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Luteolin and kaempferol promote insulin secretion in islet and improve hyperglycemia through IL18-NCC signaling in mice

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ABSTRACT: Interleukin 18 (IL18)-Na-Cl cotransporter (NCC) signaling triggers β cell proliferation, development survival and insulin production in islets. Targeting islet insulin production is a potential strategy for hyperglycemia treatment. Dietary flavonoids possess the substantial anti-hyperglycemia activity. However, whether these flavonoids improve hyperglycemia through directly promoting islet insulin production and the underlying mechanism remains unclear. In this study, islets are isolated from wild-type (WT) and *Ncc*$^{-/-}$ mice and treated with dietary flavonoids *ex vivo*. Among the six dietary flavonoids of chrysin, apigenin, luteolin, quercetin, myricetin and kaempferol, 10 μM luteolin and kaempferol exhibit the highest activities in stimulating insulin production in *ex vivo* islet. Furthermore, luteolin and kaempferol treatments increase IL18 production and NCC expression, enhance β cell proliferation and survival, protect against streptozocin (STZ)-induced apoptosis and damage in islet. However, the deficiency of NCC eliminate these activities of luteolin and kaempferol. Moreover, STZ-induced hyperglycemic mice are fed a diet supplemented with 0.01% luteolin or kaempferol. In vivo studies have shown that dietary supplementation with luteolin or kaempferol improves glucose tolerance and insulin production, alleviates the apoptosis of islet β cells, and elevates IL18 expression in STZ-induced hyperglycemic mice. In conclusion, dietary luteolin and kaempferol promote islet insulin production, proliferation and survival, protect against STZ-induced islet apoptosis and hyperglycemia through IL18-NCC signaling.

Keywords: Dietary flavonoid; luteolin; kaempferol; IL18-NCC signaling; insulin production; hyperglycemia

1. Introduction

Diabetes is characterized by persistent hyperglycemia resulting from abnormalities in insulin secretion or action. The progressive loss or dysfunction of insulin-secreting islet β cell mass due to environmental, genetic and immunological factors ultimately leads to type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM), respectively [1]. The total pancreatic β cell mass is the result of a balance between β cell formation and death during development. Therefore, β cell mass is determined by a balance among proliferation from existing β cells, neogenesis of β cells from stem cells or progenitors, and death through β

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cell apoptosis [2]. However, β cells are intrinsically vulnerable to killing, and are more prone to death under endoplasmic reticulum stress and immune stress conditions than other islet endocrine cells [3, 4]. T1DM is primarily caused by an autoimmune response that destroys β cells, leading to an absolute deficiency of insulin [5, 6]. Targeting β cell to control hyperglycemia by promoting β cell proliferation or preserving functional β cell mass is proposed as potential strategy for diabetes treatment [7].

Interleukin-18 (IL18) is a multifunctional cytokine and predominantly expressed in T cells, macrophages and adipocytes [8, 9]. IL18 levels are elevated in plasma and islets of diabetic patients and mice [10, 11]. Notably, IL18 has been reported to increase islet insulin production and suppress the development of hyperglycemia [12, 13]. Deficiency of IL18 enhances spontaneous hyperglycemia and insulin resistance, and administration of IL18 improves insulin sensitivity in *Il18*$^{-/-}$ mice [14]. IL18 receptor (IL18r) and Na-Cl co-transporter (NCC) are two receptors of IL18 [15, 16]. We reported that IL18 is expressed on islet α cells and NCC on β cells in human and mouse islets. NCC deficiency in β cells impairs glucose tolerance and insulin sensitivity, reduces islet size and β cell proliferation, and increases β cell apoptosis. Mechanistically, IL18, together with glucagon-like peptide-1 (GLP1), uses the NCC and glucagon-like peptide-1 receptor (GLP1r) on β cells to trigger β cell proliferation, development and insulin secretion [12]. Therefore, targeting IL18-NCC signaling in islets might prove an attractive target for prevention of β cell destruction and diabetes.

Flavonoids are the secondary metabolites in fruits and vegetables, and possess antioxidant, anti-inflammatory, and anti-hyperglycemic activities [17]. In chemical structure, flavonoids have a basic structure of 15-carbon skeleton consisting of two benzene rings (A ring and B ring) and a connected heterocyclic pyran ring (C ring). Based on structural differences, flavonoids can be generally classified into flavonols, flavones, isoflavones, flavanones, dihydroflavonols, and dihydroflavones [18]. Dietary flavonoids have been reported as effective supplements for prevention of diabetes based on in vitro and animal models [19]. It has been demonstrated that supplementation of dietary flavonoids, such as apigenin, quercetin, and luteolin, ameliorate streptozocin (STZ)-induced and high-fat diet (HFD)-induced hyperglycemia [19-21]. However, whether dietary flavonoids improve hyperglycemia through directly promoting insulin production in islet β cell and the underlying mechanism remains unclear.

In this study, we firstly compared the effect of six dietary flavonoids, including the flavones chrysin, apigenin, luteolin and flavonols quercetin, myricetin, kaempferol, on the insulin production in *ex vivo* islet, and revealed that luteolin and kaempferol possess the highest activities among the six dietary flavonoids. Next, whether luteolin and kaempferol promote islet insulin production and survival, inhibit STZ-induced islet apoptosis through IL18-NCC signaling were fully investigated in islet isolated from wild-type (WT) and *Ncc*$^{-/-}$ mice. Finally, the role of luteolin and kaempferol on glucose tolerance, insulin production, and IL18 expression were studied in STZ-induced hyperglycemic mice.

2. Materials and methods

2.1 Mice

WT (C57BL/6J, Vital River Laboratory Animal Technology Co., Beijing, China) and *Ncc*$^{-/-}$ [16] mice were housed in ventilated cages in a pathogen free barrier facility with a 12-hlight: 12-h dark cycle. 7 week-old WT mice were divided into three groups (n = 10): 1) the Con group, the mice received a single intraperitoneal injection of a high dose of streptozotocin (STZ, S8050, Solarbio, Beijing, China) at 8 week-old; 2) the Lu group, the mice fed on a luteolin-containing chow diet (0.01% luteolin [L107329, Aladdin, Shanghai, China]) for 5 weeks from 7 week-old, and received an injection of STZ at 8 week-old; 3) the Ka group, the mice fed on a kaempferol-containing chow diet (0.01% kaempferol [K107145, Aladdin]) for 5 weeks from 7 week-old, and received an injection of STZ at 8 week-old. At 12-week-old, the mice were sacrificed by the exposure to CO_2, and the plasma and pancreas were harvested.

2.2 STZ-induced hyperglycemia

A single intraperitoneal injection of a high dose of STZ (180 mg/kg body weight) was administered to induce β cell depletion and hyperglycemia. Male mice (8 weeks old) were weighed and fasted for 4-6 hours prior to the STZ injection. STZ was dissolved in freshly prepared sodium citrate buffer (pH 4.5) at a concentration of 10 mg/mL before injection. Mouse body weight, blood glucose, and blood insulin levels were monitored weekly.

