Antihyperglycemic effects of *N*-containing sugars derived from Mulberry leaves in streptozocin-induced diabetic mice

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(*Received July 27, 1995. Accepted October 25, 1995.*)

Abstract

The antihyperglycemic effects of six compounds, *N*-containing sugars 1 deoxynojirimycin (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, 2α, 3β, 4α-tetrahydroxynortropane (calystegin B_2) derived from mulberry leaves (*Morus alba* L.) were investigated in streptozocin (STZ) induced diabetic mice. GAL-DNJ and fagomine 6 hr after injection, lowered the blood glucose level in a dose-dependent manner. The ED_{50} values (95 % confidence limits) were 115.0 (96.8-136.7) μmol/kg and 142.4 (130.5-155.3) μmol/kg, respectively. The ED_{50} values with 95 % confidence limits were 41.0 (31.8 52.7) mg/kg for hot water extract and 33.9 (26.6 43.1) mg/kg for ethanol-insoluble extract from mulberry leaves, respectively. GAL-DNJ and fagomine are the most active among six compounds to produce antihyperglycemic effects. Fagomine (3 mM) but not GAL-DNJ (3 mM) potentiated 8.3 mM glucose-induced immunoreactive insulin release from isolated perfused rat pancreas. The mechanism of antihyperglycemic effect by fagomine may be the potentiation of the insulin release.

Key words *N*-containing sugars, fagomine, 2-O-α-D-galactopyranosyl-1-deoxynojirimycin, antihyperglycemic effect, mulberry leaves (*Morus alba* L.), streptozocin-diabetic mice.

Abbreviations A2, ethanol-insoluble extract ; calystegin B_2, 1, 2α, 3β, 4α tetrahydroxynortropane ; DNJ, 1-deoxynojirimycin ; DAB, 1,4-dideoxy-1,4-imino-D-arabinitol ; GAL-DNJ, 2-O-α-D galactopyranosyl-1-deoxynojirimycin ; IRI, immunoreactive insulin ; *N* Me DNJ, *N*-methyl-1-deoxynojirimycin ; STZ, streptozocin ; W, hot water extract.

Introduction

Mulberry leaves (*Morus alba* L.) traditionally have been used to cure "Xiao ke" (diabetes) in traditional Chinese medicine.[1] We have reported that the extracts from mulberry leaves showed a potent antihyperglycemic activity in streptozocin (STZ)-induced diabetic mice. The activity of the ethanol insoluble fraction was more potent than that of the ethanol-soluble fraction.[2,3] Rutin, inokosterone, ectdysterone and moracetin are contained in mulberry leaves.[4,5,6] *N*-containing sugars derived from mulberry leaves were isolated and identified by Asano *et al.*[7,8] However, antihyperglycemic activities of these compounds have never been reported. In the present study, our aim is to investigate antihyperglycemic activities of *N*-containing sugars, derived from hot water extract and ethanol insoluble fraction in STZ-induced diabetic mice, and its mechanism in perfused pancreas of normal rat.

Materials and Methods

Animals : Male mice of ddY strain (4 weeks of age, 18-23 g) were injected with 150 mg/kg of STZ

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(Sigma, St. Louis, MO, USA) in saline via the tail vein, and were used 4 weeks after its injection. The blood glucose levels of these mice were more than 200 mg/dl 12-14 hr before each experiment. Normal male rats (Wistar, body weight, 200-250 g ; blood glucose levels, 92-122 mg/dl) were used. The animals were purchased from SLC Ltd. (Shizuoka, Japan). The animals were maintained in an airconditioned room with light from 7 a.m. to 7 p.m.. The room temperature (23 ± 1°C) and humidity (55 ± 5 %) were controlled automatically.

Determination of blood glucose levels[9] : The blood samples (20 μl) were obtained from the orbital venous plexus using capillary glass tubes before and 2, 4 and 6 hr after injection. The blood glucose levels were measured by the glucose oxidase method using a glucose analyzer II (Beckman Instruments, Inc., CA, U.S.A.).

Evaluations of antihyperglycemic activity : The antihyperglycemic activity in STZ-diabetic mice was evaluated as previously described.[9]

$$\text{Fall } (\%) = \frac{b - a}{b - 85} \times 100$$

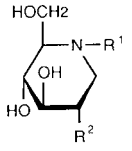
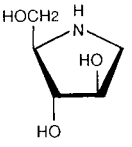
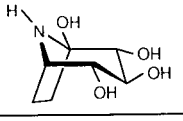
where b and a are blood glucose levels before and after injection of extracts and compounds, respectively.

