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Small molecule kaempferol modulates PDX-1 protein expression and subsequently promotes pancreatic β -cell survival and function via CREB

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Abstract

Chronic hyperlipidemia causes β -cell apoptosis and dysfunction, thereby contributing to the pathogenesis of T2D. Thus, searching for agents to promote pancreatic β -cell survival and improve its function could be a promising strategy to prevent and treat T2D. We investigated the effects of kaempferol, a small molecule isolated from ginkgo biloba, on apoptosis and function of β -cells and further determined the mechanism underlying its actions. Kaempferol treatment promoted viability, inhibited apoptosis, and reduced caspase-3 activity in INS-1E cells and human islets chronically exposed to palmitate. In addition, kaempferol prevented the lipotoxicity-induced down-regulation of anti-apoptotic proteins Akt and Bcl-2. The cytoprotective effects of kaempferol were associated with improved insulin secretion, synthesis, and PDX-1 expression. Chronic hyperlipidemia significantly diminished cAMP production, PKA activation, and CREB phosphorylation and its regulated transcriptional activity in β -cells, all of which were restored by kaempferol treatment. Disruption of CREB expression by transfection of CREB siRNA in INS-1E cells or adenoviral transfer of dominant-negative forms of CREB in human islets ablated kaempferol protection of β -cell apoptosis and dysfunction caused by palmitate. Incubation of INS-1E cells or human islets with kaempferol for 48 h induced PDX-1 expression. This effect of kaempferol on PDX-1 expression was not shared by a host of structurally related flavonoid compounds. PDX-1 gene knockdown reduced kaempferol-stimulated cAMP generation and CREB activation in INS-1E cells. These findings demonstrate that kaempferol is a novel survivor factor for pancreatic β -cells via up-regulating the PDX-1/cAMP/PKA/CREB signaling cascade.

Keywords

kaempferol; palmitate; β -cells; islets; cAMP; CREB; PDX-1

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1. Introduction

Diabetes mellitus is a major chronic disease, presently affecting at least 23.6 million, or 7.8%, of the American population and about 150 million people worldwide [1], and this number is projected to double by 2025 [2]. Loss of β -cell mass and function through apoptosis is central to the development of both type 1 and type 2 diabetes (T2D) [3-5]. While peripheral insulin resistance is common during obesity in rodents and people, its progression to T2D is largely due to insulin secretory dysfunction and significant apoptosis of functional β -cells [3, 6-9], leading to an inability to compensate for insulin resistance. Indeed, those with T2D always manifest increased β -cell apoptosis and reduced β -cell mass [7-8, 10-12]. Past studies have established that sustained hyperlipidemia in obese individuals plays an important role in causing β -cell apoptosis and dysfunction, thereby contributing to the deterioration of glycemic control and the overt development of T2D [13-15]. Hence, a strategy that promotes β -cell survival and mass can potentially provide a therapeutic means to prevent the onset of diabetes.

Kaempferol is a flavonol that is present in various plants [16], including grapefruit, ginkgo biloba, and some edible berries [17]. While studies regarding the health benefits of this compound are limited, it has been reported that kaempferol has anti-oxidative [18], anti-inflammatory [19], lipolytic [20], and anti-cancer effects [21-22]. Studies on whether this compound possesses an anti-diabetic property are lacking.

In this study, we investigated the effects of kaempferol on apoptosis and function of β -cells and further determined the mechanism underlying its actions. The results show that kaempferol treatment prevented apoptosis and improved insulin biosynthesis and secretion in β -cells and human islets with lipotoxicity. These beneficial effects of kaempferol are mediated through enhancing the activity of the cAMP-responsive element binding-protein (CREB). We further show that kaempferol up-regulates pancreatic and duodenal homeobox-1 (PDX-1) expression, which then mediates kaempferol-elicited cAMP signaling.

2. Materials and Methods

2.1. Reagents

Cell culture medium, supplements, palmitate, fatty acid-free bovine serum albumin (BSA), and protease and phosphatase inhibitor cocktails were from Sigma (St. Louis, MO); kaempferol was purchased from Tauto Biotech Co. (Shanghai, China, purity 98.0%). Its purity and identity were verified by HPLC and nuclear magnetic resonance spectroscopy, respectively; insulin ELISA kits were obtained from Mercodia Inc. (Winston-Salem, NC); cell viability assay kits, cAMP-dependent protein kinase A (PKA) assay reagents, and the dual-reporter luciferase assay system were purchased from Promega (Madison, WI); cyclic AMP EIA kits were from Cayman Chemical Company (Ann Arbor, MI); caspase-3 assay kits were from Invitrogen (Carlsbad, CA); terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kits were from Trevigen Inc. (Gaithersburg, MD); adenoviral cAMP-response reporter gene construct (Ad-CRE-Luc) and renilla control virus (Ad-pRL-Luc) were purchased from Vector Biolabs (Philadelphia, PA); replication-deficient adenovirus containing dominant-negative CREB (MCREB) that is mutated at the phosphorylation site (S133) was provided by Jane Reusch (University of Colorado, Denver, CO); CREB ShortCut® siRNA and transfection reagent were from New England Biolabs (Ipswich, MA); PDX-1 siRNA was from Santa Cruz (Santa Cruz, CA); antibodies against Akt, Bcl-2, PDX-1, phospho-CREB, CREB, and β -actin were bought from Cell Signaling Technology (Beverly, MA). Stock solutions of kaempferol at 100 mM dissolved in dimethyl sulfoxide (DMSO) were stored at -20°C .

