Evaluation of sphingolipid metabolism in the renal cortex of rats with streptozotocin-induced diabetes and the effects of rapamycin

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Abstract

Background. Abnormal lipid metabolism contributes to the pathogenesis of diabetes, but it is uncertain whether it plays a role in the development of diabetic nephropathy (DN). While rapamycin was shown to prevent DN development in streptozotocin (STZ)-induced diabetic rats in our previous studies, it is unknown if it intervenes with lipid metabolism.

Methods. We divided the rats into four groups: normal control rats, rapamycin-treated normal rats, diabetic rats and rapamycin-treated DN rats. The apoptosis was evaluated by immunohistochemistry. The crude lipid and sphingolipid were extracted from rat renal cortex and analysed by matrix-assisted laser desorption ionization-time of flight mass spectrometry. The expression of the three key enzymes in sphingolipid metabolism including serine palmitoyltransferase, acid sphingomyelinase and sphingomyelin synthase was measured by western blot and immunohistochemistry in rat renal cortex.

Results. The level of apoptosis was increased in diabetic rats, and rapamycin treatment reduced apoptosis. STZ treatment significantly increased formation of many sphingolipids species through elevated de novo synthesis. These changes were inhibited by treatment with rapamycin.

Conclusions. Accumulation of sphingolipids contributes to STZ-induced diabetes, and the therapeutic effect of rapamycin on diabetic nephropathy is partly through suppression of sphingolipid abnormality.

Keywords: diabetes; kidney; rapamycin; rat; sphingolipids

Introduction

Diabetic nephropathy (DN) is one of the most frequent causes of chronic renal dysfunction. The early abnormalities in the kidney detected after the onset of diabetes in-
But systematic study of the different lipid metabolism under health and disease conditions became possible only after the advent of ‘lipidomics’, in which individual lipid species can be analyzed simultaneously by mass spectrometry (MS). It can collect vast amounts of information regarding the changes of lipids in different conditions [3,9–11]. Different MS techniques, including matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS and liquid chromatography-electrospray ionization (LC-ESI)-MS/MS, are crucial for lipidomic study [12,13].

Rapamycin, an immunosuppressive macrolide, binds to FK-binding protein 12. This complex then inhibits the mammalian target of rapamycin (mTOR) signalling pathway [8]. A previous study showed that rapamycin can attenuate compensatory renal hypertrophy after unilateral nephrectomy [14]. Another study indicated that low-dose rapamycin inhibits MC proliferation, collagen IV production and laminin synthesis [15]. The blockade of mTOR by low-dose rapamycin has a beneficial effect in DN [16]. Our group reported previously that rapamycin can reduce albuminuria, glomerular enlargement, glomerular basement membrane thickening and renal macrophage recruitment in STZ-induced diabetes [17]. Therefore, rapamycin can prevent the early renal structural changes and halt the early steps of the development of DN, but the mechanism needs further in-depth investigation.

Since sphingolipids play important roles at the MC proliferation and hypertrophy of DN, and rapamycin treatment can attenuate the proliferation and hypertrophy, in the present study, we investigated the changes of sphingolipids in renal cortex of STZ-induced diabetic rats using MALDI-TOF MS to identify the changes of sphingolipid metabolism in diabetes and the influence of rapamycin treatment.

Materials and methods

Animal

Experiments involving animals were conducted following the regulation of the Guide for the Care and Use of Laboratory Animals, Zhejiang University, according to the protocols that we reported before [17]. Eight-week-old male Sprague–Dawley rats (200–250 g body weight) were purchased from Zhejiang University Animal Center (Hangzhou, China) and housed under controlled environmental conditions (temperature 22°C, 12-h darkness period). Water was given ad libitum.

Rats were divided into four groups: normal control rats (NC, n = 6), rapamycin-treated normal rats (NC + RAPA, n = 6), diabetic rats (DN, n = 6) and rapamycin-treated DN rats (DN + RAPA, n = 6) at random by weight. Diabetes was induced by intraperitoneal injection of STZ (Sigma-Aldrich, Germany) at 60 mg/kg body weight in 0.1 mmol/L citrate buffer (pH 4.5) after 12 h of food deprivation. Three days after STZ administration and twice a week thereafter, the rats were weighted, and tail-vein blood glucose was examined. Rats with blood glucose levels >16.7 mmol/L were considered to have diabetes. Rapamycin (1 mg/kg) was administered daily by gavage starting 3 days after STZ injection.

