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Neuroscience Letters 434 (2008) 195–199
Neuroscience Letters
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Release of acetylcholine by syringin, an active principle of *Eleutherococcus senticosus*, to raise insulin secretion in Wistar rats

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Received 12 July 2007; received in revised form 13 January 2008; accepted 23 January 2008

Abstract

The present study is designed to screen the effect of syringin, an active principle purified from the rhizome and root parts of *Eleutherococcus senticosus* (Araliaceae), on the plasma glucose and investigate the possible mechanisms. Plasma glucose decreased in a dose-dependent manner 60 min after intravenous injection of syringin into fasting Wistar rats. In parallel to the decrease of plasma glucose, increases of plasma insulin level as well as the plasma C-peptide was also observed in rats receiving same treatment. Both the plasma glucose lowering action and the raised plasma levels of insulin and C-peptide induced by syringin were also inhibited by 4-diphenylacetoxy-*N*-methylpiperdine methiodide (4-DAMP), the antagonist of the muscarinic M_3 receptors, but not affected by the ganglionic nicotinic antagonist, pentolinium or hexamethonium. Moreover, disruption of synaptic available acetylcholine (ACh) using an inhibitor of choline uptake, hemicholinium-3, or vesicular acetylcholine transport, vesamicol, abolished these actions of syringin. Also, physostigmine at concentration sufficient to inhibit acetylcholinesterase enhanced the actions of syringin. Mediation of ACh release from the nerve terminals to enhance insulin secretion by syringin can thus be considered. The results suggest that syringin has an ability to raise the release of ACh from nerve terminals, which in turn to stimulate muscarinic M_3 receptors in pancreatic cells and augment the insulin release to result in plasma glucose lowering action.

Keywords: Acetylcholine; Insulin; Muscarinic M_3 receptors; Syringin; Wistar rats

A key feature of type-2 diabetes is that glucose fails to stimulate adequate release of insulin from pancreatic β-cells [14]. This metabolic disorder often leads to disability from the vascular complications of coronary artery disease and cerebrovascular disease, renal failure, blindness, and limb amputation in addition to neurological complications and premature death [13]. In order to reverse the plasma glucose level near to the normal, dietary restrictions, exercise, and oral glucose-lowering agents are widely applied [17]. Sulfonylureas and related insulin secretagogues merit consideration as first-line therapy for diabetic patients [15]. Additional oral glycemic control agents with insulinotropic action through alternative mechanisms of action than sulfonylureas would be desirable.

Eleutherococcus senticosus (Rupr. & Maxim.) Maxim., a plant which is also known as ciwujia or *Siberian ginseng*, belongs to the Araliaceae family but is a distant relative of *Panax ginseng* C.A. Meyer (Araliaceae), the true ginseng. This plant has been used extensively in Russia, China, Korea and Japan as an adaptogen to help the body adapt to stress by supporting healthy adrenal gland function [5]. Generally, *E. senticosus* serves as a preventive medicine and general tonic. The active ingredients, eleutherosides have been shown to be responsible for the adaptogenic properties of *E. senticosus* [4,16].

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doi:10.1016/j.neulet.2008.01.054

Syringin (4-(3-hydroxyprop-1-enyl)-2,6-dimethoxyphenyl β-D-glucopyranoside) is widely regarded to be the important constituents of eleutherosides and been applied to protect the damage from radiation in mice after X-ray irradiation [20]. In addition to possess both anti-inflammatory and antinociceptive activities, immunopharmacological effect of syringin has been also documented [4,20]. Moreover, syringin has been documented to be responsible for the plasma glucose lowering actions of *E. senticosus* in normal and alloxan-induced hyperglycemic mice [11]; this component seems helpful in the handling of type-1 diabetes. However, little information is available regarding the effects of syringin on type-2 diabetes. It is of particular interest to clarify whether syringin possesses the insulinotropic efficacy to be of therapeutic benefit in type-2 diabetes.