2.2. Culture of INS-1E cells and human islets

INS-1E cells (provided by Dr. Pierre Maechler, University of Geneva Medical School) were cultured in RPMI-1640 medium containing 11.1 mM D-glucose, 10% FBS, 10mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 µM 2-mercaptoethanol as previously described [23]. Human islets were isolated from cadaver organ donors in the Islet Cell Resource Centers and provided through the Integrated Islet Distribution Program, which is funded by National Institute of Health and the Juvenile Diabetes Research Foundation International and administrated by Administrative and Bioinformatics Coordinating Center (City of Hope Medical Center, Duarte, CA). The islet purity was 80-90 % and viability was 90-97 %. The islets were maintained in CMRL-1066 medium containing 10% FBS. Palmitate was dissolved to 7 mM in medium containing 7% fatty acid-free BSA, sonicated, and sterile-filtered before use (final fatty acid/BSA molar ratio=6.6) [24]. For control experiments, BSA in the absence of palmitate was prepared in the same way as stated above. The control condition contained the same concentration of BSA, which alone had no effect on any of the variables examined in this study (data not shown). For glucose-stimulated insulin secretion (GSIS), INS-1E cells and human islets were pre-incubated in glucose-free Krebs-Ringer bicarbonate (KRB) buffer (129 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 5 mM NaHCO₃, 0.1% BSA, 10 mM HEPES, pH 7.4) for 1 h at 37°C before assay [25].

2.3. Cell viability assay

INS-1E cells or human islets were cultured in complete RPMI-1640 medium with or without 0.5 mM palmitate in the absence or presence of kaempferol (0.1, 1, and 10 µM) for 4 d in 96-well plates, with the medium replaced every other day. The cell viability was determined by measuring reduction of resazurina to resorufin using a cell viability assay kit.

2.4. Cell apoptosis assay

For apoptosis experiments, cells or islets were treated as above. Cellular apoptosis was measured with a TUNEL assay kit, which provides colorimetric detection of DNA fragmentation and quantification of apoptotic cells.

2.5. Caspase-3 activity assay

Caspase-3 activity in the cells or islets treated was measured using a caspase-3 assay kit according to the manufacturer's instruction. The caspase-3 activity in the cell lysates was recorded with a fluorescence plate reader and was normalized to the cellular protein concentration, measured by using a Bio-Rad assay kit.

2.6. Intracellular ATP and cAMP measurements

For intracellular ATP and cAMP measurements, cells or islets treated as above were washed in KRB buffer. Intracellular ATP was measured using a bioluminescence ATP assay kit per the manufacturer's instruction. Intracellular cAMP levels were measured by an EIA kit as previously described [26]. All data were normalized to the cellular protein concentration.

2.7. PKA activity assay

Cells were collected in phosphate-buffered saline (PBS) supplemented with protease (1:500) and phosphatase inhibitor (1:200) cocktails. Protein was extracted and harvested by sonication and centrifugation. The enzymatic activity of PKA in lysates was assessed by measuring phosphorylation of kemptide as we previously described [26].

2.8. Insulin secretion and content assays

INS-1E cells or human islets were cultured in RPMI-1640 medium with or without 0.5 mM palmitate in the absence or presence of 10 μ M kaempferol for 4 d. Cells and islets were then incubated in KRB buffer containing 3 mM or 20 mM glucose at 37°C for 30 min. Insulin secreted in supernatants was measured by an ELISA kit. For intracellular insulin content measurement, cells or islets were lysed in lysis buffer (50 mM HEPES, 0.1% (v/v) Triton X-100, 1 mM PMSF, 10 mM E-64, 10 mM pepstatin A, 10 mM TLCK, 100 mM leupeptin, pH 7.4), and cellular proteins were extracted and harvested by sonication and centrifugation. Insulin in cell extracts was measured as stated above. All data were normalized to the cellular protein concentration.

2.9. Western blot analysis

Equal amounts of protein from cell extracts were subjected to Western blot analysis. Membranes were probed with antibodies against Akt, Bcl-2, PDX-1, phosphorylated-CREB, or CREB. The immunoreactive proteins were detected by chemiluminescence. Nitrocellulose membranes were then stripped and reprobed with β -actin. The protein bands were digitally imaged for densitometric analysis with a software program (ImageJ, NIH). All proteins levels were normalized to those of β -actin from the same samples.

2.10. siRNA transfection

Exponentially growing INS-1E cells were transfected with a heterogeneous mixture of target-specific CREB siRNA (50 nM), PDX-1 siRNAs (80 nM), or corresponding amounts of scrambled siRNA by using siRNA transfection reagents according to the manufacturers' protocols. After 2 d of transfection, cells were used for immunoblot analysis of CREB and PDX-1 protein expression, or treated with palmitate (0.5 mM) in the presence or absence of kaempferol (10 μ M) for 4 d followed by assays for cellular apoptosis, GSIS, or immunoblot analysis as described above.

2.11. Adenoviral infection of β -cells and human islets

INS-1E cells were co-infected with Ad-CRE-luc or Ad-pRL-Luc at 50 multiplicity of infection (MOI)/cell in RPMI medium. After 12 h of infection, cells were cultured in complete RPMI-1640 medium containing 0.5 mM palmitate with or without kaempferol (0.1, 1, 10 μ M) for 4 d. Luciferase activity, normalized to pRL activity in the cell extracts, was determined by using the dual luciferase assay system. Adenovirus containing MCREB were infected as previously described [27]. For determining infection efficiency, human islets were exposed to purified adenovirus at 10-500 MOI/cell in RPMI medium for 1 h at 37 °C and then cultured in RPMI containing 5% FBS for 24 h. For MOI calculation, it was assumed that each islet equivalent consists of 1000 cells. After infection, the islets were lysed for measuring the phosphorylation of CREB. For function analysis, islets were infected with adenoviral MCREB or β -gal at 50 MOI/cell, followed by treatment with 0.5 mM palmitate in the presence or absence of 10 μ M kaempferol for 3 d. Cellular apoptosis and insulin secretion were measured.

