

ORIGINAL

Protein kinase C- δ signaling regulates glucagon secretion from pancreatic islets

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Abstract : Accumulating evidence supports the “glucagonocentric hypothesis”, in which antecedent α -cell failure and inhibition of glucagon secretion are responsible for diabetes progression. Protein kinase C (PKC) is involved in glucagon secretion from α -cells, although which PKC isozyme is involved and the mechanism underlying this PKC-regulated glucagon secretion remains unknown. Here, the involvement of PKC δ in the onset and progression of diabetes was elucidated. Immunofluorescence studies revealed that PKC δ was expressed and activated in α -cells of STZ-induced diabetic model mice. Phorbol 12-myristate 13-acetate (PMA) stimulation significantly augmented glucagon secretion from isolated islets. Pre-treatment with quercetin and rottlerin, PKC δ signaling inhibitors, significantly suppressed the PMA-induced elevation of glucagon secretion. While Go6976, a Ca²⁺-dependent PKC selective inhibitor did not suppress glucagon secretion. Quercetin suppressed PMA-induced phosphorylation of Tyr³¹¹ of PKC δ in isolated islets. However, quercetin itself had no effect on either glucagon secretion or glucagon mRNA expression. Our data suggest that PKC δ signaling inhibitors suppressed glucagon secretion. Elucidation of detailed signaling pathways causing PKC δ activation in the onset and progression of diabetes followed by the augmentation of glucagon secretion could lead to the identification of novel therapeutic target molecules and the development of novel therapeutic drugs for diabetes. *J. Med. Invest.* 64 : 122-128, February, 2017

Keywords : glucagon secretion, pancreatic α -cells, PKC δ , streptozotocin-induced diabetic mice

INTRODUCTION

Diabetes is a metabolic disease characterized by persistent hyperglycemia caused by dysfunction of insulin secretion and deteriorated insulin resistance. Long-standing persistent hyperglycemia causes various diabetic complications, such as nephropathy, retinopathy, neuropathy, and various macrovascular diseases. Thus, the control of blood glucose levels is essential for diabetes therapy.

Most conventional diabetes therapies target insulin. However, the emergence of incretin-related drugs, such as dipeptidyl peptidase-4 inhibitors, which reduce plasma glucagon and are thought to contribute to lowering blood glucose, elucidated that the mechanism of diabetes onset is closely related to glucagon (1-3). It was reported that glucagon suppression in type 1 diabetic patients permits better control of hyperglycemia with reduced doses of insulin (4). Moreover, recent reports showed that glucagon suppression induced by glucagon-like peptide-1 (GLP-1) and GLP-1 receptor agonist administration ameliorates blood glucose levels in type 1 diabetic patients without residual insulin secretion (5). Thus, glucagon suppression might play a key role in glucose homeostasis and the “glucagonocentric hypothesis” was proposed, which states that antecedent α -cell failure and inhibition of glucagon secretion are responsible for diabetes progression (6, 7). This hypothesis was further supported by many studies using glucagon receptor gene knockout mice and anti-glucagon receptor antibody (8, 9).

Protein kinase C (PKC) is a serine/threonine kinase and it consists of 11 isoforms : conventional PKC (α , β , and γ), novel PKC (δ , ϵ , η , and θ), and atypical PKC (ζ and ι/λ) (10, 11). It was reported that activation of PKC by phorbol 12-myristate 13-acetate (PMA) stimulated glucagon secretion from mouse and human pancreatic islets, which was abolished by treatment with PKC inhibitors (12-14). However, in these studies, the PKC isozyme involved in the PMA-induced glucagon secretion was not identified. It was also reported that PKC δ was involved in the onset and progression of diabetes, and the inhibition of PKC δ expression or its activation prevents β -cell dysfunction and apoptosis, thereby improving diabetic symptoms (15, 16). However, the effect of PKC δ on the function of α -cells has not yet been elucidated.

We have demonstrated that PKC δ signaling is involved in PMA-induced upregulation of histamine H₁ receptor (H1R) gene expression in HeLa cells and compounds, including quercetin and (-)-maackiain, suppressed H1R gene expression through the inhibition of PKC δ signaling (17-19). Thus, suppression of PKC δ signaling by these compounds could improve diabetic symptoms and help to further elucidate the mechanisms underlying these actions to better understand the role of PKC δ signaling in diabetes onset and progression. In fact, it was demonstrated that quercetin ameliorated the symptoms of streptozotocin (STZ)-induced diabetes (20, 21). However, in these studies, antioxidant activity was thought to be the mechanism of action of quercetin and the effect of PKC δ signaling on diabetic symptoms was not addressed.

Here we elucidated the involvement of PKC δ signaling in diabetes onset and progression. We found that PKC δ is activated in α -cells after the onset of diabetes using STZ-induced diabetic model mice. We also showed that activation of PKC δ signaling augmented glucagon secretion and PKC δ signaling inhibitors

Received for publication January 19, 2017 ; accepted January 19, 2017.

