Effects of all-trans-retinoic acid on the treatment of type 2 diabetic nephropathy

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Effects of all-\textit{trans}-retinoic acid on the
treatment of type 2 diabetic
nephropathy

Directed by Professor Ahn Chul Woo

The Doctoral Dissertation
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This certifies that the Doctoral Dissertation of Kim Chul Sik is approved.

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The Graduate School
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June 2007
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I have completed this paper with a lot of support and encouragement from many people around me.

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Lastly, I would like to dedicate this paper to my family: my parents, my parents-in-laws, my brothers, my dear wife Kim Eun Kyung who always provides me with support and devotion, and my precious daughters, Ye Won and Ye Jin.

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ABSTRACT

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Diabetic nephropathy is the leading cause of kidney disease in patients starting renal replacement therapy and affects approximately 40% of patients with diabetes mellitus. It increases the risk of death, mainly from cardiovascular causes, and is defined by increased urinary albumin excretion (UAE) in the absence of other renal diseases. About fifty percent of diabetic subjects develop microalbuminuria, which progresses towards established diabetic nephropathy
in one third of the patients. Hyperglycemia, advanced glycation end-product (AGE), increased polyol pathway, oxidative stress, and the activation of transforming growth factor-β1 (TGF-β1) are interrelated in the pathogenesis of diabetic nephropathy.

All-trans-retinoic acid (ATRA) has been reported to suppress interstitial proliferation as well as glomerular inflammation, and to prevent renal damage in diabetic rats. Retinoic acid has also been reported to block lipid peroxidation in streptozocin-induced diabetic rats, and there are studies that also support the protective effect of retinoic acid on the progression of type 2 diabetic nephropathy.

In this study, we examined the effect of ATRA on improving diabetic nephropathy by measuring the amount of UAE after administrating ATRA to Otsuka Long-Evans Tokushima Fatty (OLETF) rats. In order to understand the mechanism of action of ATRA, we administrated ATRA to see its inhibitory action on the production of TGF-β1 after confirming the increased production of TGF-β1 in response to high or control glucose media in cultured rat mesangial cells (RMCs). Moreover, we examined changes in protein kinase C (PKC) and reactive oxidative stress (ROS), which are located in the upstream of intracellular signaling pathway of TGF-β1 after the administration of ATRA.

From 28 weeks of age, the OLETF rats weighed more than the LETO rats of
the same age, while there was no difference in weights between the non-treated OLETF rat group and the ATRA-treated OLETF rat group. At 44 weeks of age, 16 weeks after the administration of ATRA, fasting glucose levels were significantly lower in the LETO rats (105.4 ± 14.4 mg/dL) than in the non-treated OLETF rats (178.5 ± 38.3 mg/dL, \( P < 0.01 \)) or in the ATRA-treated OLETF rats (151.0 ± 25.1 mg/dL, \( P < 0.01 \)). A decrease in serum glucose was observed in the ATRA-treated OLETF rats when compared with the non-treated OLETF rats. Compared with the LETO rats, the OLETF rats showed increased levels of total cholesterol and triglyceride. However, no significant differences in total cholesterol and triglyceride levels were found between the ATRA-treated OLETF rats and the non-treated OLETF rats. The non-treated OLETF rats might have been in the greater insulin resistance status, as demonstrated by increased insulin, C-peptide and HOMA-IR levels than the other two groups. Also, there were no remarkable differences in AST, ALT, creatinine, hemoglobin levels, white blood cell count, or platelet count between the three groups. The OLETF rats showed a higher daily UAE than the LETO rats at 44 weeks of age. In the ATRA-treated OLETF rats, daily UAE was lower than that of non-treated the OLETF rats (0.07 ± 0.03 vs. 0.17 ± 0.15 mg/mgCr, \( P < 0.01 \)).

After incubation of quiescent mesangial cells in media containing 30 or 5 mM of glucose, treatment with ATRA showed time- and dose-dependent decreases
in TGF-β1 levels. Moreover, ATRA treatment under both low and high glucose conditions showed a dose-dependent decrease in PKC activity. Lastly, treatment with ATRA showed dose- and time-dependent decreases in DCF-sensitive cellular ROS in the RMCs.

In this study, we demonstrated that the administration of ATRA resulted in a reduction of UAE in OLETF rats, cultured RMCs increased TGF-β1 synthesis in response to high glucose stimuli, and that ATRA treatment suppressed TGF-β1 synthesis induced by high glucose stimulation. It is of interest that ATRA treatment suppressed UAE and TGF-β1 synthesis, which was mediated by a significant reduction of PKC activity and ROS production. Our results suggest that ATRA has a potential therapeutic role for diabetic nephropathy.

Key words: all-trans-retinoic acid, diabetic nephropathy, transforming growth factor-β1, protein kinase-C, reactive oxygen species, Otsuka Long-Evans Tokushima Fatty rats
Effect of all-trans-retinoic acid on the treatment of type 2 diabetic nephropathy

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I. INTRODUCTION

Diabetic nephropathy is the leading cause of chronic kidney disease in patients starting renal replacement therapy\(^1\) and is associated with increased cardiovascular mortality\(^2\). Diabetic nephropathy has been classically defined by the presence of proteinuria > 0.5 g/24 h. This stage has been referred to as overt
nephropathy, clinical nephropathy, proteinuria, or macroalbuminuria. In the early 1980s, seminal studies from Europe revealed that small amounts of albumin in the urine, not usually detected by conventional methods, were predictive of the later development of proteinuria in type 1\textsuperscript{3-5} and type 2\textsuperscript{6} diabetic patients. This stage of renal involvement is termed microalbuminuria or incipient nephropathy.

Initial changes in diabetic nephropathy include hypertrophy of basement membrane and proliferation of glomerular interstitial cells, and later, glomerular sclerosis and interstitial fibrosis may occur. Hyperglycemia, advanced glycation end-product (AGE), increased polyol pathway, oxidative stress, and the activation of transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)) are interrelated in the pathogenesis of diabetic nephropathy\textsuperscript{7,8}.

Achieving the best metabolic control, treating hypertension, using drugs with blockade effect on the renin-angiotensin-aldosterone system, and treating dyslipidemia are effective strategies for preventing the development of microalbuminuria, delaying the progression to more advanced stages of nephropathy, and reducing cardiovascular mortality in patients with type 1 and type 2 diabetes. However, the above measures might not be effective in some patients with diabetes, and novel therapeutic strategies are warranted.

