Effect of Micardis on the Expression of Renal Medulla Aquaporin-2 in Diabetic Mice*

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Summary: In current study, the effect of angiotensin receptor blocker Micardis on the localization and expression of aquaporin-2 (AQP2) was investigated in the renal medullary collecting duct of mice with diabetic nephropathy (DN). Mice were divided into three groups: normal group, DN group and Micardis-treated group. Six weeks after establishment of STZ-induced DN model in mice, the expression of AQP2 in renal medulla was detected measured by semiquantitative immunofluorescence histochemistry and Western blot techniques, and the localization of AQP2 by confocal immunofluorescence laser scanning microscopy. The results showed that the urinary osmolality was decreased in DN group as compared with normal group (2.39±0.11 vs 3.16±0.16, P<0.05). Although the localization of AQP2 on the renal medulla was unchanged, the expression of AQP2 was increased significantly in DN group as compared with normal group. Micardis could partly attenuate above changes. It was concluded that treatment with Micardis could partly rectify the abnormal expression of AQP2 in renal medulla of DN mice, which suggested that rennin-angiotensin system (RAS) is implicated in the pathogenesis of DN by regulating the expression of AQP2.

Key words: diabetic nephropathy; aquaporin 2; Micardis; mouse

Polyuria is a common clinic complaint presented in most diabetic patients, which was believed to be the result of osmotic diuresis produced by hyperglycaemia in the past. Recently, several researches reported the urine concentration and metabolic disorder presented under diabetic condition was caused by aquaporin family (AQPs). However, AQPs consists of at least 10 subunits, each one points to a specific tissue localization and biologic function. Since some reports showed that diabetic rats have elevated plasma vasopressin (AVP) levels[1] and unlike other AQP subunit, AQP2, which is mainly localized in the kidney, is the main water channel regulated by AVP, it is a crucial issue to investigate the expression of AQP2 in DM kidney and explore a drug to interfere with this procedure.

Micardis is a kind of angiotensin receptor blocker (ARB) which is generally used in clinic and has been proved to be a notable curative drug for diabetic nephropathy (DN), but whether it can implement its nephroprotective effects through influencing the expression of AQP2 is seldom known, thus we investigate the influence of Micardis on the expression of AQP2 in DN renal medulla.

1 MATERIALS AND METHODS

1.1 Materials and Reagents

Streptozotocin (STZ) was purchased from Sigma (USA); BCA protein ELISA kit was purchased from Biyun Bio. Co. (China). Donkey anti-goat IgG conjugated with Texas Red was purchased from San-Ying Co. (China). Horseradish peroxidase labeled donkey anti-goat second antibody and goat anti-mouse AQP2 antibody were purchased from Santa Cruze Co. (USA). ARB Micardis was kindly provided by Boehringer Ingelheim Pharmaceuticals Co. (China). Major apparatus included cryosection machine (Leica CM3050S, Germany) and confocal laser-scanning microscopy (Olympus FV500, Japan).

1.2 Animals

Twenty C57BL/6 male mice with body weight from 25 to 35 g were purchased from Organ Transplantation Center, Tongji Medical College, Tongji Hospital, HUST (China). After adaptation to metabolic cages over a period of 2 weeks, the mice were divided into 3 groups randomly. Murine DN model was induced by intraperitoneal injection of STZ (100 mg/kg of body weight, dissolved in cold citrate buffer, pH 4.0) every other day. One week later, the glucose concentration was determined in tail blood samples, and only those with blood glucose above 16.7 mmol/L were considered as DN mice. Nondiabetic mice initially injected with STZ vehicle served as controls (normal group, n=6). The DN mice then received Micardis (9 mg·kg⁻¹·d⁻¹ po, Micardis-
treated group, n=7) or vehicle (DN group, n=7) by gastric tube. During the whole experiment, mice got free access to standard mouse chow and tap water. Two mice, each from DN group and Micardis-treated group, died on the day 21 and 26 after the last administration of STZ. On the day 41, 24-h urine samples were collected in metabolic cages. The protein concentration in the urine was determined by BCA protein ELISA. Forty-two days after application of STZ or vehicle, the following experiments were performed on the surviving mice.

1.3 Samples

Experimental mice were anesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg). After opening of the abdominal cavity, the left renal artery was clamped and the left kidney was excised. The renal medullae were dissected and immediately immersed into liquid nitrogen for Western blot analysis later. Then the heart was exposed. The tip of the perfusion system was inserted into the left ventricle, and the arterial system was perfused for 1 min with 3–5 mL PBS to clear blood of the kidney, and subsequently for 10 min with 10–15 mL fixation solution (4% paraformaldehyde and 3% sucrose in phosphate buffer) at room temperature. Then the right kidney was removed, weighted and incubated for 15 min in the fixation solution at 4°C. After being rinsed in PBS for 15 min, kidney slices were dehydrated in 30% sucrose in PBS for 6 h at 4°C. Thereafter, kidney slices were frozen in isopentane precooled by liquid nitrogen and stored at –80°C until further use. Cryosections of 8 μm were made at –20°C.