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suppressed it in isolated islets from mice.

Materials and Methods

Materials

Streptozotocin, PMA, Histopaque1077, Histopaque1199, rotlerin, cOmplete Mini, and PhosSTOP phosphatase inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin was from Wako Pure Chemical (Osaka). Ro-31-8220 and Go6976 were from Merck Millipore (Billerica, MA, USA). Celastrol was from Cayman Chemical (Ann Arbor, MI, USA). RPMI-1640 medium and HBSS were from Invitrogen (Carlsbad, CA, USA) and Thermo Fisher Scientific (Waltham, MA, USA), respectively. Collagenase type IV was from Worthington Biochemical Corporation (Lakewood, NJ, USA). A glucagon assay kit was from Cisbio Bioassays (Codolet, France). Mouse insulin assay kit was from Morinaga Institute of Biological Science (Yokohama). RNeasy Mini Kit was from QIAGEN (Tokyo). PrimeScript RT Reagent Kit, and Premix ExTaq were from Takara Bio Inc. (Kyoto). Smear Gell was from GenoStaff (Tokyo). DeadEnd Fluorometric TUNEL System and RQ1 RNase-Free DNase were from Promega (Madison, WI, USA). All other chemicals were of analytical grade.

Preparation of STZ-induced diabetic model mice

Male C57BL/6JmsSlc mice (8-15-week-old, Japan SLC, Hamamatsu) were housed in a room at a constant temperature of $25 \pm 2^\circ\text{C}$ and humidity of $55 \pm 10\%$ under a 12-h light/dark cycle with free access to food and water. Mice were intraperitoneally injected with STZ (200 mg/kg) freshly dissolved in 0.1 M citrate buffer (pH 4.5). Four days after STZ injection, blood glucose was measured and mice with blood glucose levels > 300 mg/dl were considered diabetic. Mice were divided into two groups: a control group ($n=5$) and a STZ-treated group ($n=15$). After 6 weeks, no mouse died. Experiments using similar conditions were previously conducted, in which no mice died (7). All animal experiments were approved by the Ethical Committee for Animal Studies of Tokushima University School of Medicine.

Immunohistochemical analysis

Pancreatic tissues were fixed in 10% neutral buffered-formalin, embedded in paraffin, and cut into $5 \mu\text{m}$ -thick sections. The sections were deparaffinized, blocked with 5% normal donkey serum for 45 min, and stained with anti-insulin antibody (Abcam Japan, Tokyo), anti-glucagon (C-18) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PKC δ antibody (Cell Signaling Technology Japan, Tokyo), and anti-phospho-Tyr³¹¹ PKC δ antibody (Cell Signaling). Antibodies and 4,6-diamidino-2-phenylindole (DAPI) were diluted in Can Get Signal[®] immunostain Solution A (Toyobo, Osaka). The sections were observed using LSM510 confocal laser-scanning microscope (Carl Zeiss AG, Oberkochen, Germany). RGB profile plots were detected using Image J image processing software (<http://imagej.nih.gov/ij/>).

Pancreatic islet isolation

Mice were anesthetized by the intraperitoneal injection of pentobarbital. Then, the stomach and duodenum were removed and placed to the right of the body cavity. The common bile duct was clipped and collagenase IV solution (1.5 mg/ml) was injected into the pancreatic duct using a stereoscopic microscope. The entire pancreas was removed and placed in a 15-ml tube containing collagenase IV solution and heated for 40 min at 37°C . HBSS solution (1% bovine serum albumin (BSA), 100 IU/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin) was added to the cell suspension and centrifuged at 1,200 rpm for 2 min. After the supernatant was discarded, Histopaque solution (5 ml of Histopaque1077 and 6 ml of Histopaque1119) was

added to the cells and centrifuged at 1,200 rpm for 20 min. Then the supernatant was transferred to a new 50-ml tube and 25 ml of HBSS solution was added. The cell suspension was centrifuged at 1,500 rpm for 4 min, the precipitated cells were re-suspended in HBSS solution, passed through a 100- μm cell strainer (BD Biosciences, San Jose, CA, USA), and collected into a 10-cm dish. Pancreatic islets were handpicked and cultured at 37°C under a humidified atmosphere of 5% CO_2 and 95% air in islet medium (RPMI-1640 medium containing 2.0 g/l NaHCO_3 , 10 mM HEPES solution, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10% FBS, 100 IU/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin) (22).

