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Release of acetylcholine to raise insulin secretion in Wistar rats by oleanolic acid, one of the active principles contained in *Cornus officinalis*

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Abstract

The plasma glucose lowering action of fruits of cornus (*Cornus officinalis*), the major active constituent of Die-Huang-Wan, has been documented to mediate acetylcholine (ACh) release, which in turn to stimulate muscarinic M_3 receptors resulting in the enhancement of insulin secretion in rats with functional pancreatic β-cells. The present study was conducted to investigate the effect of oleanolic acid, one of the active principles of cornus fruit, on the release of insulin in rats. After an intraperitoneal injection into the fasting Wistar rats for 90 min, oleanolic acid decreased the plasma glucose in a dose-dependent manner in parallel to an increase of plasma levels of insulin as well as C-peptide. Moreover, disruption of synaptic ACh using an inhibitor of choline uptake, hemicholinium-3, or vesicular acetylcholine transport, vesamicol, abolished these actions of oleanolic acid. Also, physostigmine at concentration sufficient to inhibit acetylcholinesterase enhanced the actions of oleanolic acid. Both the plasma glucose lowering action and the raised plasma levels of insulin and C-peptide induced by oleanolic acid were also inhibited by 4-diphenylacetoxy-*N*-methylpiperdine methiodide (4-DAMP), but not affected by the ganglionic nicotinic antagonist, pentolinium or hexamethonium. The results suggest that oleanolic acid has an ability to raise the release of ACh from nerve terminals, which in turn to stimulate muscarinic M_3 receptors in the pancreatic cells and augment the insulin release to result in plasma glucose lowering action. Thus, oleanolic acid is one of the active principles responsible for the increase of plasma insulin produced by cornus fruit in rats.

Keywords: Acetylcholine; Cornus fruit; Muscarinic M_3 receptors; Wistar rats; Oleanolic acid

A key feature of type 2 diabetes is that glucose fails to stimulate adequate release of insulin from pancreatic β-cells [11]. Characteristically, the β-cell eventually fails to compensate for the gradually development of insulin resistance, resulting in overt hyperglycemia. Sulfonylureas and related compounds stimulate insulin release to use as first-line therapy for diabetic patients [11]. However, the sulfonylurea-based drugs fail to control normal blood glucose levels and cause weight gain [11]. Additional oral hypoglycemic agents with insulinotropic action through different mechanisms of sulfonylureas would therefore be desirable in recent.

In Chinese traditional medicine (CTM), Die-Huang-Wan, has widely been used to treat diabetic disorders [14]. Previous study has demonstrated that the plasma glucose lowering action of Die-Huang-Wan is associated with an increase of insulin secretion via the release of acetylcholine (ACh) from nerve terminals to stimulate the muscarinic cholinoceptors in pancreatic islets of Wistar rats [14]. Die-Huang-Wan is a mixture of six herbs: dioscorea (*Dioscoreae rhizoma*), cornus (*Cornus officinalis*), alisma (*Rhizoma alismatis*), holelen (*Poria coccos*), rehmannia (*Rehmanniae radix*), and tree peony bark (*Moutan radicis cortex*). Actually, cornus is introduced as the major herb of Die-Huang-Wan for the plasma glucose lowering action in rats [13]. Cornus, also named as "San-Zu-Yee" in mandarin, is used in CTM for its tonic, analgesic, and diuretic actions [8]. In addition to reducing tinnitus, this herb has beneficial effect on the urogenital system, such as improving impotence and decreasing excessive urination [12]. Tannins, galloylated glycosides, gallotannins, organic acids, and furan derivatives have been reported from fruits of cornus [10]. In fact, triterpenoids, mainly oleanolic acid and ursolic acid and the glycosides derived from these have been identified in cornus fruit [20]. Merits of triterpenoids have been reported including their beneficial effects on cardiovascular systems, interaction with cytochrome P450s, pro-

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tection against kainate-induced excitotoxicity in rat hippocampal neurons and immunomodulatory effects in addition to the effects on intracellular redox balance and osteoclast formation [15]. In addition, oleanolic acid glycosides were mentioned to show plasma glucose lowering action in oral glucose-loaded rats [21]. However, ursolic acid (3β-hydroxy-olea-12-en-28-oic acid), but not its isomer oleanolic acid, showed the ability to lower blood sugar in streptozotocin-induced diabetic rats [20]. Thus, oleanolic acid is possible to work as an active principle for the action of cornus in rats with functional pancreatic β-cells. Based on this hypothesis, the present study was designed to investigate the effect of oleanolic acid on insulin release *in vivo*.