Male Wistar rats, aged 8–10 weeks (200–250 g body weight), were obtained from the Animal Center of National Cheng Kung University Medical College. Rats were housed in a temperature ($25 \pm 1\,^{\circ}C$) and humidity ($60 \pm 5\%$)-controlled room and kept on a 12:12 light-dark cycle (light on at 06:00 h). Water and standard laboratory diet were freely available throughout. All animal procedures were performed according to the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. The powder of syringin (purity = 98.6%), isolated from the rhizome and root part of *E. senticosus*, was gifted from Professor F.L. Hsu (Department of Pharmacology, School of Pharmacy, Taipei Medical University, Taipei City, Taiwan), which were dissolved in warm water with 0.9% sodium chloride for intravenous injection at the desired doses into fasted Wistar rats. In the preliminary experiments in Wistar rats receiving an intravenous injection of syringin at a dose of 100 g/kg, the plasma glucose lowering effect was observed after 10 min and reached a plateau within 60 min which was maintained for 120 min or more (Fig. 1). Thus, the effect of syringin on the concentrations of glucose and insulin in plasma were determined using blood samples collected at 60 min later of treatment. Control Wistar rats received similar intravenous injection of vehicle of the same volume. Pharmacological interventions were carried out using drugs injected intravenously into fasted rats 30 min before intravenous injection of syringin. In the present study, 4-diphenylacetoxy-*N*-methylpiperdine methiodide (4-DAMP) (Tocris Cookson Inc., MO, USA), hexamethonium bromide (Sigma–Aldrich. Louis, MO, USA), pentolinium tartrate (Sigma–Aldrich), hemicholinium-3 (Sigma–Aldrich), 2-[4-phenylpiperidino] cyclohexanol (vesamicol) (Sigma–Aldrich) and physostigmine (Sigma–Aldrich) were used as pharmacological inhibitors.

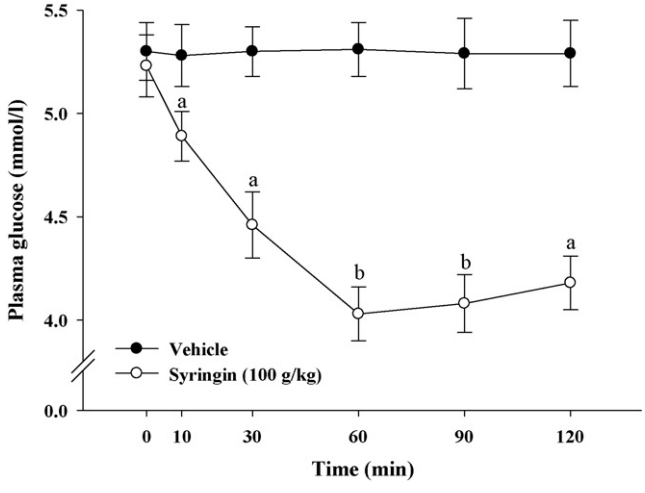

Fig. 1. Changes of plasma glucose in Wistar rats receiving an intravenous injection of syringin at 100 μg/kg (open circles). Values (mean ± S.E.M.) were obtained from each group of eight animals. Vehicle of distilled water used to dissolve syringin was treated at same volume (closed circles). [a]$P < 0.05$ and [b]$P < 0.01$ compared with value before treatment (0 min) in each group, respectively.

The levels of plasma glucose were estimated with an analyzer (Quik-Lab, Ames, Miles Inc., Elkhart, Indiana 46515, USA) by a commercial kit (Catalog #COD12503) from BioSystem (Costa Brava, Barcelona, Spain) run in duplicate. The plasma insulin was determined by rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Catalog #EZRMI-13K) of LINCO Research Inc. (St. Charles, MO, USA). Sample were analyzed in triplicate and results expressed as pmol of insulin-like immunoreactivity per liter of plasma. Plasma C-peptide level was also estimated using a commercial rat C-peptide ELISA kit (Catalog #295-57401) from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Samples from each individual were analyzed in triplicate at the same time. The obtained values were indicated as pmol of peptide-like immunoreactivity per liter of plasma. The test compounds used in the present study did not affect the binding of peptide with antibodies. In addition, systolic blood pressure (SBP) was measured using a noninvasive tail-cuff monitor (UR-5000, Ueda Company, Tokyo, Japan) in conscious rats expressing as the mean of at least four measurements.