Retinoic acid is a derivative of vitamin A, which is a fat-soluble essential
vitamin supplied to human through fiber-rich diets. Vitamin A is stored in the liver as retinol, and then is converted to retinoic acid and delivered to organs involved in normal growth, integration of tissues, and immunity\textsuperscript{9,10}. Vitamin A is converted to two active forms, \textit{trans} and \textit{cis}, and enters nucleus after forming heterodimer with the retinoic acid A receptor (RAR) and the retinoic X receptor (RXR) in cellular cytoplasm. By stimulating the transcription of retinoic acid response element, it is known to have anti-proliferative and anti-inflammatory effects\textsuperscript{11}.

When all-\textit{trans}-retinoic acid (ATRA), a pan-retinoic acid agonist, was administered orally to an anti-Thy nephropathy model rats, it suppressed interstitial proliferation and glomerular inflammation, and prevented renal damage\textsuperscript{12}. Moreover, retinoic acid has been reported to block lipid peroxidation in streptozocin-induced diabetic rats, and there are studies that also support the protective effect of retinoic acid on the progression of type 2 diabetic nephropathy\textsuperscript{13,14}.

Otsuka Long-Evans Tokushima Fatty (OLETF) rat, derived from Long-Evans rats, is a spontaneously developed model of type 2 diabetes mellitus (T2DM) with obesity, insulin resistance, and impaired insulin secretion at a later age\textsuperscript{15}. The rats gain weight around 8 weeks of age and develop a slight degree of glucose intolerance at the same time. These changes predominantly occur in
male rats, with approximately 90% developing diabetes and 7% showing impaired glucose tolerance by 30 weeks of age. Male OLETF rats develop glomerulopathy similar to that in diabetic nephropathy, mesangial proliferation around 22 weeks of age, and fibrin cap and periodic acid shiff (PAS)-positive deposits in a part of the glomerulus. Functionally, albuminuria starts before 20 weeks of age and then becomes prominent after 30 weeks of age.

Therefore, in this study, we examined the effect of ATRA on improving diabetic nephropathy by measuring the amount of urine albumin excretion (UAE) after administrating ATRA to OLETF rats. In order to understand the mechanism of action of ATRA, we administered ATRA to see its inhibitory action on the expression of TGF-β1 after verifying the increased expression of TGF-β1 in response to high or control glucose media in cultured rat mesangial cells (RMCs). Moreover, we examined changes in protein kinase C (PKC) and reactive oxidative stress (ROS) which are located in the upstream of intracellular signaling pathway of TGF-β1 after the administration of ATRA.
II. MATERIALS AND METHODS

1. Animal study and experimental design

Six-week old male OLETF rats and Long-Evans-Tokushima-Otsuka (LETO) rats were obtained as a generous gift from the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. Each rat was housed in a metabolic cage and maintained on a 12-h light-dark cycle at 24°C and 40~60% humidity. Tap water and pelleted rat chow were available ad libitum throughout experiments. We measured the body weight of the rats every 4 week after an overnight fast.

In order to evaluate the effect of ATRA on diabetic nephropathy, we orally administered 10 mg/kg body weight of ATRA (All-Trans-Retinoic acid, Promega, Madison, WI, USA) dissolved in cellulose and 5% DMSO to 20 OLETF rats with diabetes for 16 weeks (ATRA-treated OLETF group). We administered cellulose and DMSO as a vehicle to 20 OLETF rats (Non-treated OLETF group) and 10 LETO rats (LETO group) instead of ATRA for the same period.

The fasting blood glucose (FBG, Hexokinase method, Advia® 1650, Bayer, Berkley, CA, USA) levels were measured at 28 weeks of age utilizing whole blood obtained from a tail vein after overnight fasting. An oral glucose
tolerance test (OGTT, 2 gram glucose per kg body weight) was performed at 28 weeks of age for the diagnosis of diabetes, and their blood glucose levels were measured before and 2 hour after administering the glucose load.

At 44 weeks of age, the rats were anesthetized with tiletamine/zolazepam (Zoletin®, 30mg/kg, intraperitoneally, Virbac Lab., Carros, France) as well as xylazine (Rompen® 10 mg/kg, intraperitoneally, Bayer, Berkley, CA, US) and subsequently blood samples were collected via cardiac puncture. Serum was promptly prepared from the blood samples by centrifugation at 3000 rpm for 10 min at room temperature and stored at −70 °C until analyses. We then measured serum glucose (Hexokinase method, Advia® 1650, Bayer, Berkley, CA, USA), C-peptide (Radioimmunoassay, RAT C-peptide RIA kit, Linco, St. Charles, Missouri, USA), insulin (Radioimmunoassay, RAT Insulin RIA kit, Linco, St. Charles, Missouri, USA), total cholesterol (Enzymatic assay, Advia® 1650, Bayer, Berkley, CA, USA), triglyceride (Enzymatic assay, Advia® 1650, Bayer, Berkley, CA, USA), HDL-cholesterol (Selective inhibition method, Advia® 1650, Bayer, Berkley, CA, USA), and LDL-cholesterol (calculated by total cholesterol - triglyceride/5 - HDL-cholesterol). For the evaluation of adverse reactions, we did peripheral blood examination (LH 750, Coulter, Miami, FL, USA) after overnight fasting, including serum creatinine (Jaffe, Alkaline Picrate, Kinetic method, Advia® 1650, Bayer, Berkley, CA, USA), and aspartate
aminotransferase (IFCC UV method, Advia® 1650, Bayer, Berkley, CA, USA), and alanine aminotransferase (IFCC UV method, Advia® 1650, Bayer, Berkley, CA, USA) levels. Twenty-four hour urine samples were collected at 44 weeks of age, and albumin excreted into the urine was measured by an immunoturbidmetric method (Cobas Integra, Roche, Basel, Switzerland) using rat albumin as a standard. For control group, we kept the LETO rats under the same conditions and performed the same tests. All procedures were performed according to institutional guidelines for animal research.

2. Culture of rat mesangial cells (RMCs)

We cultured commercial RMCs in DMEM (Dulbecco's Modified Eagle Medium), low glucose (1g/L), 1% Penicillin, 5% Fetal Bovine Serum, and exchanged the culture media every 2-3 days for 2 weeks. When the media were almost completely filled with RMCs, we then subcultured them using trypsin/EDTA (0.5%/0.53 nM). The RMCs that underwent 6-10 cycles of subculture for the following experiment.