2.12. Statistical analysis

Data were analyzed with one-way ANOVA, using the Proc Mixed procedure of SAS program, and are expressed as mean $\pm$ standard error of mean (SEM). Treatment differences were subjected to the Tukey's test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Kaempferol protects β -cells and human islets from palmitate-induced apoptosis

To determine whether kaempferol is a survival factor for β -cells, we first evaluated whether kaempferol protects INS-1E cells exposed to chronic hyperlipidemia. Incubation of β -cells with palmitate (0.5 mM) for 4 d significantly reduced cell viability. Addition of kaempferol restored palmitate-induced reductions in cell viability (Fig. 1A), with 1 μ M kaempferol exerting a significant effect, suggesting that kaempferol may be capable of protecting against hyperlipidemia-induced β -cell toxicity. We further examined the effect of kaempferol on palmitate-induced β -cell function alterations by measuring mitochondrial ATP generation. Consistent with the impaired viability, chronic exposure to high concentrations of palmitate ablated intracellular ATP production in INS-1E cells, but this detrimental effect was prevented by treatment with kaempferol (Fig. 1B). However, intracellular ATP production in INS-1E cells exposed to unconditioned medium was not altered by kaempferol (data not shown). To further confirm the protective effect of kaempferol on lipotoxicity, we performed TUNEL assays to directly measure apoptotic cells. Exposure of INS-1E cells to palmitate for 4 d increased apoptosis, which was significantly reduced by treatment with kaempferol (Fig. 1C). The caspase family proteins are critical components of the apoptotic signaling cascade, and caspase-3 is one of the key proteases involved in the convergence of disparate apoptotic signaling pathways [28]. In parallel to decreased cell viability, the cellular caspase-3 activity was significantly increased after chronic exposure of INS-1E cells to palmitate. Co-incubation of INS-1E cells with kaempferol significantly reduced caspase-3 activity (Fig. 1D). This result further supports the concept that kaempferol exerts an anti-apoptotic effect on insulin-secreting cells.

To determine whether kaempferol has an anti-apoptotic effect on islets, we cultured freshly isolated human islets with palmitate in the presence or absence of kaempferol for 4 d. The results show that kaempferol promoted viability (Fig. 2A), preserved ATP generation (Fig. 2B), prevented apoptosis (Fig. 2C), and inhibited caspase-3 activity (Fig. 2D) in the islets, suggesting that the protective effects of kaempferol is not restricted to insulinoma cells. Consistent with the observations in INS-1E cells, intracellular ATP production in human islets cultured in basal medium was not altered by kaempferol treatment (data not shown).

3.2. Kaempferol restores palmitate-mediated reduction of Akt And Bcl-2 proteins in β -cells and human islets

As up-regulation of anti-apoptotic proteins Akt and Bcl-2 has been identified as a critical mechanism by which growth factors protect cells against apoptosis [29-31], we next tested whether kaempferol has an effect on Akt and Bcl-2 protein expression in cells and islets cultured in palmitate. Consistent with its effect on cellular apoptosis, kaempferol mitigated the effects of palmitate on abundance of Akt and Bcl-2 proteins in INS-1E cells (Figs. 3A and 3B) and human islets (Figs. 3C and 3D).

3.3. Kaempferol reverses palmitate-induced impairment of β -cell function

We evaluated whether the cytoprotective effect of kaempferol on β -cells is concomitant with improved cellular function. Chronic exposure of INS-1E cells to 0.5 mM palmitate for 4 d abolished acute glucose-stimulated insulin secretion (GSIS) (Fig. 4A). Addition of kaempferol completely restored GSIS. Insulin content of INS-1E cells cultured with palmitate was decreased by 67% compared with the control and was partly restored by treatment with kaempferol (Fig. 4B). In addition, kaempferol restored palmitate-attenuated PDX-1 protein expression (Fig. 4C), a transcription factor that regulates insulin transcription in β -cells [32]. Consistent with these beneficial effects on INS-1E cells, addition of kaempferol also improved GSIS (Fig. 4D), insulin content (Fig. 4E), and PDX-1 protein

expression in human islets cultured with palmitate (0.5 mM) for 4 d. However, kaempferol had no effects on GSIS in INS-1E cells and human islets exposed to the basal medium for the same length (data not shown).

3.4. Kaempferol restores palmitate-mediated impairment of the cAMP/PKA/CREB Signaling Pathway in β -cells

The cAMP signaling pathway plays a critical role in promoting β -cell survival and in regulating insulin synthesis and secretion. To further investigate the underlying mechanism by which kaempferol promotes β -cell survival and function, we evaluated whether treatment with this compound influenced the cAMP signaling pathway. Incubation of INS-1E cells in the presence of palmitate for 4 d led to a reduction in intracellular cAMP production (Fig. 5A). However, addition of kaempferol significantly attenuated this detrimental effect caused by palmitate. To investigate whether the restoration of cAMP production by kaempferol subsequently improves its downstream signals, we first assessed the activity of PKA in INS-1E. As shown in Fig. 5B, kaempferol improved the palmitate-blunted PKA activity. To investigate whether increased activation of PKA by kaempferol yields higher CREB phosphorylation, we performed Western blots using a phospho-CREB antibody that only recognizes CREB when phosphorylated at serine 133. In INS-1E cells incubated with palmitate (0.5 mM) for 4 d, there was a 57% reduction in CREB phosphorylation, which was improved at 1 μ M kaempferol and completely restored to the normal level at 10 μ M (Fig. 5C). However, neither palmitate nor addition of kaempferol affected the total CREB protein content. Pretreatment of cells with PKA inhibitor H89 completely abolished the CREB phosphorylation stimulated by kaempferol (data not shown), suggesting that PKA mediates the kaempferol-stimulated phosphorylation of CREB. Since activation of CREB mediates cAMP-regulated gene expression, we then tested whether this change in cAMP and subsequent activation of CREB by kaempferol is sufficient to affect cAMP-regulated gene transcription. INS-1E cells were infected with Ad-CRE-Luc containing a series of repeats of the consensus binding site for CRE in a synthetic promoter fused to luciferase and control virus Ad-pRL-Luc, followed by incubation with palmitate and kaempferol. Consistent with the effects on cAMP production and CREB phosphorylation, lipotoxicity reduced CREB-mediated gene transcription in β -cells, which was completely prevented by kaempferol (Fig. 5D). These data demonstrate that chronic palmitate-induced down-regulation of CREB-mediated transcriptional activity is due to impaired CREB phosphorylation, and that kaempferol prevents this deleterious effect by protecting cAMP signaling.

3.5. Kaempferol protection of β -cells from apoptosis and dysfunction is mediated by CREB

Next, we examined whether kaempferol protection of cell apoptosis is mediated by CREB. To that end, we used siRNA to abolish the expression of CREB in INS-1E cells. Transfection of β -cells with CREB siRNA reduced CREB protein expression by $79 \pm 4\%$ whereas the scrambled siRNA showed no effect. As a result, CREB phosphorylation was decreased by $71 \pm 5\%$ (Fig. 6A). Accordingly, ablation of CREB blocked the effects of kaempferol on CREB phosphorylation (Fig. 6B), apoptosis (Fig. 6C), GSIS (Fig. 6D), and insulin content (Fig. 6E), in INS-1E cells, whereas transfection with a scrambled siRNA did not alter these effects of kaempferol. Additionally, disruption of CREB expression with siRNA blunted the effects of kaempferol on Bcl-2 protein expression in INS-1E cells (Fig. 6F).

