Potentiating Effects on Pilocarpine-Induced Saliva Secretion, by Extracts and N-Containing Sugars Derived from Mulberry Leaves, in Streptozocin-Diabetic Mice

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The effects of hot water extracts and six compounds of N-containing sugars, 1-deoxyxojirimycin (DNJ), N-methyl-DNJ (N-Me-DNJ), 2-O-α-d-galactopyranosyl-DNJ (GAL-DNJ), fagomine, 1,4-dideoxy-1,4-imino-D-arabinitol (DAB), and 1,2x,3β,4x-tetrahydroxynortropane (calystegin B2), derived from mulberry leaves (Morus alba L.), were investigated on pilocarpine-induced saliva secretion in streptozocin (STZ)-induced diabetic mice. The extracts (100 and 200 mg/kg, i.p.) significantly potentiated the pilocarpine-induced salivary flow but not the protein content. The component compounds (37.5—300 μmol/kg) potentiated the saliva secretion, and the potency order was DAB > fagomine > GAL-DNJ. Only fagomine significantly increased the protein content in the saliva. The potentiation of pilocarpine-induced salivary flow was correlated with anti-hyperglycemic effects by the extract and GAL-DNJ from mulberry leaves in the same dose ranges.

Key words anti-hyperglycemic saliva secretion; pilocarpine; STZ-diabetic mouse; mulberry leaf; GAL-DNJ; fagomine

Mulberry leaves (Morus alba L.), a traditional Chinese herb, are used to treat and prevent diabetes mellitus ("Xiao-ke") and to alleviate thirst. 1) The component compounds are flavones, steroids, triterpenes, amino acids, vitamins and other trace amounts of minerals. 2, 3) Two N-containing sugars, N-methyl-1-deoxyxojirimycin (N-Me-DNJ) and 2-O-α-D-galactopyranosyl-DNJ (GAL-DNJ), have recently been isolated and identified by Asano et al. 4) The yields of six component compounds range from only 0.0016 to 0.11% for the crude drug, from 0.018 to 0.21% for its hot water extract, and from 0.008 to 0.18% for the ethanol-insoluble fraction of mulberry leaves. 5, 6) Fagomine and 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) have also been isolated from Fagopyrum and the fruits of aegonicly cucubitus. We have found anti-hyperglycemic effects of hot water extracts (25—200 mg/kg, i.p.) and its component compound N-containing sugars (75—300 μmol/kg, i.p.) from mulberry leaves on STZ-diabetic mice. Among six N-containing sugars (Table 1), GAL-DNJ and fagomine have the most potent anti-hyperglycemic effects. 5—7) Feeding with a diet containing 2.5—5% mulberry leaves (dried powders) for 48 weeks significantly lowers blood glucose and increases the plasma insulin level, and improves the glucose tolerance ability in WBN/Kob diabetic rats. 8

Diabetes mellitus is a disease involving the abnormal metabolism of saccharides, protein and fat, which is caused by deficient insulin release or reduced sensitivity to insulin receptors. Xerostomia is one of the complications to noninsulin-dependent diabetes mellitus (NIDDM) in human patients. This thirst is caused by secretory dysfunction of the salivary gland and by hyperglycemia and abnormal metabolism of water and electrolytes. Therefore, the secretory function of saliva has been considered to be intrinsically regulated by blood glucose level and insulin release. The regulation of blood glucose level may be connected with saliva secretion. 10

In the present study, we investigated the potentiation by, hot water extract and N-containing sugars derived from mulberry leaves, of pilocarpine-induced saliva secretion and its correlation with their anti-hyperglycemic action.

