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Apigenin potentiates glucose-stimulated insulin secretion through the PKA-MEK kinase signaling pathway independent of K-ATP channels

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ABSTRACT

Aim: Apigenin, a natural bioflavonoid, is reported as an anti-diabetic agent since it possesses the ability to inhibit α-glucosidase activity, cause stimulation of insulin action and secretion, manage ROS, and prevent diabetes complications. Apigenin was identified as a new insulin secretagogue that enhances glucose-stimulated insulin secretion and seems like a better antidiabetic drug candidate. Here we explored the insulinotropic mechanism(s) of apigenin *in vitro* in mice islets and *in vivo* in diabetic rats.

Methods: Size-matched pancreatic islets were divided into groups and incubated in the presence or absence of apigenin and agonists or antagonists of major insulin signaling pathways. The secreted insulin was measured by ELISA. The intracellular cAMP was estimated by cAMP acetylation assay. The acute and chronic effects of apigenin were evaluated in diabetic rats.

Results: apigenin dose-dependently enhanced insulin secretion in isolated mice islets, and its insulinotropic effect was exerted at high glucose concentrations distinctly different from glibenclamide. Furthermore, apigenin amplified glucose-induced insulin secretion in depolarized and glibenclamide-treated islets. Apigenin showed no effect on intracellular cAMP concentration; however, an additive effect was observed by apigenin in both forskolin and IBMX-induced insulin secretion. Interestingly, H89, a PKA inhibitor, and U0126, a MEK kinase inhibitor, significantly inhibited apigenin-induced insulin secretion; however, no significant effect was observed by using ESI-05, an epac2 inhibitor. Apigenin improved glucose tolerance and increased glucose-stimulated plasma insulin levels in diabetic rats. Apigenin also lowered blood glucose in diabetic rats upon chronic treatment.

Conclusion: Apigenin exerts glucose-stimulated insulin secretion by modulating the PKA-MEK kinase signaling cascade independent of K-ATP channels.

1. Introduction

The evaluation of recently developed, safer, efficacious plant-based active moieties as novel drug targets for the regulation of glucose-dependent insulinotropic effects, would provide valuable information for anti-diabetic drug development. Over the past thirty years, approximately 65 % of all small-molecule drugs that got marketing approval have originated from plants, with excellent examples being artemisinin and metformin [1]. The other important factor that leads us to the identification of new natural drug candidates and targets is the increasing trend of genetic variations in already discovered drug targets, which might lead to the loss of function of existing marketed drugs. Notably, according to the Kyoto Declaration, Asian type 2 diabetes subjects have more β-cell dysfunction than insulin resistance [2]. The available therapeutic interventions for β-cell function are sulfonylureas and glinide. Unfortunately, these drugs increase the risk of hypoglycemia and other severe consequences in diabetic patients [3]. The main targets of sulfonylurea are its "K-ATP" channels, which upon activation,

Abbreviations: PKA, protein kinase A; IBMX, 3-isobutyl-1-methylxanthine; FSK, forskolin.

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secrete insulin irrespective of glucose concentration [4]. Therefore, the naturally derived compound(s) that stimulate glucose-induced insulin secretion delivers a better therapeutic alternative to address the issues reported with the marketed synthetic drugs.

The calcium-activated glucose stimulation triggers various protein kinases, including AKAP, cAMP, PKA, PKC, Epac2, and MEK kinase ERK½ [5,6]. Elevated calcium levels result in increased cAMP concentration, leading to ERK1/2 phosphorylation by PKA activation in pancreatic β-cells [6]. Similarly, activation of the p21-activated kinase 1 (PAK1) pathway by GLP-1, mediated through PKA, demonstrated phosphorylation of ERK1/2 via mitogen-activated protein kinase (MEK) activation. Activated ERK1/2 plays a role in glucose-induced F-actin modulation, contributing to the secretion of insulin from β-cells [7,8]. These pathways play crucial roles in glucose-induced insulin secretion and represent promising targets for novel insulin secretagogues. Hence, we aim to fulfill the urgent requirement for novel insulin secretory candidates capable of enhancing insulin secretion exclusively at stimulatory glucose.

In search of insulin secretagogues that are innovative, effective with enhanced safety, and also have a glucose-dependent insulinotropic effect, we selected apigenin due to its cost-effectiveness, safety, and broad pharmacological usage. Apigenin is abundant in numerous fruits, vegetables, and medicinal plants [9]. Apigenin is reported to be an anti-diabetic agent since it possesses the ability to inhibit α-glucosidase activity, cause stimulation of insulin action as well as secretion, manage ROS, and prevent diabetes complications [10,11]. Reports suggest apigenin is expected to show promising benefits for the management of diabetes and preventing its complications [12]. It has been reported that the apigenin derivative, apigenin-6-C-beta-L-fucopyranoside, exerts antihyperglycemic activity by stimulating the secretion of insulin and insulin mimetic activity by stimulating glycogen synthesis in the liver [13]. Furthermore, Suh et al. demonstrated apigenin protective action by damaging pancreatic β-cells by suppressing oxidative stress-related signaling [14]. In our previous study, apigenin was found to have a glucose-dependent insulinotropic effect, and it was observed computationally that it has a binding affinity with PKA but not to its active hot spot residues, so the details of the experiments to identify the target still need to be investigated [14]. Most importantly, acute and chronic *in vivo* investigations in diabetic conditions still need to be explored. The well-established historical use and glucose-dependent insulinotropic properties of apigenin make it worthy to explore its detailed insulin secretory mechanism(s) by evaluating its potential in both *in vitro* and *in vivo* settings. Given apigenin's extensive use as a promising pharmacologically active compound with proven safety and glucose-lowering effects, our investigation seeks to unveil the detailed glucose-dependent insulin secretory mechanisms and assess its chronic and acute anti-diabetic effects.