Glucagon and insulin secretion assay

Isolated islets were pre-incubated for 24 h in islet medium. After pre-incubation, the medium was replaced with Krebs-Ringer modified buffer (KRB)-HEPES-BSA solution consisting of NaCl (120 mM), NaHCO_3 (25 mM), KCl (4.7 mM), MgSO_4 (1.2 mM), CaCl_2 (2.5 mM), KH_2PO_4 (1.2 mM), HEPES (10 mM), BSA (1 mg/ml), D-Glucose (2 mg/ml), penicillin (100 IU/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$) at pH 7.4. Three islets were transferred to a 1.5-ml tube with 300 μl of KRB-HEPES-BSA solution (22) and then treated with quercetin (15, 30, and 60 μM), rottlerin (1-10 μM), Ro-31-8220 (1 μM), and Go6976 (1 μM), and celastrol (1 μM) for 1 h before stimulation with 100 nM PMA. The supernatant was collected 1 h after PMA stimulation and glucagon concentration was determined using the HTRF[®] glucagon assay kit. For the detection of insulin secretion, three islets in a 1.5-ml tube with 300 μl of KRB-HEPES-BSA solution were treated with quercetin (60 μM) for 1 h before PMA stimulation. After 1 h stimulation with PMA, the supernatant was collected and insulin concentration was determined using the insulin assay kit.

Real-time quantitative RT-PCR

After 24 h pre-incubation in islet medium, 100 islets were transferred to a 24-well plate with 500 μl of the islet medium and treated with quercetin (60 μM) for 4 h before PMA stimulation. Islets were collected 4 h after PMA stimulation and total RNA was prepared. Then, 5 μg of total RNA was reverse-transcribed to cDNA using the PrimeScript RT Reagent Kit. Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). Glucagon mRNA was determined using TaqMan gene amplification primers and probes (Mm01269055_m1, Applied Biosystems). To standardize the starting material, rodent GAPDH mRNA was determined using TaqMan rodent GAPDH Control Reagents (Applied Biosystems). Data are expressed as the ratio of glucagon mRNA to GAPDH mRNA.

Apoptosis Assays

Isolated islets were pre-incubated for 24 h in islet medium. After pre-incubation, the medium was replaced with new islet medium. Islets were transferred to a 24-well plate with 500 μl of islet medium and then treated with quercetin (60 μM) for 1 h before stimulation with 100 nM PMA. Islets were collected using Smear Gell at 23 h after PMA stimulation. Apoptosis was measured using the DeadEnd Fluorometric TUNEL System. As a positive control, cells were treated with RQ1 RNase-Free DNase as described in the manufacturer's protocol. Sections were observed using LSM510 confocal laser-scanning microscope.

Immunoblot analysis

For immunoblot analysis, isolated islets from 8 mice were pre-incubated for 24 h in islet medium. After pre-incubation, islets were transferred to a 24-well plate with 500 μl of islet medium and then treated with quercetin (60 μM) for 1 h before stimulation with 100 nM PMA. Islets were collected at 10 min after PMA stimulation and then homogenized in 20 mM Tris-HCl, pH 7.5 containing

100 mM NaCl, 0.5% Triton X-100, cOmplete Mini and PhosSTOP. Whole cell lysates were prepared by sonication and 30 μ g of each protein was separated on a 10% SDS-PAGE gel. Immunoblot analysis was conducted using primary antibodies (PKC δ (C-20), Santa Cruz Biotechnology; phospho-Tyr³¹¹ PKC δ ; β -actin). Proteins were visualized with an Immobilon Western Chemiluminescent HRP substrate (Merck Millipore).

Statistical analysis

Results are expressed as the mean \pm SEM. Statistical analyses were performed using analysis of variance with the Dunnett's test and *t*-test using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Localization of PKC δ in islets after the onset of diabetes

Before STZ injection, β -cells, represented as insulin-immunoreactive cells, were observed throughout the islets of normal mice, whereas α -cells, represented as glucagon-immunoreactive cells, were localized in the penumbra of islets (Fig. 1A, control). After STZ treatment for 4 days to induce diabetes, β -cells were eliminated in the islets (Fig. 1A, 0 day). On the other hand, α -cells were distributed throughout the islets (Fig. 1A, 0 day and 6 weeks).

Before STZ injection, PKC δ expression levels were low in the islets (Fig. 1A, control). However, after the onset of STZ-induced diabetes, PKC δ expression was increased (Fig. 1A). The localization of PKC δ -immunoreactive cells was different from that of β -cells (Fig. 1A and 1B) and was consistent with that of α -cells (Fig. 1A and 1B). Next, we investigated the effect of STZ on the phosphorylation of PKC δ at Tyr³¹¹ that is crucial for its activation (11). Phospho-Tyr³¹¹ PKC δ -immunoreactive cells increased after STZ stimulation and co-localized with α -cells (Fig. 1C and 1D).