After diabetes induction, rats were placed in metabolic cages for collection of 24-h urine specimens on day 0 and on day 30. Blood pressure was measured by tail-cuff method. Serum and urine creatinine levels were determined by standard methods using an autoanalyzer (Hitachi 7600, Japan). Urinary albumin excretion was determined by immunoturbidimetry (Hitachi 7020).

After a follow-up of 4 weeks, rats were sacrificed and kidney tissue samples were processed and stored as needed.

Histological studies

Tissue sections of kidney, 3–4 μm thick, were made for paraffin embedding and subsequent staining with periodic acid-Schiff (PAS). All samples were evaluated by a pathologist who was blind to group assignment. Mean glomerular volume was evaluated in PAS sections.

The following antibodies were used in immunohistochemical staining: rabbit anti-cleaved caspase 3 monoclonal antibody (1:200, Cell Signaling, USA), rabbit anti-ASM polyclonal antibody (1:200, Santa Cruz, USA), mouse anti-SMS polyclonal antibody (1:100, Santa Cruz, USA) and rabbit anti-SPT (1:200, Santa Cruz, USA). The number of positive stained tubules was calculated in 10 consecutive high power (×400) fields in all biopsies and expressed as the average percentage of the positive stained tubules over the total tubules in the field.

Crude lipid extraction

Crude lipids were extracted according to the method described by Folch et al. [18] as following: frozen renal tissues were chopped and homogenized in chloroform/methanol (2:1) and finally diluted to 20 times the volume of the original tissue sample volume using chloroform/methanol (2:1). The extract was then mixed thoroughly with 0.2 volume of water, and the mixture was separated into two phases and used for further analysis.

Sphingolipid extraction

The extraction of sphingolipids was conducted as described before [19]. Briefly, 1 mL lipid-containing lower phase, described above, was dried by vacuum centrifugation in a centrifugal evaporator (Speed-Vac, Thermo savant, Holbrook, NY). Five hundred microlitres of methanol containing...
DE MALDI-TOF MS analysis
Each sample obtained from the above procedure was dissolved in 5 μL chloroform–methanol (v/v, 2:1), followed by the addition of 5 μL matrix solution [0.5 M 2,5-dihydroxybenzoic acid (Sigma) solution in ethylacetate containing 0.1% TFA] in a 0.5 mL EP tube. The tube was vortexed vigorously and then centrifuged in a microcentrifuge for 1 min. One microlitre of mixture was directly spotted onto the sample plate and rapidly dried under a moderate warm stream of air in order to remove the organic solvent within seconds.

All samples were analysed using a 4700 MALDI-TOF/TOF Analyzer (Applied Biosystems, USA) with a 377 nm N2 UV laser. The mass spectra of samples were obtained in positive ion mode. Mass/charge (m/z) ratio was measured in the reflector/delayed extraction mode with an accelerating voltage of 20 kV, grid voltage of 67% and delay time of 100 ns.

Establishment of reference mass spectrum and relative quantification
Reference mass spectra (RMS) were established, and relative quantification was processed as described before [20]. For analysing sphingolipids, we use the RMS to deal with problems of inter-animal variation, as well as the problem associated with the poor quantification ability of MALDI-TOF MS. Only those peaks presented in at least five mass spectra from the same group were chosen and put into the RMS. It was found that in all the mass spectra obtained, peak 725.62, which corresponds to SM [d16:1C18:0+Na] +, always had the relative intensity of 100%, meaning it was the highest peak in the mass spectra obtained, peak 725.62, then the mean and standard deviation of the relative intensity of each peak was established for each sample, and the particular lipid giving rise to the peak was identified by comparing their validated masses in lipid mass spectrum analysis database (http://lipid.zju.edu.cn). All the comparisons were then conducted using these RMS.

Protein extraction and western blot
Renal cortexes were homogenized and centrifuged in lysis buffer (1% NP40, 10% glycerol, 50 mmol/L HEPES, 137 mmol/L NaCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 2 mmol/L Na₃NO₂, 10 mmol/L Na₂P₂O₅, 100 mmol/L NaF, 5 μg/mL aprotinin, 1 mmol/L PMSF and 1 μg/mL leupeptin) at 4°C. The protein concentration in the supernatant was measured with a BCA protein assay kit (Pierce, Rockford, USA). Fifty micrograms of protein from each sample was blotted by rabbit anti-ASM polyclonal antibody (Santa Cruz, USA) and mouse anti-SPT (Santa Cruz, USA) at 1:100 dilution and incubated in a centrifugal evaporator after removing the upper phase to discard the salt. The residue, considered the crude sphingolipid fraction, was stored at −70°C for MALDI-TOF MS analysis.