2. Materials and methods

2.1. Materials

Collagenase V, bovine serum albumin (BSA), glibenclamide (GB), streptozotocin (STZ), and nicotinamide were purchased from Sigma. 3-isobutyl-1-methylxanthine (IBMX), forskolin, diazoxide, U0126, H89, ESI-05, verapamil, SQ22536, U73122. Rat and mouse insulin ELISA kits were purchased from Crystal Chem.

2.2. Animal

Eight-weeks old male BALB/c mice (30–40 g), and Sprague Dawley (SD) rats (180–250 g) were used in this study. The animals were kept under controlled conditions of humidity and temperature (50–55 % humidity; 25 ± 2 °C), with a 12-hour dark and light cycle. Free access to food ad libitum and water was provided by maintaining a clean environment throughout the experiments. All the animal experiments were performed with prior approval from the Ziauddin University Animal Ethics Committee (Protocol no. 2023–02/FS/FHS).

2.3. Mice pancreatic islet isolation

The mouse pancreas was distended by injecting 3 ml of collagenase V (1 mg/ml) through the common bile duct. The pancreas was separated as a whole from the other tissues and digested at 37°C for 15 minutes in a collagenase solution. The digested tissue containing islets was washed 2–3 times in Hank's Balanced Salt Solution (HBSS). The islets were picked under a Nikon SMZ-745Ti stereomicroscope, Japan [15].

2.4. Effect of apigenin on cell viability and cytotoxicity

For evaluation of cell viability in islets cells, isolated islets were treated with 0.05 % trypsin in PBS to disperse the islets in the primary pancreatic islet cells. These cells were cultured in RPMI 1640 medium with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin. The cells were treated with different concentrations of apigenin for 1 hour followed by treatment with trypan blue dye for 15 minutes to assess dye uptake or exclusion (trypan blue exclusion test). Additionally, the cellular cytotoxicity of apigenin was evaluated in the MIN6 and βTC6 using MTT (3-[4,5-dimethylthiazole-2-yl]-2,5- 121 diphenyl-tetrazolium bromide) assay.

2.5. Insulin secretion

The freshly isolated islets were pre-incubated in Krebs-Ringer bicarbonate buffer (KRBB; 4.7 mM KCl; 118 mM NaCl; 1.2 mM MgSO4; 1.9 mM CaCl2; 25 mM NaHCO3, 10 mM HEPES, 1.2 mM KH2PO4, and 0.1 % BSA, pH 7.4) with 3 mM glucose at 37°C for 45 min. After pre-incubation, the batches (3 islets per batch) were incubated at 37°C for 60 min in KRBB at 16.7 mM (stimulatory) and 3 mM (basal) glucose with the test substance(s). The batches of mouse islets were treated as follows: Different apigenin concentrations (0, 5, 10, 50, 100, 200 μM) were tested at 3 mM and 16.7 mM glucose; different glucose concentrations (3, 6, 11.2, 16.7, and 25 mM) with or without apigenin (50 μM) and glibenclamide (10 μM); 16.7 mM glucose, in the presence of diazoxide (50 μM), with or without apigenin; 16.7 mM glucose with 200 μM verapamil with or without apigenin; 16.7 mM glucose with 25 mM KCl+50 μM Diazoxide with or without apigenin; 16.7 mM glucose with 10 μM glibenclamide with or without apigenin; 16.7 mM glucose with 100 μM isobutylmethylxanthine (IBMX), an inhibitor of phosphodiesterase with or without apigenin; 16.7 mM glucose with H-89 (50 μM), a PKA inhibitor, with or without apigenin; 16.7 mM glucose with U0126 (20 μM), a MEK kinase inhibitor with or without apigenin. 16.7 mM glucose plus SQ22536 (25 μM), an adenylate cyclase inhibitor with or without apigenin. The mouse insulin ELISA kit was used to measure the amount of secreted insulin after incubation [15].

2.6. Intracellular cAMP assay

A group of nine islets were cultured in KRBB medium containing 16.7 mM glucose for a duration of 60 minutes. This incubation was done with or without apigenin alone as well as in combination with IBMX (100 μM), a phosphodiesterase inhibitor, and 10 μM forskolin (adenylyl cyclase activator). After the incubation period, the media was removed and the cells rinsed with KRBB, and then 300 μL of 0.1 N HCl was introduced to the islets. After freeze thaw cycle and sonication, the intracellular cAMP levels of the islets were quantified using an ELISA kit [15].

2.7. Development of Nicotinamide Streptozotocin-induced diabetic model

The male SD rats, fasted overnight, were given nicotinamide (120 mg/kg, IP) dissolved in distilled water 15 minutes prior to injecting

55 mg/kg streptozotocin intravenously (dissolved in citrate buffer 0.1 mM concentration). Rats with fasting blood glucose (150–200 mg/dl) following 7 days of streptozotocin induction were chosen for inclusion in this study [16,17].

2.8. Effects of glibenclamide and apigenin on oral glucose tolerance test (OGTT)

The impact of apigenin on tolerance to glucose and insulin in plasma was examined *in vivo* using SD rats (diabetic and non-diabetic). Twenty-four rats were divided into four groups (n=6 per group); Group I, served as the negative control, receiving solely distilled water. Group II, the positive control, was administered glibenclamide (5 mg/kg). Groups III and IV were respectively given 15 mg/kg and 30 mg/kg doses of apigenin. The experimental groups received oral administration of apigenin and glibenclamide in a 1 ml water suspension. Before the oral glucose tolerance test (OGTT), all rats were put on overnight fasting (12–14 h). Sixty (60) minutes after the administration of apigenin or glibenclamide, each rat received an oral glucose load (3 g/kg). Blood samples from the lateral tail vein of the rats were obtained at −60 min (immediately before the administration of apigenin or glibenclamide) and 0 minutes (before the glucose load), including at various time points following the glucose load. A glucometer was used to measure the fasting blood glucose levels. The plasma insulin levels were measured using an insulin ELISA kit [16].