1.4 HE Staining and Glomeruli Morphometry

Sections were deparaffinized with xylene, hydrated in graded ethanol and then stained with hematoxylin and eosin (HE). Twenty glomeruli were randomly selected from sections of each subject at an original magnification of ×400. Mean glomeruli area and perimeter were determined from light microscopic images using HMIAS-2000 system (Qian-Ping Co., China).

1.5 Immunohistochemistry

Immunofluorescence staining and confocal laser scanning microscopy were performed as previously described. Briefly, after preincubation for 30 min in PBS containing 4% bovine serum albumin and 0.25% Triton X-100, cryosections were incubated overnight at 4°C with goat anti-mouse AQP2 antibody (2 μg/mL). After washed three times for 5 min in PBS, the sections were incubated for 2 h with donkey anti-goat IgG conjugated with Texas Red (final concentration 5 μg/mL, Sigma, USA). Sections were then washed twice for 10 min in PBS and the fluorescence intensity was determined by Image-Pro Plus version 5.0 System (Media Cybernetics, USA).

1.6 Western Blotting

As described before, total cellular proteins of renal medulla were obtained separately by pulverizing the tissue and dissolving the powder in lysis buffer. Homogenization was followed by centrifugation (1000 g, 10 min, at 4°C). The protein content was determined by the method of Bradford. The following steps were performed at room temperature. Samples of 10 μg protein were subjected to SDS gel electrophoresis using 12% acrylamide gels in a Mini-PROTEAN II Electrophoresis Cell. For determination of molecular weight, a prestained protein ladder was used. After gel electrophoresis (60 mA/gel, 70 min), proteins were transferred to PVDF membrane of 0.45 μm pore size. Membranes were blocked for 90 min with blocking buffer and rinsed twice with TBS containing 0.1% Tween 20 (TBST). Thereafter, the AQP2 antibody (0.5 μg/mL) was incubated overnight in TBST. The secondary horseradish peroxidase-conjugated antibody was incubated at a concentration of 0.1 μg/mL for 2 h in TBST. Immunoreactive proteins were detected using ECL-Western blotting detection system according to the manufacturer’s instructions. Membranes were exposed to Hyperfilm-ECL autoradiography films for 2 min and quantified with the software analysis system of MGIAS-1000 image.

1.7 Statistics

Data were presented as x̄±s. Statistical analysis was carried out by means of t-test using SPSS 10.0, and values of P<0.05 were considered statistically significant.

2 RESULTS

2.1 General Characterization

More than 16.7 mmol/L blood glucose persisted in STZ-induced DN mice during the 6-week studying period, which could not be attenuated by Micardis treatment. Body weight, right kidney weight, right kidney-to-body weight ratio, 24-h urine protein excretion, mean glomeruli area and perimeter were increased, while the urinary osmolality decreased in DN group as compared normal group (all P<0.05). The above changes could be attenuated by Micardis treatment (P< 0.05 vs. normal and DN groups). The results were shown in table 1.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal group</th>
<th>DN group</th>
<th>Micardis-treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>7.10±0.68</td>
<td>33.33±0</td>
<td>31.55±3.44</td>
</tr>
<tr>
<td>24-h urinary protein excretion (mg)</td>
<td>0.32±0.03</td>
<td>5.52±0.49*</td>
<td>3.98±0.28</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>24.58±2.82</td>
<td>17.58±2.63*</td>
<td>21.08±2.08</td>
</tr>
<tr>
<td>Right kidney weight (mg)</td>
<td>123.30±10.95</td>
<td>155.33±13.89*</td>
<td>137.08±3.11</td>
</tr>
<tr>
<td>Right kidney weight/body weight (×10^-5)</td>
<td>5.1±0.57</td>
<td>9.1±12*</td>
<td>6.5±0.57</td>
</tr>
<tr>
<td>Mean glomeruli area (×10^-2 μm^2)</td>
<td>96.05±5.34</td>
<td>158.35±12.91*</td>
<td>125.60±8.75</td>
</tr>
<tr>
<td>Mean glomeruli perimeter (×10^-1 μm)</td>
<td>34.70±0.97</td>
<td>44.57±1.81*</td>
<td>39.70±1.38</td>
</tr>
<tr>
<td>Urinary osmolality (osmol/kg)</td>
<td>3.16±0.16</td>
<td>2.39±0.11*</td>
<td>2.85±0.14*</td>
</tr>
</tbody>
</table>

*P<0.05 vs normal group; ▲P<0.05 vs DN group