Results

Metabolic data and biochemical parameters

Table 1. Metabolic data and biochemical parameters and structural change of kidney

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>NC + rapamycin</th>
<th>DN</th>
<th>DN + rapamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat, total number</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>450.00 ± 31.88</td>
<td>435.00 ± 26.11</td>
<td>249.00 ± 47.49*</td>
<td>250.00 ± 11.88*</td>
</tr>
<tr>
<td>Kidney/body weight (g/100 g)</td>
<td>0.78 ± 0.03</td>
<td>0.78 ± 0.04</td>
<td>1.37 ± 0.05*</td>
<td>1.11 ± 0.06***</td>
</tr>
<tr>
<td>MGV (× 10⁶μm²)</td>
<td>0.90 ± 0.02</td>
<td>0.89 ± 0.01</td>
<td>1.38 ± 0.12*</td>
<td>0.93 ± 0.03**</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>7.40 ± 0.61</td>
<td>7.30 ± 0.46</td>
<td>25.10 ± 2.96*</td>
<td>23.40 ± 2.74*</td>
</tr>
<tr>
<td>Urine volume (mL/24 h)</td>
<td>16.00 ± 2.64</td>
<td>15.00 ± 1.67</td>
<td>146.00 ± 22.12*</td>
<td>154.00 ± 14.5*</td>
</tr>
<tr>
<td>Urine protein (mg/24 h)</td>
<td>0.94 ± 0.13</td>
<td>0.94 ± 0.13</td>
<td>28.65 ± 4.53*</td>
<td>28.65 ± 4.53*</td>
</tr>
<tr>
<td>SCr (mg/dL)</td>
<td>107.90 ± 9.03</td>
<td>109.13 ± 7.60</td>
<td>104.05 ± 5.70</td>
<td>104.05 ± 5.70</td>
</tr>
<tr>
<td>Ccr (μL/min per 100 g body weight)</td>
<td>930.13 ± 55.72</td>
<td>917.12 ± 66.25</td>
<td>1264.32 ± 240.26*</td>
<td>1040.92 ± 101.25</td>
</tr>
</tbody>
</table>

Note: Data are mean ± SEM; SBP, systolic pressure; Ccr, creatinine clearance.

*Significantly different versus NC (P < 0.05).
**Significantly different versus DN (P < 0.05).

Statistical analysis
Each experiment was conducted at least three times. Statistical analysis was performed using a Student’s t-test. A probability level of P < 0.05 was considered significant. Data are presented as mean ± S.D.

Rapamycin reduced apoptosis in diabetic kidney

It has been reported that apoptosis is involved in the development of diabetic nephropathy [21]. Therefore, we tested whether apoptosis was influenced by rapamycin treatment. We conducted immunohistochemical staining for activated caspase 3. The results showed that activated caspase 3 was increased in the tubular interstitial regions in diabetic kidney (Figure 3, Table 2). Rapamycin treatment significantly reduced the number of caspase 3-positive cells. These data indicate that rapamycin can inhibit apoptosis in DN.
Crude lipid analysis of rat renal cortex

Since the above results and our previous study [17] showed that rapamycin treatment can reduce apoptosis and inflammation in diabetic kidney, and sphingolipids such as ceramides are important mediators in these biological processes, we hypothesized that the sphingolipid profile might change in diabetic nephrology and in re-

Fig. 2. Effects of rapamycin treatment on renal glomerular enlargement. NC, normal control; NC + rapamycin, normal control treated with rapamycin; DN, diabetic nephrology; DN + rapamycin, diabetic nephrology treated with rapamycin. Tissue sections of kidney were made for paraffin embedding and subsequent staining with PAS. Mean glomerular volume was evaluated in PAS sections, magnification ×400. The mean glomerular volume was increased in diabetes rats and reduced by rapamycin treatment.