In the equation 100 % fall means that blood glucose levels in STZ-diabetic mice are decreased to 85 mg/dl, the mean blood glucose level in fasted (14 hr) state of normal mice.

Determination of immunoreactive insulin release from perfused rat pancreas : Perfusion of the isolated pancreas[10] and determination of immunoreactive insulin (IRI) levels in the perfusate[11] were carried out as described previously. The perfusate was a basal medium of Krebs-Ringer bicarbonate buffer solution (pH 7.4) containing 0.5 % bovine serum albumin (Fraction V ; Sigma, St. Louis, MO. USA), 2 % dextran (T-70 ; Pharmacia, Uppsala, Sweden) and 2.8 mM D-glucose saturated with gas mixture of 95 % O_2 and 5 % CO_2. The flow rate was kept at 1 ml/min. Every 1 min, 1 ml of perfusate was collected from a portal vein catheter. The levels of IRI in perfusate collected from a portal vein catheter were determined by radioimmunoassay.

Compounds used : The extraction procedures of hot water extracts (W) and ethanol-insoluble extracts

Table I The chemical structures of *N* containing sugars derived from mulberry leaves.

No.	Compounds	Abbreviation	Chemical structures[a]		
				R^1	R^2
1	1-Deoxynojirimycin	DNJ		H	OH
2	*N*-Methyl-1-deoxynojirimycin	*N*-Me-DNJ		CH_3	OH
3	2-O-α-D-Galactopyranosyl-1-deoxynojirimycin	GAL-DNJ		H	O-α-D-galactosyl
4	Fagomine	—		H	H
5	1, 4-Dideoxy-1, 4-imino-D-arabinitol	DAB			
6	Calystegin B_2 (Nortropanoline)	—			

[a]The chemical structures are from Asano *et al.*[12]

(A2) from mulberry leaves (Folium Mori)[2] and the isolation procedures of *N*-containing sugars[7,8] are already reported. The six compounds of *N*-containing sugars used are 1-deoxynojirimycin (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 1,2α, 3β, 4α-tetrahydroxynortropane (calystegin B_2) (Table I).

Compounds and extracts tested were dissolved in saline and were *i.p.* injected into the STZ-diabetic mice 14 hr after fasting. Glibenclamide (Research Biochemicals Inc., MA, U.S.A.) was suspended in saline and was also *i.p.* injected.

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

Results

Antihyperglycemic effects of N-containing sugars derived from mulberry leaves on STZ-diabetic mice

Antihyperglycemic effects of DNJ, *N*-Me-DNJ, GAL-DNJ, fagomine, calystegin B_2 (300 μmol/kg), and DAB (350 μmol/kg) were examined 2, 4 and 6 hr after injection on STZ-diabetic mice. Glibenclamide (30 μmol/kg) was used as a positive control. Significant antihyperglycemic effects were obtained by *N*-Me-DNJ, GAL-DNJ, fagomine at 300 μmol/kg and glibenclamide at 30 μmol/kg, compared with saline control. GAL-DNJ and fagomine exhibited the most potent antihyperglycemic effects (Table II).

Time-dependent antihyperglycemic effects of GAL-DNJ, fagomine and crude extracts from mulberry leaves on STZ-diabetic mice

The antihyperglycemic effects of GAL-DNJ and fagomine were investigated with 75, 150, and 300 μmol/kg in fasted STZ-diabetic mice. GAL-DNJ showed significant antihyperglycemic effects at 300 μmol/kg 4 and 6 hr after its injection, at 150 μmol/kg 6 hr after its injection, but not at 75 μmol/kg. The fall of blood glucose levels for GAL-DNJ (300 μmol/kg) was 69.3±11.8 % and 72.4±8.7 % 4 and 6 hr after its injection, respectively. Fagomine showed significant antihyperglycemic effects 6 hr after injection with 75 and 150 μmol/kg. Fagomine at a high dose (300 μmol/kg) showed antihyperglycemic effects 2 hr after its injection. The effects became more prominently to be 58.6±12.6 % and 66.9±10.9 % fall of blood glucose levels 4 and 6 hr after its injection, respectively (Fig. 1A, B).

The antihyperglycemic effects of hot water extracts (W) and ethanol-insoluble extracts (A2) derived from mulberry leaves at doses of 25-200 mg/kg in STZ-diabetic mice were examined. The extracts W and A2 showed antihyperglycemic effects at doses of 100 and 200 mg/kg 4 hr after injection

Table II Antihyperglycemic effects of *N*-containing sugars on blood glucose levels in STZ diabetic mice.