3.6. Kaempferol stimulates cAMP generation and CREB phosphorylation through up-regulation of PDX-1 expression

To determine how kaempferol improves the cAMP signaling pathway, we examined if kaempferol directly activates cAMP signaling in INS-1E cells. Incubation of INS-1E cells with kaempferol for up to 24 h had no significant effect on cAMP synthesis (data not

shown). Interestingly, cAMP generation was greatly increased in cells treated with kaempferol for 48 h (Fig. 7A). The effect of chronic exposure of INS-1E cells to kaempferol on intracellular cAMP levels was also accompanied by increased PDX-1 protein expression (Fig. 7B). We then examined the role of PDX-1 in kaempferol-stimulated cAMP signaling by transfecting INS-1E cells with siRNA specific to PDX-1, which ablated PDX-1 protein expression (Fig. 7C). PDX-1 knockdown with siRNA in INS-1E cells diminished kaempferol-induced increases in intracellular cAMP levels (Fig. 7D), whereas inhibition of the PI3 kinase/Akt pathway had no effect (data not shown). Furthermore, kaempferol-induced CREB phosphorylation was blocked by PDX-1 knockdown (Fig. 7E).

3.7. Kaempferol increases PDX-1 expression, improves cAMP signaling, and subsequently prevents apoptosis of human islets exposed to chronic palmitate

Exposure of human islets to kaempferol for 48 h increased PDX-1 expression (Fig. 8A). In addition, kaempferol improved intracellular cAMP production impaired by chronic palmitate in human islets (Fig. 8B). To further confirm the role of CREB in mediating the kaempferol effects on islets, human islets were infected with adenoviral MCREB mutated at the phosphorylation site (S133) [27]. As shown in Fig. 8C, over-expression of MCREB in human islets clearly reduced the phosphorylation of CREB, whereas no such a change was detected in islets infected with β -gal. Accordingly, mutation of the CREB phosphorylation greatly reduced the anti-apoptotic effect of kaempferol in human islets (Fig. 8D), whereas the control virus was inactive. These results further support a central role of PDX-1 and CREB in mediating kaempferol's protective actions in β -cells.

3.8. The effect of kaempferol on PDX-1 expression is not shared by other structurally related flavonoid compounds

To determine the specificity of this novel effect of kaempferol on PDX-1, we first tested a host of structurally related natural flavonoid compounds in parallel to kaempferol on PDX-1 protein expression in INS-1E cells and human islets, including flavones (apigenin, luteolin), flavanones (naringenin, hesperetin), flavanols (epicatechin, epigallocatechin gallate), and flavonols (quercetin, kaempferol). We found that only kaempferol significantly increased the expression of PDX-1 in both INS-1E cells (Fig. 9A) and human islets (Fig. 9B). These results demonstrated the unique effect of kaempferol on PDX-1 expression in β -cells that is possibly not shared by other structurally related flavonoid compounds.

4. Discussion

Insulin resistance is common in experimental animals and people with obesity. However, chronic insulin resistance will not progress to T2D unless β -cells are unable to secrete adequate amounts of insulin to compensate for decreased insulin sensitivity, which is largely due to insulin secretory dysfunction and significant loss of functional β -cells [3, 6-9]. Chronic exposure of β -cells to high levels of saturated fatty acids has been shown to induce β -cell apoptosis and impair cellular function, thereby contributing to the pathogenesis of T2D [33-35]. We demonstrated for the first time that kaempferol at relatively low concentrations (1-10 μ M) promoted survival and function of INS-1E cells and human islets upon lipotoxicity. This finding is important and may be physiologically relevant since the attainable plasma concentrations of flavonoids in both humans and rodents through dietary supplementation are typically within the several micromolar range. Therefore, it is intriguing to speculate that the beneficial effects of kaempferol on islet survival and function *ex vivo* could also be achieved in the relevant environment *in vivo*.

Akt and Bcl-2 are well known anti-apoptotic proteins and up-regulation of these molecules has been identified as a critical mechanism by which some growth factors protect cells

against apoptosis [30-31]. Our data showed that the expression of Akt and Bcl-2 was down-regulated in INS-1E cells and human islets chronically exposed to palmitate, which is concomitant with palmitate-induced apoptosis. Consistent with the cytoprotective effect of kaempferol, kaempferol restored Akt and Bcl-2 protein expression. These findings suggest that kaempferol likely prevents β -cell apoptosis by preserving these critical pro-survival molecules.

Recent studies showed that the transcriptional factor CREB plays a pivotal role in β -cell survival [30-31, 36]. However, it is unclear whether lipotoxicity in β -cells is mediated by changes in CREB expression and activity. In the present study, we demonstrated that chronic exposure of β -cells to palmitate severely impaired CREB phosphorylation and its transcriptional activity associated with apoptosis and impaired β -cell function. Notably, we found that these deleterious effects of palmitate were completely prevented by co-incubation with kaempferol. This result suggests that kaempferol protection of β -cells may be mediated by the preservation of functional integrity of CREB. Indeed, depletion of CREB expression with siRNA completely blocked the protective effects of kaempferol on survival and function in INS-1E cells and human islets, suggesting that the preservation of CREB function is essential for maintenance of β -cell survival and function. Consistent with this, we found that virus- or siRNA-mediated disruption of CREB function ablated the reversal of palmitate-mediated down-regulation of Bcl-2 in INS-1E cells and human islets, demonstrating that Bcl-2 is a downstream effector of CREB in the kaempferol signaling cascade. Indeed, a cAMP-response-element (CRE) site is present within promoter region of Bcl-2 and the activated CREB binds to the CRE site to positively regulate Bcl-2 transcriptional activity [37-38].