Fig. 3. The immunohistochemistry of activated caspase 3 in renal cortex (magnification ×400). NC, normal control; NC + rapamycin, normal control treated with rapamycin; DN, diabetic nephrology; DN + rapamycin, diabetic nephrology treated with rapamycin. Caspase 3-positive cells were increased in DN rats and reduced by rapamycin treatment.
### Table 2. The quantification of the immunohistological tests

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC (mean ± SD)</th>
<th>NC + rapamycin (mean ± SD)</th>
<th>DN (mean ± SD)</th>
<th>DN + rapamycin (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat, total number</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Caspase 3 positive percent (%)</td>
<td>13.3 ± 16.3</td>
<td>8.3 ± 13.3</td>
<td>55.8 ± 21.3 *</td>
<td>34.3 ± 21.5 **</td>
</tr>
<tr>
<td>ASM positive percent (%)</td>
<td>11.0 ± 15.0</td>
<td>8.8 ± 7.8</td>
<td>15.0 ± 13.1</td>
<td>12.9 ± 14.1</td>
</tr>
<tr>
<td>SMS positive percent (%)</td>
<td>8.2 ± 6.7</td>
<td>5.2 ± 4.8</td>
<td>7.6 ± 7.2</td>
<td>4.7 ± 4.8</td>
</tr>
<tr>
<td>SPT positive percent (%)</td>
<td>7.3 ± 7.6</td>
<td>7.2 ± 8.5</td>
<td>22.4 ± 18.8 *</td>
<td>12.9 ± 10.2 **</td>
</tr>
</tbody>
</table>

Note: ASM, acid sphingomyelinase; SMS, sphingomyelin synthase; SPT, serine palmitoyltransferase.

*Significantly different versus NC group (P < 0.05).

**Significantly different versus DN group (P < 0.05).

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**Fig. 4.** Representative MALDI-TOF mass spectra of crude lipids in renal cortex of different rats. NC, normal control rats; NC + rapamycin, normal control rats treated with rapamycin; DN, STZ-induced diabetes rats; DN + rapamycin, STZ-induced diabetes rats treated with rapamycin. Crude lipid was extracted from rat renal cortex and analysed by MALDI-TOF MS. In the MS profiles of all these renal cortexes, no significant differences were observed.
response to rapamycin treatment. Thus, we used MALDI-TOF MS to investigate both the crude lipids and sphingolipids in normal control, rapamycin-treated control, diabetic kidney and rapamycin-treated diabetic kidney.

The crude lipid MALDI-TOF MS profiles of renal cortices from four groups were similar and no obvious differences were observed; the representative mass spectra of crude lipids are shown in Figure 4. Based on the pattern shown in previous reports [22–24], two major classes of phospholipids [phosphatidyl cholines (PC), sphingomyelins (SM)] were identified. It was found that m/z ions corresponding to PC comprised the main ions in the spectra. The dominant mass peaks corresponded to PC 34:1. Ions 756.64 ([PC32:0+Na]+), 810.64 ([PC38:4+H]+), 834.64 ([PC40:6+H]+) and 1542.22 ([2(PC34:1)+Na]+) were also identified.

Sphingolipid profile changes dramatically in STZ-induced diabetes rats

Since PCs comprise the majority of crude lipid, the existence and change of other phospholipid classes at lower concentrations might be masked when detected in MALDI-TOF positive ion mode [25,26]. In order to detect sphingolipids in rat renal cortex extracts, it is necessary to separate sphingolipids from crude lipids. Therefore, crude lipids were treated by mild alkalization, and sphingolipids were extracted and analysed by MALDI-TOF MS. Reference mass spectra for sphingolipids in the rat renal cortices of normal control, rapamycin treatment, STZ-induced diabetes and STZ-induced diabetes treated with rapamycin were established with 21 identified major peaks (Figure 5). Three classes of sphingolipids, ceramide (Cer), sphingomyelin (SM) and ceramide monohexoside (CMH), were identified for these 21 peaks. After comparing with the lipid mass spectrum analysis database (http://lipid.zju.edu.cn), molecular ions [M]+, [M+Na]+, [M+H]+ and [M+H2O+H]+ for Cer, SM and CMH species, were assigned. The major ion assignments in the mass spectra are listed in Table 3.