2.9. Chronic effect of apigenin treatment in diabetic rats

The SD rats were divided into four groups, each consisting of five rats.: Group I consisted of diabetic rats without treatment (Db); Group II included diabetic rats administered apigenin at a dosage of 15 mg/kg (AP-15); Group III comprised diabetic rats treated with apigenin at a dosage of 30 mg/kg (AP-30); and Group IV consisted of diabetic rats induced with glibenclamide at a dosage of 5 mg/kg (GB), administered for 15 days once daily. Rats in all groups consume an equivalent amount of water. The drugs dissolved in 2.5 % DMSO were administered orally with an oral gavage. A glucometer was used to measure the blood glucose levels on days 1, 7, and 15, respectively.

2.10. Statistical analysis

The statistical analyses were conducted using SPSS 22.0 Statistical Package for Windows (SPSS, Inc., Chicago, IL, USA). The values were presented as mean ± S.E.M. *In vitro* comparisons were performed using the student t-test and one-way ANOVA, as applicable. Statistical significance was determined by P values <0.05.

3. Results

3.1. Effect of apigenin on insulin secretion from isolated mice islets

Apigenin, a safe natural flavonoid, induces a specific insulin secretion response in isolated islets upon glucose stimulation (Fig. 1A). Furthermore, the cell viability assays in the MIN6, β-TC6 cells (Fig. 1B), and islets cells showed that apigenin is safe even at the dose of 400 μM, cell viability was found >90 %. Further, evaluating the dose-dependent effect, apigenin (5–200 μM), showed no insulin secretory activity at basal glucose concentration; however, it exerts insulin secretion dose-dependently at stimulatory glucose. (Fig. 2A). Apigenin showed significant insulin secretory activity at 50 μM (28.13 ± 1.87 ng/islet/h; $P<0.001$) and 100 μM (30.56 ± 2.27 ng/islet/h; $P<0.001$) concentrations, when compared with 16.7 mM glucose alone (10.11 ± 1.37 ng/islet/h). No further elevation in insulin secretion was observed beyond 100 μM of apigenin.

Furthermore, the exclusive glucose-dependent insulinotropic effect of apigenin was evaluated at different glucose concentrations (3, 6, 11.2, 17, and 20 mM), and compared with glibenclamide, a marketed sulfonylurea drug (Fig. 2B). Apigenin exerts no insulin secretory activity at both 3- and 6-mM glucose concentrations. However, it significantly stimulated insulin secretion at 11.2 mM, 16.7 mM, and 25 mM glucose, with the optimum stimulation of insulin secretion by apigenin observed at 16.7 mM glucose concentration (about 2-fold; $P < 0.001$). In contrast to apigenin, glibenclamide induced insulin secretion at both basal and stimulatory glucose concentrations. Interestingly, apigenin-induced insulin secretion was found higher at 16.7 mM compared to glibenclamide. Furthermore, 16.7 mM was used to investigate the insulinotropic mechanism(s) of apigenin.

A.

Addition to the medium	Glucose (mM)	
	3	16.7
None	2.29 ± 0.55 ng/islet/h	11.29 ± 0.95 ng/islet/h
Glibenclamide	9.33 ± 1.05 ng/islet/h	27.55 ± 3.05 ng/islet/h; P<0.001
Apigenin	2.09 ± 0.35 ng/islet/h	26.25 ± 2.55 ng/islet/h; P<0.001

B.

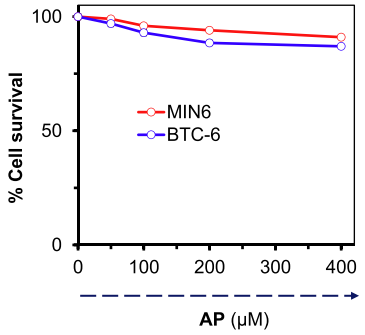

Fig. 1. Effect of AP on insulin secretion in isolated mice islets. (*A*) AP stimulated insulin secretion in isolated mice islets (*B*) effect of AP on cell viability in MKIN-6 and β-TC-6 cells. Values are mean ± SEM from 3 to 5 independent experiments each in triplicates. ***$P<0.001$, a significant change over the respective control value.

3.2. Apigenin effect is glucose-dependent, independent of K_{ATP} channel

We further explored the mechanism of apigenin by evaluating whether the glucose-induced insulin release of apigenin is dependent and/or independent of K-ATP channels. It was observed that insulin secretion of apigenin was abolished (2.55 ± 0.3 *vs.* 31.21 ± 1.9 ng/islet/h) using diazoxide (50 μM). (Fig. 3A). Furthermore, we observed that the apigenin-induced insulin secretion was enhanced further (53.33 ± 3.9 *vs.* 31.61 ± 2.7 ng/islet/h) using islets depolarized by KCl (25 mM) with diazoxide (Fig. 4A). Interestingly, apigenin also exerted an incremental effect on glibenclamide-induced insulin secretion (43.44 ± 3.1 *vs.* 32.61 ± 3.1 ng/islet/h) (Fig. 4B). These results suggest the role of apigenin as a potential insulin secretagogue on the glucose-dependent amplifying pathway without its direct effect on K-ATP channels.