MATERIALS AND METHODS

Animals Male ddY mice (4-week-old) weighing 18—23 g were purchased from Japan Shizuoka Laboratory Center, Ltd. (Shizuoka, Japan). The mice were injected intravenously (i.v.) with a bolus dose of streptozocin (STZ, 150 mg/kg; Sigma, St. Louis, MO, U.S.A.), and used 4—5 weeks (34—42 g) after its injection. The animals were maintained in an air-conditioned room with light from 7 a.m. to 7 p.m. The room temperature (23 ± 1°C) and humidity (55 ± 5%) were controlled automatically. Blood glucose levels were measured, using the glucose oxidase method, on a glucose analyzer (Type 2, Beckman, CA, U.S.A.). The blood glucose levels were more than 200 mg/dl after fasting for 13—14 h before the experiment.

Collection of Total Saliva Hot water extracts and N-containing sugars were dissolved in saline and intraperitoneally (i.p.) injected into STZ-diabetic mice after they had fasted for 13—14 h. After 2 h, the mice were anesthetized with sodium pentobarbital (Nembutal, Abbott Lab., North Chicago, IL, U.S.A.) (50 mg/kg, i.p.) and were placed on a heating pad maintained at 37°C. A polyethylene tube (i.d.: 1.5 mm, length: 20 mm) was surgically cannulated into the trachea to keep the airways open. A second stimulation with pilocarpine (1.0 mg/kg, i.v.) was made 10 min after the first (basal) stimulation with pilocarpine (0.1 mg/kg, i.v.). For 20 min after the second stimulation, the total saliva was collected into microcapillary tubes (Microcaps, 20 μl, Drummond, PA, 1995 Pharmaceutical Society of Japan
Table 1. The Chemical Structures of N-Containing Sugars Derived from Mulberry Leaves

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>Abbreviation</th>
<th>Chemical structure†</th>
<th>Yield† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-Deoxynojirimycin</td>
<td>DNJ</td>
<td>HOCH₂</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>N-Methyl-1-deoxynojirimycin</td>
<td>N-Me-DNJ</td>
<td>HOCH₂</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2-O-α-D-galactopyranosyl-1-deoxynojirimycin</td>
<td>GAL-DNJ</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fagomine</td>
<td></td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1,4-Dideoxy-1,4-imino-ß-arabinitol</td>
<td>DAB</td>
<td>H</td>
<td>0.011</td>
</tr>
<tr>
<td>6</td>
<td>Calystegnin B₂ (Nortropanoline)</td>
<td></td>
<td>H</td>
<td>0.0042</td>
</tr>
</tbody>
</table>

† The chemical structures are from Asano et al.⁴³

U.S.A.), which had been placed under the tongue, at 1 min-intervals. The total volume of saliva for 20 min was measured. At the end of saliva collection, bilateral sides of the submandibular, parotid and sublingual glands were isolated from the connective tissue, fat and lymph nodes, and were weighed. Salivary flow was measured as the volume of saliva per wet total weight of total salivary glands per min. The protein concentration in saliva was determined by the method described by Lowry et al.¹² using bovine serum albumin as a standard.

**Materials** The purification procedures of N-containing sugars from mulberry leaves have already been reported.⁴,¹³ The six compounds of N-containing sugars used were 1-deoxynojirimycin (DNJ), N-methyl-DNJ (N-Me-DNJ), 2-O-α-D-galactopyranosyl-DNJ (GAL-DNJ), fagomine, 1,4-dideoxy-1,4-imino-ß-arabinitol (DAB), and 1,2,3,ß,4ß-tetrahydroxynortropine (calystegin B₂) (Table 1). The extraction procedure for hot water extracts from mulberry leaves has been reported previously.⁷ The compounds and extracts tested were dissolved in saline and were injected into the STZ-diabetic mice 14 h after fasting.

Pilocarpine hydrochloride (Wako Pure Chemical Co., Osaka, Japan) was used as a salivary secretagogue.

**Statistical Analysis** Significant differences between the mean values of the saline control and treatment were statistically analyzed by one-way ANOVA and then the Scheffe multiple-comparison test. All data are expressed as the means ± S.E.M.