As shown in Figures 5 and 6 and Table 3, the composition of ceramide was dramatically changed in the renal cortex extracts of DN rats compared with NC rats. In diabetic kidney, we identified eight new species of ceramide,
one species of ceramide significantly increased and three species of ceramide disappeared (Figures 5 and 6, Table 3). For example, ions 512.55 (Cer [d18:1C13:1+H2O+H]+) and 559.57 (Cer [d18:2C16:0+Na]+) appeared, and the intensity of ion 540.58 (Cer [d18:0C16:0+H]+) increased in DN rats. In accordance with the change of ceramide, SM and CMH, which are synthesized from ceramide, were also changed. Three new species of SM and one new species of CMH appeared. Two species of SM and one species of CMH increased.

Rapamycin treatment attenuates the change of sphingolipid profile in DN

Although rapamycin itself only caused a slight change in sphingolipid profile in rat renal cortex (Figures 5 and 6, Table 3), the composition of sphingolipids in DN rats was greatly altered by rapamycin treatment. The relative intensities of ions 540.58 (Cer [d18:0C16:0+H]+), 685.60 (CMH [d18:1C15:0]+), 703.62 (SM [d16:1C18:0+H]+) and 753.65 (SM [d18:1C18:0+Na]+), which were increased in DN rats, were decreased after rapamycin treatment. Rapamycin treatment also inhibited the generation of many new ceramide and sphingomyelin species induced by STZ (Figures 5 and 6, Table 3), such as 559.57 (Cer [d18:2C16:0+Na]+), 659.34 (SM [d18:1C10:0+H2O+Na]+) and 850.74 (CMH [d18:1C24h:0+Na]+).

The expression of SPT is up-regulated in STZ-induced diabetes rats, but can be attenuated by rapamycin treatment

In order to investigate why sphingolipid profile changed greatly in DN and by rapamycin treatment, we examined

### Table 3. The average relative intensity of major sphingolipids in different rat groups

<table>
<thead>
<tr>
<th>Sphingolipid species (m/z)</th>
<th>NC (mean ± SD)</th>
<th>NC + rapamycin (mean ± SD)</th>
<th>DN (mean ± SD)</th>
<th>DN + rapamycin (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>512.55 Cer [d18:1C13:1+H2O+H]+</td>
<td>41.53 ± 6.93*</td>
<td>17.71 ± 7.06**</td>
<td>6.17 ± 0.70</td>
<td>19.95 ± 6.69*</td>
</tr>
<tr>
<td>537.58 Cer [d16:1C18:0]+</td>
<td>7.37 ± 2.35</td>
<td>7.62 ± 1.71*</td>
<td>19.95 ± 6.69*</td>
<td>19.95 ± 6.69*</td>
</tr>
<tr>
<td>540.58 Cer [d18:0C16:0+H]+</td>
<td>34.13 ± 3.49*</td>
<td>11.42 ± 3.28*</td>
<td>5.24 ± 0.57</td>
<td>7.39 ± 1.85**</td>
</tr>
<tr>
<td>551.27 Cer [d18:1C17:0]+</td>
<td>19.95 ± 6.69*</td>
<td>19.95 ± 6.69*</td>
<td>34.13 ± 3.49*</td>
<td>17.71 ± 7.06**</td>
</tr>
<tr>
<td>559.57 Cer [d18:2C16:0+Na]+</td>
<td>34.13 ± 3.49*</td>
<td>11.42 ± 3.28*</td>
<td>19.95 ± 6.69*</td>
<td>19.95 ± 6.69*</td>
</tr>
<tr>
<td>577.36 SM [d18:1C7:0+H]+</td>
<td>6.32 ± 2.39*</td>
<td>6.32 ± 2.39*</td>
<td>11.42 ± 3.28*</td>
<td>11.42 ± 3.28*</td>
</tr>
<tr>
<td>596.65 Cer [d18:1C19:0+H]+</td>
<td>7.62 ± 1.71*</td>
<td>7.62 ± 1.71*</td>
<td>19.95 ± 6.69*</td>
<td>19.95 ± 6.69*</td>
</tr>
<tr>
<td>652.61 Cer [d18:2C23:0+H2O]+</td>
<td>7.28 ± 1.31</td>
<td>7.28 ± 1.31</td>
<td>6.32 ± 2.39*</td>
<td>6.32 ± 2.39*</td>
</tr>
<tr>
<td>659.34 SM [d18:1C10:0+H2O+Na]+</td>
<td>7.28 ± 1.31</td>
<td>7.28 ± 1.31</td>
<td>19.95 ± 6.69*</td>
<td>19.95 ± 6.69*</td>
</tr>
<tr>
<td>664.46 Cer [d18:2C24:1+H2O+H]+</td>
<td>8.35 ± 1.09</td>
<td>8.35 ± 1.09</td>
<td>7.62 ± 1.71*</td>
<td>7.62 ± 1.71*</td>
</tr>
<tr>
<td>675.48 Cer [d18:2C26:0]+</td>
<td>6.26 ± 1.93*</td>
<td>6.26 ± 1.93*</td>
<td>7.74 ± 2.24</td>
<td>7.74 ± 2.24*</td>
</tr>
<tr>
<td>677.35 Cer [d18:1C26:0]+</td>
<td>9.12 ± 3.51*</td>
<td>9.12 ± 3.51*</td>
<td>11.38 ± 1.63</td>
<td>11.38 ± 1.63*</td>
</tr>
<tr>
<td>685.60 CMH [d18:1C15:0]+</td>
<td>33.87 ± 7.02*</td>
<td>33.87 ± 7.02*</td>
<td>7.54 ± 2.24</td>
<td>7.54 ± 2.24*</td>
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<tr>
<td>703.62 SM [d16:1C18:0+H]+</td>
<td>43.48 ± 6.66*</td>
<td>43.48 ± 6.66*</td>
<td>30.04 ± 4.84</td>
<td>30.04 ± 4.84*</td>
</tr>
<tr>
<td>725.62 SM [d16:1C18:0+Na]+</td>
<td>7.62 ± 1.71*</td>
<td>7.62 ± 1.71*</td>
<td>56.55 ± 27.64</td>
<td>56.55 ± 27.64*</td>
</tr>
<tr>
<td>753.65 SM [d18:1C18:0+Na]+</td>
<td>34.45 ± 6.69*</td>
<td>34.45 ± 6.69*</td>
<td>7.62 ± 1.71*</td>
<td>7.62 ± 1.71*</td>
</tr>
<tr>
<td>813.74 SM [d18:1C24:0+H]+</td>
<td>10.96 ± 3.52</td>
<td>10.96 ± 3.52</td>
<td>13.91 ± 8.97</td>
<td>13.91 ± 8.97</td>
</tr>
<tr>
<td>850.74 CMH [d18:1C24h:0+Na]+</td>
<td>21.64 ± 6.47**</td>
<td>21.64 ± 6.47**</td>
<td>15.36 ± 1.52*</td>
<td>15.36 ± 1.52*</td>
</tr>
</tbody>
</table>