The plasma glucose lowering activity was calculated as percentage decrease of the initial value according to the formula: $((G_i - G_t)/G_i) \times 100$ where G_i was the initial glucose level and G_t was the plasma glucose concentration after treatment of syringin. Data are expressed as the mean ± S.E.M. for the number (n) of animals in the group as indicated in tables and figures. Repeated measures analysis of variance (ANOVA) was used to analyze the changes in plasma glucose and other parameters. The Dunnett range post-hoc comparisons were used to determine the source of significant differences where appropriate. A P-value <0.05 was considered statistically significant.

Sixty minutes after treatment, a dose-dependent lowering of plasma glucose was observed in Wistar rats receiving an i.v. injection of syringin for 60 min (Table 1). The maximal plasma glucose lowering activity of syringin at 100 μg/kg in Wistar rats received treatment for 60 min was about $22.62 \pm 2.13\%$. Although it has been indicated that *E. senticosus* extract caused concentration-dependent relaxation from in phenylephrine-induced contracted arterial rings, and the relaxation was primarily endothelium-dependent [16], 60 min after an intravenous injection of syringin failed to modify the SBP in normal rats (Table 1). The results indicated that plasma glucose lowering effect of syringin in rats with functional pancreas seems not related to the decrease of blood pressure. In fact, prediabetics show the impaired glucose tolerance depending on insulin

Table 1
Changes of the plasma levels of glucose, insulin-like immunoreactivity (IRI) and C-peptide as well as systolic blood pressure (SBP) in Wistar rats receiving an intravenous injection of syringin for 60 min

Wistar rats	Plasma glucose (mmol/l)	Plasma IRI (pmol/l)	Plasma C-peptide (pmol/l)	SBP (mmHg)
Vehicle	5.21 ± 0.17	185.93 ± 7.62	188.52 ± 15.21	102.73 ± 3.28
Syringin (μg/kg, i.v.)				
50	4.64 ± 0.13 a	266.41 ± 10.23 a	271.72 ± 15.48 a	97.59 ± 2.74
75	4.41 ± 0.16 a	340.25 ± 14.17 b	357.47 ± 16.11 b	98.44 ± 3.17
100	4.03 ± 0.14 b	587.83 ± 11.34 b	592.09 ± 17.28 b	96.61 ± 2.96

Values (mean ± S.E.M.) were obtained from each group of eight animals. Vehicle of distilled water used to dissolve syringin was treated at same volume. [a] $P < 0.05$ and [b] $P < 0.01$ compared with data from vehicle-treated group.

secretion. The demand for insulin production-induced insulin resistance probably due to the excessive stress on the pancreatic β-cells, which can lead to complete β-cells failure [14]. Additionally, the values of plasma insulin from normal rats were dose-dependently raised by the similar treatment of syringin (Table 1). Also, plasma C-peptide level in Wistar rats was increased as the plasma insulin response to syringin (Table 1). Plasma C-peptide has been considered as an indicator of insulin secretion [18]. The parallel increase of plasma insulin and C-peptide by syringin seems helpful to rule out the inhibition of insulin turnover. Thus, the plasma glucose-lowering action of syringin through insulin secretion is undoubtedly.