In β -cells, CREB phosphorylation at S133, which is required for CRE-regulated transcription, can be induced by activation of the PI3K/Akt pathway [29, 37-38]. In this study, the effect of kaempferol on CREB phosphorylation should be mainly dependent on activation of PKA because this effect was completely prevented by inhibition of PKA activity. PKA is generally activated in response to elevated levels of intracellular cAMP. Consistently, intracellular cAMP production was significantly reduced in both INS-1E cells and human islets exposed to chronic hyperlipidemia, but this effect was greatly attenuated by treatment with kaempferol.

Experimental evidence shows that islets have relatively low levels of intrinsic antioxidant enzyme expression than other cell types [13], and that fatty acid-induced β -cell lipotoxicity is mediated at least partially via the formation of reactive oxygen species (ROS) [39]. As various flavonoids are reportedly ROS scavengers, we considered the possibility that the protective effects of kaempferol on cAMP signaling and subsequent cellular survival might be due to a secondary action whereby it scavenges ROS as an antioxidant. However, kaempferol is not an effective anti-oxidant at physiologically relevant doses used in the present study as determined by measuring its ability to scavenge ROS (data not shown).

As we observed that intracellular cAMP content in kaempferol-treated cells under lipotoxicity was even higher than that in normal control cells, we speculate that kaempferol may directly stimulate cAMP production. However, incubation of INS-1E cells with kaempferol for up to 24 h had no significant effect on intracellular cAMP levels. Interestingly, cAMP contents were greatly increased in cells treated with kaempferol for 48 h (Fig. 7A), suggesting an indirect effect of kaempferol on cAMP production that may be mediated via a genomic mechanism. We demonstrated that kaempferol also increased PDX-1 protein expression in INS-1E cells and human islets cultured without palmitate, suggesting that kaempferol may have a direct regulatory effect on PDX-1. However, it is unclear how kaempferol is able to regulate PDX-1 in β -cells. It was shown that kaempferol

is an activator of SIRT1 [40], the mammalian ortholog of yeast silent information regulator (Sir2) and a protein deacetylase. SIRT1 is involved in energy metabolism and glucose homeostasis. In pancreatic β -cells, activation of SIRT1 was able to increase PDX-1 expression [41]. Therefore, it is speculative that kaempferol may up-regulate PDX-1 protein via activation of SIRT1. Another possibility is that kaempferol may enhance PDX-1 expression through the Akt/Forkhead transcription factor O1 (FoxO1) pathway. FOXO1 is a negative regulator of *Pdx-1* transcription through competing with forkhead box protein A2 for the binding sites in the *Pdx-1* promoter [42-43]. The transcriptional activity of FoxO1 is inhibited upon phosphorylation by Akt and subsequent nuclear exclusion [44]. However, whether Akt-dependent inactivation of FoxO1 was responsible for kaempferol effect on PDX-1 expression remains to be determined.

This effect of kaempferol is novel and important, given that its structurally related flavonoids had no such an action. Studies in INS-1E cells indicated that PDX-1 played an essential role in mediating kaempferol's effects on cAMP signaling and subsequent cytoprotective effect as siRNA-mediated deletion of PDX-1 diminished kaempferol-up-regulated cAMP production and CREB activation. These findings are consistent with previous observations that up-regulation of PDX-1 expression is required for exendin-4-stimulated cAMP production in pancreatic β -cells [45-46].

It is well characterized that glucose induces insulin secretion through glycolysis and mitochondrial oxidation in β -cells. These events cause the increase in intracellular ATP/ADP ratio, which sequentially leads to closure of K_{ATP} channels, depolarization of voltage-gated L-type Ca^{2+} channels on the plasma membrane, Ca^{2+} influx, and exocytosis of insulin-containing granules [47-48]. Therefore, the effect of kaempferol on insulin secretion may be partially mediated through improving chronic palmitate-impaired β -cell survival, thereby mitochondria metabolism and subsequent ATP generation. We show that kaempferol restored the palmitate-induced reduction in insulin content, which could also contribute to the improved insulin secretion by this compound, given that the decreased insulin expression impairs insulin release [49]. Cyclic AMP induces insulin transcription through the CRE site within the gene promoter [50]. In addition, PDX-1 also stimulates transcription of insulin gene [51]. Thus, the effect of kaempferol on insulin content in β -cells and islets cultured under elevated palmitate conditions is likely due to the increase in the transcriptional activation of insulin mediated by PDX-1 and cAMP signaling. Given the critical roles of PDX-1 in β -cell differentiation, proliferation, and normal function and the evidence that PDX-1 reduction impairs glucose homeostasis in both rodents and humans [52], it is tempting to speculate that kaempferol may have therapeutic potential for T2D prevention and treatment.

In summary, we provide evidence for the first time that kaempferol can protect pancreatic β -cells from palmitate-induced apoptosis and dysfunction. Kaempferol modulates PDX-1 expression and subsequently restores variables associated with enhanced β -cell survival and function, including improved cAMP signaling and anti-apoptotic protein expression. These studies demonstrate that the naturally occurring small molecule kaempferol may be a novel agent targeting PDX-1 to promote survival and function of pancreatic β -cells.