Note: Cer, ceramide; SM, sphingomyelin; d, dihydroxy-sphingosine; h, there is hydroxyl group on the seventh carbon atom in side chain.
*Significantly different versus NC (P < 0.05).
**Significantly different versus DN (P < 0.05).
three key enzymes in the synthesis pathway for ceramide, including ASM, SMS and SPT. Immunohistochemical results showed that these three enzymes were mainly localized in the tubular interstitial region (Figure 7). The positive cells were tubular epithelial cell and microvascular endothelial cell. There was no significant change in the number of ASM- and SMS-positive cells, but the percentage of SPT-positive cells was greatly increased in DN rats and such elevation was inhibited by rapamycin treatment (Table 2). Western blot analysis also revealed that the expression of SPT was significantly increased in the renal cortex of DN rats compared with NC rats and this increase was reversed by rapamycin treatment, whereas no significant change was found for the levels of ASM and SMS in renal cortex between different experimental groups (Figure 8). These results suggest that during development of DN, ceramide profile changes as a result of an elevated expression of SPT, and rapamycin treatment can reverse this change through regulating SPT.

Discussion

STZ-induced DN showed some signs of early stage DN including hyperglycaemia, albuminuria, high ratio of kidney-to-body weight and high kidney filtration. While treatment with rapamycin had no effect on hyperglycaemia, it significantly decreased albuminuria and the ratio of kidney-to-body weight. The effect of rapamycin on proteinuria is consistent with our previous study [17] and the report by Lloberas et al. [16]. This is also in accordance with the inhibitory effect of rapamycin on proteinuria in
chronic hyperfiltration and inflammatory model [27]. Our group previously showed that cell proliferation and macrophage recruitment were elevated in STZ-induced DN and such changes were reduced by rapamycin [17]. In this study, we further tested apoptosis, which is also an important process in the early stage of DN in this model [28], and the effect of rapamycin treatment. The immunohistochemical results for activated caspase 3 indicated that apoptosis was increased in diabetic kidney. Rapamycin treatment significantly reduced apoptosis.