Similar to glucose stimulation, parasympathetic nervous activity as plays an important role in the regulation of insulin secretion from pancreatic β-cells [10]. Several animal models of type-2 diabetes are characterized by the change of autonomic nervous regulation especially an increase of parasympathetic activities to result in hyperinsulinemia and cholinergic agonists are introduced to be helpful for insulin secretion and glucose homeostasis in type-2 diabetic patients [10]. The effects of acetylcholine (ACh) on pancreatic insulin release are known to be mediated by an activation of muscarinic receptors [9]. Muscarinic receptors exit in multiple subtypes, the muscarinic M_3 receptor appears to be the predominant subtype in pancreatic β-cells for the regulation of insulin secretion [9,12]. In addition to raise the possibility of a novel pharmacology altering the progressive course of type-2 diabetes, it has been also documented that the drugs, which can activate muscarinic M_3 receptors, may be of therapeutic benefit in the treatment of type-2 diabetes [6,9]. Thus, we used the pharmacological inhibitors or antagonists to clarify whether ACh or muscarinic M_3 receptor activation was involved in the action of syringin.

4-DAMP is introduced to selectively inactivate muscarinic M_3 receptors without influence of other muscarinic cholinoceptors [3]. We found that prior occupancy of muscarinic M_3 receptors by 4-DAMP impeded the plasma glucose lowering

Table 2
Effects of cholinergic inhibitors on the plasma levels of glucose, insulin-like immunoreactivity (IRI) and C-peptide in Wistar rats receiving an intravenous injection of syringin for 60 min

	Plasma glucose (mmol/l)	Plasma IRI (pmol/l)	Plasma C-peptide (pmol/l)
Basal	5.22 ± 0.16[d]	186.76 ± 10.21[d]	189.86 ± 14.71[d]
Syringin (100 μg/kg)			
Vehicle	4.01 ± 0.15 b	567.47 ± 14.36 b	572.20 ± 15.62 b
4-DAMP (μg/kg)			
0.001	4.37 ± 0.18 a	477.28 ± 13.26 b,c	481.55 ± 15.28 b,c
0.01	4.63 ± 0.19 a,c	340.75 ± 14.19 b,d	345.28 ± 16.32 b,d
1.0	5.20 ± 0.17 d	202.17 ± 16.21 d	208.16 ± 17.59 d
Hemicholinium-3 (μg/kg)			
1.0	4.52 ± 0.16 b,c	467.29 ± 14.32 b,c	476.68 ± 16.93 b,c
5.0	4.92 ± 0.17 a,c	328.31 ± 13.15 b,d	334.22 ± 15.68 b,d
10.0	5.15 ± 0.19 d	192.12 ± 15.42 d	195.96 ± 16.27 d
Vesamicol (mg/kg)			
1.5	4.42 ± 0.19 b,c	481.72 ± 16.28 b,c	491.35 ± 15.61 b,c
2.5	4.96 ± 0.16 a,c	336.23 ± 17.26 b,c	342.81 ± 17.52 b,c
3.5	5.25 ± 0.18 d	194.71 ± 14.35 d	198.60 ± 16.97 d
4-DAMP (1.0 μg/kg)	5.20 ± 0.21 d	187.14 ± 15.29 d	190.88 ± 15.75 d
Hemicholinium-3 (10.0 μg/kg)	5.24 ± 0.19 d	188.25 ± 14.29 d	192.02 ± 17.39 d
Vesamicol (3.5 mg/kg)	5.23 ± 0.22 d	190.12 ± 13.64 d	193.92 ± 18.75 d

The antagonists were given by intravenous injection 30 min before the intravenous injection of syringing (100 μg/kg). Vehicle of distilled water was used to dissolve the antagonists and given in the same volume. Values (mean ± S.E.M.) were obtained from each group of 8 animals. Basal level shows the value from animals received a similar treatment of the same volume of vehicle. [a] $P < 0.05$ and [b] $P < 0.01$ compared with basal value, respectively. [c] $P < 0.05$ and [d] $P < 0.01$ compared with animals receiving an intravenous injection of syringin (100 μg/kg) only, respectively.