**RESULTS**

**Potentiating Effects on Pilocarpine-Induced Saliva Secretion by Hot Water Extract from Mulberry Leaves** The hot water extract from mulberry leaves (100 and 200 mg/kg, i.p.) significantly potentiated pilocarpine-induced saliva secretion (Fig. 1A), and tended to increase the protein content in the saliva (Fig. 1B). The extract alone stimulated neither the basal saliva secretion nor the basal protein contents in the saliva.
Potentiating Effects on Pilocarpine-Induced Saliva Secretion by N-Containing Sugars Derived from Mulberry Leaves

N-Containing sugars derived from mulberry leaves were injected into STZ-diabetic mice at a dose of 300 μmol/kg (i.p.). The effects on pilocarpine-induced saliva secretion were compared among the 6 component compounds. The potentiating effects on the salivary flow were in the order of DAB > fagomine > GAL-DNJ. Only fagomine increased the protein content. These effective compounds alone stimulated neither the basal saliva secretion nor the basal protein content in saliva. An oral anti-hyperglycemic drug, glibenclamide (30 μmol/kg, i.p.), did not potentiate the effect of salivary flow, but increased the protein content at a dose which lowered blood glucose (Fig. 2).

Time-Dependent Potentiating Effect of Fagomine on Pilocarpine-Induced Saliva Secretion

When fagomine (75—300 μmol/kg, i.p.) was injected into STZ-diabetic mice, the salivary flow rate reached the peak value immediately, within 1—2 min, and then gradually decreased. Not only the peak value, but also the duration of saliva secretion at the dose of 300 μmol/kg, was higher and longer than that of the control, respectively (Fig. 3).

Fig. 2. Histogram of Potentiating Effects on Pilocarpine-Induced Salivary Flow (A) and Protein Content in Saliva (B) by N-Containing Sugars from Mulberry Leaves in Streptozocin-Diabetic Mice

Saliva was collected over a 20-min period after the second stimulation with pilocarpine (1.0 mg/kg, i.v.). The effects of N-containing sugars (300 μmol/kg, i.p.) and glibenclamide (30 μmol/kg, i.p.), an effective dose to lower the blood glucose level (Z), were compared with the saline control (open bar). The N-containing sugars are 1-deoxynojirymycin (DNJ) (●), N-methyl-DNJ (N-Me-DNJ) (■), 2-O-α-D-galactopyranosyl-DNJ (GAL-DNJ) (▲), fagomine (□), 1,4-dideoxy-1,4-manno-porabiose (DAB) (△), and 1,2α,3β,4α-tetrahydronortropane (calystegine B3) (■). *p<0.05, and **p<0.01: Significantly different from the control by one-way ANOVA and then Scheffe multiple-comparison test. All values are the means±S.E.M. of 5—10 mice.

Fig. 3. Time-Course of Potentiating Effects by Fagomine (75—300 μmol/kg, i.p.) on Pilocarpine-Induced Saliva Secretion in Streptozocin-Diabetic Mice

Saliva was collected over a 20-min period after the second stimulation with pilocarpine (1.0 mg/kg, i.v.). Open circles are the saline control. Closed symbols represent the dose of fagomine: 75 (▼), 150 (▲) and 300 (●) μmol/kg, respectively. Each symbol indicates the amount of salivary flow per min and the means±S.E.M. of 5—10 mice.

Fig. 4. Dose-Response Curves of N-Containing Sugars from Mulberry Leaves on Pilocarpine-Induced Salivary Flow (A) and Protein Content in Saliva (B) in Streptozocin-Diabetic Mice

Saliva was collected over a 20-min period after the second stimulation with pilocarpine (1.0 mg/kg, i.v.). The N-containing sugars are 2-O-α-D-galactopyranosyl-DNJ (GAL-DNJ, ▲), 1,4-dideoxy-1,4-manno-porabiose (DAB, △), and fagomine (●) (37.5—300 μmol/kg, i.p.). *p<0.05, and **p<0.01: Significantly different from the saline control (open symbol) by one-way ANOVA and then Scheffe multiple-comparison test. All values are the means±S.E.M. of 5—12 mice.