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. Under anesthesia with sodium pentobarbital (30 mg/kg, i.p.), blood samples (0.1 ml) were collected from the tail vein of rats for measurement of plasma parameters. 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. Oleanolic acid was obtained from Prof. Hsu F.L. (Institute of Pharmacognosy, School of Pharmacy, Taipei Medical University, Taipei City, Taiwan) with the purity higher than 98% and it was suspended in 95% ethanol to prepare the stock solution for further dilution with saline solution. Because oleanolic acid is water-insoluble, i.v. injection is not suitable. In the preliminary experiments, the plasma glucose lowering effect was found to reach the plateau within 90 min and maintained for 120 min or more in fasted rats received an i.p. injection of oleanolic acid at 20 mg/kg. However, oleanolic acid produced the plasma glucose lowering activity in fasted rats about $18.56 \pm 4.32\%$ at the oral dosage of 100 mg/kg until 120 min later. Thus, the effect of oleanolic acid on the plasma levels of glucose and insulin as well as C-peptides were determined using blood samples collected at 90 min later of an i.p. injection. Control rats received similar injection of vehicle at same volume. Moreover, 4-diphenylacetoxy-*N*-methylpiperdine methiodide (4-DAMP) (Tocris Cookson Inc., Ellisville, MO, USA), hexamethonium bromide (Sigma-Aldrich, Louis, MO, USA), pentolinium tartrate (Sigma-Aldrich), 2-[4-phenylpiperidino]cyclohexanol (vesamicol) (Sigma-Aldrich) and physostigmine (Sigma-Aldrich) were used as pharmacological inhibitors and injected intravenously into fasted rats 30 min before an i.p. injection of oleanolic acid except hemicholinium-3 (Sigma-Aldrich) that was i.p. injected for 3 h before receiving oleanolic acid treatment [22].

The levels of plasma glucose were estimated with an analyzer (Quik-Lab, Ames, Miles Inc., Elkhart, IN 46515, USA) by a commercial kit (Catalog #COD12503) from BioSystem (Costa Brava, Barcelona, Spain). 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) and results expressed as pmol of insulin-like immunoreactivity (IRI) 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) and the obtained values were indicated as pmol of peptide-like immunoreactivity per liter of plasma. Samples from each individual were analyzed in triplicate at the same time. The test compounds used in the present study did not affect the binding of peptide with antibodies.

The plasma glucose lowering activity was calculated as percentage decrease of the initial value according to the formula: $[(Gi - Gt)/Gi] \times 100$ where Gi was the initial glucose level and Gt was the plasma glucose concentration after treatment of oleanolic acid. 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.

Ninety minutes after the i.p. injection, a dose-dependent lowering of plasma glucose by oleanolic acid was observed in Wistar rats (Fig. 1A) in a manner similar to that of cornus fruit [13]; the maximal effective dose of oleanolic acid was 20 mg/kg. Also, the value of plasma insulin in Wistar rats was dose dependently raised by the similar treatment of oleanolic acid (Fig. 1B). The plasma C-peptide level in Wistar rats was also raised as the response of plasma insulin to oleanolic acid (Fig. 1C). Actually, plasma C-peptide has been considered as an indicator of insulin secretion [17]. The parallel increase of plasma insulin and C-peptide by oleanolic acid seems helpful to rule out the inhibition of insulin turnover. Thus, the plasma glucose-lowering action of oleanolic acid through insulin secretion is undoubtedly.

Two metabolic characteristics of type 2 diabetes including the alterations of insulin secretion and/or the inability of peripheral tissues responding to insulin were mentioned [9]. In fact, prediabetics show the impaired glucose tolerance depending on insulin 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. In fact, similar to glucose stimulation, parasympathetic nervous activity as plays an important role in the regulation of insulin secretion from pancreatic β-cells [7]. 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 [7]. The effects of acetylcholine (ACh) on pancreatic insulin release are known to be mediated by an activation of muscarinic receptors [5]. Thus, we used the pharmacological inhibitors to clarify the role of ACh or muscarinic receptor activation in this action of oleanolic acid.