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References

1. ADA. Total Prevalence of Diabetes & Pre-diabetes. 2008. <http://www.diabetes.org/diabetes-statistics/prevalence.jsp>
2. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care*. 1998; 21:1414-31. [PubMed: 9727886]
3. Stoffers DA. The development of beta-cell mass: recent progress and potential role of GLP-1. *Horm Metab Res*. 2004; 36:811-21. [PubMed: 15655713]
4. Donath MY, Halban PA. Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. *Diabetologia*. 2004; 47:581-9. [PubMed: 14767595]
5. Muoio DM, Newgard CB. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat Rev Mol Cell Biol*. 2008; 9:193-205. [PubMed: 18200017]
6. Turrel C, Bailbe D, Lacorne M, Meile MJ, Kergoat M, Portha B. Persistent improvement of type 2 diabetes in the Goto-Kakizaki rat model by expansion of the beta-cell mass during the prediabetic period with glucagon-like peptide-1 or exendin-4. *Diabetes*. 2002; 51:1443-52. [PubMed: 11978641]
7. Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S. Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia*. 2002; 45:85-96. [PubMed: 11845227]
8. Marchetti P, Del Guerra S, Marselli L, Lupi R, Masini M, Pollera M, Bugliani M, Boggi U, Vistoli F, Mosca F, Del Prato S. Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. *J Clin Endocrinol Metab*. 2004; 89:5535-41. [PubMed: 15531508]
9. Cozar-Castellano I, Fiaschi-Taesch N, Bigatel TA, Takane KK, Garcia-Ocana A, Vasavada R, Stewart AF. Molecular control of cell cycle progression in the pancreatic beta-cell. *Endocr Rev*. 2006; 27:356-70. [PubMed: 16638909]
10. Clark A, Wells CA, Buley ID, Cruickshank JK, Vanhegan RI, Matthews DR, Cooper GJ, Holman RR, Turner RC. Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes. *Diabetes Res*. 1988; 9:151-9. [PubMed: 3073901]
11. Yoon KH, Ko SH, Cho JH, Lee JM, Ahn YB, Song KH, et al. Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea. *J Clin Endocrinol Metab*. 2003; 88:2300-8. [PubMed: 12727989]
12. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes*. 2003; 52:102-10. [PubMed: 12502499]
13. Robertson RP, Harmon J, Tran PO, Poitout V. Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. *Diabetes*. 2004; 53(Suppl 1):S119-24. [PubMed: 14749276]
14. Bakker SJ, RG IJ, Teerlink T, Westerhoff HV, Gans RO, Heine RJ. Cytosolic triglycerides and oxidative stress in central obesity: the missing link between excessive atherosclerosis, endothelial dysfunction, and beta-cell failure? *Atherosclerosis*. 2000; 148:17-21. [PubMed: 10580166]
15. Lazar MA. How obesity causes diabetes: not a tall tale. *Science*. 2005; 307:373-5. [PubMed: 15662001]
16. Miesan KH, Mohamed S. Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. *J Agric Food Chem*. 2001; 49:3106-12. [PubMed: 11410016]
17. Hakkinen SH, Karenlampi SO, Heinonen IM, Mykkanen HM, Torronen AR. Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. *J Agric Food Chem*. 1999; 47:2274-9. [PubMed: 10794622]

18. Suh KS, Choi EM, Kwon M, Chon S, Oh S, Woo JT, Kim SW, Kim JW, Kim YS. Kaempferol attenuates 2-deoxy-d-ribose-induced oxidative cell damage in MC3T3-E1 osteoblastic cells. *Biol Pharm Bull.* 2009; 32:746–9. [PubMed: 19336918]
19. Crespo I, Garcia-Mediavilla MV, Gutierrez B, Sanchez-Campos S, Tunon MJ, Gonzalez-Gallego J. A comparison of the effects of kaempferol and quercetin on cytokine-induced pro-inflammatory status of cultured human endothelial cells. *Br J Nutr.* 2008; 100:968–76. [PubMed: 18394220]
20. da-Silva WS, Harney JW, Kim BW, Li J, Bianco SD, Crescenzi A, Christoffolete MA, Huang SA, Bianco AC. The small polyphenolic molecule kaempferol increases cellular energy expenditure and thyroid hormone activation. *Diabetes.* 2007; 56:767–76. [PubMed: 17327447]
21. Zhang Y, Chen AY, Li M, Chen C, Yao Q. Ginkgo biloba extract kaempferol inhibits cell proliferation and induces apoptosis in pancreatic cancer cells. *J Surg Res.* 2008; 148:17–23. [PubMed: 18570926]
22. Mylonis I, Lakka A, Tsakalof A, Simos G. The dietary flavonoid kaempferol effectively inhibits HIF-1 activity and hepatoma cancer cell viability under hypoxic conditions. *Biochem Biophys Res Commun.* 2010; 398:74–8. [PubMed: 20558139]
23. Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P. Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology.* 2004; 145:667–78. [PubMed: 14592952]
24. Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. *Diabetes.* 2003; 52:726–33. [PubMed: 12606514]
25. Fu Z, Zhang W, Zhen W, Lum H, Nadler J, Bassaganya-Riera J, Jia Z, Wang Y, Misra H, Liu D. Genistein induces pancreatic beta-cell proliferation through activation of multiple signaling pathways and prevents insulin-deficient diabetes in mice. *Endocrinology.* 2010; 151:3026–37. [PubMed: 20484465]
26. Liu D, Zhen W, Yang Z, Carter JD, Si H, Reynolds KA. Genistein acutely stimulates insulin secretion in pancreatic beta-cells through a cAMP-dependent protein kinase pathway. *Diabetes.* 2006; 55:1043–50. [PubMed: 16567527]
27. Jambal P, Masterson S, Nesterova A, Bouchard R, Bergman B, Hutton JC, Boxer LM, Reusch JE, Pugazhenth S. Cytokine-mediated down-regulation of the transcription factor cAMP-response element-binding protein in pancreatic beta-cells. *J Biol Chem.* 2003; 278:23055–65. [PubMed: 12679364]
28. Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol.* 2004; 5:897–907. [PubMed: 15520809]
29. Pugazhenth S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, Reusch JE. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem.* 2000; 275:10761–6. [PubMed: 10753867]
30. Li L, El-Kholy W, Rhodes CJ, Brubaker PL. Glucagon-like peptide-1 protects beta cells from cytokine-induced apoptosis and necrosis: role of protein kinase B. *Diabetologia.* 2005; 48:1339–49. [PubMed: 15902400]
31. Granata R, Settanni F, Gallo D, Trovato L, Biancone L, Cantaluppi V, Nano R, Annunziata M, Campiglia P, Arnoletti E, Ghè C, Volante M, Papotti M, Muccioli G, Ghigo E. Obestatin promotes survival of pancreatic beta-cells and human islets and induces expression of genes involved in the regulation of beta-cell mass and function. *Diabetes.* 2008; 57:967–79. [PubMed: 18162507]
32. Sun Y, Zhang L, Gu HF, Han W, Ren M, Wang F, Gong B, Wang L, Guo H, Xin W, Zhao J, Gao L. Peroxisome proliferator-activated receptor- α regulates the expression of pancreatic/duodenal homeobox-1 in rat insulinoma (INS-1) cells and ameliorates glucose-induced insulin secretion impaired by palmitate. *Endocrinology.* 2008; 149:662–71. [PubMed: 17991720]
33. Zhou YP, Grill VE. Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. *J Clin Invest.* 1994; 93:870–6. [PubMed: 8113418]
34. Zhou YP, Grill V. Long term exposure to fatty acids and ketones inhibits B-cell functions in human pancreatic islets of Langerhans. *J Clin Endocrinol Metab.* 1995; 80:1584–90. [PubMed: 7745004]