For the experiment, D-glucose (Sigma Chemical Co. St. Louis, MO, USA) was added to the DMEM culture media to make the final glucose concentrations of 30 and 5 mM for high glucose and control glucose groups, respectively. When the cells reached a subconfluent state, we replaced the media with media
containing 0.2% FBS, and after 48 hours, we cultured in the media containing 30 or 5 mM of glucose. To exclude osmolar effect of high glucose concentration, we added 25 mM of mannitol into the media containing 5 mM of glucose.

After incubation of quiescent RMCs with $10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ M of ATRA for the given periods (6, 24, and 48 hrs) in the media containing 30 or 5 mM of glucose, the changes in TGF-β₁, PKC, and ROS from these cells were measured.

3. Measuring TGF-β₁, PKC, ROS

We collected the entire culture media and then centrifuge them. When cell suspension was activated using 1N HCl and 1.2N NaOH, we measured concentrations of TGF-β₁ with the quantitative sandwich enzyme immunoassay technique from R&D system (Minneapolis, MN, USA).

PKC assay were performed using SDS-PAGE and immunoblotted with anti-PKC isoforms. In brief, we washed the culture cells ($5 \times 10^6$~$1 \times 10^7$) with PBS buffer two times and suspended them in 0.5ml of whole cell lysate buffer. Equal amounts of protein were separated by SDS-PAGE (8% resolving), electroblotted to nitrocellulose, and probed with antibodies against PKC-α, PKC-β, PKC-δ (1:500 dilution, Santa Cruz, CA, USA), or β-actin (1:5,000 dilution, Sigma, St. Louis, MO, USA) overnight in a 4°C cold room. The PKC antibodies recognized mouse and rat PKCs. The proteins were visualized by
chemiluminescent detection (ECL, Amersham Biosciences, Bucks, UK).

A peroxidase-sensitive fluorescent indicator, 2',7'-dichlorofluorescin diacetate (DCF, 10 mmol/L; Molecular Probes Inc., Eugene, Oregon, USA) was incubated within mesangial cells for 30 minutes and washed before the addition of experimental conditions. ROS oxidized the probe, rendering compound, 2', 7'-dichlorofluorescin, whose fluorescence was quantified by E-max ELISA reader (Molecular Device Corp., Sunnyvale, USA). In addition, we examined oxidative activity after H$_2$O$_2$ (Sigma Chemical Co., St. Louis, MO, USA) stimulation.

4. Statistical analyses

Results are expressed as mean ± SE or SD, with n as the number of experiments. We used non-parametric analysis because most of the variables were not normally distributed. The Kruskal-Wallis multiple comparison nonparametric test (or ANOVA) was performed and a post hoc was applied to determine individual differences between means. The changes in the various parameters following the administration of ATRA at different concentrations and with different treatment periods were analyzed using the trend analysis (linear by linear association method). A $p$ value of < 0.05 was considered significant. All statistical analyses were performed using SPSS for Windows.
11.5 (SPSS, Chicago, IL, USA).
III. RESULTS

1. *In vivo* study

Table 1 shows the monthly measured weights for each group starting at 10-week old. There was no significant difference in weights among three groups at first. From 28 weeks of age, the OLETF rats weighed more than the LETO rats of the same age, while there was no statistical difference in weights between the non-treated OLETF rat group and the ATRA-treated OLETF rat group.

<table>
<thead>
<tr>
<th>Table 1. Body weight of rats from 10 to 44 weeks of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>LETO rats</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>10 weeks (g)</td>
</tr>
<tr>
<td>28 weeks (g)</td>
</tr>
<tr>
<td>32 weeks (g)</td>
</tr>
<tr>
<td>36 weeks (g)</td>
</tr>
<tr>
<td>40 weeks (g)</td>
</tr>
<tr>
<td>44 weeks (g)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD, Statistical difference was performed
among groups with the same duration of experimental period. *P < 0.05 versus the LETO rats, **P < 0.01 versus the LETO rats, †P < 0.05 versus the non-treated OLETF rats, ‡P < 0.01 versus the non-treated OLETF rats.

At 28 weeks of age, immediately before the administration of ATRA, a fasting glucose level was significantly lower in the LETO rats (104.1 ± 4.2 mg/dL, mean ± SD) than in the OLETF rats (115.6 ± 9.8 mg/dL, P < 0.01). A postprandial 2-hr glucose level was also significantly lower in the LETO rats (143.1 ± 6.7 mg/dL) than in the OLETF rats (334.9 ± 9.8 mg/dL, P < 0.01). However, both fasting and postprandial 2-hr blood glucose levels were not different between the non-treated and ATRA-treated OLETF rats (Table 2).

Table 2. Glucose levels of control and diabetic rats at 28 weeks of age

<table>
<thead>
<tr>
<th></th>
<th>LETO rats</th>
<th>Non-treated OLETF rats</th>
<th>ATRA-treated OLETF rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>104.1 ± 4.2</td>
<td>118.1 ± 9.2**</td>
<td>113.5 ± 10.3†</td>
</tr>
<tr>
<td>OGTT 2hr glucose (mg/dL)</td>
<td>143.9 ± 6.7</td>
<td>353.0 ± 81.0**</td>
<td>321.4 ± 55.3**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD, Statistical difference was performed among groups with the same duration of experimental period. *P < 0.05 versus the LETO rats, **P < 0.01 versus the LETO rats, †P < 0.05 versus the non-
treated OLETF rats, \(^{‡}P < 0.01\) versus the non-treated OLETF rats, OGTT; oral glucose tolerance test.

At 44 weeks of age, 16 weeks after the administration of ATRA, a fasting glucose level was significantly lower in the LETO rats (105.4 ± 14.4 mg/dL) than in the non-treated OLETF rats (178.5 ± 38.3 mg/dL, \(P < 0.01\)) or in the ATRA-treated OLETF rats (151.0 ± 25.1 mg/dL, \(P < 0.01\)). The significant difference in serum glucose levels was observed between the ATRA-treated OLETF rats and the non-treated OLETF rats \(P < 0.05\) (Table 3).

Compared with the LETO rats (111.0 ± 7.8 mg/dL), the non-treated OLETF (199.3 ± 59.0 mg/dL) and the ATRA-treated rats (164.0 ± 63.7 mg/dL) showed increased concentrations of total cholesterol \(P < 0.01\). However, no significant difference in total cholesterol was found between the ATRA-treated OLETF rats and the non-treated OLETF rats. Moreover, the LETO rats showed decreased triglyceride levels (26.8 ± 15.1 mg/dL) compared with the non-treated (231.6 ± 149.2 mg/dL, \(P < 0.01\)) and ATRA-treated OLETF rats (201.7 ± 75.5 mg/dL, \(P < 0.01\)). However, no significant difference in triglyceride levels was found in the ATRA-treated OLETF rats when compared with the non-treated OLETF rats \(P = 0.851\). Moreover, there were no significant differences in HDL- and LDL-cholesterol levels between the three groups (all \(P\)
The non-treated OLETF rats might have been in the higher insulin resistance status, as demonstrated by increased insulin, C-peptide and HOMA-IR levels than the other two groups (Table 3).