3.3. Ca^{2+}-dependent insulin secretory effect of apigenin

We found that the amplifying effect of apigenin on the release of insulin was significantly eliminated by verapamil (200 μM), a Ca^{2+} channel blocker (4.55 ± 0.7 *vs.* 35.66 ± 3.9 ng/islet/h, $P<0.001$) (Fig. 3B). Second, by removing the extracellular Ca^{2+}, insulin secretion by apigenin was significantly inhibited (data not shown).

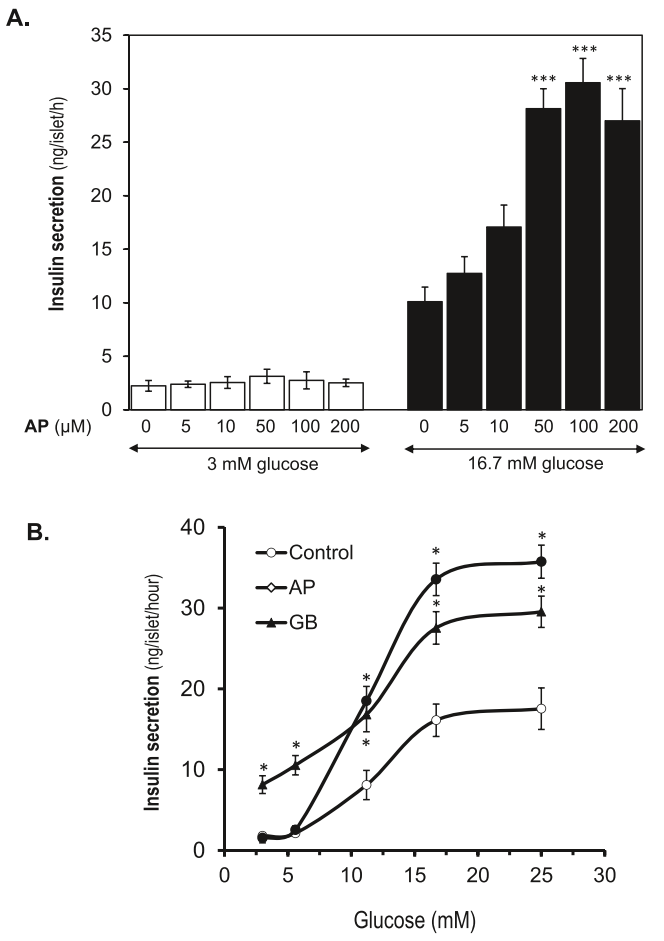

Fig. 2. AP enhances GSIS in isolated mice islets in a dose-dependent manner. (*A*) AP was added at 0, 5, 10, 50, 100, and 200 μM concentrations in the presence of 16.7 mmol/L (Black Bar) or 3 mM (white Bar) glucose. (*B*) Effect of indicated glucose concentrations on insulin secretory pattern in islets treated with AP and GB. Insulin secretion was induced by 50 μM AP (●), 10 μM GB (▲), and glucose alone (control) (○). Values are mean ± SEM from 3 to 5 independent experiments. *$P<0.05$, ***$P<0.001$, a significant change over the respective control value.

3.4. Apigenin-induced amplification of insulin secretion through inhibition of cAMP hydrolysis and/or activation of cAMP production

cAMP, an intracellular second messenger, plays a vital role in the secretion of insulin by the cAMP-dependent PKA signaling cascade (Liu *et al.*, 2006). We therefore determined whether apigenin can induce intracellular cAMP, thereby elevating glucose-modulated insulin secretion. To validate whether apigenin's mode of action is inhibition of cAMP hydrolysis or activation of cAMP production, the effect of apigenin was evaluated on forskolin, an adenylate cyclase activator, and IBMX-induced insulin secretion, respectively. We found that apigenin amplified both forskolin (69.93 ± 3.4 vs. 49.83 ± 3.9 ng/islet/h) and IBMX-induced insulin secretion (63.93 ± 6.1 *vs.* 45.57 ± 3.8 ng/islet/h) in comparison with forskolin and IBMX alone (Fig. 5AB). Furthermore, apigenin exerts no effect on intracellular cAMP concentration in comparison to stimulatory glucose alone (1.48 ± 0.14 *vs.* 1.31 ± 0.12 pmole/islet/h). Interestingly, in contrast to the enhanced effect in insulin secretion, apigenin showed no additive effect in both forskolin (7.11 ± 0.48 *vs.* 6.97 ± 0.34 pmole/islet/h) and IBMX (3.97 ± 0.27 *vs.* 3.92 ± 0.38 pmole/islet/h)-induced intracellular cAMP concentration. (Fig. 5CD)

