Effects of ursolic acid on glucose metabolism, the polyol pathway and dyslipidemia in non-obese type 2 diabetic mice

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Ursolic acid (UA) is a pentacyclic triterpenoid compound that naturally occurs in fruits, leaves and flowers of medicinal herbs. This study investigated the dose-response efficacy of UA (0.01 and 0.05%) on glucose metabolism, the polyol pathway and dyslipidemia in streptozotocin/nicotinamide-induced diabetic mice. Supplement with both UA doses reduced fasting blood glucose and plasma triglyceride levels in non-obese type 2 diabetic mice. High-dose UA significantly lowered plasma free fatty acid, total cholesterol and VLDL-cholesterol levels compared with the diabetic control mice, while LDL-cholesterol levels were reduced with both doses. UA supplement effectively decreased hepatic glucose-6-phosphatase activity and increased glucokinase activity, the glucokinase/glucose-6-phosphatase ratio, GLUT2 mRNA levels and glycogen content compared with the diabetic control mice. UA supplement attenuated hyperglycemia-induced renal hypertrophy and histological changes. Renal aldose reductase activity was higher, whereas sorbitol dehydrogenase activity was lower in the diabetic control group than in the non-diabetic group. However, UA supplement reversed the biochemical changes in polyol pathway to normal values. These results demonstrated that low-dose UA had preventive potency for diabetic renal complications, which could be mediated by changes in hepatic glucose metabolism and the renal polyol pathway. High-dose UA was more effective anti-dyslipidemia therapy in non-obese type 2 diabetic mice.

Keywords: Dyslipidemia, Kidney, Liver, Polypol pathway, Ursolic acid

Type 2 diabetes mellitus (T2DM) has become an epidemic in Asia and occurs even at a young age and low body mass index¹. When lean Asian people develop T2DM, diabetic progression is faster than in obese people because of reduced insulin secretion capacity to compensate for insulin resistance². Lean subjects with T2DM reportedly have reduced β-cell mass and defective insulin secretion compared with obese patients³. The non-obese T2DM phenotype is characterized by disproportionately reduced insulin secretion and less insulin resistance compared with obese T2DM patients⁴. Meanwhile, the increased T2DM risk in the Asian population may be attributed to higher abdominal and visceral adiposity compared with Europeans who have the same BMI and waist circumference⁵. Increased waist circumference has been associated with substantially increased T2DM risk⁶, as well as cardiovascular and all-cause mortality, independent of BMI⁷. Therefore, non-obese patient treatment strategy is important for T2DM management in Asia.

Ursolic acid (UA) has many bioactivities, including antioxidant⁸, hepatoprotective⁹, anticancer¹⁰ and antidiabetic activity¹¹. UA supplement improves hyperglycemia and the immune system in both type 1 diabetic mice fed a high-fat diet¹² and non-obese type 2 diabetic mice¹³. Accordingly, in the present study, the dose-response effect of UA has been compared with metformin on glucose metabolism, the polyol pathway and dyslipidemia in streptozotocin/nicotinamide (STZ/NA)-induced type 2 diabetic mice.

Materials and Methods

Animals and experimental design—Male 8-week-old ICR mice were purchased from Biogenomics, Inc. (Seoul, Korea). The mice were individually housed in polycarbonate cages at 22 ± 2 °C with a 12:12 h L:D cycle. The mice were acclimatized under laboratory conditions for 7 days and were randomly divided into non-diabetic and diabetic groups.

Diabetes was induced by a single intra-peritoneal injection of STZ (50 mg/kg body weight/day; Sigma,
St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.2) on two consecutive days. Nicotinamide (NA, 120 mg/kg body weight; Sigma, St. Louis, MO, USA) was dissolved in saline and injected ip 15 min before STZ administration on the first day\(^\text{14}\). The non-diabetic mice were injected with citrate buffer or saline alone. After 7 days, only the STZ/NA-treated mice that exhibited a fasting blood glucose level \(\geq 11\) mmol/L were used in the study. Diabetic mice were randomly subdivided into 4 groups of 9 mice each; the untreated diabetic (DM) group, the diabetic-low-dose ursolic acid (DM-lowUA) group, the diabetic-high-dose ursolic acid (DM-highUA) group and the diabetic-metformin (positive control, DM-Metformin) group. The mice were fed an AIN-76 semisynthetic diet\(^\text{15}\) with ursolic acid (0.01 and 0.05 g/100 g diet, TCI Co., Ltd, Japan) or metformin (0.5 g/100 g diet, Sigma, St. Louis, MO, USA) for 4 weeks. The dose of metformin was based on previous data (unpublished data) that was based on previous human\(^\text{16}\) and animal\(^\text{17}\) studies. The mice had \textit{ad libitum} access to food and water, and their food consumption and weight gain were measured daily and weekly, respectively.