Twenty four-hour urine amount was lower in the LETO rats compared with the non-treated and ATRA-treated OLETF rats (17.7 ± 3.0 mL vs. 37.7 ± 18.8 vs.30.4 ± 9.7, respectively, all $P < 0.01$). The OLETF rats showed a higher daily UAE than the LETO rats (0.01 ± 0.01 mg/mgCr) at 44 weeks of age. In the ATRA-treated OLETF rats, daily UAE was lower than in the non-treated OLETF rats (0.07 ± 0.03 vs. 0.17 ± 0.15 mg/mgCr, $P < 0.01$) (Table 3).

There were no significant differences in AST, ALT, creatinine, hemoglobin levels, white blood cell count, or platelet count between the three groups (Table 3).

Table 3. Biochemical data of control and diabetic rats at 44 weeks of age

<table>
<thead>
<tr>
<th></th>
<th>LETO rats</th>
<th>Non-treated OLETF rats</th>
<th>ATRA-treated OLETF rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>105.4 ± 14.4</td>
<td>178.5 ± 38.3**</td>
<td>151.0 ± 25.1**†</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>111.0 ± 7.8</td>
<td>199.3 ± 59.0**</td>
<td>164.0 ± 63.7**</td>
</tr>
<tr>
<td>Parameter</td>
<td>Group 1</td>
<td>Group 2</td>
<td>Group 3</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>26.8 ± 15.1</td>
<td>231.6 ± 149.2**</td>
<td>201.7 ± 75.5**</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>55.1 ± 14.1</td>
<td>55.0 ± 16.0</td>
<td>48.2 ± 12.7</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>62.6 ± 7.0</td>
<td>97.9 ± 45.2</td>
<td>77.3 ± 51.8</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.43 ± 0.56</td>
<td>2.39 ± 0.34**</td>
<td>1.93 ± 0.74†</td>
</tr>
<tr>
<td>C-peptide (ng/mL)</td>
<td>0.79 ± 0.19</td>
<td>1.06 ± 0.56</td>
<td>0.75 ± 0.59</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.37 ± 0.13</td>
<td>1.06 ± 0.28**</td>
<td>0.73 ± 0.32*,†</td>
</tr>
<tr>
<td>Urine amount (mL/day)</td>
<td>17.7 ± 3.0</td>
<td>37.7 ± 18.8**</td>
<td>30.4 ± 9.7**</td>
</tr>
<tr>
<td>Urine albumin (mg)</td>
<td>0.15 ± 0.07</td>
<td>2.42 ± 2.15***</td>
<td>0.96 ± 0.52*,†</td>
</tr>
<tr>
<td>Urine creatinine (mg)</td>
<td>13.9 ± 2.1</td>
<td>15.2 ± 3.2</td>
<td>14.6 ± 2.8</td>
</tr>
<tr>
<td>UAE (mg/mgCr)</td>
<td>0.01 ± 0.01</td>
<td>0.17 ± 0.15**</td>
<td>0.07 ± 0.03*,‡</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.95 ± 0.13</td>
<td>0.88 ± 0.09</td>
<td>0.83 ± 0.15</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>64.5 ± 33.5</td>
<td>74.7 ± 42.3</td>
<td>68.3 ± 26.1</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>56.9 ± 9.2</td>
<td>55.6 ± 18.3</td>
<td>61.6 ± 19.8</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.4 ± 1.0</td>
<td>14.3 ± 0.9</td>
<td>13.5 ± 1.1</td>
</tr>
<tr>
<td>WBC (10³/mm³)</td>
<td>2.28 ± 1.68</td>
<td>3.28 ± 1.45</td>
<td>3.17 ± 1.48</td>
</tr>
<tr>
<td>Platelet (10³/mm³)</td>
<td>725.3 ± 212.1</td>
<td>829.1 ± 231.8</td>
<td>905.1 ± 84.1</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. Statistical difference was performed among groups with the same duration of experimental period. *P < 0.05 versus the LETO rat, **P < 0.01 versus the LETO rats, †P < 0.05 versus the non-treated OLETF rats, ‡P < 0.01 versus the non-treated OLETF rats. TC, total cholesterol;
HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment for insulin resistance; UAE, urine albumin excretion; AST, aspartate aminotransferase; ALT, alanine aminotransferase; OGTT, oral glucose tolerance test; WBC, white blood cell.
2. *In vitro* study

A. Effect of ATRA on TGF-β₁ in the RMCs

The media showed a time-dependent increase in TGF-β₁ levels under both 30 and 5 mM glucose conditions. When compared with control glucose, high glucose significantly increased TGF-β₁ secretion at 24 and 48 hours with TGF-β₁ levels being 578.3 ± 23.1 (mean ± SEM of three experiments) and 651.2 ± 29.7 pg/mL in control glucose and 798.0 ± 39.8 and 887.4 ± 43.7 pg/mL in high glucose (*P* < 0.05), respectively.

Synchronized quiescent RMCs were incubated in media containing 30 or 5 mM of glucose for 24 hrs. Consequently, treatment with ATRA under both 30 and 5 mM glucose conditions showed dose-dependent decreases in TGF-β₁ levels (*P* < 0.05 by a test for a trend, Figure 1).
Figure 1. Effect of ATRA on TGF-β₁ protein. After the incubation of quiescent mesangial cells in media containing 30 (high) or 5 mM (control) of glucose and different concentrations of ATRA, TGF-β₁ levels were measured with the quantitative sandwich enzyme immunoassay technique. Treatment with ATRA under both 30 and 5 mM glucose conditions showed dose-dependent decreases in TGF-β₁ levels ($P < 0.05$ by a test for a trend). *$P < 0.05$ compared to the vehicle, i.e. without ATRA. Values are expressed as mean ± SEM of three experiments.

In addition, TGF-β₁ was measured after incubating synchronized quiescent
RMCs with $10^{-5}$ M ATRA for up to 48 hours. Treatment with ATRA under both 30 and 5 mM glucose conditions showed time-dependent decreases in TGF-$\beta_1$ levels ($P < 0.05$, Figure 2).