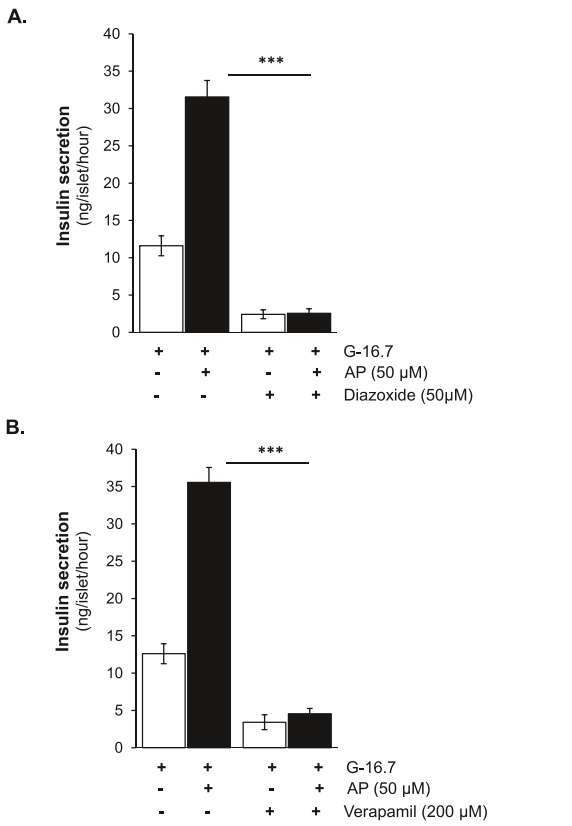

Fig. 3. AP enhances glucose-dependent insulinotropic effect independent of K-ATP channels. (*A*) Effect of AP on insulin secretion in mice islets with K-ATP channels opened by diazoxide. Mice islets were incubated in 3 mM or 16.7 mM glucose in the presence or absence of AP and/or diazoxide (K-ATP channels opener) used at the indicated concentration. (*B*) Effect of AP on insulin secretion with Ca^{2+} channels blocked by verapamil. Islets were incubated in 3 mM or 16.7 mM glucose in the presence or absence of AP and/or verapamil at the indicated concentrations. Values are mean ± S.E.M. from 3 to 5 independent experiments each in triplicate. ***P<0.001, significant changes over the respective control values.

3.5. PKA mediated apigenin-induced insulin secretion

Furthermore, to examine whether the synergic effect of apigenin on IBMX and forskolin-induced insulin secretion is due to PKA, a PKA inhibitor, H-89, was used. It was observed that H-89 (50 μM), did not alter GSIS, although the insulin, releasing activity of apigenin was inhibited significantly (14.16 ± 1.9 ng/islet/h; $P<0.001$) when compared to apigenin alone (31.7 ± 2.8 ng/islet/h) (Fig. 6A).

3.6. MEK kinase-mediated apigenin-induced insulin secretion

To further evaluate whether the additive effect of apigenin in IBMX and forskolin-stimulated insulin secretion is exclusively through PKA or due to crosstalk between PKA and the MEK kinase signaling pathway, U0126, a MEK kinase inhibitor, was used. We observed that U0126 (20 μM) was not able to alter GSIS. However, the apigenin effect on insulin release was inhibited significantly (15.09 ± 1.89 ng/islet/h; $P<0.001$) when compared to apigenin alone (33.46 ± 3.67 ng/islet/h) (Fig. 6B).

3.7. Apigenin enhances glucose tolerance and stimulation of glucose-induced plasma insulin levels in diabetic rats

Apigenin improved glucose tolerance and increased plasma insulin levels in both non-diabetic and diabetic SD rats. Using the oral glucose

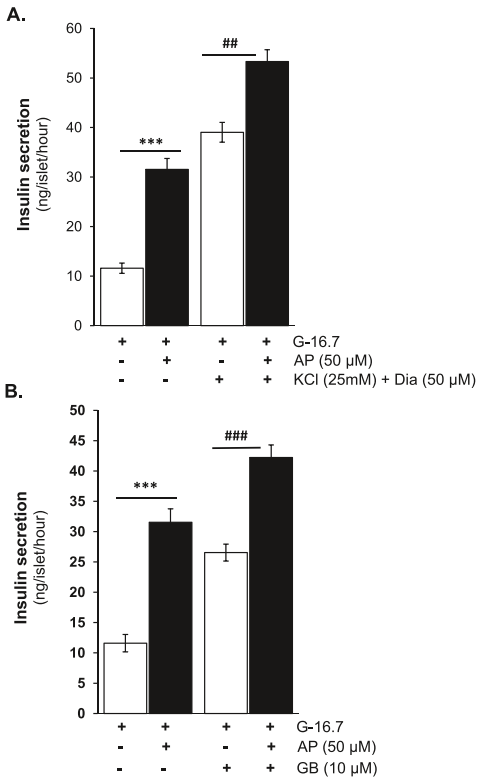

Fig. 4. Effects of AP on insulin secretion in (*A*) depolarized and (*B*) Glibenclamide (GB) treated mice islets. Islets were incubated in 16.7 mM glucose in the presence or absence of AP (50 μM) and/or diazoxide (50 μM) + KCl (25 mM) and GB (10 μM). Values are mean ± S.E.M. from 3 to 5 independent experiments each in triplicate. ***$P<0.001$, ##$P<0.01$, ###$P<0.001$, significant changes over the respective control values.

tolerance test (OGTT), a spike in blood glucose levels to 350 mg/dL was observed in diabetic rats 30 minutes after a glucose load of 3 g/kg.

After treatment with apigenin at 15 and 30 mg/kg doses, a significant reduction in glucose levels was observed. The dose of 30 mg/kg showed the highest decrease of approximately 31 % ($P<0.01$) compared to diabetic control (Fig. 7B). Apigenin also improved glucose tolerance in non-diabetic rats, with the greatest effect seen at the 30 mg/kg dose (Fig. 7A). Notably, apigenin lowered the levels of blood glucose in diabetic rats before and after administration, while in non-diabetic rats, no such effect was observed. In contrast, glibenclamide reduced blood glucose levels in both diabetic and normal control rats. This suggests that apigenin specifically targets the blood glucose levels in the diabetic state, consistent with our *in vitro* findings.