No. Compounds	Blood glucose levels before *i.p.* (mg/dl)	Fall of blood glucose level (%)			n
		2	4	6 (hr)	
1 DNJ	253.4±14.8	18.9± 5.1	26.0± 8.7	38.8± 9.8	7
2 *N*-Me-DNJ	271.4±26.7	39.2± 8.2	48.9± 7.2	53.2± 5.4*	7
3 GAL-DNJ	286.1±46.8	24.8±10.0	69.3±11.8**	72.4± 8.7**	7
4 Fagomine	297.4±31.1	46.9±14.8*	58.6±12.5**	66.9±10.9**	7
5 DAB[a]	315.6±19.2	5.6± 3.7	22.9±11.1	29.7±12.0	5
6 Calystegin B_2	278.9±21.9	41.7± 6.1	44.4± 7.2	38.7± 6.6	7
Glibenclamide[b]	270.0±22.9	25.4± 9.9	43.0±14.3	71.3± 5.8**	5
Saline control	252.4±22.8	12.1± 5.0	16.5± 4.7	16.1± 4.9	8

The samples were administered with 300 μmol/kg except, [a]350 μmol/kg and [b]30 μmol/kg. Fall (%) = (b-a)/(b-85)×100, where b : blood glucose level just before injection, a : the level after injection, and 85 : the mean blood glucose level (mg/dl) in normal ddY strain mice 14 hr after fasting. *$p<0.05$, and **$p<0.01$: vs. saline control by one way ANOVA and Scheffe multiple comparison test. All data are means ±S.E.M.. n : The number of observations.

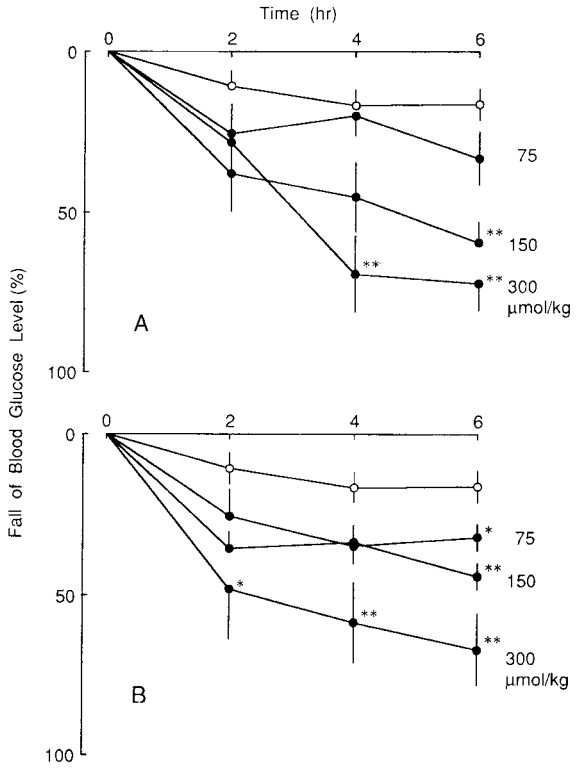


Fig. 1 Time dependent antihyperglycemic effects of GAL-DNJ (A) and fagomine (B) on streptozocin (STZ)-diabetic mice. The fall of blood glucose level (%) is presented as in Table II caption.
● : GAL DNJ and fagomine (75-300 μmol/kg, *i.p.*). $*p<0.05$, and $**p<0.01$: Significantly different from the saline control (○) by one way ANOVA and then Scheffe multiple comparison test. All values are the means±S.E.M. of 5-10 mice.

(data not shown). The effects of W and A2 at dose of 200 mg/kg become more prominent to be 74.7±12.8 % and 78.6±8.1 % (n=10-20) fall of blood glucose levels 6 hr after the injections, respectively.

Dose-dependent antihyperglycemic effects of component compounds and extracts from mulberry leaves

The dose-dependent antihyperglycemic effects of extracts (W and A2) were compared with those of GAL-DNJ and fagomine. All curves of component compounds (GAL-DNJ and fagomine) and extracts (W and A2) are overlapped. Both GAL-DNJ and fagomine lowered the blood glucose level in a dose-dependent manner, and the ED_{50} (95 % confidence limits) were 115.0 (96.8-136.7) μmol/kg and 142.4 (130.5-155.3) μmol/kg, respectively. The ED_{50} values (95 % confidence limits) were 41.0 (31.8-52.7) mg/kg for extract W and 33.9 (26.6-43.1) mg/kg for extract A2, respectively (Fig. 2).

