Maslinic Acid Reduces Blood Glucose in KK-A′ Mice

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In the present study, we have examined the hypoglycemic effect of maslinic acid (MA) in KK-A′ mice, an animal model of genetic type-2 diabetes. MA (10 mg/kg body wt) reduced the blood glucose levels in KK-A′ mice at 4 h after a single oral dose. KK-A′ mice receiving MA at daily dosages of 10 mg/kg and 30 mg/kg for 2 weeks showed a significant reduction in the blood glucose levels. Furthermore, the results also showed that MA might modulate glucose metabolism partially through reducing insulin resistance in KK-A′ mice. Taken together, MA may hold great promise as a natural therapeutic agent for treatment of type 2 diabetes.

Key words maslinic acid; KK-A′ mice; type 2 diabetes; insulin resistance

Pentacyclic triterpenes are widely distributed throughout the plant kingdom. A variety of biological properties have been ascribed to this class of compounds including anti-inflammatory, hepatoprotective, gastroprotective, anti-ulcer, anti-HIV, anti-cancer, anti-diabetic, hypolipidemic, antiatherosclerotic and immunoregulatory effects.1 As a part of our project aimed at drug discovery based on pharmacological interference with glucose metabolism, we have been focused on research and development of pentacyclic triterpenes as natural and low-toxic anti-diabetic agents with preventive and therapeutic effects against ischemic diabetic complications.2–5

Maslinic acid (MA, 1, structure shown in Fig. 1), a pentacyclic triterpene acid abundant in olive fruit skin, has recently attracted much attention due to its anti-tumor,6) anti-HIV7) and anti-oxidation8) activities. We have previously reported that maslinic acid and related pentacyclic triterpenes represent a new class of inhibitors of glycogen phosphorylase, and their glucose-lowering activity in diabetic mice induced by adrenaline might be due to, at least in part, modulation hepatic glycogen metabolism.3,4) Very recently, Fernandez-Navarro et al.9) reported that maslinic acid exhibited modulation effects of on hepatic glycogen metabolism in the rainbow trout, and claimed that their observation was in agreement with our findings.

In the present study, the anti-diabetic effects of MA have been evaluated in KK-A′ mice because they are an excellent model that closely resembles type 2 diabetes in humans.10,11) The aim of this study is to test whether or not MA improves diabetes in obesity-linked type 2 diabetic KK-A′ mice.

MATERIALS AND METHODS

Materials MA was obtained by semi-synthesis starting from readily available oleanolic acid as previously described.3,4) MA was stored at room temperature until use. For oral administration, MA was suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na) solution. Control group was received the same volume CMC-Na solution. For subcutaneous administration, insulin 0.5 U/kg was dissolved in saline.

Animals and Treatments Animal studies were performed after obtaining prior permission and handled according to the university and institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals. KK-A′ male mice (6 weeks old, weighing 37–41 g) (Shanghai laboratory animal center, China) with a blood glucose level greater than 300 mg/dl were considered to be diabetic and were used. The mice were housed in environmentally-conditioned room at 22±2 °C with a 12 h light/dark cycle. The animals were kept in the experimental animal room for 7 d either free access to food and water. Blood samples were withdrawn from the cavernous sinus with a capillary for glucose determination. The experiments were performed between 9:00 and 10:00 a.m. Baseline measurements of body weight and food intake were obtained daily prior to the administrations.

Blood Glucose Levels Determination (Single Dose) The mice were orally given MA (3, 10, 30 mg/kg body wt) dissolved in 0.5% CMC-Na solution. The control group received an equal volume 0.5% CMC-Na solution. Blood samples were taken at 2, 4 and 7 h later for glucose determination. This experiment was performed under nonfasting conditions. Blood glucose levels were determined using the glucose oxidase method.12) Blood glucose levels in normal animals were also measured.

Blood Glucose Levels Determination (Repeated Administration for 2 Weeks) The mice orally received MA (10 mg/kg) in 0.5% CMC-Na suspension once a day for 2 weeks. The control group received an equal volume of 0.5% CMC-Na solution. Blood samples were taken for glucose determination every week. This experiment was performed under nonfasting conditions. Blood glucose levels were determined using the glucose oxidase method.12) Blood glucose levels in normal animals were also measured.

Insulin Tolerance Test At the end of the repeated administration period and overnight fasting, an insulin solution 0.5 U/kg was injected subcutaneously into the mice and blood samples were obtained for glucose determinations 0, 30, 60, and 120 min later. Plasma insulin was measured with
GLAZyme Insulin-EIA Test.\textsuperscript{13)}

Hepatic Glycogen Content Measurement Hepatic glycogen content was measured with some modifications using anthrone (Sigma, St. Louis, MO, U.S.A.).\textsuperscript{29} Briefly, 0.1 g liver tissue was resuspended in 30% KOH, and incubated for 15 min at 95°C to extract glycogen. Then 2% Na$_2$SO$_4$ was added and the glycogen was precipitated with ethanol. The precipitate was resuspended in H$_2$O: sulfuric acid (3:7.6 ratios) containing 0.15% anthrone and heated for 15 min at 95°C and the reaction was stopped by chilling the tubes in ice. The glycogen content was immediately determined spectrophotometrically at 620 nm.