Effect of quercetin on glucagon secretion from isolated islets

Immunofluorescence studies showed that STZ-induced increase in PKC δ expression and its activation in α -cells after the onset of diabetes (Fig. 1) suggesting that PKC δ plays some physiological roles in α -cells. PMA-induced PKC δ activation caused significant increase in glucagon secretion from isolated islets (Fig. 2). Pretreatment with rottlerin, a PKC δ signaling inhibitor and Ro-31-8220, a pan PKC inhibitor, significantly suppressed PMA-induced glucagon secretion. However, Go6976, a Ca²⁺-dependent PKC selective inhibitor had no effect (Fig. 2). It was recently reported that rottlerin also inhibited PKC θ another nPKC isoform, with IC₅₀ of 1.6 μ M and less than 3 μ M of rottlerin selectively inhibited PKC θ (23). PMA-induced glucagon secretion was not suppressed by less than 2.5 μ M of rottlerin, suggesting that PKC θ was not involved in PMA-induced glucagon secretion (Fig. 2).

Previously, we reported that quercetin is a PKC δ signaling

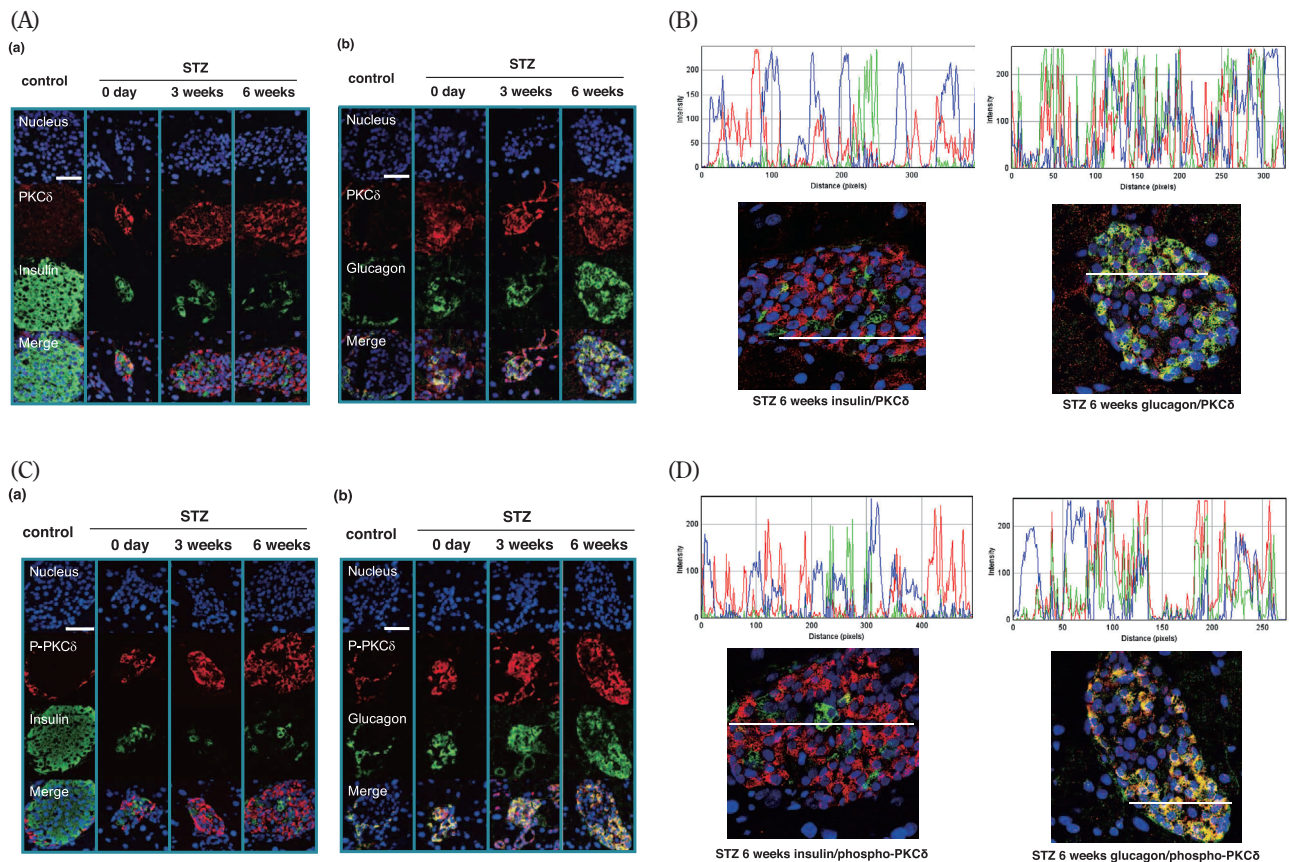


Fig 1. Localization of PKC δ in pancreatic islets.