Potency ratios of GAL-DNJ and fagomine to, and their yields in, W and A2 extracts

Potency ratios and the yields of GAL-DNJ and fagomine in W and A2 extracts were compared in Table III. The yields of GAL-DNJ and fagomine were very low. These results demonstrate that the antihyperglycemic effects of extracts are not explained only by the effects of GAL DNJ and fagomine.

Potentiating effects on glucose-induced IRI release from perfused rat pancreas by fagomine

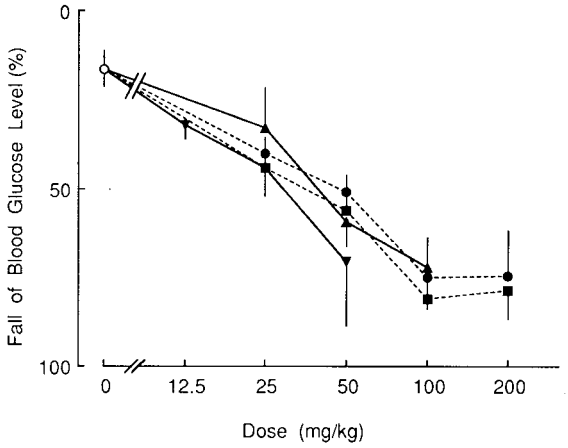


Fig. 2 Dose dependent antihyperglycemic effects 6 hr after injection of component compounds (GAL-DNJ : ▲, and fagomine : ▼, straight lines) and extracts (hot water extracts, W : ●, and ethanol-insoluble extracts, A2 : ■, broken lines) from mulberry leaves on STZ diabetic mice. ○ : saline control.

Table III The antihyperglycemic potencies of GAL DNJ and fagomine estimated for, and its yields in, hot water extracts (W) and ethanol-insoluble extracts (A2) from mulberry leaves.

	GAL-DNJ		Fagomine	
	potency[a]	yields (%)[b]	potency[a]	yields (%)[b]
W	1.10	0.141	1.96	0.086
A2	0.91	0.109	1.62	0.062

[a]Potency ratio=ED_{50B}/ED_{50A}, where ED_{50A} is ED_{50} of compounds (GAL DNJ or fagomine), and ED_{50B} is ED_{50} of extracts (W or A2).
[b]Percentage of GAL-DNJ or fagomine, contained in hot water extracts (W) and ethanol-insoluble extracts (A2) from mulberry leaves (*Morus alba* L.).

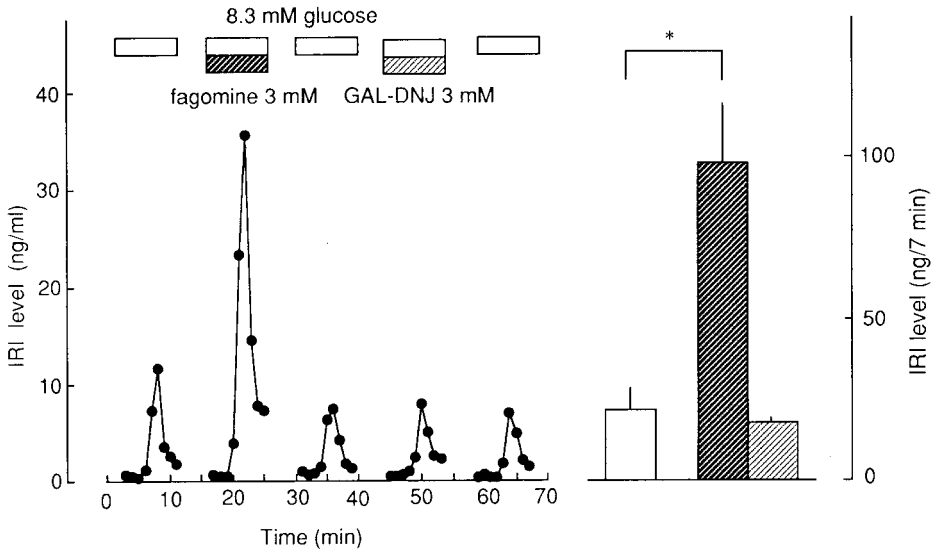


Fig. 3 Potentiating effect of fagomine (3 mM) but not GAL-DNJ (3 mM) on 8.3 mM glucose-induced immunoreactive insulin (IRI) release from perfused pancreas of normal Wistar rat. The upper columns show the 7-min period of stimulation by 8.3 mM glucose with (shaded columns) or without (open columns) fagomine and GAL-DNJ. Closed circles show the IRI level every 1 min. The right panel indicates the total amount of IRI level for the 7 min stimulation. The columns show the effects of 8.3 mM glucose with fagomine (▨) and GAL DNJ (▨), and without the compound (□), respectively. $*p<0.05$: Significantly different from the effect of the 8.3 mM glucose alone by one way ANOVA and then Scheffe multiple-comparison test. All values are the means±S.E.M. of 3 rats.