After feeding with the experimental diets for 4 weeks, mice were starved for 12 h and anestomized under anesthesia by diethyl ether, and blood samples were taken from the inferior vena cava to determine plasma biomarkers. The experimental protocols and procedures used in the present study were approved by the Sunchon National University Institutional Animal Care and Use Committee.

\textbf{Fasting blood glucose levels and plasma lipid profiles}—Fasting blood glucose levels were determined using a glucometer (GlucoDr supersensor, Allmedicus, Korea) and venous blood was drawn from the tail vein after a six-hour fast. Plasma triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) concentrations were measured using an enzymatic kit (Asan Diagnostics, Seoul, Korea). Free fatty acid (FFA) concentrations were determined using an enzymatic colorimetric method (Wako Chemicals, Richmond, VA). The amount of low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) were calculated with the Friedewald equation\(^\text{18}\) as follows.

\[
\text{LDL-C} = \text{TC-HDL-C-(TG/5)} \quad \text{and} \quad \text{VLDL-C} = \frac{\text{TG}}{5}
\]

\textbf{Hepatic glucose-regulating enzyme activities and glycogen content}—Activities of glucokinase (GK), glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) and glycogen content were determined using a spectrophotometric continuous assay as previously described\(^\text{18}\).

\textbf{Hepatic GLUT2 mRNA expression level using RT-qPCR analysis}—The liver was homogenized in Trizol reagent (Invitrogen Life Technologies, Grand Island, NY, USA) and total RNA was isolated according to the manufacturer's specifications. DNase was used to remove DNA contamination, and the RNA was re-precipitated in ethanol to ensure that there was no phenol contamination. For quality control, RNA purity and integrity were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA).

Total RNA (1 \(\mu\)g) was reverse-transcribed into cDNA using the QuantiTect\(^\text{®}\) reverse transcription kit (Qiagen, Germany). mRNA expression was quantified by real-time quantitative PCR using a SYBR green PCR kit (Qiagen, Germany) and the CFX96TM real-time system (Bio-Rad, Hercules, CA, USA). The following gene-specific primers were used: glucose transporter 2 (GLUT2), 5\' -TCACTGTGTACTGAGTTCCTTCC-3\' (forward), 5\' -CTGTTGTTGTATGCTGTTG-3\' (reverse); glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5\' -TGCAATGTGCAAGTGGAGAT-3\' (forward), 5\' -TTGAATTTGCCGTGAGTGGA-3\' (reverse). Cycle thresholds were determined based on the SYBR green emission intensity during the exponential phase. The fold changes were calculated using the 2\(^{-\Delta\Delta Ct}\) method, GAPDH transcripts were also amplified from samples to normalize real-time quantitative RT-PCR detection\(^\text{20}\).

\textbf{Renal polyol pathway enzyme activities}—Activities of aldose reductase (AR) and sorbitol dehydrogenase (SDH) were determined using a spectrophotometric continuous assay as described previously\(^\text{19}\).

\textbf{Renal morphology}—For histological analysis, kidney tissue was fixed in 10\% formalin in a buffer solution. Fixed tissues were paraffin-embedded, and 4 \(\mu\m\) sections were prepared and stained with hematoxylin and eosin. Stained areas were viewed using an optical microscope (Olympus, Japan) at 200x and 400x magnification.

\textbf{Statistical analysis}—All data are presented as mean \(\pm\) SE and were evaluated by one-way ANOVAs using SPSS (SPSS Inc., Chicago). The differences between the means were assessed using Duncan’s multiple-range test. The data were considered to be statistically significant at \(P < 0.05\).
Results

**Effects on blood glucose levels**—As shown in Table 1, there were no differences in initial fasting blood glucose levels between the diabetic groups (11.91–13.19 mmol/L). However, 4-week UA supplementation lowered blood glucose levels in STZ/NA-induced type 2 diabetic mice in a dose-independent manner, similar to metformin.