Serum Biochemical Analysis At the end of the repeated administration period and overnight fasting, the blood samples were collected and were then centrifuged at 1500 rpm for 10 min at 4°C. Plasma adiponectin levels were measured using ELIZA kits purchased from R&D Systems Inc. (Minneapolis, U.S.A.). Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) levels were also determined by using commercial kits from Jiancheng Bioengineering Inc. (Nanjing, China).

Statistical Analysis Data are expressed as mean±S.E.M. from 7 mice. Statistical analysis was conducted using the Student’s t test and ANOVA. Values of $p<0.05$ were considered statistically significant.

RESULTS

Effects of MA on Blood Glucose Levels in KK-A$^\text{y}$ Mice (Single Administration) The mean blood glucose levels in KK-A$^\text{y}$ mice at different doses (3, 10, 30 mg/kg) and at different time point (2, 4, 7 h) after a single oral administration are shown in Fig. 2. MA 10 mg/kg or 30 mg/kg reduced blood glucose levels in KK-A$^\text{y}$ mice after administration ($p<0.05$). MA 30 mg/kg treated mice showed a significant decrease in plasma glucose levels at 4 and 7 h compared to the control values ($p<0.01$) (Fig. 2).

Effects of Repeated Administration of MA on Blood Glucose Levels and Hepatic Glycogen Content in KK-A$^\text{y}$ Mice The mean blood glucose levels of KK-A$^\text{y}$ mice after repeated oral doses for 2 weeks were determined. As shown in Fig. 3, KK-A$^\text{y}$ mice exhibited reduced blood glucose levels 1 week and/or 2 weeks after administration of MA (10 mg/kg/d or 30 mg/kg/d) ($p<0.05$). Hepatic glycogen content shows MA is able to increase hepatic glycogen content ($p<0.05$), which may contribute to reducing glucose supply to blood.

Effects of MA on Plasma Insulin Levels in KK-A$^\text{y}$ Mice The effects of MA on plasma insulin in KK-A$^\text{y}$ mice are shown in Fig. 4. The plasma insulin levels in MA treated KK-A$^\text{y}$ mice decreased 2 weeks after administration.

Insulin Tolerance Test As shown in Fig. 5, KK-A$^\text{y}$ mice had basal hyperglycemia. This hyperglycemia was significantly decreased after treatment with MA (10, 30 mg/kg). The blood glucose levels at 30, 60, 120 min after insulin administration reduced significantly compared to the controls (30, 60 min, $p<0.01$; 120 min, $p<0.05$).

Effects of MA on Body Weight, Food Intake and Serum Biochemical Analysis in KK-A$^\text{y}$ Mice As shown in Table 1, oral administration of MA normalized the depressed adiponectin levels, reduced food intake and epididymal fat pads weight in KK-A$^\text{y}$ mice, which may contribute to im-

![Fig. 2. Effects of a Single Dose of MA on Blood Glucose in KK-A$^\text{y}$ Mice](image1)

MA (3, 10, 30 mg/kg body wt) was administrated orally to the mice. The control mice received the same volume of CMC-Na suspension. Blood samples were taken for glucose determinations 2, 4 and 7 h later. Each value represents the mean±S.E.M. from 7 mice. Significantly different from control, *$p<0.05$, **$p<0.01$. $p<0.05$ vs. control group (untreated KK-A$^\text{y}$ mice).

![Fig. 3. Effects of Repeated Administration of MA on Blood Glucose Levels and Hepatic Glycogen Content in KK-A$^\text{y}$ Mice](image2)

(A) MA 10 and 30 mg/kg was administrated orally to the mice for 2 weeks. The control mice received the same volume of CMC-Na suspension. Blood samples were taken for glucose determination at 0, 1 and 2 week. Each value represents the mean±S.E.M. from 7 mice. Significantly different from control, *$p<0.01$. Significantly different from 0 week, *$p<0.05$. (B) Effect of MA on hepatic glycogen content of KK-A$^\text{y}$ Mice. *$p<0.05$ vs. control group (untreated KK-A$^\text{y}$ mice).

![Fig. 4. Effects of MA on Plasma Insulin Levels in KK-A$^\text{y}$ Mice (2 Weeks)](image3)

After daily administration of MA 10 and 30 mg/kg for 2 weeks, blood samples of KK-A$^\text{y}$ mice were taken for insulin levels determination. Each value represents the mean±S.E.M. from 7 mice. Significantly different from control, **$p<0.01$, $p<0.05$. 