2.3 Glucose tolerance test (GTT)

After four weeks of STZ treatment, glucose tolerance tests (GTT) were conducted on the mice following fasting periods of 16 hours. Briefly, the mice were intraperitoneally injected with D-glucose (1 g/kg body weight, G7021, Sigma-Aldrich, Shanghai, China). Blood glucose levels were measured from the tail veins using a blood glucose meter (GH81, B.Braun, Shanghai, China) at 0, 15, 30, 45, 60, 90, and 120 mins after injection.

2.4 Glucose-stimulated insulin secretion (GSIS) assay and insulin measurements

After 16 hours fasting, mice were administered by glucose (3 g/kg body weight) via intraperitoneal injection. Blood samples were collected from tail-tip bleeding. Plasma insulin levels were determined using ELISA at 0, 2, 5, and 15 mins after injection. Insulin levels in the blood and islet culture medium were assessed using the ELISA kits (SEKM-014, Solarbio) according to the manufacturer's instructions and normalized to protein concentrations.

2.5 Pancreatic islet isolation and treatment

Pancreatic islets were isolated form WT and *Ncc*$^{-/-}$ male mice as previously reported [22]. In brief, mice were sacrificed and immediately processed for pancreatic perfusion with 3 mL of type-4 collagenase (1.4 mg/mL, LS004188, Worthington Biochemical Co. Lakewood, NJ) dissolved in HBSS. Collagenase solution was injected by inserting a 30G1/2-G needle into the common bile duct through the joint site of the hepatic duct under a dissecting microscope. After complete distension, the pancreas tissue was digested by incubation at 37 ºC for 17 min in 5 mL digestion solution. Digested tissue was vigorously shaken and subsequently washed twice in RPMI 1640 medium. Islets were purified using 450-μm nylon mesh and re-suspended in a

mixture of lymphocyte separation medium (0850494, MP Biomedicals, Irvine, CA) and RPMI 1640 medium (2:1) and centrifuged at 800 *g* for 25 min without brake. The islets were collected from the interface between the medium and lymphocyte separation medium, re-suspended in RPMI 1640 medium, and hand-picked under a dissecting microscope. Islets were cultured in RPMI 1640 medium with 10% FBS overnight after isolation, and then cultured in medium containing 2.8 mM (low glucose) or 16.7 mM glucose (high glucose) with or without 10 μM chrysin (C110078, Aladdin), 10 μM apigenin (A106675, Aladdin), 1, 10, or 50 μM luteolin, 10 μM quercetin (Q111273, Aladdin), 10 μM myricetin (M111175, Aladdin) or 1, 10, or 50 μM kaempferol for 12 hrs. For STZ-induced apoptosis assay, islets were pretreated with 10 μM luteolin or kaempferol for 24 hrs, subsequently stimulated with 50 mM STZ for 30 min.

2.6 IL18 level measurement

The levels of IL18 in the islet culture medium and plasma were assessed using mouse ELISA kits (SEKM-0019, Solarbio, Beijing, China), following the manufacturer's instructions.

2.7 Mouse islet and pancreas tissue histologic analysis

The isolated islets and pancreas tissues were fixed in 10% neutral phosphate-buffered formalin for 24 hours. The isolated islets were soaked in low-melting agar, cooled to form gel discs, wrapped in dewatering paper, and placed in cassette [23]. Isolated islets and pancreas tissues were dehydrated and then embedded in paraffin. Serial islet and pancreatic sections of 6 μm thickness were sliced. For immunohistochemical analysis, the sections were deparaffinized, rehydrated, and incubated with the following primary antibodies: rabbit anti-IL18 (1:50, ab207324, Abcam), rabbit anti-insulin (1:300, 3014, Cell Signaling Technology, Danvers, MA), mouse anti-glucagon (1:200, 14-9743-82, eBioscience, California, US) followed by appropriate biotin-conjugated secondary antibodies (1:500, Boster, Wuhan, China) and HRP-streptavidin (BA1088, Boster). After detection with AEC chromogenic agent (AR1020, Boster), slides were counterstained with hematoxylin.

For immunofluorescence analysis, the sections were deparaffinized, rehydrated, and incubated with the following primary antibodies: rabbit anti-IL18 (1:50, ab207324, Abcam), rabbit anti-insulin (1:300, 3014, Cell Signaling Technology, Danvers, MA), mouse anti-glucagon (1:200, 14-9743-82, eBioscience), rabbit anti-PDX1 (1:100, 5679, Cell Signaling Technology), and rabbit anti-cleaved caspase3 (1:200, 9661, Cell Signaling Technology), followed by detection using Alexa Fluor 550 (BA1135, Boster, Wuhan, China) and 488-labeled (BA1127, Boster) secondary antibodies. Slides were counterstained with DAPI (R37606, Invitrogen), and images were captured using a confocal microscope (ZEISS LSM980, ZEISS). The data were presented as the positive area percentage by detecting the staining intensity with Image-Pro Plus software.

2.8 Immunoblot analysis

For immunoblot analysis of mouse pancreatic islets, equal amounts of extracted proteins were separated on SDS-PAGE, blotted, and detected with the following primary antibodies: IL18 (1:1000, ab207324, Abcam), NCC (1:1000, AB3553, Millipore), pAKT (1:1000, 4060, Cell Signaling Technology), AKT (1:1000,

9272, Cell Signaling Technology), PDX1 (1:1000, 5679, Cell Signaling Technology), and GAPDH (1:1000, 2118, Cell Signaling Technology), followed by appropriate HRP-conjugated secondary antibodies.

2.9 Real-time PCR

Total RNA was extracted from islets using Trizol reagent (9109, Takala, Osaka, Japan), and reverse transcribed into cDNA using cDNA Reverse Transcriptase (R323, Vazyme, China) following the manufacturer's instructions. Real-time PCR was performed using SYBR Green dye (1725125, Bio-Rad, Hercules, CA) on the Bio-Rad MyiQ2 Real-Time PCR System. Data were analyzed using the ΔΔCT method when GAPDH was used as the reference gene. All primer sequences for mouse samples used in this study are listed in Table 1.

Table 1. Mouse primers for quantitative real time-PCR.

Gene	Forward primers (5' to 3')	Reverse primers (5' to 3')
Ins2	TGGCTTCTTCTACACACCCAAG	TGGCTTCTTCTACACACCCAAG
Il18	CAGGCCTGACATCTTCTGCAA	CTGACATGGCAGCCATTGT
Ncc	CTTCGGCCACTGGCATTCTG	GATGGCAAGGTAGGAGATGG
Nlrp1	TGGCACATCCTAGGGAAATC	TCCTCACGTGACAGCAGAAC
Caspase1	AGCTCCAACCCTCGGAGAAA	AATACAACCACTCGTACACGTC
Pdx1	CCCCAGTTTACAAGCTCGCT	CTCGGTTCCATTCGGGAAAGG
Mafa	AGGAGGAGGTCATCCGACTG	CTTCTCGCTCTCCAGAATGTG
Ki67	AATCCAACTCAAGTAAACGGGG	TTGGCTTGCTTCCATCCTCA
Caspase3	TGGTGATGAAGGGGTCATTTATG	TTCGGCTTTCCAGTCAGACTC
GAPDH	TGTCATACTTGGCAGGTTTCT	TGTCATACTTGGCAGGTTTCT

2.10. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). The statistical significance was assessed using one-way ANOVA for comparisons involving one independent variable across multiple groups, and two-way ANOVA for comparisons with two independent variables across multiple groups. GraphPad Prism 9.0 software (GraphPad Software Inc., CA, USA) was used for all statistical analyses and $P < 0.05$ was considered statistically significant.