Figure 2. Effect of ATRA on TGF-$\beta_1$ protein. After the incubation of quiescent rat mesangial cells (RMCs) with $10^{-5}$ M ATRA for the given periods (6, 24, and 48 hrs) in media containing 30 (high) or 5 (control) mM of glucose, TGF-$\beta_1$ levels were measured with the quantitative sandwich enzyme immunoassay technique. Treatment with ATRA under both 30 and 5 mM glucose conditions showed time-dependent decreases in TGF-$\beta_1$ levels ($P < 0.05$ by a test for a trend). *$P < 0.05$ compared with 0 hour. Values are expressed as mean ± SEM.
of three separate experiments.

B. Effect of ATRA on PKC activity in the RMCs

The media showed a time-dependent increase in PKC activity under both control and high glucose conditions. The increase in PKC activity was observed at 12 hrs and reached a maximum at 24 hrs. Treatment with ATRA under both control and high glucose conditions showed a dose-dependent decrease in PKC activity. Our findings demonstrated decreased PKC activity in the RMCs by ATRA (Figure 3-A, B, C).
Figure 3-A, B, C. Effect of ATRA on protein kinase C (PKC)-α (Figure 3-A), β (Figure 3-B), δ (Figure 3-C) activity at 24 hrs in rat mesangial cells (RMCs)
in media containing 30 (high) or 5 (control) mM of glucose. Equal amounts of
total cell lysate were analyzed by SDS-PAGE and immunoblotted with an anti-
PKC isoforms. The RMCs were treated with a vehicle (without ATRA, C, Lane
1), 10^{-8} M (Lane 2), 10^{-7} (Lane 3), 10^{-6} (Lane 4), or 10^{-5} M (Lane 5) of all-trans-
retinoic acid (ATRA). *P < 0.05 versus the control.

C. Effect of ATRA on ROS generation in the RMCs

Synchronized quiescent RMCs grown on cover glass were incubated in media
containing 30 or 5 mM of glucose for 24 hrs. After the incubation of the
quiescent RMCs at different concentrations of ATRA, DCF-sensitive cellular
ROS levels were measured. ATRA at 10^{-6}, 10^{-5} M significantly decreased DCF-
sensitive cellular ROS levels compared with the control. DCF-sensitive cellular
ROS in the RMCs showed a time-dependent decrease after ATRA
administration (Figure 4).
Figure 4. Effect of ATRA on dichlorofluorescein (DCF)-sensitive cellular reactive oxygen species (ROS). Synchronized quiescent rat mesangial cells (RMCs) grown on cover glass were incubated in media containing 30 (high) or 5 (control) mM of glucose for 6 hrs. After the incubation of the quiescent RMCs under different experimental conditions, DCF-sensitive cellular ROS were measured as described in the text. DCF-sensitive cellular ROS in the RMCs showed a dose-dependent decrease after ATRA administration (\( P < 0.05 \) by a test for a trend). \(^* P < 0.05 \) compared to the vehicle. Values are expressed as mean ± SEM of three separate experiments.
In addition, after incubating the synchronized quiescent RMCs with $10^{-5}$ M ATRA administration in media containing 30 or 5 mM of glucose for the given periods (6, 12, and 48 hrs), a relative increase of ROS was measured. DCF-sensitive cellular ROS in the RMCs showed a time-dependent decrease after ATRA administration (Figure 5). Also, $H_2O_2$-induced ROS production in the RMCs was decreased dose-dependently after ATRA administration (data not shown).

Figure 5. Effect of ATRA on dichlorofluorescein (DCF)-sensitive cellular reactive oxygen species (ROS). After the incubation of the quiescent rat
mesangial cells (RMCs) with $10^{-5}$ M of ATRA for the given periods (6, 24, and 48 hrs) in media containing 30 (high) or 5 (control) mM of glucose, a relative increase of ROS was measured. DCF-sensitive cellular ROS in the RMCs showed a time-dependent decrease after ATRA administration ($P < 0.05$ by a test for a trend). *$P < 0.05$ compared with 0 hour. Values are expressed as mean ± SEM of three separate experiments.
IV. DISCUSSION

Diabetic nephropathy, characterized by persistent albuminuria, tends to develop in approximately 40% of T2DM patients with poor glycemic control. Listed as the chief cause of ESRD in North America, Japan, Korea, and most industrialized European nations, diabetic nephropathy in 1998 accounted for 44.5% of incident ESRD patients covered by Medicare\textsuperscript{1}. Typically, diabetic ESRD patients have serious co-morbid conditions, especially heart, eye, and peripheral vascular diseases. It is not surprising, therefore, that caring for the afflicted individuals imposes a major financial burden on family members and governments.

14 to 24% of patients newly diagnosed with type 2 diabetes have microalbuminuria associated with hyperglycemia, elevated BP, smoking, or hyperlipidemia\textsuperscript{19-21}. There is also a significant association between microalbuminuria and total or cardiovascular mortality. Microalbuminuria raised the overall odds ratio for death to 2.4 and cardiovascular mortality to 2.0 in type 2 diabetic patients compared with those without microalbuminuria\textsuperscript{22}.

The pathogenesis of diabetic nephropathy is a multistage process starting with a genetic predisposition to injury by an elevated glucose concentration.
Genetically determined differences in the renal response to hyperglycemia may affect glucose transporter function, ROS formation, the polyol pathway, PKC activation, AGE formation, or the hexosamine pathway. These differences in renal response to hyperglycemia may also affect more distal mediators of diabetic renal pathology, such as TGF-β1 and other growth factors causing increased matrix deposition. In addition to growth factors, gene products causing altered glomerular hemodynamics may also be differentially affected. OLETF rat, derived from Long-Evans rats, is a spontaneously developed model of T2DM with obesity and insulin resistance. The rats gain weight around 8 weeks of age and develop a slight degree of glucose intolerance at the same time. These changes predominantly occur in male rats with approximately 90% developing diabetes and 7% showing impaired glucose tolerance by 30 weeks of age. More than 95% of female OLETF rats maintain a normal glucose tolerance at the same age. Male OLETF rats develop glomerulopathy similar to that in diabetic nephropathy, mesangial proliferation around 22 weeks of age, and fibrin cap as well as periodic acid shiff (PAS)-positive deposits in a part of the glomerulus. The frequency of these changes increases with age, although the changes remain segmental. In some rats, a nodule-like lesion and microaneurysmal dilatation of tuft have been observed around 50 weeks of age, and sclerosis and atrophy of the glomerulus occur at a later age, thus resulting in
a severe resolution of the kidney. Functionally, albuminuria starts before 20 weeks of age and then becomes prominent after 30 weeks of age.