action of syringin (Table 2). In parallel, the actions of syringin regarding the plasma levels of insulin and C-peptide were also 4-DAMP sensitive (Table 2). However, 4-DAMP at maximal dose of 1 μg/kg did not modify the basal plasma glucose level in normal rats. Both the plasma insulin and C-peptide levels were not influenced by 4-DAMP alone as compared with the vehicle-treated control. In contrast, the ganglionic nicotinic blocker, pentolinium or hexamethonium [8] at the effective dose of 7.5 mg/kg failed to modify the plasma glucose lowering action of syringin. The plasma glucose level was 4.04 ± 0.17 mmol/l ($n = 6$) in pentolinium (7.5 mg/kg) pretreated group or 4.06 ± 0.16 mmol/l ($n = 7$) in hexamethonium (7.5 mg/kg) pretreated group of rats receiving an i.v. injection of syringin (100 μg/kg), which was not statistically different ($P > 0.05$) from rats received same treatment of syringin (100 μg/kg) (4.03 ± 0.18 mmol/l). These results demonstrated that the insulinotropic effect of muscarinic M_3 receptor activation is mainly responsible for the plasma glucose lowering action of syringin. Thus, the role of endogenous ACh shall be elucidated.

Basically, ACh is synthesized in the cytoplasm from acetyl-CoA and choline through the catalytic action of choline acetyltransferase. The synthesized ACh is then transported from cytoplasm into the vesicles by an antiporter [1]. Hemicholinium-3-sensitive cholinergic neuron is mentioned to be rate limiting in the biosynthesis of ACh [21]. We observed that treatment with hemicholinium-3 at effective dose abolished the plasma glucose lowering action of syringin (Table 2). Also, the raised plasma level of insulin or C-peptide by Hon-Chi was surmounted by the same dosing of hemicholinium-3 (Table 2), indicating the participation of endogenous ACh in plasma glucose lowering action of syringin. This view is further supported by the blocking effect of vesamicol on the action of syringin. Vesamicol is known as an inhibitor specific to the uptake of ACh into synaptic vesicles in cholinergic nerve terminals [19]. Not only the plasma glucose lowering action, but also the increase of plasma insulin and C-peptide levels by syringin in rats were reversed by vesamicol (Table 2). Thus, release of ACh from the cholinergic nerve terminals by syringin can be considered.

After the release from nerve terminal, ACh may bind with cholinoceptors or metabolized to choline and acetate by acetylcholinesterase. The cholinesterase inhibitors act primarily where ACh is released and work as an amplifier of endogenous ACh [2]. We observed that acetylcholinesterase inhibitor, physostigmine, enhanced the plasma glucose lowering action of syringin (Fig. 2A). Also, physostigmine increased the plasma insulin and C-peptide levels raised by syringin (Fig. 2B and C). The synergistic effect of physostigmine on these actions of syringin might due to an increase of ACh accumulation to facilitate the insulin secretion through an activation of muscarinic M_3 receptors. Actually, the half-life of released ACh in synapse is very short [7], the content of released ACh in pancreas by syringin was not easy to determine and this effect of syringin was not estimated in the present study. Nevertheless, blockade the action of syringin by pharmacological inhibitors at concentrations sufficient to inhibit cholinergic neurotransmission or muscarinic M_3 receptors *in vivo* seems reliable to demonstrate this insulin secretion induced by this compound. Thus, our results could provide a new insight on the pharmacological benefits of syringin for the enhance insulin secretion, and might be useful as an adjuvant in the treatment and/or prevention of type-2 diabetes.