3. Results

3.1 Luteolin and kaempferol treatments promote insulin production in islet β cells.

To investigate the direct effects of dietary flavonoids on insulin production in islet β cells, we added six dietary flavonoids, including the flavones chrysin, apigenin, luteolin and flavonols quercetin, myricetin, kaempferol, to the cultured *ex vivo* islet isolated from WT mice. Similar to metformin at 20 μM, luteolin and kaempferol at 10 μM increased insulin secretion in WT islets cultured in medium containing basal glucose (2.8 mM) and high glucose (16.7 mM), while apigenin and myricetin promoted insulin production only in islets under high glucose challenge (Figure 1A). Thus, luteolin and kaempferol, the flavonoids with highest activities among the six dietary flavonoids, were chose to treat islets under high glucose for further investigation. Next, 1, 10, and 50 μM of luteolin and kaempferol were added to WT islets to evaluate their activities and cytotoxicity. From 1 μM to 50 μM, luteolin and kaempferol promoted insulin secretion only at 10 μM without inhibiting cell viability (Figure 1B-1D). Insulin and glucagon immunofluorescent staining

revealed that 10 μM luteolin and kaempferol treatment elevated insulin positive area in islet β cells (Figure 1E and 1F), but unaffected glucagon positive area in islet α cells (Figure 1G and 1H). Consistently, luteolin and kaempferol significantly upregulated insulin encoding gene *Ins2* expression in WT islets (Figure 1I). These results suggest that dietary luteolin and kaempferol promote insulin production in islet β cells.

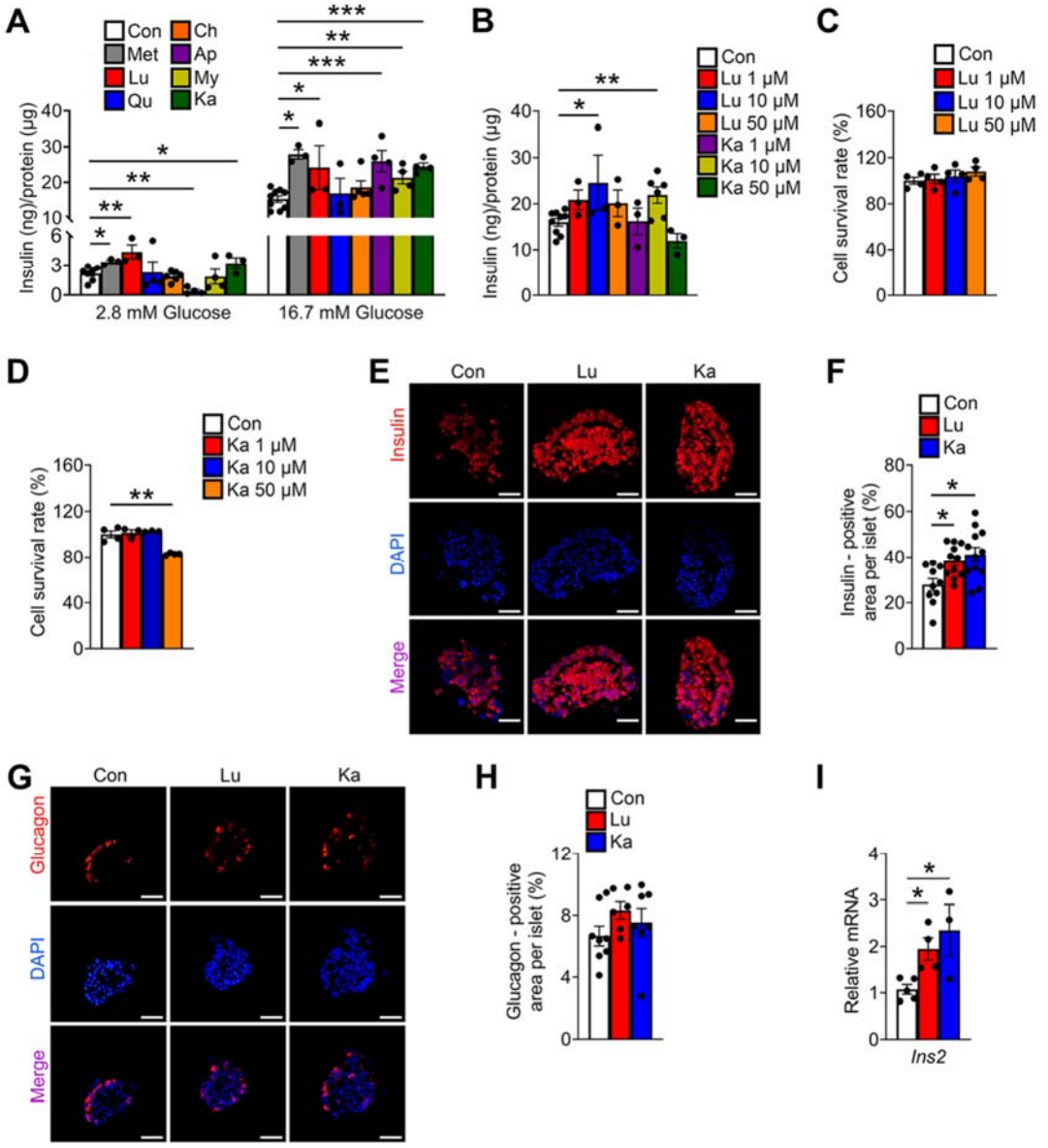

Figure 1. Luteolin and kaempferol treatments promote insulin production in WT islets. (A) Insulin levels in WT mouse islets cultured in 2.8 or 16.7 mM glucose with or without 20 μM metformin (Met), 10 μM luteolin (Lu), quercetin (Qu), chrysin (Ch), apigenin (Ap), myricetin (My), or kaempferol (Ka) for 12 hrs. (B) Insulin levels, (C) cell survival assay of Lu, and (D) cell survival assay of Ka in WT islets cultured in 16.7 mM glucose with or without 0, 10 or 50 μM Lu or Ka for 12 hrs. Immunofluorescent staining and quantification of (E and F) insulin and (G and H) glucagon, and (I) real time-PCR analysis of *Ins2* expression in islets cultured in 16.7 mM glucose with or without 10 μM Lu or Ka for 12 hrs. Scale bar, 100 μm. n = 3-4, each sample consisted of over 30 islets in (A-D), (I). n = 8-10 in (E-H). All data are represented as mean ± SEM. Statistical difference was determined by the One-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.2 Luteolin and kaempferol treatments activate IL18 signaling in islets.