The effect of apigenin on plasma insulin was evaluated in diabetic and normal rats. In diabetic rats, apigenin significantly increased plasma insulin levels at 15 minutes for both 15 mg/kg (1.3-fold; $P<0.05$) and 30 mg/kg (1.5-fold; $P<0.05$) compared to untreated diabetic rats (Fig. 7D). In non-diabetic rats, apigenin enhanced plasma insulin levels at 15 minutes, both at 15 mg/kg (approximately 1.3-fold; $P<0.05$) and 30 mg/kg (approximately 1.9-fold; $P<0.01$), compared to untreated rats (Fig. 7C). Notably, a significant increase in plasma insulin was observed in diabetic rats at 60 minutes post-apigenin administration (0 min); however, no effect was observed in non-diabetic rats (Fig. 7C).

3.8. Chronic treatment with apigenin lowers blood glucose in type 2 diabetic rats

We further examined whether apigenin maintains its ability to lower glucose levels and slow down the progression of diabetes during chronic treatment. For this study, we selected streptozotocin-nicotinamide (STZ-

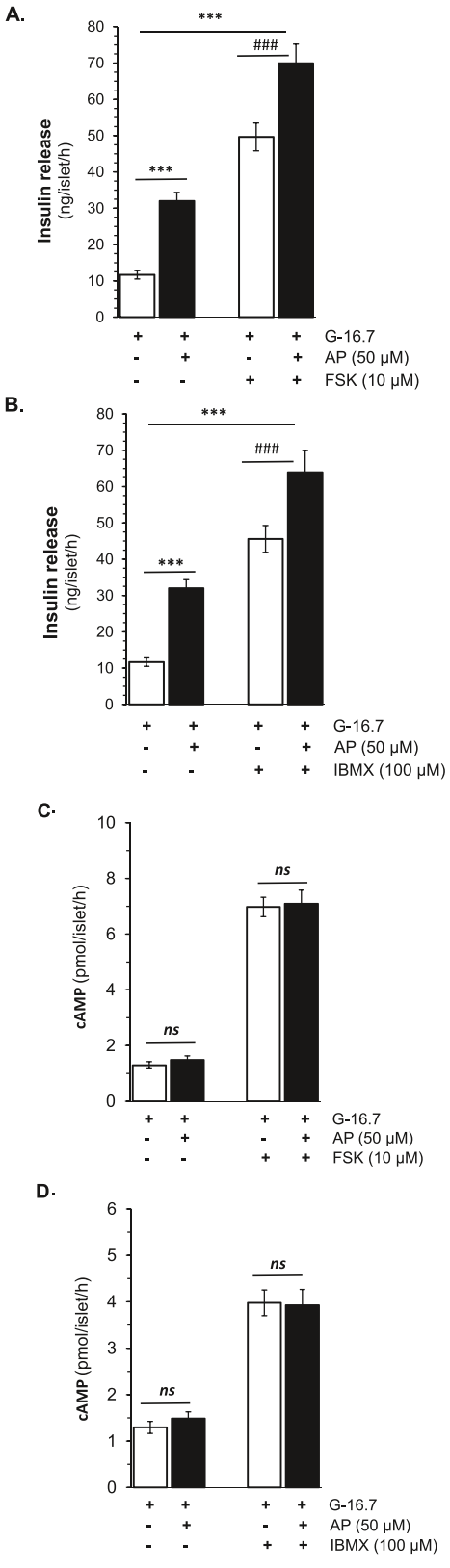

Fig. 5. Effect of AP on cAMP production and/or inhibition of cAMP hydrolysis. Effect of AP on insulin secretion (*AB*) and intracellular cAMP (*CD*), in the presence or absence of FSK, an adenylate cyclase activator, and/or IBMX, a phosphodiesterase inhibitor. Islets were incubated in 16.7 mM glucose in the presence or absence of AP and/or FSK/IBMX at the indicated concentrations. Values are mean ± S.E.M. for 3–5 independent experiments each in triplicate. ***$P<0.001$, ###$P<0.001$, significant changes when compared with control values. *ns,* not significant.

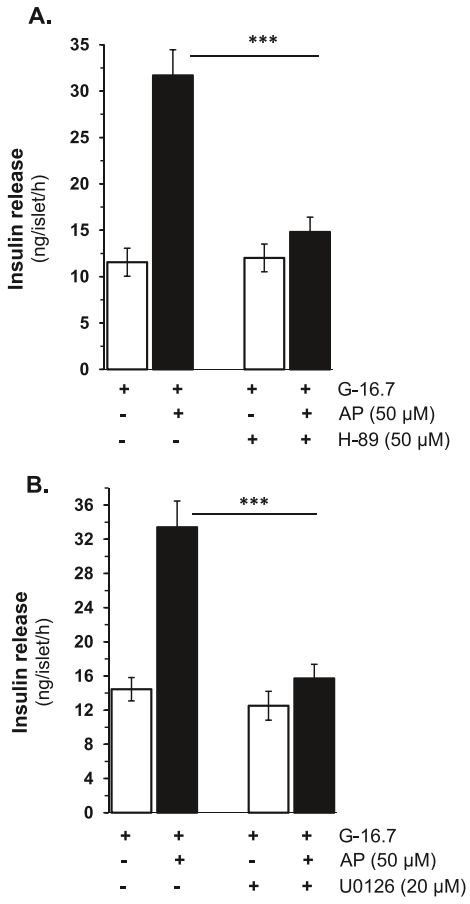

Fig. 6. Effect of AP on PKA and MEK kinase for stimulation of insulin secretion. Islets were incubated in 16.7 mM glucose in the presence or absence of AP and/or H89, a PKA inhibitor (*A*), and U0126, a MEK kinase inhibitor (*B*) at the indicated concentrations. Values are mean ± S.E.M. for 3–5 independent experiments each in triplicate. ***$P<0.001$, significant changes when compared with AP alone.