Retinoic acid is a derivative of vitamin A which is a fat-soluble essential vitamin supplied to human through fiber-rich diets. Vitamin A is stored in the liver as retinol, and then is converted to retinoic acid and delivered to organs involved in normal growth, integration of tissues, and immunity\(^9,^{10}\). Vitamin A is converted to two active forms, trans and cis, and enters nucleus after forming heterodimer with the retinoic acid A receptor (RAR) and the retinoic X receptor (RXR) in cellular cytoplasm. By stimulating the transcription of retinoic acid response element, vitamin A is known to have anti-proliferative and anti-inflammatory effects\(^{11}\). The role of retinoic acid in embryogenesis (including renal development) has been studied in detail\(^{23, 24}\). Little is known, however, about the renal effects of ATRA.

In our study, there was a trend toward a decrease in body weight (about 50 gram) in the ATRA-treated OLETF rats compared with the non-treated OLETF rats although this trend did not reach statistical significance. In a previous study, treatment with high dose ATRA (100 mg/kg) triggered a reduction of adiposity and body weight\(^{25}\). The reduction of adiposity after ATRA treatment was consistent with the well-known effect of high ATRA doses of inhibiting the accumulation of lipids in differentiating white\(^{26}\) and brown\(^{27, 28}\) preadipocytes in
culture. Moreover, ATRA treatment was reported to reduce brown adipose
tissue lipid content in intact mice\textsuperscript{27,29}. Reduction of adiposity and body weight
after ATRA treatment correlated with a general down-regulation of the
expression of PPAR\textgreek{2} in all the adipose depots examined\textsuperscript{25}. However, in our
study, we used 10 mg/kg of ATRA, which was a smaller dose than had been
used in the previous study. For that reason the effects on weight reduction might
have been different.

Elevated levels of triglyceride and cholesterol are frequently reported as
retinoic acid-induced alterations\textsuperscript{30}. But, these adverse effects of retinoic acid are
mainly due to 13 \textit{cis} retinoic acid\textsuperscript{31-33}. It is unclear if ATRA treatment alters
overall levels of cholesterol\textsuperscript{30}. The OLETF rats showed increased
concentrations of total cholesterol and triglyceride compared with the LETO
rats. These increments may have been not due to ATRA treatment but due to
obesity or insulin resistance in the OLETF rats that might be the reason why
there were no differences in serum lipid profiles between the ATRA-treated
OLETF rats and the non-treated OLETF rats.

Sixteen weeks after the administration of ATRA, fasting glucose levels were
slightly lower in the ATRA-treated OLETF rats when compared with the non-
treated OLETF rats. This result suggests ATRA has a modest glucose lowering
effect. In many previous studies, treatment with ATRA decreased glucose levels
in diabetic mice\textsuperscript{30, 34}. In terms of insulin resistance, as one might expect, the OLETF rats were in the higher insulin resistance status as demonstrated by increased insulin and HOMA-IR levels than the LETO rats. Moreover, insulin and HOMA-IR levels were slightly lower in the ATRA-treated OLETF rats than in the non-treated OLETF rats. This result implies ATRA has a beneficial effect on insulin resistance.

Retinoic acid and synthetic retinoids are also potent agonists for the peroxisome proliferator-activated receptor (PPAR) that bind to DNA as heterodimer with the retinoid X receptor (RXR) \textsuperscript{35, 36}. The thiazolidinediones are PPAR agonists, which reduce insulin resistance and hypertriglyceridemia in T2DM; PPAR\textsubscript{γ} also increases the expression of tumor necrosis factor α (TNF-α) and glucose transporter-4 (GLUT-4)\textsuperscript{37, 38}. Retinoid-like ligands of the RXR receptor have beneficial effects on diabetes and insulin resistance in rodents\textsuperscript{39, 40}. Therefore, we can speculate that the slight but significant superiority on glucose and insulin resistance observed in the ATRA-treated OLETF rats could have been due to an indirect mechanism of PPAR\textsubscript{γ}/RXR activation.

In order to evaluate the adverse effects of ATRA treatment, we performed the serum chemistry and complete blood counts in rats after 16 weeks of the treatment with ATRA. There were no remarkable differences in AST, ALT, creatinine, hemoglobin levels, WBC count, or platelet count between the three
groups, suggesting no serious hepatic, renal or hematologic alterations were induced by the ATRA treatment. Some authors have previously published similar results\textsuperscript{30,31}.

In this study, we examined the effect of ATRA on improving diabetic nephropathy by measuring the amount of urinary albumin excretion (UAE) in the OLETF rats treated with ATRA. The results were compared with those from the OLETF and LETO rats who did not receive ATRA. These results suggest that ATRA may have renoprotective effects in diabetic nephropathy. ATRA and other retinoids have beneficial effects on the treatment of diabetic and nondiabetic nephropathy\textsuperscript{41-44}. In the anti-Thy1.1 nephropathy rat model, ATRA treatment has been shown to limit glomerular cell proliferation and renal damage by the reduction of renal TGF-β\textsubscript{1} and TGF receptor II expression\textsuperscript{12}. ATRA treatment also has an anti-oxidant effect by blocking lipid peroxidation in streptozotocin (STZ)-induced diabetic rats\textsuperscript{13,14}. In a diabetic rat model, treatment with ATRA caused a drop in the urinary excretion of protein and albumin\textsuperscript{42}. ATRA regulates the expression of multiple genes by binding to and subsequently activating retinoic acid receptors (RAR) α, β, and γ and/or retinoid X receptors (RXR) α, β, and γ\textsuperscript{45}. The binding of ATRA or other retinoids to the receptors causes the dissociation or release of corepressors and recruitment of coactivators to prompt and facilitate gene transcription\textsuperscript{45}. It is speculated that
the therapeutic effect of ATRA in animal models of nondiabetic renal disease may be linked to down-regulation of genes related to inflammation, cell proliferation, and fibrosis\textsuperscript{46}. However, the mechanism of its protective effect on diabetic nephropathy is currently not known.