IL18 mediates islet β cell insulin synthesis, proliferation and apoptosis via islet NCC [12]. To test whether dietary flavonoids promote insulin production through IL18 signaling, we detected the expression of IL18-NCC signaling in luteolin and kaempferol treated islets under high glucose. Luteolin and kaempferol

treatments promoted IL18 release in the culture medium (Figure 2A) and enhanced IL18 and NCC protein expressions (Figure 2B and 2C) in islets. The immunofluorescence co-localization of IL18 and glucagon indicated that IL18 originated from the α cells in the islets (Figure 2D). Moreover, IL18 positive area was notably elevated in WT islets treated by luteolin and kaempferol (Figure 2D and 2E). IL18 is produced by inflammasome-mediated activation of caspase1, such as NLR family pyrin domain containing 1 (NLRP1) inflammasome [24]. Accompanied by the increased *Il18* and *Ncc* mRNA expressions, the expressions of *Nlrp1* and *Caspase1* were also upregulated in WT islets after luteolin and kaempferol treatments (Figure 2F). Altogether, along with higher insulin production, luteolin and kaempferol treatments obviously activate IL18 production and increase NCC expression in islet.

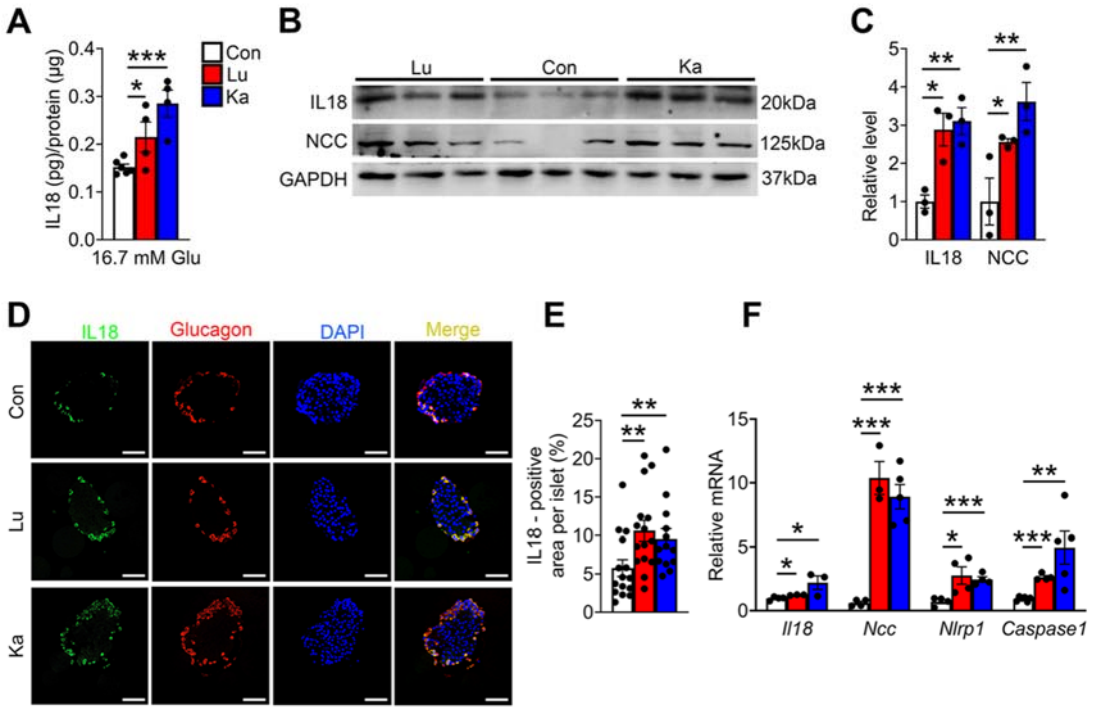

Figure 2. Luteolin and kaempferol treatments activate IL18 signaling in islets. WT islets were cultured in 16.7 mM glucose with or without 10 μM Lu or Ka for 12 hrs. (A) IL18 levels, (B) representative western blot and (C) the densitometry quantification of IL18 and NCC relative to GAPDH, (D) immunofluorescent staining of IL18 and Glucagon and (E) quantification of IL18-positive area, and (F) real time-PCR analysis of *Il18*, *Ncc*, *Nlrp* and *Caspase1* expression in WT islets. Scale bar, 100 μm. n = 3-4, each sample consisted of over 30 islets in (A-C) and (F). n = 10-12 in (D-E). All data are represented as mean ± SEM. Statistical difference was determined by the One-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.3 The deficiency of NCC impairs luteolin and kaempferol-induced insulin production and IL18 production.

We next examined the involvement of islet IL18 receptor NCC in the improvement of islet function by dietary flavonoids. We compared the insulin secretion and IL18 production in WT and *Ncc*$^{-/-}$ islets with luteolin and kaempferol treatments. Compared with the WT islets, NCC deficiency dramatically blocked islet insulin secretion, and prevented the facilitated effect of luteolin and kaempferol on insulin secretion in islets cultured in medium containing both basal glucose and high glucose (Figure 3A). Consistent with inhibition of insulin secretion in culture medium, NCC deficiency markedly depressed luteolin and kaempferol-induced *Ins2* expression (Figure 3B) and insulin positive area (Figure 3C and 3D) in cultured islets. Surprisingly, luteolin and kaempferol-induced *Il18* expression (Figure 3E) and IL18 positive area (Figure 3F and 3G) were

also prevented in *Ncc*$^{-/-}$ islets. These results demonstrate that luteolin and kaempferol treatments promote insulin production through IL18-NCC signaling.