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 [5,19]. It has been documented that the drugs which can activate muscarinic M_3 receptors may be of therapeutic benefit in the treat-

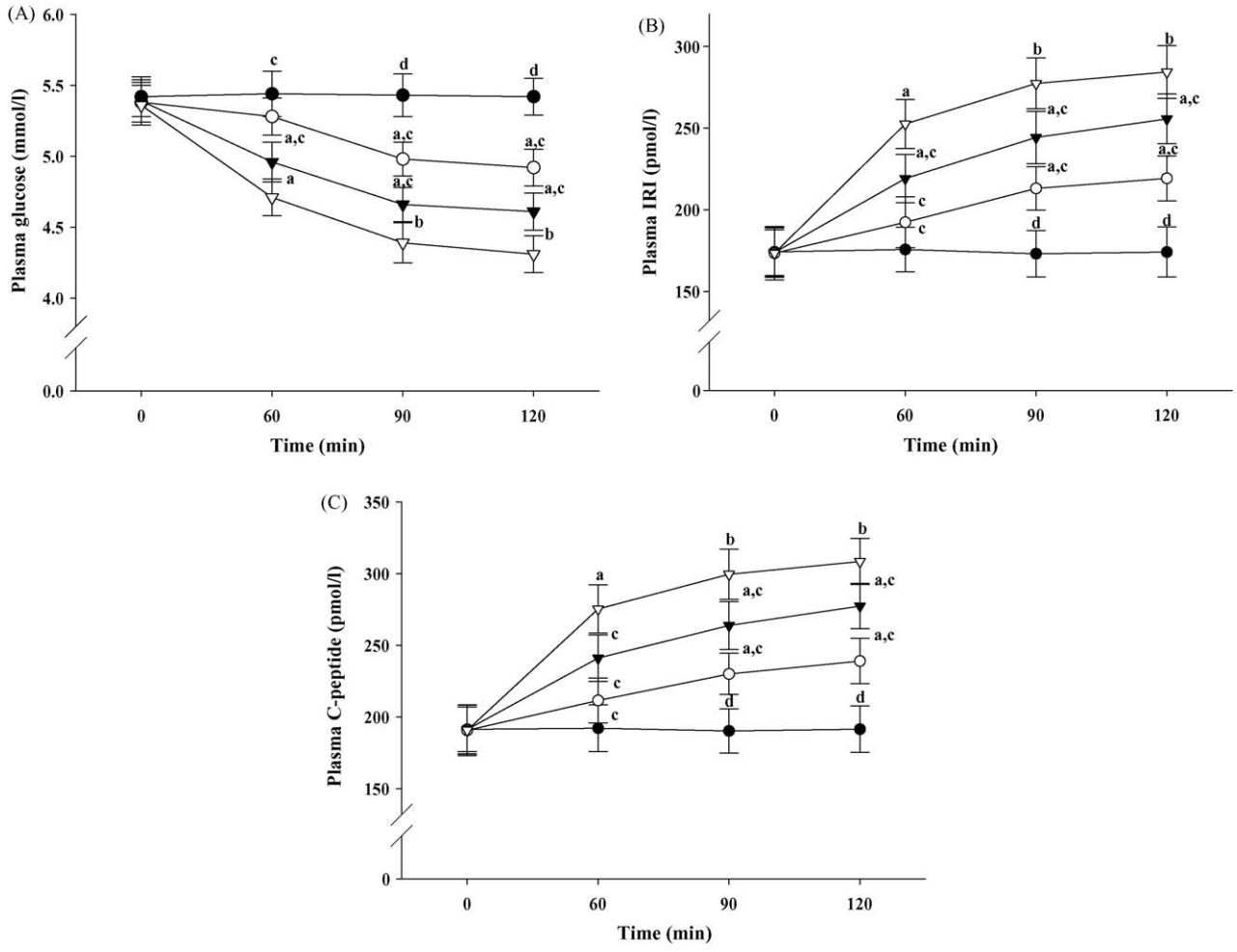

Fig. 1. Changes of the plasma levels of glucose (A), insulin-like immunoreactivity (IRI) (B) and C-peptide (C) in Wistar rats receiving an i.p. injection of oleanolic acid at 5 mg/kg (open circles), 10 mg/kg (closed triangles) or 20 mg/kg (open triangles). Values (mean ± S.E.M.) were obtained from each group of eight animals. Basal level shows the value from animals received a similar treatment of vehicle at same volume (closed circles). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. data from basal value (closed circles) at the indicated timing.