![Fig. 5. Insulin Tolerance Test](image4)
Table 1. Effects of MA in KK-Ay Mice (Means±S.D., n=7)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>10 mg/kg</th>
<th>30 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma adiponectin level (µg/ml)</td>
<td>7.3±0.5</td>
<td>8.5±1.1*</td>
<td>10.3±1.2**</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>41.3±2.9</td>
<td>42.0±1.7</td>
<td>43.5±2.0</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>6.5±0.4</td>
<td>4.7±0.3**</td>
<td>4.3±0.4**</td>
</tr>
<tr>
<td>Epididymal fat pads weight (g/100 g of body weight)</td>
<td>3.5±0.5</td>
<td>2.9±0.4*</td>
<td>2.4±0.3*</td>
</tr>
<tr>
<td>LDH (g/l)</td>
<td>6.62±0.64</td>
<td>6.54±0.58</td>
<td>6.47±0.73</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>78.8±3.4</td>
<td>76.1±2.3</td>
<td>75.3±3.0</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>35.5±1.5</td>
<td>33.7±1.8</td>
<td>33.1±1.5</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>41.3±2.9</td>
<td>39.4±2.9</td>
<td>40.3±3.0</td>
</tr>
<tr>
<td>Epididymal fat pads weight (g/100 g of body weight)</td>
<td>3.5±0.5</td>
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</tr>
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<td>4.7±0.3**</td>
<td>4.3±0.4**</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01 vs. control group (untreated KK-Ay mice).

Provision of blood glucose, insulin level and insulin resistance.

DISCUSSION

Type 2 diabetes is the most common form of diabetes. In type 2 diabetes, either the body does not produce enough insulin or the cells ignore the insulin that results in insulin resistance. Over time, insulin resistance and high blood glucose levels may cause severe damages to various organs, including eyes, kidneys, brain or heart. In this regard, novel anti-diabetic agents with insulin-sensitizing effects and preventive effects against diabetic complications are highly desirable. KK-Ay mice show a degree of obesity-related hyperglycemia, hyperlipidemia and hyperinsulinemia, and which are thought to be a reliable animal model for the study on non-insulin-dependant diabetic mellitus (NIDDM).15)

In this study, we measured the blood glucose levels after treatment with MA in KK-Ay mice. We examined the dose dependence (3, 10, 30 mg/kg) after MA treatment, and observed that antidiabetic activity at dose 10 and 30 mg/kg after oral administration (Fig. 2). Therefore, we examined the effect of repeated MA 10 and 30 mg/kg administration. The results of this study clearly show that MA produces a consistent hypoglycemic. Moreover, the hepatic glycogen levels have been also increased at dose 10 and 30 mg/kg after MA treatment for 2 weeks (Fig. 3). In our previous study, we reported that MA is a glycogen phosphorylase inhibitor.14) Glycogen phosphorylase, an enzyme that catalyzes the process of glycogenolysis, contributes to glycogen metabolism. Research with GPIs (glycogen phosphorylase inhibitors) was shown the inhibition of HGP (hepatic glucose output) in the context of developing a treatment for the hyperglycemia.16) A reduction in glycogenolysis via the inhibition of GP is one possible approach to lower the blood glucose in this study.

The results of plasma insulin levels in KK-Ay mice showed that after repeated administration of MA for 2 weeks, the plasma insulin levels were significantly reduced (Fig. 4). In addition, after being injected subcutaneous insulin (0.5 U/kg body wt), control groups did not exhibit reduced blood glucose levels due to insulin resistance in the peripheral tissues (Fig. 5). However, the blood glucose levels in MA treated groups were reduced significantly compared to the controls. These results indicate that MA maybe useful in improving hyperinsulinemia in type 2 diabetes.

Oral administrations of MA did not change the body weight of KK-Ay mice. However, the excessive food intake and fat weight may contribute to improvement of blood glucose, insulin levels and insulin resistance. On the other hand, adiponectin is abundantly present in plasma and leads to enhanced insulin action and maintains insulin sensitivity and glucose homeostasis.17) In the present study, MA normalized the plasma adiponectin levels suggests the possible mechanism by which MA improve insulin resistance in KK-Ay mice.

As a natural and low toxic compound, MA elicited excellent outcomes without inducing side-effects such as diarrhea and soft feces (data not shown). In the present study, none of the mice died. MA doesn’t change the plasma LDH, ALP, ALT and AST levels, which are marker enzymes for the hepatic tissue (Table 1). This indicated that MA did not exert appreciable toxic effects in the liver.

In summary, MA exhibited significant glucose-lowering and hypoinsulinemic effects in KK-Ay mice. The advantages of MA as a promising natural anti-diabetic agent are obvious: 1) as an abundant constituent of olive fruit skin, MA is almost non-toxic; 2) besides reducing blood glucose, MA exhibits preventive effects against diabetic complications; 3) MA also exhibits other therapeutic benefits such as anti-inflammation and antioxidiant activities, etc. To our knowledge, it is the first time for us to report that MA lowers blood...
glucose levels in KK-A^y mice. Taking into account of the results of this study and our previous studies, MA may hold great promise as a natural therapeutic agent for treatment of type 2 diabetes.

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