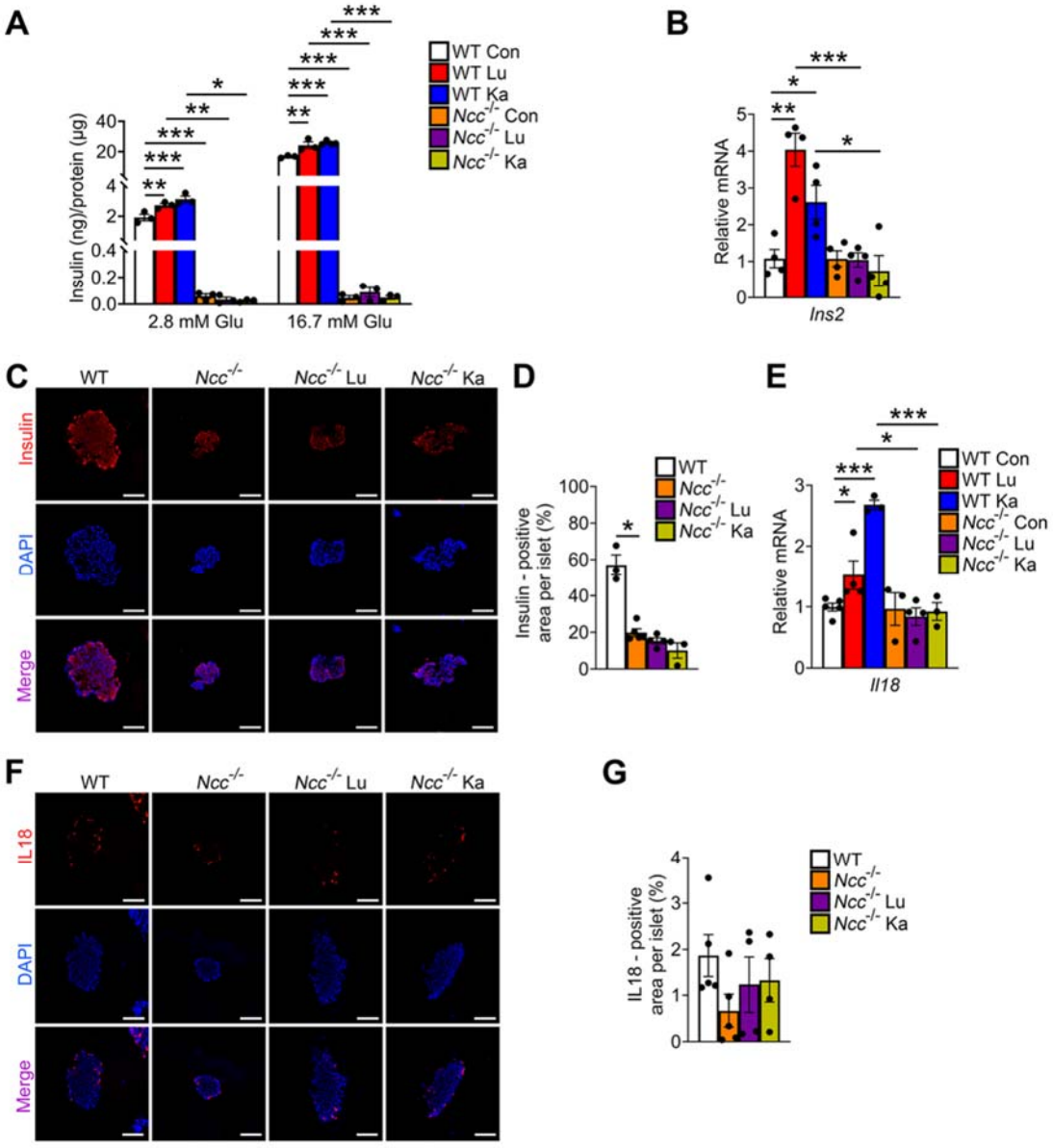

Figure 3. The deficiency of NCC impairs luteolin and kaempferol-induced insulin production and IL18 production. WT and *Ncc*$^{-/-}$ islets were cultured in 2.8 or 16.7 mM glucose with or without 10 μM Lu or Ka. (A) Insulin levels, (B) real time-PCR analysis of *Ins2* expression, (C) immunofluorescent staining and (D) quantification of insulin, (E) real time-PCR analysis of *Il18* expression, and (F) immunofluorescent staining and (G) quantification of IL18 in WT and *Ncc*$^{-/-}$ islets. Scale bar, 100 μm. n = 3-4, each sample consisted of over 30 islets in (A), (B) and (E). n = 3-5 in (C), (D), (F) and (G). All data are represented as mean ± SEM. Statistical difference was determined by the Two-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.4 Luteolin and kaempferol treatments enhance islet β cell proliferation and survival through IL18-NCC signaling.

IL18-NCC signaling activation promotes β cell proliferation and survival by activating the AKT pathway [12]. To understand the mechanism underlying luteolin and kaempferol-induced islet insulin production and IL18-NCC signaling activation, we investigated the effect of luteolin and kaempferol on islet β cell proliferation and survival, and relative AKT activity. Luteolin and kaempferol treatments increased the phosphorylation of AKT in the cultured islets from WT mice (Figure 4A and 4B). Pancreatic duodenal

homeobox protein 1 (PDX1) and V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA) are insulin transcription factors essential for β cell development and maturation [25]. Subsequent immunoblotting and immunofluorescence staining analyses showed that luteolin and kaempferol treatments augmented PDX1 expression (Figure 4A and 4C) and PDX1-positive area (Figure 4D and 4E) in the islets from WT mice. Furthermore, luteolin and kaempferol treatments enhanced the mRNA expressions of transcription factors *Pdx1* and *Mafa* (Figure 4F), and proliferation marker *Ki67* (Figure 4G) in WT islets. Surprisingly, these activates of luteolin and kaempferol were muted when the islets from *Ncc*$^{-/-}$ mice were used (Figure 4F and 4G). These observations support that luteolin and kaempferol treatments enhance β cell proliferation and survival, and relative AKT activity through IL18-NCC signaling.

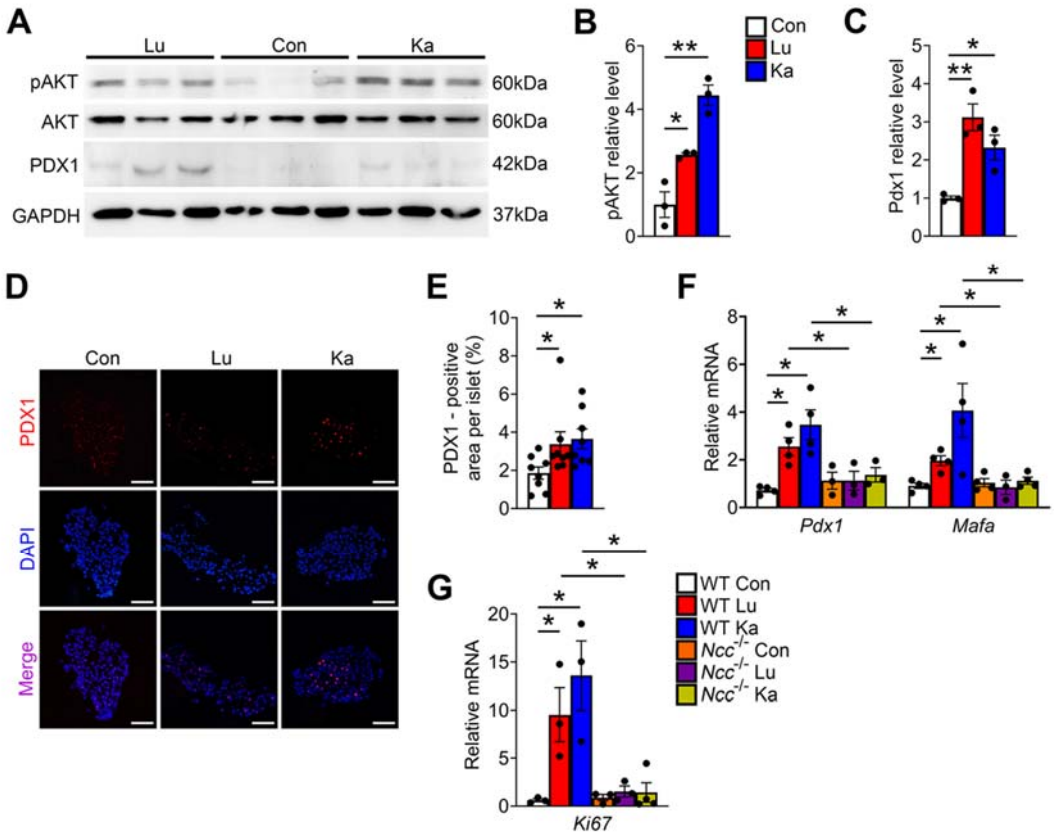

Figure 4 Luteolin and kaempferol treatments enhance islet β cell proliferation and survival through IL18-NCC signaling. (A) Representative western blot and (B) the densitometry quantification of pAKT relative to AKT, and (C) PDX1 relative to GAPDH, (D) immunofluorescent staining and (E) quantification of PDX1 in WT islets cultured in 16.7 mM glucose with or without 10 μM Lu or Ka for 12 hrs. Real time-PCR analysis of the expression of (F) islet survival marker *Pdx1* and *Mafa*, and (G) proliferation marker *Ki67* in WT and *Ncc*$^{-/-}$ islets cultured in 16.7 mM glucose with or without 10 μM Lu or Ka for 12 hrs. Scale bar, 100 μm. n = 3-4, each sample consisted of over 30 islets in (A-C), (F) and (G). n = 8 in (D) and (E). All data are represented as mean ± SEM. Statistical difference was determined by the One-way ANOVA for A-E and Two-way ANOVA for F and G, * $P < 0.05$, ** $P < 0.01$.