Pancreases were harvested on 0, 3, and 6 weeks after the onset of diabetes and subjected to immunofluorescence analysis. (A) Images of double staining of insulin and PKC δ (a) and glucagon and PKC δ (b). (B) RGB profile plot of STZ 6 weeks insulin/PKC δ and glucagon/PKC δ . (C) Images of double staining of insulin and phospho-PKC δ (a) and glucagon and phospho-PKC δ . (D) RGB profile plot of STZ 6 weeks insulin/phospho-PKC δ and glucagon/phospho-PKC δ . Images were captured using a confocal laser scanning microscope. RGB profile plot was detected with Image J image processing software. Scale bar : 50 μ m

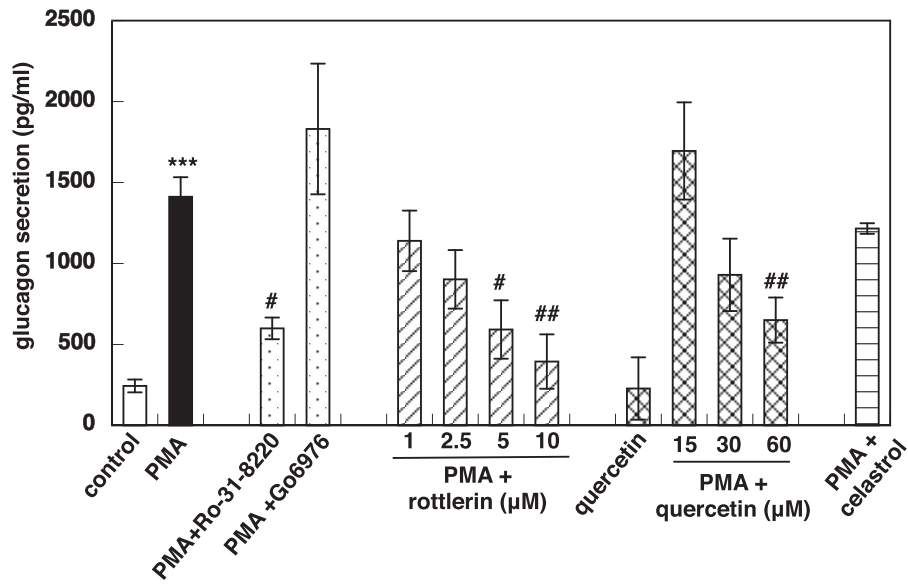


Fig 2. Effect of quercetin on glucagon secretion from pancreatic islets.

Isolated islets were pre-incubated for 24 h in islet medium. After pre-incubation, the medium was replaced with KRB-HEPES-BSA solution. Three islets were transferred to a 1.5-ml tube with 300 μl of KRB-HEPES-BSA solution and treated with quercetin (15, 30, and 60 μM), rottlerin (1-10 μM), Ro-31-8220 (1 μM), Go6976 (1 μM), and celastrol (1 μM) for 1 h before PMA stimulation. Supernatant was collected 1 h after stimulation with 100 nM PMA. Secreted glucagon was measured using a glucagon assay kit. Data are expressed as means ± SEM. Control, n = 18; 60 μM quercetin, n = 4; PMA, n = 15; PMA+Ro-31-8220, n = 4; PMA+Go6976, n = 3; PMA+10 μM rottlerin, n = 3; PMA+5 μM rottlerin, n = 4; PMA+2.5 μM rottlerin, n = 4; PMA+1 μM rottlerin, n = 4; PMA+celastrol, n = 3; PMA+60 μM quercetin, n = 9; PMA+30 μM quercetin, n = 4; PMA+15 μM quercetin, n = 4. ***p < 0.001 vs. control; ##p < 0.01, #p < 0.05 vs. PMA.

inhibitor (18). We also reported that heat shock protein 90 (Hsp90) regulated PKCδ signaling in HeLa cells (24). Therefore, we investigated the effect of quercetin and Hsp90 inhibitors on PMA-induced glucagon secretion. Quercetin suppressed the PMA-induced increase in glucagon secretion (Fig. 2), while an Hsp90 inhibitor celastrol had no effect, suggesting that Hsp90 is not involved in glucagon secretion (Fig. 2). Quercetin itself showed no effect on glucagon secretion, suggesting the effect of quercetin was not due to its anti-oxidant activity. Treatment with PMA alone and PMA with quercetin did not induce cell apoptosis (Fig. 3), suggesting that suppression of glucagon secretion by quercetin was not due to cell apoptosis.

It was reported that insulin regulates glucagon secretion (25). So, we investigated whether PMA-induced glucagon secretion from isolated islets was due to the effect of insulin secreted in response to PMA. Stimulation with PMA increased insulin secretion in isolated islets (Fig. 4). However, treatment with quercetin had no effect on its secretion, suggesting that PMA-induced glucagon secretion is not due to the effect of insulin. PMA and/or quercetin did not show any effect on glucagon gene expression in isolated islets (Fig. 5).