Dose-Dependent Potentiating Effects by DAB, Fagomine and GAL-DNJ on Pilocarpine-Induced Saliva Secretion

The dose-response curves for salivary flow (Fig. 4A) and for protein content (Fig. 4B) in pilocarpine-induced
Fig. 5. The Regression Lines and Correlation Coefficients in Anti-hyperglycemic Effects and Pilocarpine-Induced Saliva Secretion for Hot Water Extract (▲), GAL-DNJ (●), fagomine (○) and DAB (○) from Mulberry Leaves in Streptozocin-Diabetic Mice

Each value is the means ± S.E.M. of 5–20 mice. **, p<0.01; Significant correlation.

saliva secretion were plotted using 3 component compounds derived from mulberry leaves. DAB at 75, 150 and 300 μmol/kg, fagomine at 150 and 300 μmol/kg and GAL-DNJ at 300 μmol/kg (i.p.) significantly potentiated the saliva flow. Fagomine at 150 and 300 μmol/kg significantly increased the protein content in the saliva.

Correlation Analysis of Pilocarpine-Induced Saliva Secretion with Anti-hyperglycemic Effects by Hot Water Extract and GAL-DNJ from Mulberry Leaves Whether or not anti-hyperglycemic effects were closely related to the potentiation effects on pilocarpine-induced saliva secretion was examined by correlation analysis. The data of anti-hyperglycemic effects were replotted from Kimura et al. Two parameters were significantly and positively correlated in hot water extract and GAL-DNJ, but not in fagomine and DAB (Fig. 5). The correlation coefficients indicated that both parameters were strongly correlated in the extracts and GAL-DNJ.

DISCUSSION

Diabetic patients experience xerostomia, a feeling of thirst and the need to frequently drink water. This syndrome is related to a reduced flow rate of saliva and an accompanying decrease in salivary protein components. In addition to the loss of salivary gland function, the glandular tissue undergoes a significant pathologic variation in salivary glands in the diabetic animal. By stimulating the parasympathetic and sympathetic nerve in STZ-diabetic mice and rats, the salivary flow and protein content are obviously lower than those of the normal animals, respectively. With autoimmune diabetes-prone nonobese diabetic (NOD) male and female mice, salivary flow rates and total salivary protein content showed a progressive decline from non-diabetic to diabetic states. The present study indicated that the hot water extract of mulberry leaves and GAL-DNJ, a N-containing sugar, potentiated pilocarpine-induced saliva secretion at anti-hyperglycemic doses observed in STZ-diabetic mice. The strong correlation between saliva secretion and anti-hyperglycemic effect in the extract and GAL-DNJ also supported this.

Many salivary protein constituents possess antimicrobial properties and promote wound healing and epidermal tissue regeneration. The peptide P-C, consisting of 44 amino acid residues from the human parotid gland, shows anti-hyperglycemic effects on diabetic mice. We have reported the anti-hyperglycemic effects of hot water extract, ethanol-insoluble extract and their component compound N-containing sugars from mulberry leaves on STZ-diabetic mice. These extracts increase glucose uptake in the diaphragm muscles of normal mice. The inhibition of glycosidase is a mechanism involved in anti-hyperglycemic effects. The component compounds DAB, fagomine and GAL-DNJ increased the saliva flow, and fagomine raised the protein content in saliva. The stimulation of muscarinic M3- and α-receptors elicits a large volume of saliva with low protein contents, whereas the stimulation of β1-receptor elicits a small volume of saliva with high protein content. The extract and other effective components may relieve thirst during the muscarinic (pilocarpine) stimulation.

In conclusion, hot water extracts from mulberry leaves and their component compounds, DAB, fagomine, and GAL-DNJ, dose-dependently potentiated pilocarpine-induced saliva secretion in STZ-diabetic mice. The effects of the extracts and GAL-DNJ were strongly correlated with their anti-hyperglycemic effects in the same dose ranges.

REFERENCES