3.5 Luteolin and kaempferol treatments ameliorate islet apoptosis through IL18-NCC signaling.

IL18 improves β cell damage and apoptosis, and promotes insulin production in the STZ-treated islets [12]. To investigate whether luteolin and kaempferol protect against islet apoptosis through IL18-NCC signaling, islets were pretreated with luteolin and kaempferol, and subsequently stimulated with STZ to induce islet apoptosis in WT and *Ncc*$^{-/-}$ islets. As previously reported [12], STZ significantly decreased insulin secretion (Figure 5A) and aggravated β cell apoptosis (Figure 5B-5D) in cultured WT islets. Luteolin and kaempferol

treatments prevented STZ-induced decrease of insulin secretion (Figure 5A), elevation of cleaved caspase3 positive area (Figure 5B-5C), and upregulation of *Caspase3* gene expression (Figure 5D). Furthermore, STZ suppressed IL18 release (Figure 5E) and the mRNA expression of *Il18* and *Ncc* (Figure 5F), and luteolin and kaempferol treatments resisted the actions of STZ (Figure 5E and 5F) in WT islets. When the islets form *Ncc*$^{-/-}$ mice were used, the increased activities of luteolin and kaempferol on the insulin production (Figure 5G and 5H) and IL18 expression (Figure 5I and 5J) were completely blocked. These observations indicate that luteolin and kaempferol treatments protect against STZ-induced islet apoptosis and damage through IL18-NCC signaling.

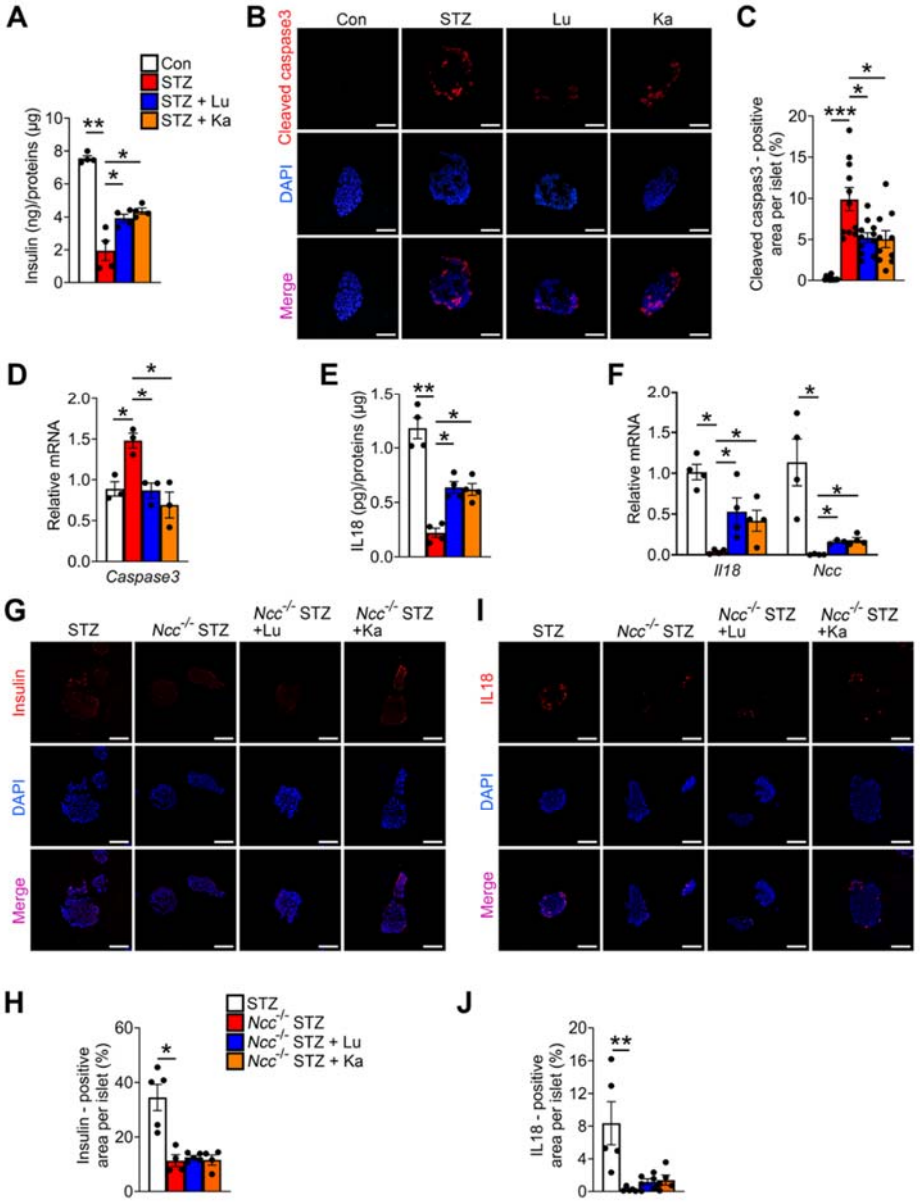

Figure 5 Luteolin and kaempferol treatments ameliorate islet apoptosis through IL18-NCC signaling. (A) Insulin levels, (B) immunofluorescent staining and (C) quantification of cleaved caspase3, (D) real time-PCR analysis of apoptosis marker *Caspase3* expression, (E) IL18 levels, and (F) real time-PCR analysis of *Il18* and *Ncc* expression in WT islets pretreated with or without 10 μM Lu or Ka for 24 hrs, subsequently stimulated with 50 mM STZ for 30 min. Immunofluorescent staining and quantification of (G and H) insulin and (I and J) IL18 in WT and *Ncc*$^{-/-}$ islets pretreated with or without 10 μM Lu or Ka for 24 hrs, subsequently stimulated with 50 mM STZ for 30 min. Scale bar, 100 μm. n = 3-4, each sample consisted of over 30 islets in (A) and (D-F). n = 5-10 in (B), (C) and (G-J). All data are represented as mean ± SEM. Statistical difference was determined by the two-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.6 Dietary luteolin and kaempferol supplementation improve glucose homeostasis and insulin production through IL18 signaling in hyperglycemic mice.