NA)-treated diabetic rats with blood glucose levels ranging from 140 to 200 mg/dL. These rats were treated with apigenin at doses of 15 and 30 mg/kg for a period of 15 days (see Fig. 8). We observed that apigenin reduced blood glucose levels in a dose- and time-dependent manners. Apigenin led to a non-significant decrease in fasting blood glucose on day 7. However, on day 15, both the 15 and 30 mg/kg doses of apigenin significantly lowered blood glucose levels by 155.2±9.5 mg/dL and 140.2±7.4 mg/dL, respectively, compared to the initial day levels.

4. Discussion

In this research, we presented evidence indicating that, unlike presently utilized insulin secretagogues such as sulfonylureas and glinides, which directly stimulate insulin secretion regardless of blood glucose, apigenin effectively reduced blood glucose, specifically in hyperglycemic conditions. Apigenin is an important, safe natural flavonoid used due to its promising broad pharmacological functions [18]. However, its detailed antidiabetic and insulin secretory mechanisms are not well known. Here, we have evaluated the insulin secretory mechanism (s) of apigenin and its acute and chronic anti-hyperglycemic effects.

During screening, apigenin stimulates the release of insulin at stimulatory glucose but no insulin release at basal glucose. Furthermore, apigenin dose-dependently enhanced exclusive glucose-stimulated insulin secretion, suggesting a low risk of hypoglycemia. To explore further mechanisms of action and identify its drug target(s), a series of experiments using mouse islets were performed.

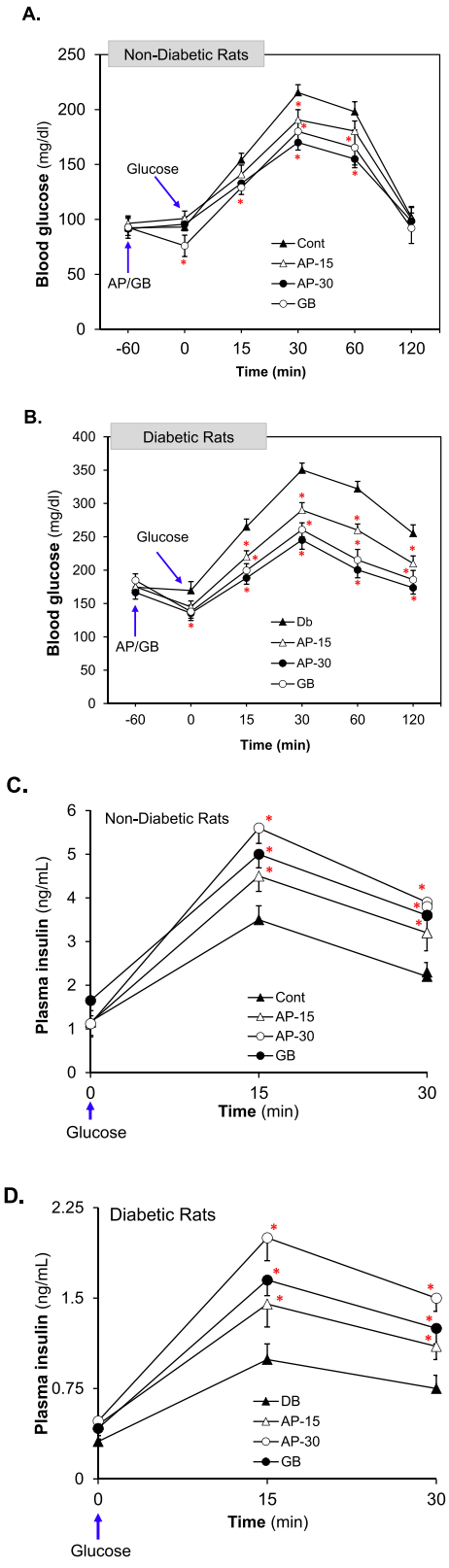

Fig. 7. Evaluation of AP effect on glycemic levels and glucose-stimulated insulin secretion (GSIS) in both diabetic and non-diabetic rats. AP and GB were administered orally 60 minutes prior to a glucose load (3 g/kg), and blood glucose (*A, B*) and plasma insulin (*C, D*) were measured at specified intervals. The data represent means ± S.E.M. for six rats in each group. Cont, non-diabetic rats; Db, diabetic control rats; AP-15 and AP-30, rats treated with AP at doses of 15 and 30 mg/kg, respectively; GB, rats treated with glibenclamide (5 mg/kg). *$P<0.05$, in comparison to respective control values.

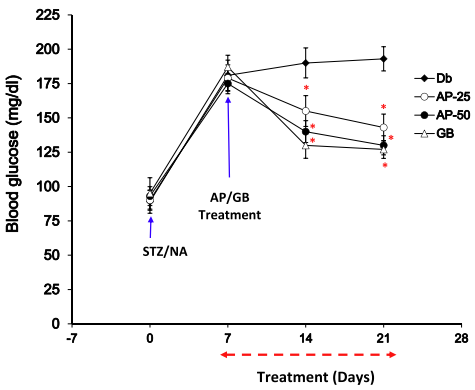

Fig. 8. The Chronic effect of AP on fasting blood glucose of non-obese diabetic rats. Diabetic rats were treated with AP for 15 days, and blood glucose was monitored weekly. Data represents means ± S.D. for five rats in each group. Db, untreated diabetic rats; AP-15, AP-30, rats treated with apigenin at 15, and 30 mg/kg dose, respectively, and GB, rats treated with glibenclamide (5 mg/kg). *$P<0.01$ compared to their respective day 1 values.