35. Frigerio F, Brun T, Bartley C, Usardi A, Bosco D, Ravnskjaer K, Mandrup S, Maechler P. Peroxisome proliferator-activated receptor alpha (PPARalpha) protects against oleate-induced INS-1E beta cell dysfunction by preserving carbohydrate metabolism. *Diabetologia*. 2010; 53:331–40. [PubMed: 19908022]
36. Jetton TL, Lausier J, LaRock K, Trotman WE, Larmie B, Habibovic A, Peshavaria M, Leahy JL. Mechanisms of compensatory beta-cell growth in insulin-resistant rats: roles of Akt kinase. *Diabetes*. 2005; 54:2294–304. [PubMed: 16046294]
37. Wilson BE, Mochon E, Boxer LM. Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. *Mol Cell Biol*. 1996; 16:5546–56. [PubMed: 8816467]
38. Pugazhenth S, Miller E, Sable C, Young P, Heidenreich KA, Boxer LM, Reusch JE. Insulin-like growth factor-I induces bcl-2 promoter through the transcription factor cAMP-response element-binding protein. *J Biol Chem*. 1999; 274:27529–35. [PubMed: 10488088]
39. Elsner M, Gehrmann W, Lenzen S. Peroxisome-generated hydrogen peroxide as important mediator of lipotoxicity in insulin-producing cells. *Diabetes*. 2011; 60:200–8. [PubMed: 20971967]
40. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature*. 2003; 425:191–6. [PubMed: 12939617]
41. Vetterli L, Brun T, Giovannoni L, Bosco D, Maechler P. Resveratrol potentiates glucose-stimulated insulin secretion in INS-1E beta-cells and human islets through a SIRT1-dependent mechanism. *J Biol Chem*. 2011; 286:6049–60. [PubMed: 21163946]
42. Kitamura T, Ido Kitamura Y. Role of FoxO Proteins in Pancreatic beta Cells. *Endocr J*. 2007; 54:507–15. [PubMed: 17510498]
43. Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH 3rd, Wright CV, Wright CV, White MF, Arden KC, Accili D. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J Clin Invest*. 2002; 110:1839–47. [PubMed: 12488434]
44. Buteau J, Spatz ML, Accili D. Transcription factor FoxO1 mediates glucagon-like peptide-1 effects on pancreatic beta-cell mass. *Diabetes*. 2006; 55:1190–6. [PubMed: 16644672]
45. Wang H, Iezzi M, Theander S, Antinozzi PA, Gauthier BR, Halban PA, Wollheim CB. Suppression of Pdx-1 perturbs proinsulin processing, insulin secretion and GLP-1 signalling in INS-1 cells. *Diabetologia*. 2005; 48:720–31. [PubMed: 15756539]
46. Song WJ, Schreiber WE, Zhong E, Liu FF, Kornfeld BD, Wondisford FE, Hussain MA. Exendin-4 stimulation of cyclin A2 in beta-cell proliferation. *Diabetes*. 2008; 57:2371–81. [PubMed: 18544709]
47. Rutter GA. Nutrient-secretion coupling in the pancreatic islet beta-cell: recent advances. *Mol Aspects Med*. 2001; 22:247–84. [PubMed: 11890977]
48. Iezzi M, Kouri G, Fukuda M, Wollheim CB. Synaptotagmin V and IX isoforms control Ca²⁺ - dependent insulin exocytosis. *J Cell Sci*. 2004; 117:3119–27. [PubMed: 15190121]
49. Kaiser N, Leibowitz G, Nesher R. Glucotoxicity and beta-cell failure in type 2 diabetes mellitus. *J Pediatr Endocrinol Metab*. 2003; 16:5–22. [PubMed: 12585335]
50. Hay CW, Docherty K. Comparative analysis of insulin gene promoters: implications for diabetes research. *Diabetes*. 2006; 55:3201–13. [PubMed: 17130462]
51. Brissova M, Shiota M, Nicholson WE, Gannon M, Knobel SM, Piston DW, Wright CV, Powers AC. Reduction in pancreatic transcription factor PDX-1 impairs glucose-stimulated insulin secretion. *J Biol Chem*. 2002; 277:11225–32. [PubMed: 11781323]
52. Brissova M, Blaha M, Spear C, Nicholson W, Radhika A, Shiota M, Charron MJ, Powers AC. Reduced PDX-1 expression impairs islet response to insulin resistance and worsens glucose homeostasis. *Am J Physiol Endocrinol Metab*. 2005; 288:E707–14. [PubMed: 15562255]

Fig. 1.

Kaempferol protects β -cells against palmitate-induced apoptosis. INS-1E β -cells were incubated in RPMI1640 medium containing 0.5 mM palmitate (P) or vehicle with or without various concentrations of kaempferol (K; 0.1, 1, 10 μ M) for 4 d. Cell viability was assessed by an MTS based assay (A) and by measuring intracellular ATP production (B). Apoptotic cells as determined by DNA fragmentation were quantified using a HT TiterTACS™ assay kit (C), and enzymatic activity of caspase-3 in cell extracts was measured (D). Values are mean $\pm$ SEM obtained from four independent experiments performed in triplicate. *, $p < 0.05$ vs. normal control; #, $p < 0.05$, ##, $p < 0.01$ vs. Palmitate-alone treated cells.

Fig. 2.

Kaempferol protects human islets against palmitate-induced apoptosis. Human islets (200 islets/well) were incubated in RPMI1640 medium containing 0.5 mM palmitate (P) with or without various concentrations of kaempferol (K; 0.01, 0.1, 1, 10 μM) for 4 d. Islet viability was assessed by an MTS based assay (A) and by measuring intracellular ATP production (B). Apoptotic cells were quantified with a TUNEL assay kit (C), and enzymatic activity of caspase-3 in cell extracts was measured (D). Values are mean ± SEM, obtained from three independent experiments performed in triplicate. *, $P < 0.05$ vs. normal control; #, $p < 0.05$, $p < 0.01$ vs. palmitate-alone treated islets.