We further assessed the actions of dietary luteolin and kaempferol on glucose intolerance and hyperglycemia in mice, along with the relative IL18 signaling activity. To induce β cell depletion and establish hyperglycemia, a single intraperitoneal injection of a high dose of STZ was employed in WT mice [22]. 0.01% luteolin or kaempferol were supplemented to chow diet one week prior to STZ administration (Figure 6A). Compared with the control mice, dietary supplementation with luteolin or kaempferol did not affect body weight gain (Figure 6B), but obviously ameliorated hyperglycemia (Figure 6C), glucose intolerance (Figure 6D) and glucose-stimulated insulin secretion (GSIS) (Figure 6E), and significantly promoted insulin level in plasma (Figure 6F). Consistently, pancreatic insulin and glucagon immunostainings revealed that a higher insulin-positive area (Figure 6G and 6H), but unchanged glucagon-positive area (Figure 6G and 6I) in dietary luteolin and kaempferol supplemented mice. Along with the improvement of hyperglycemia, dietary supplementation with luteolin and kaempferol elevated IL18-positive area (Figure 6G and 6J) in islets and IL18 level in plasma (Figure 6K). Immunofluorescence double staining of insulin and cleaved caspase3 showed that dietary supplementation with luteolin and kaempferol inhibited STZ-induced β cell apoptosis (Figure 6L and 6M). Altogether, these findings suggest that dietary luteolin and kaempferol supplementation improve glucose homeostasis, insulin production, and β cell apoptosis in STZ-induced hyperglycemic mice through IL18-NCC signaling.

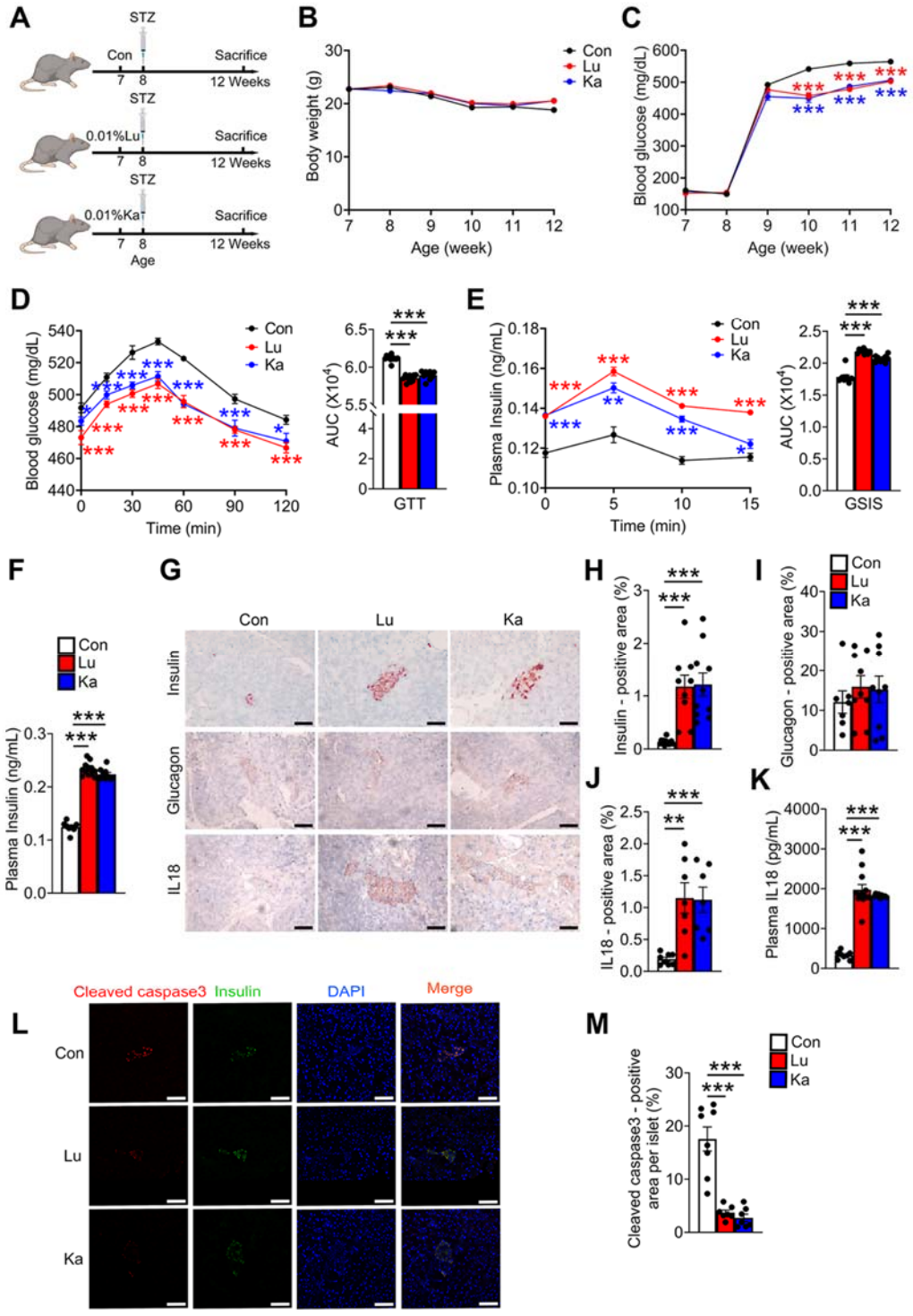

Figure 6 Dietary luteolin and kaempferol supplementation improve glucose homeostasis and insulin production through IL18 signaling in hyperglycemic mice. (A) WT mice were fed on a 0.01% Lu or 0.01% Ka-containing chow diet for 5 weeks from 7-week-old, and received an injection of a 180 mg/kg body weight STZ at 8-week-old. (B) Body weight, (C) blood glucose levels, (D) GTT, (E) GSIS, (F) plasma insulin levels, (G) immunohistochemical staining and (H) quantification of insulin, (I) glucagon and (J) IL18, (K) plasma IL18 levels, and (L) immunofluorescent double staining and (M) quantification of insulin and cleaved caspase3 in WT mice. Scale bar, 100 μm. n = 8. All data are represented as mean ± SEM. Statistical difference was determined by the One-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4. Discussion

Dietary flavonoids have been reported as effective supplements for prevention of diabetes based on in vitro and animal models [19]. It has been demonstrated that supplementation of dietary flavonoids, such as

apigenin, quercetin, and luteolin, ameliorate STZ-induced and HFD-induced hyperglycemia [19-21]. However, whether dietary flavonoids improve hyperglycemia through directly promoting insulin production in islet and the underlying mechanism remains unclear. In this study, among the six dietary flavonoids, luteolin and kaempferol showed the highest activities in stimulating insulin production in *ex vivo* islet. Meanwhile, luteolin and kaempferol treatment effectively promoted islet β cell insulin production and survival, inhibited STZ-induced islet apoptosis through IL18-NCC signaling. Finally, dietary supplementation with luteolin and kaempferol ameliorated glucose tolerance and insulin production, and increased islet IL18 expression in STZ-induced hyperglycemic mice.

