; b = p < 0.05 vs Diabetic control; c = p< 0.05 vs Daidzein in diabetic control; d= p<0.05 vs Hemin in diabetic control; e = p<0.05 vs L-arginine + Hemin in diabetic control. f = p<0.05 vs Daidzein + Hemin in diabetic control.
GP 1 (Normal Control)  GP 2 (L-Arginine per se)  GP 3 (Daidzein per se)  GP 4 (Hemin per se)  GP 5 (Diabetic)  GP 6 (DMSO treated)  GP 7 (Daidzein treated Diabetic group)  GP 8 (Hemin treated Diabetic group)  GP 9 (L-Arginine + Hemin diabetic)  GP 10 (Daidzein+ Hemin treated)  GP 11 (Daidzein+L-NAME treated)  GP 12 (Hemin +LNAME treated diabetic)  GP 13 (L-Arginine + Hemin + L-NAME diabetic gp)  GP 14 (Daidzein+Hemin+L NAME treated diabetic group)
Table-1 Effect of various pharmacological interventions on serum glucose, urinary output and serum nitrite/nitrate ratio on 56 day.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Groups</th>
<th>Serum glucose (mg/dl)</th>
<th>Urine output (ml/24 h)</th>
<th>Serum nitrite/nitrate (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal control</td>
<td>106.13 ± 7.30</td>
<td>9.12 ± 2.42</td>
<td>11.53 ± 0.38</td>
</tr>
<tr>
<td>2</td>
<td>L-Arginine per se</td>
<td>105.96 ± 7.99</td>
<td>9.58 ± 2.68</td>
<td>11.84 ± 0.50</td>
</tr>
<tr>
<td>3</td>
<td>Daidzein per se</td>
<td>109.16 ± 8.28</td>
<td>9.53 ± 2.84</td>
<td>11.55 ± 0.39</td>
</tr>
<tr>
<td>4</td>
<td>Hemin per se</td>
<td>104.41 ± 8.24</td>
<td>9.79 ± 2.55</td>
<td>11.53 ± 0.68</td>
</tr>
<tr>
<td>5</td>
<td>Diabetic control</td>
<td>365.88 ± 10.04</td>
<td>88.86 ± 4.11</td>
<td>2.54 ± 0.28</td>
</tr>
<tr>
<td>6</td>
<td>DMSO diabetic rat</td>
<td>364.31 ± 7.75</td>
<td>88.04 ± 4.16</td>
<td>2.22 ± 0.46</td>
</tr>
<tr>
<td>7</td>
<td>Daidzein in diabetic group</td>
<td>341.65 ± 10.34</td>
<td>64.26 ± 2.94</td>
<td>5.44 ± 0.72</td>
</tr>
<tr>
<td>8</td>
<td>Hemin in diabetic gp</td>
<td>345.93 ± 9.63</td>
<td>51.21 ± 2.56</td>
<td>7.21 ± 0.49</td>
</tr>
<tr>
<td>9</td>
<td>L-Arginine+ hemin in diabetic gp</td>
<td>339.31 ± 10.29</td>
<td>43.35 ± 3.76</td>
<td>8.72 ± 0.32</td>
</tr>
<tr>
<td>10</td>
<td>Daidzein+ hemin in diabetic gp</td>
<td>345.7 ± 9.87</td>
<td>34.39 ± 3.13</td>
<td>9.59 ± 0.43</td>
</tr>
<tr>
<td>11</td>
<td>Daidzein + L-NAME in diabetic gp</td>
<td>339.9 ± 11.46</td>
<td>89.01 ± 3.57</td>
<td>3.69 ± 0.70</td>
</tr>
<tr>
<td>12</td>
<td>Hemin + L-NAME in diabetic gp</td>
<td>339.13 ± 14.30</td>
<td>87.67 ± 3.45</td>
<td>3.30 ± 0.63</td>
</tr>
<tr>
<td>13</td>
<td>L-Arginine + Hemin + L-NAME in diabetic gp</td>
<td>344.41 ± 11.66</td>
<td>85.30 ± 2.67</td>
<td>3.60 ± 0.59</td>
</tr>
<tr>
<td>14</td>
<td>Daidzein + hemin + L-NAME in diabetic gp</td>
<td>340.05 ± 11.81</td>
<td>89.01 ± 3.57</td>
<td>3.77 ± 0.82</td>
</tr>
</tbody>
</table>