The effects of fagomine and GAL-DNJ (3 mM) on the glucose (8.3 mM)-induced IRI release from perfused rat pancreas were examined. Fagomine potentiated markedly IRI release (Fig. 3). The total IRI level for 7 min induced by fagomine was 4-fold high for 8.3 mM glucose alone. GAL-DNJ did not change the IRI level.

Discussion

Mulberry leaves (Folium Mori) are used as a traditional medicine with anti-inflammatory and antihyperglycemic actions.[2, 3, 12] The component compounds are flavones, steroids, triterpenes, amino acids, vitamins, trace amounts of mineral,[13, 14] and an acidic polysaccharide which consists of galacturonic acid and rhamnose.[15] In this study, the antihyperglycemic effects of six N-containing sugars derived (DNJ, GAL-DNJ, *N*-Me-DNJ, fagomine, DAB, and calystegin B_2) from mulberry leaves were investigated. Among them, GAL-DNJ and *N*-Me-DNJ have been recently isolated. Fagomine and DAB are also isolated from *Fagopyrum esculentum* and from the fruits of *Angylocalyx boutiqueanus*.[16, 17] Polysaccharide-like compounds from mulberry leaves showed antihyperglycemic effects in alloxan-diabetic mice.[12] In the present study, we at first found that GAL-DNJ and fagomine showed antihyperglycemic effects on STZ-diabetic mice. The values of the antihyperglycemic potencies of GAL-DNJ and fagomine to extract W and A2 were too small (0.91-1.96) to explain the antihyperglycemic effects of the extracts, because the yields of GAL-DNJ and fagomine were low in the extracts as shown in Table III. Unidentified compounds seem to exert mainly the antihyperglycemic action of extracts from mulberry leaves.

An oral antihyperglycemic drug glibenclamide potentiates insulin release from isolated perfused pancreas of the normal rats.[18] Our data demonstrated that fagomine potentiated the glucose-induced IRI release from perfused pancreas of normal rats. These results suggested that the mechanism of antihyperglycemic action by fagomine was similar to that by glibenclamide. The effects of glibenclamide and fagomine reached the maximum 6 hr after injection, indicating that they may be slowly adsorbed. GAL-

DNJ did not change the glucose-induced IRI level. It has been reported that N-containing sugars from mulberry leaves inhibit the functions of α-glucosidase, α-mannosidase, and β-galactosidase.[19] The mechanisms may contribute to the antihyperglycemic action of GAL-DNJ.

Hot water extracts (W) and ethanol-insoluble extracts (A2) from mulberry leaves increased the glucose uptake by diaphragm muscles of normal mice.[3] The total polysaccharide fraction from mulberry leaves (100 mg/kg, *i.p.*) increased the plasma insulin level in normal rats.[12] The mechanisms of antihyperglycemic action by extracts and its component compound from mulberry leaves are not completely clear.

In conclusion, fagomine and GAL-DNJ are most active among the six compounds to produce antihyperglycemic effects. Fagomine may induce the effect by potentiating the glucose-induced insulin release.

和文抄録

Streptozocin (STZ) 糖尿病マウスを用いて，桑葉から単離された6化合物について血糖下降作用を検討した。それらの成分は 1-deoxynojirimycin (DNJ), *N*-methyl-DNJ (*N*-Me-DNJ), 2-O-α-D-galacto-pyranosyl-DNJ (GAL-DNJ), fagomine, 1,4-dideoxy-1,4-imino-D-arabinitol (DAB), 1,2α, 3β, 4α-tetrahydroxynortropane (calystegin B_2) である。そのうち，GAL-DNJ と fagomine は投与した6時間後にそれぞれ用量依存的な血糖下降作用がみられた。その ED_{50} (95 % confidence limits) はそれぞれ 115.0 (96.8-136.7) μmol/kg と 142.1 (130.5-155.3) μmol/kg であった。桑葉水エキス (W) 及びエタノール不溶部エキス (A2) の血糖下降作用にも用量依存性がみられ，ED_{50} はそれぞれ 41.0 (31.8-52.7) mg/kg と 33.9 (26.6-43.1) mg/kg であった。以上の結果から，それらの6化合物の中で fagomine と GAL-DNJ は最も強い血糖下降作用を示すことが明らかになった。正常ラット膵灌流において fagomine には 8.3 mM glucose 刺激による insulin 放出促進作用がみられた。これは fagomine の血糖下降作用のメカニズムの一つと考えられる。

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