The fact that apoptosis was elevated in STZ-induced DN and rapamycin inhibited it suggests that apoptosis was caused by the factors that sensitive to rapamycin. Our finding that ceramide increased greatly in diabetic kidney and decreased after rapamycin treatment and the long established relationship of ceramide and apoptosis indicate that ceramide is a good candidate. Reports from several groups showed that inhibition of ceramide generation by inhibition of de novo synthesis such as inhibiting SPT or ceramide synthase effectively reduces cell death caused by chemical hypoxia, hypoxia-reoxygenation and radiocontrast media in tubular epithelial cells in kidney [29–31]. Our results showed that the increased expression of SPT, which represents the increased de novo synthesis of ceramide, was located mainly in tubular epithelial cells and microvascular endothelial cells, where the apoptosis mainly occurred, supporting the idea that increased ceramide induced apoptosis in DN.

Besides ceramide formation, glucosylceramide generation was also induced in DN. This is consistent with the study from Zador et al. [32] that the increase of renal gangliosides GM3 was found in diabetic rats. Our previous study showed that Akt/mTOR pathway was activated in DN and rapamycin treatment suppressed its activation [17]. Recently, Natoli et al. reported that glucosylceramide accumulated in kidneys of polycystic kidney disease mouse model and inhibition of such accumulation effectively block the disease by blocking the activation of Akt/mTOR [33]. This gives us a clue that the increased glucosylceramide may also participate in regulating Akt/mTOR pathway in DN. And although rapamycin directly inhibits mTOR complex, its suppression on glucosylceramide may also contribute to Akt/mTOR inhibition. The relationship between Akt/mTOR and sphingolipids in DN and rapamycin treatment requires further study.

In conclusion, in STZ-induced DN rat model, we found increased apoptosis in tubular interstitial regions, and rapamycin treatment reduced it. Using the lipidomic strategy, we analysed the sphingolipid composition and found that ceramide increased through de novo synthesis, which was also reversed by rapamycin treatment in the same kind of cell apoptosis occurred in diabetic kidney. These results indicate that elevated de novo synthesis of ceramide and ceramide accumulation participate in apoptosis and thus development of DN, and can be inhibited by rapamycin treatment.

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Conflict of interest statement. None declared.

References

25. Petkovic M, Schiller J, Muller M et al. Detection of individual phospholipids in lipid mixtures by matrix-assisted laser desorption/ionization...
Early and delayed effects of AST-120 on chronic cyclosporine nephropathy

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Abstract

Background. Removal of uraemic toxins by AST-120 (Kremezin®) decreases the progression of chronic kidney disease by reducing oxidative stress. We performed this study to evaluate whether AST-120 has a similar effect on progression of cyclosporine (CsA)-induced renal injury.

Methods. Two separate studies were performed in adult Sprague–Dawley rats. First, AST-120 was administered with CsA (15 mg/kg) for 4 weeks (early treatment). Second, AST-120 was administered to the rats for 3 weeks after treatment with CsA for 3 weeks (delayed treatment). Uraemic toxin and oxidative stress were evaluated with plasma indoxyl sulphate (IS) levels and urinary 8-OHdG excretion. The effects of AST-120 on CsA-induced renal injury were evaluated in terms of renal function, interstitial fibrosis, inflammation, and apoptotic cell death.

Results. CsA treatment for 4 weeks showed 2-fold increase in plasma IS and urinary 8-OHdG levels compared with the VH group. Early treatment with AST-120 significantly decreased both parameters, and this was accompanied by improved renal function and decreased interstitial inflammation, fibrosis, and apoptotic cell death compared with those of rats that received CsA alone. Delayed treatment with AST-120 also decreased the plasma IS and urinary 8-OHdG levels, and reduced the progression of chronic CsA nephropathy. Furthermore, delayed AST-120 treatment decreased the epithelial–mesenchymal transition in chronic CsA nephropathy.

Conclusions. Removal of uraemic toxins with AST-120 treatment is effective in decreasing the progression of CsA-induced renal injury by reducing oxidative stress.

Keywords: AST-120; chronic cyclosporine nephropathy; indoxyl sulphate; oxidative stress