ment of type 2 diabetes [4]. In recent, 4-DAMP is introduced to selectively inactivate muscarinic M_3 receptor 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 action of oleanolic acid (20 mg/kg) (Table 1). In parallel, the actions of oleanolic acid (20 mg/kg) regarding the plasma levels of insulin and C-peptide were also 4-DAMP sensitive (Table 1). However, 4-DAMP at maximal dose of 10 μg/kg did not modify the basal plasma glucose level in normal rats. Also, both the plasma insulin and C-peptide levels were not influenced by 4-DAMP alone as compared with the vehicle-treated rats. However, pentolinium or hexamethonium, the ganglionic nicotinic blocker, at the effective dose of 7.5 mg/kg failed to modify the plasma glucose lowering action of oleanolic acid (20 mg/kg). The plasma glucose level was 5.41 ± 0.18 mmol/l ($n = 7$) in pentolinium (7.5 mg/kg) pretreated group or 5.39 ± 0.21 mmol/l ($n = 8$) in hexamethonium (7.5 mg/kg) pretreated group of rats receiving a i.p. injection of oleanolic acid (20 mg/kg), which was not statistically different ($P > 0.05$) from rats only treated with 20 mg/kg oleanolic acid (5.43 ± 0.17 mmol/l). Also, plasma glucose levels were 5.38 ± 0.23 mmol/l ($n = 8$) and 5.42 ± 0.18 mmol/l ($n = 8$) in rats only treated with pentolinium (7.5 mg/kg) and hexamethonium (7.5 mg/kg), respectively. These results demonstrated that the insulinotropic effect of muscarinic M_3 receptor activation is mainly responsible for the plasma glucose lowering action of oleanolic acid; the involvement of nicotinic cholinoceptor in the action of oleanolic acid is unlikely. These results are similar to the action of cornus fruit [13]. The obtained findings highlight the possibility of oleanolic acid as one of the active principles in cornus fruit for insulin release.

Acetylcholine 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 [16]. We observed that treatment with hemicholinium-3 abolished the plasma glucose lowering action of oleanolic acid (20 mg/kg) in a dose-dependent manner (Table 1). Also, the raised plasma level of insulin or C-peptide by oleanolic acid (20 mg/kg) was surmounted by the same dosing of hemicholinium-3 (Table 1), indicating the participation of

Table 1
Effects of cholinergic inhibitors on the plasma levels of glucose, insulin-like immunoreactivity (IRI) and C-peptide in Wistar rats receiving an i.p. injection of oleanolic acid for 90 min

	Plasma glucose (mmol/l)	Plasma IRI (pmol/l)	Plasma C-peptide (pmol/l)
Basal	5.41 ± 0.16[d]	173.27 ± 15.28[d]	207.41 ± 15.61
Oleanolic acid (20 mg/kg)			
+ Vehicle	4.39 ± 0.15[b]	278.16 ± 16.24[b]	343.53 ± 16.13[b]
+ 4-DAMP (μg/kg)			
5	4.59 ± 0.17[a,c]	267.83 ± 19.34[b]	328.14 ± 16.38[b,c]
7	4.87 ± 0.18[a,c]	248.36 ± 18.21[a,c]	273.18 ± 17.59[a,d]
10	5.35 ± 0.19[d]	179.75 ± 16.52[d]	220.31 ± 16.24[d]
+ Hemicholinium (μg/kg)			
1	4.62 ± 0.17[b,c]	262.11 ± 14.32[b]	295.72 ± 18.21[b]
5	5.02 ± 0.16[a,c]	226.41 ± 15.17[a,d]	260.43 ± 17.51[a]
10	5.25 ± 0.18[d]	182.26 ± 16.24[d]	220.98 ± 16.31
+ Vesamicol (mg/kg)			
1.5	4.77 ± 0.16[b]	241.89 ± 18.23[b]	288.83 ± 17.42[b]
2.5	5.11 ± 0.18[a,c]	217.83 ± 16.41[a,c]	241.54 ± 16.13[a]
3.5	5.32 ± 0.19[d]	186.71 ± 17.12[d]	211.68 ± 18.58
4-DAMP (10 μg/kg)	5.40 ± 0.17[d]	173.56 ± 15.62[d]	206.43 ± 14.92[d]
Hemicholinium (10 μg/kg)	5.38 ± 0.18[d]	170.15 ± 15.32[d]	203.43 ± 17.32
Vesamicol (3.5 mg/kg)	5.32 ± 0.17[d]	172.73 ± 14.53[d]	206.21 ± 15.36

The antagonists were given by an i.v. injection 30 min before the i.p. injection of oleanolic acid except hemicholinium-3 that was i.p. injected for 3 h before administration of oleanolic acid. Values (mean ± S.E.M.) were obtained from each group of eight 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 animal treated with oleanolic acid only, respectively.