It is of interest that retinoic acid treatment suppressed proteinuria, which was shown by the significant inhibition of TGF-\(\beta_1\), PKC, and ROS in the RMCs. TGF-\(\beta_1\) is a prototype of a profibrogenic cytokine\textsuperscript{47}. TGF-\(\beta_1\) stimulates the transcription of many extracellular matrix genes in renal cells\textsuperscript{48, 49}. In several models of renal disease, TGF-\(\beta_1\) has been implicated as a primary mediator for cell growth and accumulation of extracellular matrix, e.g., diabetic nephropathy, experimental glomerulonephritis, or unilateral ureteral obstruction\textsuperscript{50, 51}. TGF-\(\beta_1\) might be a critical mediator for diabetic nephropathy. Interrupting this system may hold promise for amelioration of diabetic nephropathy. Existing approaches to renoprotection, including glycemic control, lowering dietary protein, and the administration of angiotensin conversion enzyme (ACE) inhibitors and angiotensin receptor blockers, may act, at least in part, via the inhibition of TGF-\(\beta_1\). ATRA have been previously shown to down-regulate TGF-\(\beta_1\) via AP-1 binding sites on the TGF-\(\beta_1\) promoter\textsuperscript{52}. Furthermore, Wagner \textit{et al.} demonstrated that the beneficial effects of ATRA on glomerular damage were presumably due to a marked reduction in renal TGF-\(\beta_1\) and TGF receptor
II expression\textsuperscript{12}. Similarly, in our study, the treatment with ATRA showed a dose- and time-dependent decrease in TGF-β\textsubscript{1} production in the RMCs. As in previous studies, our present observations suggest that ATRA treatment may exert beneficial effects on diabetic renal disease by inhibiting TGF-β\textsubscript{1} production.

The inappropriate activation of PKC has been implicated as a putative mediator in the pathogenesis of diabetic nephropathy based on evidence from both in vivo experimental animal diabetic models and in vitro studies with cultured glomerular cells\textsuperscript{53-58}. Activation of PKC contributes to increased accumulation of microvascular matrix protein by inducing the expression of TGF-β\textsubscript{1} in both cultured mesangial cells\textsuperscript{59,60} and the glomeruli of diabetic rats\textsuperscript{61}. PKC-α, β, δ, and ε are activated in the glomeruli of diabetic rats\textsuperscript{62}. Inhibition of PKC-β is nephroprotective in diabetes mellitus\textsuperscript{63}. Treatment with ruboxistaurin mesylate, PKC inhibitor, in genetically diabetic mice prevented mesangial expansion and glomerular dysfunction\textsuperscript{64}. Retinoids can function as antioxidants and promote differentiation, providing a protective effect against altered activation of PKC\textsuperscript{65}. In many systems, retinoic acid reduces or inactivates PKC\textsuperscript{66-68}. In our study, we have shown that ATRA treatment has beneficial effect on diabetic nephropathy and high glucose-induced PKC activation is effectively inhibited by ATRA administration.
PKC activation might regulate the overexpression of TGF-β₁ at a transcriptional level since its promoter contains activator protein 1 (AP-1) sites. AP-1 sites are activated by the proto-oncogenes complex, fos-jun homodimers or heterodimers. Retinoid suppresses important inflammatory transcriptional factors, activator protein (AP)-1, by activation of the RXR. Therefore, we can speculate the beneficial effect of ATRA on diabetic nephropathy could be due to either its direct effect on PKC inactivation or through the suppression of TGF-β₁. However, to clarify its mechanism, further investigations are needed.

Increased oxidative stress due to increased oxygen free radical production is an important mechanism proposed to explain why poor glycemic control in diabetes results in vascular complications. High glucose induces ROS and up-regulates TGF-β₁ expression in the glomerular mesangial cells. ROS also up-regulates TGF-β₁ expression in mesangial cells. In this study, we have demonstrated that high glucose induces DCF-sensitive ROS in the RMCs. Moreover, we showed that high glucose-induced ROS generation in the RMCs was effectively blocked by ATRA in a dose- and time-dependent manner. Therefore, along with previous studies, our present observations suggest that ATRA treatment may exert beneficial effects on diabetic renal disease by lowering blood glucose and inhibiting TGF-β₁ production through the inhibition of PKC activation and ROS synthesis in mesangial cells.
Besides hyperglycemia, hypertension is another potentially modifiable factor to prevent the initiation and progression of diabetic nephropathy in susceptible individuals\textsuperscript{80}. The beneficial effects of ATRA on diabetic nephropathy in our study might have been due to the antihypertensive effect of ATRA. The antihypertensive mechanism of ATRA has not yet been clarified, but has been considered to include the alleviation of renal damage by retinoic acid and blockade of angiotensin II action\textsuperscript{81}. Although we did not measure blood pressure in the experimental rats, it has been reported that most of the antihypertensive effect of retinoids was observed in the acute and chronic experimental nephritic rat models\textsuperscript{43, 82}.

Diabetic nephropathy is the leading cause of ESRD, requiring dialysis therapy in the Western and Asian countries\textsuperscript{1}. About 25–40\% of patients with type 1 and type 2 diabetes develop diabetic nephropathy 25 years after the onset of diabetes\textsuperscript{83–87}. Based on the results of cumulative epidemiological studies\textsuperscript{88, 89}, it is evident that the most effective therapy to prevent the development and progression of nephropathy is to maintain normoglycemia. However, from these epidemiological studies, it is also evident that the long-term maintenance of normoglycemia is difficult in most subjects with type 1 or type 2 diabetes. Therefore, efforts have been directed to clarify the responsible mechanisms by which diabetes causes diabetic nephropathy and identify the therapeutic
strategies that could abrogate the development and progression of diabetic nephropathy.

Our findings in the present study provide the evidence that ATRA treatment can influence the development of diabetic nephropathy without causing any obvious adverse effect. The beneficial effects of ATRA on diabetic nephropathy in the RMCs were in part due to reduction of TGF-β₁ synthesis, which was mediated by a significant reduction of PKC activity and ROS production. Also, our findings clearly warrant further studies to assess the potential therapeutic effects of ATRA in diabetic nephropathy, to delineate the mechanism of action of ATRA, and to identify retinoid receptor-specific pathways in the kidney.
V. CONCLUSION

In this study, we demonstrated that administration of ATRA resulted in a reduction of UAE in OLETF rats, cultured RMCs increased TGF-β_{1} synthesis in response to high glucose stimuli, and that ATRA treatment suppressed TGF-β_{1} synthesis induced by high glucose stimulation. It is of interest that ATRA treatment suppressed UAE and TGF-β_{1} synthesis, which was mediated by a significant reduction of PKC activity and ROS production. Our results suggest that ATRA has a potential therapeutic role for diabetic nephropathy. We elucidated the effect of ATRA on suppressing the progression of diabetic nephropathy and its pharmacologic mechanism of action. Our results also suggest that ATRA treatment might be a novel approach for the treatment of diabetic nephropathy.
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ABSTRACT (IN KOREAN)

제 2형 당뇨병성 신증의 치료에 있어서 all-trans-retinoic acid의 효과

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당뇨병성 신증은 만성 신부전이 가장 흔한 원인 질환으로 초기에 기저막 비후와 사구체 간질세포의 증식을 보이며 사구체 경화와 세뇨관 간질의 섬유화를 동반하는데 고혈당, advanced glycation end-product (AGE), polyol 대사 이상, 산화성 스트레스, transforming growth factor-β₁ (TGF-β₁)의 활성화 등 다양한 인자들이 서로 복합적으로 이 질환의 생성에 관여한다고 알려져 있다.