Fig. 3.

Kaempferol restores palmitate-mediated reductions in Akt and Bcl-2 protein expression. INS-1E cells (A and B) and islets (C and D) were treated as described in Fig. 1. The levels of Akt and Bcl-2 protein in cell protein extracts was measured by immunoblot and normalized to β -actin content. Data are mean $\pm$ SEM. from four separate experiments performed in duplicate. *, $p < 0.05$ vs. normal control; #, $p < 0.05$, ##, $p < 0.01$ vs. palmitate-alone treated cells or islets.

Fig. 4.

Kaempferol improves palmitate-induced dysfunction of INS-1E cells and human islets. INS-1E cells (A-C) and human islets (D-F) were incubated in RPMI1640 medium containing 0.5 mM palmitate (P) with or without 10 μM kaempferol (K) for 4 d. Cells were then washed and further cultured in KRB buffer containing either 3 (LG) or 20 mM glucose (HG) for 30 min at 37°C. Insulin secreted into KRB buffer (A and D) and inside the cells (B and E) was measured by an ELISA kit. The levels of PDX-1 protein in cell extracts was measured by immunoblot and normalized to β-actin content (C and F). Values are means ± SEM derived from four separate experiments performed in duplicate. *, $p < 0.05$ vs. normal control; #, $p < 0.05$, ##, $p < 0.01$ vs. palmitate-alone treated cells.

Fig. 5.

kaempferol restores cAMP signaling impaired by palmitate in INS-1E β -cells. INS-1E cells were treated as stated in Fig. 1. (A) Intracellular cAMP levels were measured by EIA. (B) PKA activity in cell extracts was determined by measuring phosphorylation of fluorescent-labeled PKA substrate kemptide. (C) The phosphorylation of CREB (p-CREB) in the cells was detected by immunoblot analysis using a phospho-specific CREB antibody. The membrane was stripped and reprobed with a CREB antibody. (D) Cells were infected with adenoviral construct containing CRE-luc prior to above treatment. Firefly luciferase activity was measured with the dual reporter luciferase assay system and normalized to activity of the renilla control construct in the cell extracts. Values are mean $\pm$ SEM from three experiments performed in duplicate. *, $p < 0.05$ vs. normal control; #, $p < 0.05$, ##, $p < 0.01$ vs. palmitate-alone treated cells.

Fig. 6.

Kaempferol prevents INS-1E β-cells from lipotoxicity-induced apoptosis and dysfunction via CREB. INS-1E cells transfected with scrambled siRNA (S) or siRNA directed against CREB were treated with 0.5 mM palmitate (P) or vehicle in the presence or absence of 10 μM kaempferol (K) for 4 d. The efficiency of the knockdown was determined by measuring total and phosphorylated CREB (p-CREB) protein levels in transfected cells (A and B). Cell apoptosis (C), GSIS (D), insulin content (E), and Bcl-2 protein expression (F) in the cells were determined. Values are mean ± SEM from four experiments performed in duplicate. *, $p < 0.05$ vs. normal control; #, $p < 0.05$, ##, $p < 0.01$ vs. palmitate-alone treated cells; and §, $p < 0.05$ vs. P+K-treated cells.

Fig. 7.

Kaempferol protects the cAMP signaling pathway in INS-1E β -cells exposed to palmitate via stimulation of PDX-1 expression. INS-1E cells were treated with kaempferol (K; 0.1, 1, 10 μ M) for 48 h. Intracellular cAMP production was measured by EIA (A), and PDX-1 protein expression in cell extracts was determined by immunoblot analysis and normalized to β -actin content (B). INS-1E cells transfected with scrambled siRNA (S) or siRNA directed against PDX-1 (I) were treated with 10 μ M kaempferol (K) for 48 h. siRNA-mediated knockdown of PDX-1 was verified by immunoblot analysis of PDX-1 protein (C). Kaempferol-stimulated intracellular cAMP generation (D) and CREB phosphorylation (P-CREB) (E) in control and transfected cells were determined. Values are mean $\pm$ SEM from 4 experiments determined in duplicate. *, $p < 0.05$ vs. normal control; #, $p < 0.05$ vs. kaempferol-alone treated cells.

Fig. 8.

Kaempferol increases PDX-1 expression, maintains cAMP signaling, and subsequently prevents apoptosis of human islets after chronic exposure to palmitate. (A) Human islets treated with kaempferol (K; 1, 10 μM) for 48 h. PDX-1 protein expression in cell extracts was measured by immunoblot analysis and normalized to β-actin content. (B) Human islets were incubated with 0.5 mM palmitate (P) in the presence or absence of kaempferol (K; 0.1, 1, 10 μM) for 4 d, followed by measuring intracellular cAMP content. (C) Human islets were infected with 0, 25, 50 MOI MCREB virus/cell, or β-gal control virus. CREB phosphorylation in the cell lysates was measured with loading control monitored by measuring β-actin content. (D) Human islets were infected with 50 MOI MCREB virus/cell or the same number of control virus and then incubated with 0.5 mM palmitate (P) in the presence of 10 μM kaempferol (K) or vehicle for 4 d. Apoptosis of islet cells was measured as described in Fig. 2. Data are expressed as mean ± SEM obtained from three to four independent experiments measured in triplicate each. *, $p < 0.05$ vs. control; #, $p < 0.05$, ##, $p < 0.01$ vs. palmitate-alone treated cells.

Fig. 9.

The stimulatory effect of kaempferol on PDX-1 expression may be structure-specific. INS-1E cells (A) and human islets (B) were cultured with various flavonoids (10 μ M) in RPMI medium for 48 h. PDX-1 protein expression in cell extracts was measured by immunoblot analysis and normalized to β -actin content. (C) Chemical structures of tested compounds. Data are expressed as mean $\pm$ SE derived from three independent experiments. ##, $P < 0.01$ vs. vehicle alone-treated cells. C: control, N: naringenin, H: hesperetin, E: epicatechin, G: epigallocatechin gallate, A: apigenin, L: luteolin, Q: quercetin, K: kaempferol.