**Effects on hepatic glucose metabolism**—Both doses of UA and metformin elevated GK activity compared with the diabetic control. In non-obese type 2 diabetic mice, G6Pase activity was higher than the non-diabetic mice; however, both doses of UA and metformin suppressed its activity. The PEPCK activity did not alter by UA or metformin supplementation. Thus, UA and metformin supplement increased the hepatic GK/G6Pase ratio that had been decreased by hyperglycemia (Fig. 1).

UA supplementation dose-independently increased hepatic glycogen contents compared with the diabetic control (Fig. 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fasting blood glucose level (mmol/L)</th>
<th>Relative organ weights (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>NonDM</td>
<td>7.02 ± 0.30a</td>
<td>7.22 ± 0.24a</td>
</tr>
<tr>
<td>DM</td>
<td>13.19 ± 0.83b</td>
<td>16.95 ± 1.71c</td>
</tr>
<tr>
<td>DM-lowUA</td>
<td>11.91 ± 0.68b</td>
<td>12.00 ± 1.53b</td>
</tr>
<tr>
<td>DM-highUA</td>
<td>12.40 ± 0.76b</td>
<td>13.24 ± 1.91b</td>
</tr>
<tr>
<td>DM-Metformin</td>
<td>11.94 ± 0.88b</td>
<td>11.89 ± 0.83b</td>
</tr>
</tbody>
</table>

Means in the same column not sharing a common superscript letter differ significantly; *P* < 0.05.

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Fig. 1—Effect of ursolic acid supplement on hepatic glucose metabolic enzyme activities, glycogen content and hepatic GLUT2 gene expression in STZ/NA-induced diabetic mice. [Values are mean±SE. The values not sharing a common letter differ significantly; *P* < 0.05]
Effects on hepatic GLUT2 mRNA expression—To examine the effect of UA supplementation on hepatic glucose transport gene expression, GLUT2 mRNA levels were measured (Fig. 1). Both doses of UA and metformin supplement increased hepatic GLUT2 mRNA levels compared with the diabetic control group.

Effects on renal polyol pathway—In non-obese type 2 diabetic mice, AR was increased and SDH was decreased by 17 and 22%, respectively, compared with the non-diabetic mice. However, both doses of UA or metformin significantly decreased AR activity compared with the diabetic control mice. Only UA supplementation increased SDH activity to normal value at both doses. Thus, UA or metformin supplement improved the renal polyol pathway by decreasing the AR/SDH ratio (Fig. 2).

Effects on renal hypertrophy and histological changes—In the non-obese type diabetic mice, relative liver and relative kidney weights were increased compared with the non-diabetic mice. However, kidney weight was significantly decreased after supplement with both doses of UA and metformin, while liver weight tended to decrease in the UA group at both doses compared with the diabetic control group (Table 1).

Renal tissue histological changes are presented in Fig. 3. Normal histology was observed in the non-diabetic group. The diabetic group demonstrated expansion in capsular spaces of glomeruli and tubular lumen compared with the non-diabetic group. However, UA or metformin supplement alleviated the hyperglycemia-induced histological changes.

Effects on lipid profiles—Plasma lipid content including TG, TC and LDL-C concentrations was significantly higher in the diabetic control group than in the non-diabetic group. Supplementation of 0.01 and 0.05% UA lowered FFA, TC and VLDL-C concentrations in a dose-dependent manner, while they lowered TG and LDL-C concentrations in a dose-independent manner compared with the diabetic control mice. HDL-C concentrations were not different between the groups, but the HDL-C/TC ratio was increased by UA supplementation (Table 2).

Discussion
Liver is a critical organ for glucose homeostasis because glucose utilization and production occurs there during feed and fasted states, respectively\(^{21}\). The present study demonstrated that both doses of UA and metformin effectively inhibited hepatic G6Pase activity and elevated GK activity and glycogen content, which reduced blood glucose levels in the non-obese type 2 diabetic mice. The glucose-lowering effect of UA at both doses was similar to that of metformin. In the liver, GK facilitates glucose uptake during hyperglycemia and is essential for regulating a network of glucose-responsive genes that are involved in glycolysis, glycogen synthesis and lipogenesis\(^{22}\). GK catalyzes ATP-dependent phosphorylation of glucose to glucose-6-phosphate as the first rate-limiting enzyme in glucose utilization\(^{23}\). Therefore, there is a growing interest in antidiabetic drugs that activate hepatic GK. In the present study, GK was not different between the STZ/NA-induced diabetic mice and the non-diabetic mice; however, UA supplementation increased the activity in a dose-dependent manner compared with the diabetic control. An increase in GK activity can stimulate glucose utilization, promote glycogen synthesis and reduce glucose levels\(^{24}\). In previous studies, GK activators effectively lowered fasting and postprandial

![Fig. 2—Effect of ursolic acid supplement on renal polyol pathway enzyme activities in STZ/NA-induced diabetic mice. (Values are mean±SE. The values not sharing a common letter differ significantly; *P* < 0.05)](image-url)
glucose levels in T2DM patients\textsuperscript{25}. Thus, UA seems to be a potential hepatic GK activator. Although the hepatic glycogen synthase activity was not been shown, UA and metformin supplement increased hepatic glycogen stores simultaneous with elevated GK activity in non-obese type 2 diabetic mice.