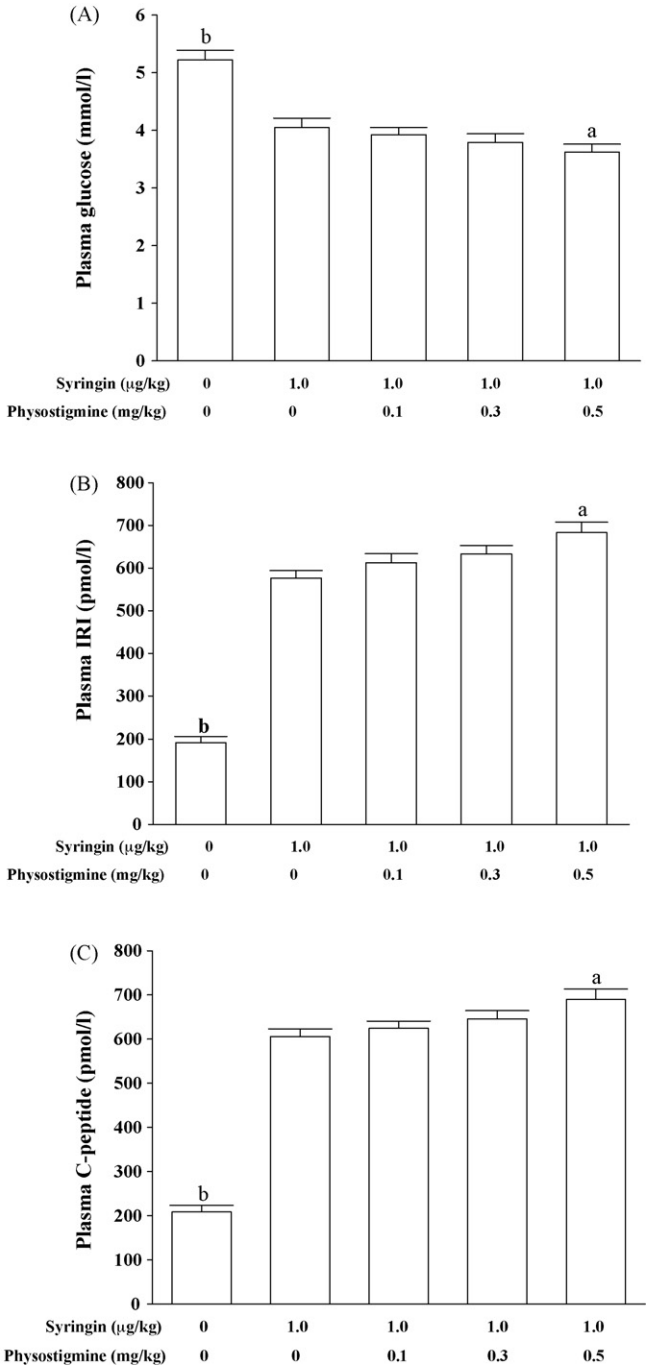

Fig. 2. Effects of physostigmine on the plasma levels of glucose (A), insulin-like immunoreactivity (IRI) (B) and C-peptide (C) in Wistar rats receiving an intravenous injection of syringin (100 μg/kg) for 60 min. Physostigmine was given by an intravenous injection at 30 min before syringin treatment. Values (mean ± S.E.M.) were obtained from each group of seven animals. [a] $P < 0.05$ and [b] $P < 0.01$ compared with animals receiving an intravenous injection of syringin (100 μg/kg) only, respectively.

In conclusion, the present study demonstrated that plasma glucose lowering action of syringin, the active component of

E. senticosus, appears to be related with the increase of insulin secretion induced by an activation of muscarinic M_3 receptors in functional pancreatic β-cells via the release of ACh from cholinergic nerve terminals. Syringin seems valuable as insulin secretagogues.

Acknowledgements

We appreciate Professor F.L. Hsu (Department of Pharmacology, School of Pharmacy, Taipei Medical University, Taipei City, Taiwan) for the kind supply of syringin. Thanks are also due to Professor Y.C. Tong (Department of Urology, College of Medicine, National Cheng Kung University, Tainan, Taiwan) for editing and Miss M.J. Wang for technical assistance.

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