Effect of quercetin on Tyr³¹¹ phosphorylation of PKCδ in isolated islets

As PKCδ is phosphorylated at Tyr³¹¹ upon activation (11), we investigated the effect of quercetin on PMA-induced Tyr³¹¹ phosphorylation of PKCδ in isolated islets. Quercetin suppressed PMA-induced Tyr³¹¹ phosphorylation of PKCδ (Fig. 6). It was reported that PKCδ was also activated by proteolytic cleavage, in which PKCδ is cleaved by caspase-3 and released the 41-kDa catalytic fragment (26). However, immunoblot analysis showed no proteolytic cleavage of PKCδ in response to PMA.

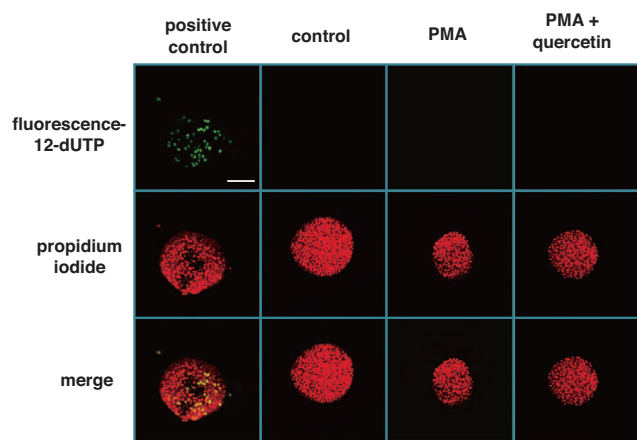


Fig. 3. Assessment of apoptosis by TUNEL staining.

Isolated islets were pre-incubated for 24 h in islet medium. After pre-incubation, the medium was replaced with new islet medium. Islets were transferred to a 24-well plate with 500 μl of islet medium and then treated with quercetin (60 μM) for 1 h before stimulation with 100 nM PMA. Islets were collected using Smear Gell at 23 h after PMA stimulation. Images of double staining of fluorescein-12-dUTP and propidium iodide. As a positive control, cells were treated with RQ1 RNase-Free DNase as described in the manufacturer's protocol. Sections were observed using a LSM510 confocal laser scanning microscope. Scale bar : 100 μm

DISCUSSION

In the present study, we showed that PKCδ was activated in α-cells after the onset and progression of STZ-induced diabetes. We also showed that PMA-induced PKCδ activation augmented glucagon secretion and inhibition of PKCδ signaling suppressed PMA-induced glucagon secretion in isolated islets.

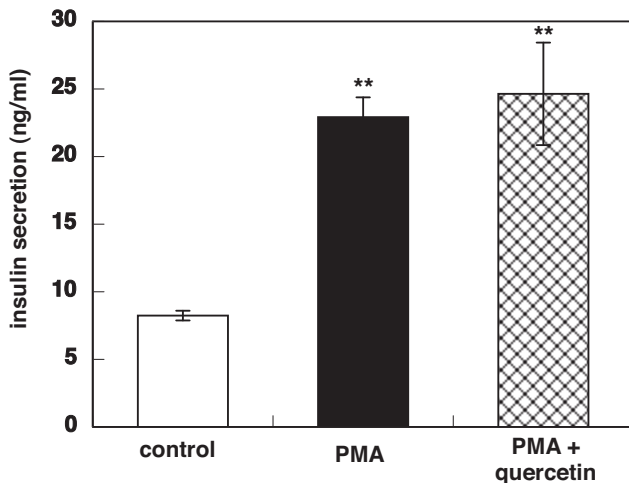


Fig. 4. Effect of quercetin on insulin secretion from pancreatic islets. Isolated islets were pre-incubated for 24 h in islet medium. After pre-incubation, the medium was replaced with new islet medium. Three islets were transferred to a 1.5-ml tube with 300 μ l of KRB-HEPES-BSA solution and then treated with quercetin (60 μ M) for 1 h before stimulation with 100 nM PMA. The supernatant was collected 1 h after PMA stimulation for the measurement of insulin concentration using an insulin assay kit. Data are expressed as means \pm SEM. (n=3) ** p < 0.01 vs. control.

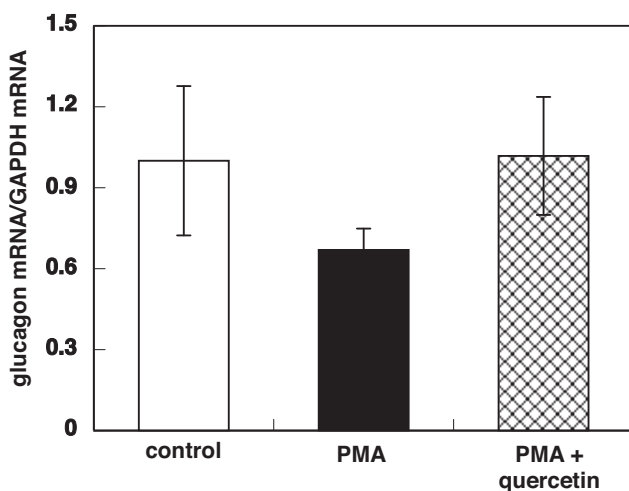


Fig. 5. Effect of quercetin on glucagon gene expression in pancreatic islets.