Recently, GLUT2 mRNA levels were reportedly significantly decreased in liver-specific GK knockout mice\textsuperscript{26}. GLUT2 is the facilitative glucose transporter that is predominantly expressed in the liver and pancreatic β-cells, which play a pivotal role in glucose homeostasis by mediating bidirectional glucose
transport. GLUT2 has been suggested to be the actual glucose sensor in the liver portal vein, indicating its essential nature for the rapid glucose response of that organ to glucose27. We found that STZ/NA induced GLUT2 mRNA down-regulation compared with the non-diabetic mice; however, both doses of UA and metformin up-regulated GLUT2 mRNA levels compared with the diabetic control mice. These results indicated that reduced blood glucose levels and elevated GK activity might increase hepatic glucose flux, although we currently cannot provide evidence for this hypothesis. The current study observed that metformin and both doses of UA significantly lowered STZ/NA injection-induced increases in G6Pase activity; however, supplement with UA or merformin did not alter the activity of PEPCK, which is another gluconeogenic enzyme. Thus, low-dose UA significantly elevated the hepatic GK/G6Pase activity ratio and glycogen content.

Diabetic nephropathy is a diabetic complication that can exacerbate diabetes severity and mortality28. Hyperglycemia plays an important role in the pathogenesis of diabetic complications by enhancing the AR-related polyol pathway29. The polyol pathway is a collateral glycolytic process that converts glucose to sorbitol and sorbitol to fructose. AR is a NADPH-dependent oxidoreductase and is the first rate-limiting enzyme in this polyol pathway that catalyzes the reduction of glucose to sorbitol, which metabolized to fructose by SDH29. Increased fructose formation leads to the generation of reactive dicarbonyl compounds, which are a key factors that are involved in advanced glycation endproduct (AGE) formation30. In diabetes, a surge in the rate of the AR-related polyol pathway augments the intracellular concentration of sorbitol and its metabolites, which accumulate in cells because of their poor penetration across membranes and inefficient metabolism, thus resulting in diabetic complications31. Increased polyol pathway activity with excessive NADPH consumption decreases nitric oxide production and reduces glutathione levels. Reduced nitric oxide and glutathione levels in turn causes ischemia and vessel hyperpermeability as well as increased free radical production32. Recent studies reported an inhibitory effect of UA on AR both in vitro and in vivo28,29. Wang et al.28 revealed that UA effectively inhibited renal and plasma AGE formation. In this study, UA and metformin supplement markedly suppressed renal AR activity and increased SDH activity, which resulted in a decreased AR/SDH ratio compared with the diabetic control group. These results are supported by previous study28 that demonstrated that 0.1 and 0.2% UA supplement significantly decreased renal AR activity in STZ-induced diabetic mice. Thus, the UA or metformin-induced renal polyol pathway improvement may contribute to recovery from diabetes-induced kidney damage.

Next, the effect UA or metformin were examined on the histological changes that occurred in the STZ/NA-induced diabetic mice. Early diabetic nephropathy changes include increased kidney weight and capsular space of glomeruli and tubular lumen expansion in STZ/NA-induced diabetic mice compared with the non-diabetic group. However, both doses of UA and metformin supplement restored kidney weight and histological changes. UA and metformin supplement preserved renal architectural