Studies have demonstrated that luteolin and kaempferol could protect islet β cell from damages induced by high glucose, fatty acids, uric acid, and other factors. 10 μM luteolin improves glucose stimulated insulin secretion in uric acid-treated Min6 β cells and cultured mouse islets [20]. Meanwhile, 10 μM kaempferol promotes insulin secretion in palmitate-treated INS-1E β cells, RIN 5F β cells, human islets, and isolated rat pancreatic β cells and protects these cells from lipotoxicity-induced apoptosis and dysfunction [26, 27]. Similarly, 10 μM kaempferol improves insulin secretion and synthesis in INS-1E β cells and human islets under high glucose condition [28, 29]. In addition, kaempferol dose-dependently inhibits caspase-3 activity and apoptosis in INS-1E β cells and human islets under high glucose condition, with maximal effect observed at 10 μM [29]. Consistently, in this study, 10 μM luteolin or kaempferol significantly promotes insulin production in isolated mouse islet, and alleviates STZ-induced islet damage and apoptosis, whereas 50 μM luteolin reduces cell viability in islet (Figure 1). In mice, dietary supplementation with 0.01% luteolin improves HFD-induced insulin resistance and glucose intolerance [30]. Administration of 50 mg/kg/day of kaempferol by oral gavage for 12 weeks ameliorates STZ-induced hyperglycemia and reduces the incidence of diabetes in mice [31]. Here, we demonstrate that dietary supplementation with 0.01% luteolin or kaempferol promotes insulin production and IL18 expression in islets, and inhibites islet β cell apoptosis in STZ-induced hyperglycemic mice (Figure 6). Therefore, at above-mentioned doses, luteolin and kaempferol not only directly stimulate insulin production in β cell lines and *ex vivo* isolated rodent and human islets, but also protects against metabolic stress factors-induced islet damage and hyperglycemia. We found that chrysin, apigenin, quercetin, and myricetin were less effective than luteolin and kaempferol in promoting insulin secretion from islet β cells at 10 μM. Further analysis revealed that the efficacy of these flavonoids was concentration dependent. The efficacy of these flavonoids, however, was concentration- and context-dependent. For instance, 30 μM apigenin significantly enhanced glucose-stimulated insulin secretion and attenuated endoplasmic reticulum stress-mediated β cells apoptosis, thereby improving β cells survival and function [32] . Moreover, under a 16.7 mM glucose challenge, apigenin stimulated insulin secretion in a dose-dependent manner, with the most pronounced effect at 100 μM [33]. For quercetin, a 50 μM treatment restored the viability of INS-1 cells impaired by high glucose For quercetin, a 50 μM treatment restored the viability of INS-1 cells impaired by high glucose [34], while at 20 μM, it enhanced glucose-induced insulin secretion and protected INS-1 β cells from oxidative damage via the ERK1/2 pathway [35]. Finally, kaempferol, which did not stimulate insulin

secretion at 3 mM glucose, exhibited a significant promoting effect at the higher concentration of 16.7 mM, aligning with our observations [36].

We previously report that islet α cells is the major source of IL18 in the pancreas. IL18 together with the GLP1, uses the NCC and GLP1r on β cells to trigger β cell development and insulin secretion [12]. GLP1 directly regulates the proliferation of β cells and the expression of transcription factor PDX1. PDX1 interacts with GLP1r-dependent signaling pathways and cooperatively regulates the survival and growth of islet β cells [37, 38]. Here, we demonstrate that luteolin and kaempferol treatments induces islet insulin production, and enhances islet β cell proliferation and survival through IL18-NCC signaling. These findings suggest that luteolin and kaempferol may potentially activate both IL18 and GLP1 from α cells, and subsequently act on NCC and GLP1r in islets to regulate β cell functions, a hypothesis needs further verification. Moreover, IL18-NCC signaling increases PDX1 expression and β cells insulin secretion in islets by activating AKT and STAT3 [12]. The STAT3 signaling pathway regulates β-cell regeneration, proliferation and insulin secretion [39]. Overexpression of AKT1 in mouse β cells alleviates STZ-induced hyperglycemia and reduces β cell apoptosis [40]. Accordingly, in this study, luteolin and kaempferol treatments increase the expression of PDX1 and the phosphorylation of AKT in islet cells (Figure 4). Therefore, further investigation is required to determine whether the STAT3 signaling pathway is involved in luteolin and kaempferol-stimulated insulin production in islets.

Kaempferol has been reported to protect against palmitic acid-induced pancreatic β cell death through modulation of autophagy via AMPK/mTOR signaling pathway [41], and promote β cell survival through upregulating the PDX-1/cAMP/PKA/CREB signaling cascade [26]. Moreover, kaempferol treatment improves the expression of anti-apoptotic proteins Akt and Bcl-2 in β cells and human islets chronically exposed to high glucose [29], and suppresses 2-deoxy-D-ribose-induced intracellular ROS production, apoptosis, and lipid peroxidation in β cells [42]. In addition, kaempferol inhibits NF-κB-mediated iNOS gene transcription in β cells under exposure to interleukin-1β [43]. Similarly, luteolin attenuated uric acid-stimulated iNOS expression, NO formation, and activation of the NF-kB pathway in pancreatic β cells [20]. These findings indicate that kaempferol and luteolin also directly target β cells and effectively improve β cells survival and damage by regulating multiple signaling pathways involved in autophagy, apoptosis, oxidative stress, and inflammation. In this study, luteolin and kaempferol treatment promotes islet β cell insulin production, survival and proliferation, inhibits STZ-induced β cell apoptosis through IL18-NCC signaling. However, whether luteolin- and kaempferol-regulated IL18-NCC signaling interacts with the aforementioned pathways remains unknown. The potential crosstalk between IL18-NCC signaling and these pathways should be elucidated.

T1DM is a chronic autoimmune disease, during which the pancreatic β cell are selectively destroyed [6], and STZ is used experimentally to cause islet β cell destruction and produce a model of T1DM [44]. The development of TIDM involves a complex interaction between β cell and both the innate and adaptive immune systems. $CD4^+$ and $CD8^+$ T cells, as well as macrophages have been demonstrated to infiltrate to islets and contribute to β cell death [5]. $CD8^+$ T cells kill pancreatic β cells through MHC class I-mediated cytotoxicity,

and both $CD4^+$ and $CD8^+$ T cells produce interferon-γ (IFNγ) that induce chemokine production by β cells [5]. Macrophages have been shown not only produce IL12 and promote efficient differentiation of $CD8^+$ T cells leading to T1DM onset [45], but also produce IL-1β, TNF and ROS that can cause β cell death [5]. Our previous research has revealed that IL18 also uses the IL18r on acinar cells to block hyperglycemic pancreas macrophage expansion [12]. Studies have shown that luteolin and kaempferol effectively inhibits the proliferation and differentiation of $CD4^+$ and $CD8^+$ T cells [46, 47]. In this study, luteolin and kaempferol significantly promotes the synthesis of IL18 in islets, suggesting that luteolin and kaempferol may inhibit the pancreatic expansion of macrophages through the IL18-IL18r signaling pathway. Moreover, the anti-inflammatory activities of luteolin and kaempferol demonstrating that luteolin and kaempferol may also improve islet β cell function by inhibiting the activation and proliferation of macrophages, $CD4^+$ and $CD8^+$ T cells in islets. However, these hypotheses require further experimental investigation.

In summary, dietary luteolin and kaempferol promote insulin production in islets, enhance β cell proliferation and survival, protect against STZ-induced islet apoptosis and hyperglycemia through IL18-NCC signaling. These findings provide insights into the role of luteolin and kaempferol in islet insulin production and elucidating a novel mechanism underlying the anti-hyperglycemia actions of flavonoids. Targeting IL18-NCC signaling may provide a potential therapeutic strategy for ameliorating hyperglycemia and diabetes.

Conflict of interest

None declared.

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