Retinoic acid는 비타민 A의 유도체로, 식이 섬유 중에 포함되어 있는 지유성 편수 인체 구성물질인 비타민 A가 retinol 형태로 간에 저장된
후 retinoic acid로 전환되어 조직으로 이동하여 정상적인 성장이나 조직의 유지, 면역기능에 관여한다. 비타민 A는 인체 내에서 trans와 cis 두 가지 활성형으로 전환되어 세포질내의 retinoic acid A receptor (RAR)와 retinoic X receptor (RXR)와 반응하여 복합체를 형성한 후 핵 내로 들어가 retinoic acid response element를 자극하는 전사인자의 역할을 통해 항증식, 항염증 작용을 한다고 알려져 있다. 최근 all trans retinoic acid (ATRA)가 당뇨병쥐에서 사구체 간질세포의 증식을 억제하며, 사구체의 염증 반응을 감소시켜 신장의 손상을 방지하는 것으로 보고되었다. 또한 ATRA는 지질의 산화를 억제하는 증 제2형 당뇨병의 진행을 예방해주는 효과가 있는 것으로 보고되고 있다.

본 연구에서는 제 2형 당뇨병 동물모델인 Otsuka Long Evans Tokushima Fatty (OLETF) 쥐에서 ATRA를 투여하여 요증 알부민 배설량을 측정하여 당뇨병성 신증의 진행이 호전되는지를 알아보고, ATRA의 작용 기전을 이해하고자 배양된 백서의 사구체 간질세포(rat mesangial cells, RMCs)에서 고혈당 자극을 주었을 때 TGF-β1이 증가하는지, 그리고 증가된 TGF-β1이 ATRA 투여로 억제되는지를 알아보았다. 또한 ATRA 투여 후 TGF-β1의 세포 내 신호전달경로의 상부에 있는 protein kinase-C (PKC), 산화성 스트레스(reactive oxygen species, ROS)의 변화를 관찰하였다.

생후 6주된 OLETF 쥐를 온도가 25℃, 실내습도가 40~60%로 잘 유지되는 사육실에서 충분한 사료 및 식수를 제공하면서 사육하고
28주령이 되는 시기에 경구 당부하검사를 시행하여 자연 발생적으로 당뇨병이 발생하였음을 확인하였다. 제2형 당뇨병이 발생한 28주령의 OLETF 쥐 20마리에 16주 동안 kg 당 10 mg의 ATRA를 DMSO 및 설탕로오스에 녹여 Sonde를 이용하여 투여하였고 같은 기간 동안 OLETF 쥐 20마리와 정상 대조군인 LETO 쥐군에 vehicle를 투여하였다.

28주령의 공복 및 식후 혈당은 OLETF 쥐군에서 LETO 쥐군에 비해 높았다. 한편 44주령 때의 체중은 LETO 쥐(561.0 ± 40.4 gram)보다 OLETF 쥐의 체중이 높았으며 ATRA를 투여한 군의 체중(648.0 ± 67.7 gram)이 투여하지 않은 군의 체중(697.5 ± 88.4 gram)에 비해 낮았으나 통계적인 의미는 없었다. ATRA 투여 16주 후의 혈당은 LETO 쥐의 혈당(105.4 ± 14.4 mg/dL)보다 OLETF 쥐의 혈당이 높았고 ATRA를 투여한 OLETF 쥐의 혈당(151.0 ± 25.1 mg/dL)이 투여하지 않은 쥐(178.5 ± 38.3 mg/dL)에 비해 의미있게 낮았다 (P < 0.05). 또한 LETO 쥐에 비해 OLETF 쥐의 콩글레스테롤, 중성지방이 높았으나 OLETF 쥐에서 ATRA를 투여한 군과 투여하지 않은 군의 차이는 없었다. ATRA를 투여한 군 16주가 되는 44주령의 시기에 하루 요중 알부민 배설량을 측정한 결과 LETO 쥐의 요중 알부민 배설량(0.01 ± 0.01 mg/mgCr)이 OLETF 쥐보다 적었으며 ATRA를 투여한 OLETF 쥐의 요중 알부민 배설량(0.07 ± 0.03 mg/mgCr)이 투여하지 않은 OLETF 쥐(0.17 ± 0.15 mg/mgCr)보다 적었다 (P < 0.01). 한편 약제의 부작용을

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알아보기 위해 시행한 혈액 및 생화학 검사 결과 ATRA가 신장, 간, 혈액에 미치는 부작용은 관찰되지 않았다.

배양된 백서의 사구체 간질세포에서 고농도의 포도당은 저농도의 포도당에 비해 TGF-β₁을 증가시켰다. ATRA를 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ M의 농도로 각각 처리를 하고 또한 0시간, 6시간, 24시간, 48시간 동안 ATRA (10⁻⁵ M)를 처리한 결과 TGF-β₁의 농도를 시간 및 농도 의존적으로 감소시켰다. 또한 같은 세포에서 ATRA는 PKC-α, β, δ의 발현을 용량 의존적으로 감소시켰으며 세포 내 ROS 또한 용량, 시간 의존적으로 감소시켰다.

이 연구결과 제2형 당뇨병 모델 동물에서 16주 동안 ATRA를 투여한 결과 투여하지 않은 대조군에 비해 혈당 및 인슐린저항성이 개선됨이 관찰되었으며 요중 알부민 배설량이 낮게 나타났다. 또한 배양된 백서의 사구체 간질세포에서 ATRA는 고농도 포도당에 의해 증가된 TGF-β₁ 생산을 감소시켰다. 또한 이러한 ATRA의 TGF-β₁ 감소 기전에는 PKC, ROS의 감소와 관련이 되어있음을 알았다. 우리의 연구 결과는 ATRA가 당뇨병성 신증의 치료제로서의 가능할가 있음을 시사한다.

핵심되는 말: all-trans-retinoic acid, 당뇨병성 신증, transforming growth factor-β₁, protein kinase-C, 반응성산소족, Otsuka Long-Evans Tokushima Fatty rats