### Table 2—Effect of ursolic acid supplement on plasma lipid profiles in STZ/NA-induced diabetic mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>FFA (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>TC (mmol/L)</th>
<th>VLDL-C (mmol/L)</th>
<th>LDL-C (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>HTR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NonDM</td>
<td>0.67 ± 0.04a</td>
<td>1.19 ± 0.05a</td>
<td>3.40 ± 0.28a</td>
<td>0.23 ± 0.01ab</td>
<td>1.09 ± 0.17a</td>
<td>2.06 ± 0.11a</td>
<td>60.60±2.31a</td>
</tr>
<tr>
<td>DM</td>
<td>0.77 ± 0.05b</td>
<td>1.67 ± 0.12b</td>
<td>4.25 ± 0.34b</td>
<td>0.29 ± 0.03b</td>
<td>2.02 ± 0.14b</td>
<td>1.80 ± 0.06b</td>
<td>42.37±1.46b</td>
</tr>
<tr>
<td>DM-lowUA</td>
<td>0.60 ± 0.04ab</td>
<td>1.40 ± 0.11a</td>
<td>3.84 ± 0.20ab</td>
<td>0.25 ± 0.03ab</td>
<td>1.47 ± 0.16a</td>
<td>2.00 ± 0.05b</td>
<td>51.99±1.74b</td>
</tr>
<tr>
<td>DM-highUA</td>
<td>0.48 ± 0.07a</td>
<td>1.18 ± 0.07a</td>
<td>3.43 ± 0.15a</td>
<td>0.18 ± 0.00a</td>
<td>1.46 ± 0.07a</td>
<td>1.79 ± 0.07a</td>
<td>52.70±1.72b</td>
</tr>
<tr>
<td>DM-Metformin</td>
<td>0.62 ± 0.07ab</td>
<td>1.24 ± 0.07a</td>
<td>4.19 ± 0.15b</td>
<td>0.22 ± 0.01ab</td>
<td>1.99 ± 0.16b</td>
<td>1.97 ± 0.09b</td>
<td>47.18±2.44ab</td>
</tr>
</tbody>
</table>

*Means in the same column not sharing a common superscript letter differ significantly; *P* < 0.05.

FFA=fatty acid, TG=triglyceride, TC=total cholesterol, VLDL-C=VLDL-cholesterol, HDL-C=HDL-cholesterol, HTR=(HDL-cholesterol/total cholesterol)×100.
structure. Thus, UA may be a potential supplement in diabetic nephropathy, which is consistent with results from Zhou et al., who showed that low-dose UA supplementation (0.01% in food) of STZ-induced diabetic mice for 3 months ameliorated glomerular hypertrophy and type IV collagen accumulation.

In obese T2DM patients, metformin is currently the drug of first choice because of its bilateral effect on glycemic regulation and cardiovascular protection. However, obese and non-obese T2DM patients experience similar cardiovascular risk, and metformin supplement might even be beneficial in non-obese T2DM patients. Lund et al. reported that metformin was more effective than repaglinide reducing inflammation and endothelial dysfunction biomarkers in non-obese T2DM patients despite similar glycemia between treatments. Major causes of diabetic dyslipidemia are increased plasma TG levels and decreased HDL-C levels. These components are closely linked to each other because of bidirectional lipid exchange between TG-rich lipoprotein and HDL particles. There is growing evidence that fasting and non-fasting TG levels are associated with increased cardiovascular disease risk in all populations, including the Asian population. Prolonged hyperglycemia significantly increases serum TG and TC levels, which may be attributed to insulin deficiency. Insulin activates lipoprotein lipase, which hydrolyzes TG; thus, this enzyme is not activated in the absence of insulin, thereby causing hypertriglyceridemia. Blood glucose level normalization resulted in a significant reduction of serum cholesterol, TG and protein levels. In the present study, elevated plasma TC, TG, VLDL-C and decreased HDL-C/TC ratio were observed in diabetic control mice. However, UA supplementation lowered TC and VLDL-C concentrations in a dose-dependent manner and lowered TG and LDL-C levels in a dose-independent manner, which may contribute to the prevention of diabetic complications.

Further, elevated FFA levels in T2DM patients impair glucose effectiveness. In the present study, plasma FFA concentrations were not significantly different between the normal mice and the non-obese diabetic mice. However, UA supplementation dose-dependently lowered plasma FFA levels compared with the diabetic control mice. Some studies demonstrated important effects of increased FFA on glucose effectiveness and gluconeogenesis up-regulation in humans. Given that hyperglycemia inhibits net glycogenolysis, it is important to determine the impact of elevated FFA on this regulation in T2DM patients.

Conclusion
The results of the present study suggest that the beneficial effects of 0.01% UA supplementation could be attributable to changes in hepatic glucose metabolism and the renal polyl pathway in non-obese type 2 diabetic mice. In addition, high-dose UA supplement has potential anti-dyslipidemic efficacy in non-obese type 2 diabetic mice. UA demonstrated similar anti-hyperglycemic potency and protection against diabetic renal complications compared with metformin.

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References