Isolated islets were pre-incubated for 24 h in islet medium. After pre-incubation, the medium was replaced with new islet medium and 100 islets were transferred to a 24-well plate with 500 μ l of islet medium and then treated with quercetin (60 μ M) for 4 h before stimulation with 100 nM PMA. Islets were collected 4 h after PMA stimulation. Glucagon mRNA was determined by real-time quantitative RT-PCR. Data are expressed as means \pm SEM (n=3).

Immunohistochemical studies showed that STZ treatment resulted in an increase in the abundance of α -cells concurrent with a decrease in β -cells. Quantification analyses of the islet area revealed that the β -cell area was significantly decreased after the onset of diabetes, while the α -cell area tended to increase just after the onset of diabetes and was significantly increased at 6 weeks (data not shown). These results suggest that the α -cell mass gradually increased with time after the onset of STZ-induced diabetes

and may be involved in the progression of diabetic symptoms. Similar results were observed in pancreas-specific Forkhead box O1 (FoxO1) transgenic mice, which also developed diabetes (27). In addition, a recent study reported that FoxO1 downregulation resulting from hyperglycemia contributed to the dedifferentiation of β -cells and the increased number of α -cells (28). Immunohistochemical studies also showed an increase in PKC δ expression in islets of STZ-induced diabetic mice, although its expression was low before induction of diabetes. Double staining studies revealed that PKC δ localization was virtually consistent with that of glucagon, but not insulin. The data suggest that the increase in PKC δ observed after STZ treatment is due to increased α -cell mass to some extent (Fig. 1). However, the fluorescence intensity of PKC δ was increased after STZ treatment, suggesting the PKC δ was induced in response to STZ stimulation. The fluorescence intensity of phospho-PKC δ was also increased in response to STZ stimulation. Because of the different conditions to detect immunoreactivities for total and phospho-PKC δ , we could not compare the amount by means of fluorescence intensity. In addition, activation of PKC δ (i.e. Tyr³¹¹ phosphorylation) is not always concomitant with an increase in total PKC δ expression. Therefore, we consider that the increase in phospho-PKC δ observed after STZ treatment was not simply due to increased α -cell mass, but rather activation of PKC δ in response to STZ stimulation and that PKC δ plays an unknown pathophysiological role in α -cells of STZ-induced diabetic mice.

Several studies have reported the involvement of PKC δ in the pathogenesis of diabetes and that PKC δ activation induced β -cell apoptosis (13-16). PKC δ is a major contributor to hepatic insulin resistance (29, 30). These reports focused on the effect of PKC δ on β -cell function and insulin signaling because β -cell dysregulation and insulin resistance are considered major factors in the onset of diabetes (31, 32). However, recent accumulating evidence suggests that dysregulation of glucagon secretion can also induce hyperglycemia, which is ameliorated by inhibition of glucagon function (6-9). Moreover, it was reported that treatment with high glucose increased glucagon secretion from isolated islets and PKC is involved in this event, although the responsible PKC isozyme was not identified (12-14). Our data demonstrate that PKC δ signaling is responsible for the PMA-induced augmentation of glucagon secretion in the isolated islets. We failed to detect PKC δ activation (i.e. Tyr³¹¹ phosphorylation) in the pancreas. However, we observed an increase in PKC δ - and phospho-Tyr³¹¹ PKC δ -immunoreactive cells in glomeruli in response to STZ stimulation (Mizuguchi *et al.* unpublished results). We confirmed this finding by immunoblot analysis using the glomeruli of STZ-treated diabetic mice, in which phosphorylation of PKC δ Tyr³¹¹ was increased in the glomerular mesangial cells (Mizuguchi *et al.* unpublished results). So, our data suggest that hyperglycemia caused by STZ treatment could activate PKC δ . In this study, we used rottlerin as a PKC δ selective inhibitor, although it was reported that rottlerin had a number of non-PKC δ -related effects (33). Rottlerin has been used to confirm the possible role of PKC δ isoform in several signaling pathways. Moreover, the results obtained with rottlerin were compatible with those obtained utilizing more selective approaches to inhibit PKC δ in a previous report (17). It was recently reported rottlerin also inhibited PKC θ (23). However, our data indicates that PKC θ is not involved PMA-induced glucagon secretion. Together with these findings, we believe that rottlerin is still accepted as a PKC δ inhibitor, although rottlerin is reported to have a number of non-PKC δ -related effects.