endogenous ACh in plasma glucose lowering action of oleanolic acid. This view is further supported by the blocking effect of vesamicol on the action of oleanolic acid (20 mg/kg); both the plasma glucose lowering action and the increase of plasma levels in insulin as well as C-peptide by oleanolic acid (20 mg/kg) in rats were reversed by vesamicol (Table 1). Vesamicol is known as an inhibitor specific to the uptake of ACh into synaptic vesicles in cholinergic nerve terminals [18]. However, like the effect of hemicholinium-3, vesamical at the highest dose did not affect the basal plasma glucose of Wistar rats (Table 1). Otherwise, treatment with hemicholinium-3 or vesamical alone failed to modify the basal plasma levels of insulin and C-peptide in Wistar rats (Table 1). Thus, release of ACh from the cholinergic nerve terminals by oleanolic acid 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, could dose dependently enhance the plasma glucose lowering action of oleanolic acid (20 mg/kg) (Table 2). Also, the elevation of plasma levels in insulin and C-peptide by oleanolic acid (20 mg/kg) was markedly enhanced by this treatment of physostigmine (Table 2). The synergistic effect of physostigmine on these actions of oleanolic acid 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 [6]. Thus, the content of released ACh in pancreas by oleanolic acid was not easy to determine, and this effect of oleanolic acid was not estimated in the present study. Blockade the action of oleanolic acid by pharmacological inhibitors at concentrations sufficient to inhibit cholinergic neurotransmission or muscarinic M_3 receptor *in vivo* seems reliable to demonstrate the involvement of ACh in the insulin secretion induced by oleanolic acid. Although oleanolic acid could be considered as one of the active principles in cornus fruit for the release of insulin, the role of another active principle(s) contained in cornus fruit cannot be excluded. The essential role of oleanolic acid needs to be characterized using the method to remove this principle from cornus fruit in the future. Nevertheless, oleanolic acid exerts an insulinotropic effect through an increase of ACh release to activate muscarinic M_3 receptors has been provided in the present study.

Oleanolic acid glycosides were found to have neither insulin-like nor insulin-releasing activity; the hypoglycemic action produced by oral administration of oleanolic acid glycoside in glucose-loaded rats was indicated as a result of reductions in gastric emptying rate and glucose absorption in small intestine [21]. Different to the glycoside product, we observed that oleanolic acid itself produced a plasma glucose lowering action either by oral administration or by injection into the intraperitoneal area of rats; although the dosage for oral administration was about five times higher than that for i.p. injection. It seems that application of oleanolic acid by i.p. injection might be useful as an adjuvant for the enhance insulin secretion in the treatment and/or prevention of type 2 diabetes. Also, isolated and purified oleanolic acid from cornus fruits may be useful to treat diabetic patients.

In conclusion, our results suggest that oleanolic acid is one of the active principles from cornus possess the ability to increase

Table 2
Effects of physostigmine on the plasma levels of glucose, insulin-like immunoreactivity (IRI) and C-peptide in Wistar rats receiving an i.p. injection of oleanolic acid for 90 min

Wistar rats	Plasma glucose (mmol/l)	Plasma IRI (pmol/l)	Plasma C-peptide (pmol/l)
Basal	5.31 ± 0.16[d]	174.08 ± 16.32[d]	190.34 ± 17.83[d]
Oleanolic acid (20 mg/kg)			
+ Vehicle	4.35 ± 0.14[b]	277.41 ± 18.73[b]	309.86 ± 16.13[b]
+ Physostigmine (mg/kg)			
0.1	4.16 ± 0.15[b]	298.74 ± 17.23[b]	327.64 ± 18.21[b]
0.3	4.08 ± 0.17[b]	332.27 ± 15.39[b,c]	365.28 ± 17.92[b,c]
0.5	3.82 ± 0.16[b,c]	356.72 ± 19.51[b,c]	392.39 ± 16.56[b,c]

Physostigmine was given by an i.v. injection 30 min before the i.p. injection of oleanolic acid. Values (mean ± S.E.M.) were obtained from each group of seven 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 animal treated with oleanolic acid only, respectively.

insulin secretion via an activation of muscarinic M_3 receptors in pancreatic β-cells through the released ACh from cholinergic nerve terminals. Therefore, oleanolic acid seems valuable as insulin secretagogues for potential application in the prevention and treatment of type 2 diabetes

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