When looking for novel, potential antidiabetic medication leads, the β-cell K-ATP channel is a potential target. However, the impact of commercially available synthetic insulin secretagogues, such as sulfonylurea, occurs via the K-ATP channels and is independent of the surrounding glucose concentration [19]. The apigenin insulin secretory mechanism(s) was compared with glibenclamide, a 2nd-generation sulfonylurea drug, to know more precisely whether the mode of action of apigenin is glucose-dependent or K- channels-dependent. Our detailed experimental findings deduced that apigenin potentiates insulin secretion at stimulatory glucose levels without the direct influence of K-ATP channels. First, apigenin's distinct pattern of insulin secretion at stimulatory glucose concentration is different from sulfonylurea. Second, the apigenin-potentiated insulin secretion effect was inhibited completely using diazoxide and verapamil. Third, apigenin-potentiated insulin secretion was amplified in depolarized islets. Fourth, apigenin-potentiated insulin secretion was amplified further in islets treated with glibenclamide. Current research findings show absolutely no effect of apigenin on basal insulin secretion, highlighting its potential glucose-dependent role distal to K-ATP channels. These results suggest the role of apigenin as a potential insulin secretagogue in amplifying glucose-dependent insulin secretion without the direct influence of K-ATP channels.

The intracellular Ca^{2+} concentration contributes as an integral factor in signaling and a slight shift in its equilibrium significantly modulates insulin exocytosis and β-cell functionality [19]. Based on our findings, it is speculated that apigenin acts independently of the direct effect of L-type Ca^{2+} channels and K_{ATP}-channels to amplify insulin secretion, coupled with stimulatory glucose concentration. First, using verapamil, the stimulated insulin secretion by apigenin was inhibited significantly. Secondly, in the absence of extracellular $Ca^{2+,}$ stimulation of insulin secretion by apigenin was completely inhibited. Third, stimulation of insulin secretion by apigenin was completely inhibited by diazoxide. Fourth, absolutely no stimulation of insulin secretion was observed by apigenin at basal glucose. Fifth, apigenin-induced insulin secretion was further triggered in glibenclamide and depolarized islets. These results highlight no direct involvement of K-ATP and Ca^{2+} channels in apigenin stimulated insulin release; however, Ca^{2+} is required to amplify the apigenin-induced insulin secretion. An alternative explanation is that apigenin acts distal to the K-ATP and Ca^{2+} channels on the targets that are directly dependent on stimulatory glucose [16].

The cAMP-PKA signaling pathway is the key signal transducer of glucose-mediated insulin release, operating in coordination with Ca^{2+} to regulate insulin secretion [20]. The cAMP amplifies the insulinotropic response in PKA and Epac2-dependent mechanisms [21,22]. However, PKA predominantly plays the key role in insulin granular exocytosis in response to Ca^{2+}, coupled with stimulatory glucose [8,23]. Therefore, cAMP-PKA is considered an important drug target for the identification of new therapies. There is reported evidence of crosstalk between the PKA and MEK-ERK½ signaling pathways, contributing to insulin exocytosis [24]. Compounds such as GLP-1 and forskolin, which influence cAMP-PKA signaling, also enhance glucose-stimulated insulin secretion through MEK-ERK½ signaling. In addition to their role in insulin secretion, these pathways are crucial for the survival of β-cells [25]. Our experimental findings demonstrated that apigenin exhibits an insulinotropic effect through the PKA-MEK signaling cascade in response to stimulatory glucose. First, according to reported evidence, PKA exerts a predominant role in response to cAMP to induce insulin exocytosis [26]. Second, apigenin exerts an additive effect on insulin secretion induced by IBMX and forskolin. Third, apigenin exhibited no effect on intracellular cAMP and did not contribute additively to forskolin- and IBMX-induced intracellular cAMP concentrations. Fifth, the insulinotropic effect induced by apigenin was significantly inhibited when PKA and MEK kinase inhibitors were employed. These findings suggest that apigenin exerts its insulinotropic effect by modulating the PKA-MEK signaling cascade coupled with stimulatory glucose. Beside the insulin secretion, the PKA-MEK signaling cascade also plays a potential role in the β-cells protection, so it can be speculated that apigenin seems to have potential in the β-cells protection in response to hyperglycemia in diabetic conditions, that needs to be further explored. Notably, apigenin exerts an acute insulinotropic *in vivo* effect in diabetic rats by improving glucose tolerance and glucose-induced plasma insulin levels mimicking the *in vitro* results. Importantly, apigenin showed a promising chronic effect by decreasing blood glucose in diabetic patients. This *in vivo* data suggests that apigenin may offer a new addition as a drug candidate to address the issues with insulin secretory impairments in diabetic patients.

5. Conclusion

The present study showed that apigenin a natural bioflavonoid reduces blood glucose effectively and exclusively in hyperglycemic conditions by amplifying the GSIS, showing a low risk of hypoglycemia. Apigenin amplifies glucose-stimulated insulin secretion without directly affecting K-ATP channels and exerts a distinct behavior compared to sulfonylureas. Apigenin potentiates the glucose-stimulated insulin secretion through the PKA-MEK signaling cascade. Importantly, mimicking the *in vitro* results *in vivo*, apigenin showed promising results by improving glucose tolerance, increases glucose-stimulated plasma insulin levels in diabetic rats. Furthermore, the chronic *in vivo* study showed the lowering of blood glucose by apigenin in diabetic rats. Collectively, the promising findings suggest apigenin as a possible drug seed to restore insulin secretion in diabetic conditions and could be a potential therapeutic candidate for diabetes.

CRediT authorship contribution statement

Rehan Imad: Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **M. Hafizur Rahman:** Writing – review & editing, Formal analysis, Data curation. **Falak Shahab:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Akhtar Ali:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Abdul Hameed:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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