Quercetin is a well-characterized flavonoid and abundantly found in tea, onions, mulberry, and many fruits (34). High oxidative stress due to persistent and chronic hyperglycemia is a hallmark of diabetes and promotes the pathogenesis and progression of tissue damage in diabetes. Therefore, anti-oxidants, such as quercetin,

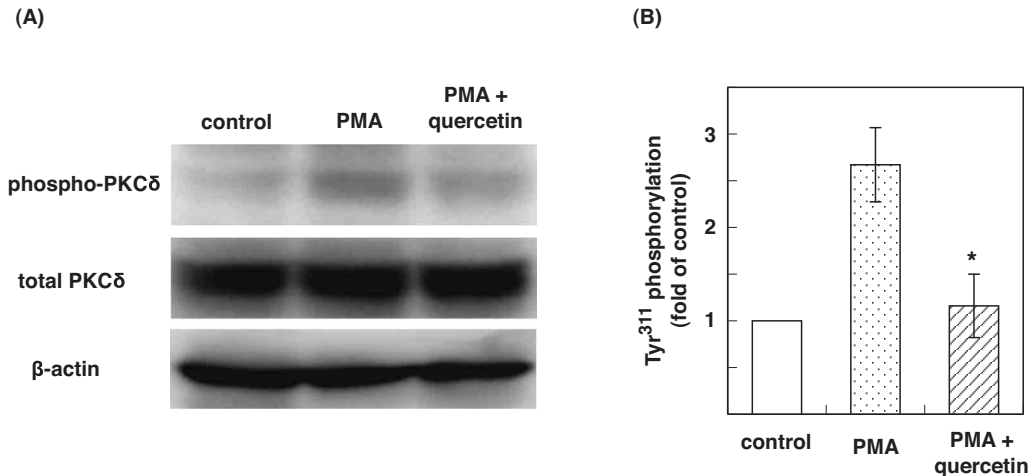


Fig. 6. Effect of quercetin on PMA-induced Tyr³¹¹ phosphorylation of PKC δ in isolated islets.

(A) Isolated islets from 8 mice were homogenized in 20 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 0.5% Triton X-100, cOmplete Mini and PhosSTOP. Whole cell lysates were prepared by sonication. Thirty μ g of each protein sample was separated on a 10% SDS-PAGE gel and then transferred onto a PVDF membrane. The membrane was briefly rinsed in Tris-buffered saline containing 0.1% Tween 20 (TBS) and then incubated for 1 h at room temperature in TBS containing 3% BSA. The membrane was then incubated with anti-PKC δ or anti-phospho-PKC δ (Tyr³¹¹) antibodies overnight at 4°C. Proteins were visualized with an Immobilon Western Chemiluminescent HRP substrate. (B) Quantification of Tyr³¹¹ phosphorylation of PKC δ . Densitometric analysis was performed using ImageJ software. Data are expressed as means \pm SEM. (n=3). **p* < 0.05 vs. PMA.

are thought to be useful to ameliorate the symptoms of STZ-induced diabetes. In fact, several reports demonstrated the antidiabetic effects of quercetin. For example, quercetin treatment significantly decreased blood glucose levels in STZ-induced rats (20). It was also reported that quercetin is not only effective against insulin sensitivity, but also protects β -cells (21). However, the effect of quercetin on α -cell function has not yet been reported. We showed that treatment with PMA significantly increased glucagon secretion from isolated islets and quercetin treatment suppressed the PMA-induced increase in glucagon secretion in α -cells. We reported that quercetin suppressed PMA-induced Tyr³¹¹ phosphorylation of PKC δ in HeLa cells (17). In the present study, we showed that quercetin also suppressed PMA-induced Tyr³¹¹ phosphorylation of PKC δ in isolated islets. Quercetin itself had no effect on glucagon secretion in isolated islets. Also, quercetin did not suppress PMA-induced insulin secretion in isolated islets. Furthermore, immunofluorescence analyses showed that PKC δ was expressed and activated in α -cells in response to PMA stimulation. Together with these findings, we consider that the suppressive effect of quercetin on glucagon secretion is not due to its anti-oxidative effect, but rather the suppression of PKC δ activation.

In conclusion, our data suggest that PKC δ signaling is activated in α -cells after the onset of diabetes, which augments glucagon release, resulting in hyperglycemia and progression of diabetic symptoms. These data also suggest that the suppression of PKC δ signaling could reduce excessive glucagon secretion in diabetes and ameliorate symptoms. A detailed elucidation of the signaling pathway underlying PKC δ activation and enhanced glucagon secretion could help to development of drugs targeting PKC δ signaling, as an alternative strategy to overcome diabetes.

DISCLOSURE

The authors declare no financial conflicts of interest.

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