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 also reported that PKCδ signaling is involved in the PMA-induced glucagon secretion was not identified. However, quercetin itself had no effect on either glucagon secretion or glucagon mRNA expression. Our data suggest that PKCδ signaling inhibitors suppressed glucagon secretion. Elucidation of detailed signaling pathways causing PKCδ 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.

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

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 PKCδ 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.

Here we elucidated the involvement of PKCδ signaling in diabetes onset and progression. It was also reported that PKCδ was involved in the onset and progression of diabetes, and the inhibition of PKCδ expression or its activation prevents β-cell dysfunction and apoptosis, thereby improving diabetic symptoms (15, 16). However, the effect of PKCδ on the function of α-cells has not yet been elucidated.

We have demonstrated that PKCδ 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 PKCδ signaling (17-19). Thus, suppression of PKCδ signaling by these compounds could improve diabetic symptoms and help to further elucidate the mechanisms underlying these actions to better understand the role of PKCδ 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 PKCδ signaling on diabetic symptoms was not addressed.

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

Methods and Materials

Materials

Streptozotocin, PMA, Histopaque1077, Histopaque1199, rottiner, 2′-O-MeThioStop phosphatase inhibitor cocktail was 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 Moringina Institute of Biological Science (Yokohama). RNase Mini Kit was from QIAGEN (Tokyo). PrimeScript RT Reagent Kit, and Premix ExTaq were from Takara Bio Inc. (Kyo). Snee Cerfl was from GenoStafl (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±2°C and humidity of 55±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 μm-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-PKCδ antibody (Cell Signaling Technology Japan, Tokyo), and anti-phospho-Tyr32 PKCδ 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 clamped 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 μg/ml streptomycin) was added to the cells and centrifuged at 1,200 rpm for 2 min. After the supernatant was discarded, Histopaque solution (5 ml of Histopaque1077 and 6 ml of Histopaque1199) 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% CO2 and 95% air in islet medium (RPMI-1640 medium containing 2.9 g/l NaHCO3, 10 mM HEPES solution, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10% FBS, 100 IU/ml penicillin, and 50 μg/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), NaHCO3 (25 mM), KCl (4.7 mM), MgSO4 (1.2 mM), CaCl2 (2.5 mM), KH2PO4 (1.2 mM), HEPES (10 mM), BSA (1 mg/ml), D-Glucose (2 mg/ml), penicillin (100 IU/ml), and streptomycin (50 μg/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), rottiner (1-10 μM), Ro-31-8229 (1 μM), and Go6976 (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 (Mm01290655_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 μg of each protein was separated on a 10% SDS-PAGE gel. Immunoblot analysis was conducted using primary antibodies (PKCδ (C-20), Santa Cruz Biotechnology; phospho-Tyr311 PKCδ; β-actin). Proteins were visualized with an Immobilon Western Chemiluminescent HRP substrate (Merck Millipore).

Statistical analysis
Results are expressed as the mean ± 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 PKCδ 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, PKCδ expression levels were low in the islets (Fig. 1A, control). However, after the onset of STZ-induced diabetes, PKCδ expression was increased (Fig. 1A). The localization of PKCδ-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 PKCδ at Tyr311 that is crucial for its activation (11). Phospho-Tyr311 PKCδ-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 PKCδ expression and its activation in α-cells after the onset of diabetes (Fig. 1) suggesting that PKCδ plays some physiological roles in α-cells. PMA-induced PKCδ activation caused significant increase in glucagon secretion from isolated islets (Fig. 2). Pre-treatment with rottlerin, a PKCδ 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 PKCθ another nPKC isoform, with IC50 of 1.6 μM and less than 3 μM of rottlerin selectively inhibited PKCθ (23). PMA-induced glucagon secretion was not suppressed by less than 2.5 μM of rottlerin, suggesting that PKCθ was not involved in PMA-induced glucagon secretion (Fig. 2).

Previously, we reported that quercetin is a PKCδ signaling...
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 induce cell apoptosis (Fig. 3), suggesting that suppression of glucagon secretion by quercetin was not due to cell apoptosis.

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.

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.
Isolated islets were pre-incubated for 24 h in islet medium. After pre-incubation, the medium was replaced with new islet medium and then treated with quercetin (60 μM) for 4 h before stimulation with 100 nM PMA. Glucagon mRNA was determined by real-time quantitative RT-PCR. Data are expressed as means±SEM. (n=3) **p< 0.01 vs. control.

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 PKCδ expression in islets of STZ-induced diabetic mice, although its expression was low before induction of diabetes. Double staining studies revealed that PKCδ localization was virtually consistent with that of glucagon, but not insulin. The data suggest that the increase in PKCδ observed after STZ treatment is due to increased α-cell mass to some extent (Fig. 1). However, the fluorescence intensity of PKCδ was increased after STZ treatment, suggesting that PKCδ was induced in response to STZ stimulation. The fluorescence intensity of phospho-PKCδ was also increased in response to STZ stimulation. Because of the different conditions to detect immunoreactivities for total and phospho-PKCδ, we could not compare the amount by means of fluorescence intensity. In addition, activation of PKCδ (i.e. Tyr311 phosphorylation) is not always concomitant with an increase in total PKCδ expression. Therefore, we consider that the increase in phospho-PKCδ observed after STZ treatment was not simply due to increased α-cell mass, but rather activation of PKCδ in response to STZ stimulation and that PKCδ plays an unknown pathophysiological role in α-cells of STZ-induced diabetic mice.

Several studies have reported the involvement of PKCδ in the pathogenesis of diabetes and that PKCδ activation induced β-cell apoptosis (13-16). PKCδ is a major contributor to hepatic insulin resistance (29, 30). These reports focused on the effect of PKCδ 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 PKCδ is involved in this event, although the responsible PKC isozyme was not identified (12-14). Our data demonstrate that PKCδ signaling is responsible for the PMA-induced augmentation of glucagon secretion in the isolated islets. We failed to detect PKCδ activation (i.e. Tyr311 phosphorylation) in the pancreas. However, we observed an increase in phospho-PKCδ- and phospho-Tyr311 PKCδ-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 PKCδ 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 PKCδ. In this study, we used rottlerin as a PKCδ selective inhibitor, although it was reported that rottlerin had a number of non-PKCδ-related effects (23). Rottlerin has been used to confirm the possible role of PKCδ isozym in several signaling pathways. Moreover, the results obtained with rottlerin were compatible with those obtained utilizing more selective approaches to inhibit PKCδ in a previous report (17). It was recently reported rottlerin also inhibited PKCδ (23). However, our data indicates that PKCδ is not involved PMA-induced glucagon secretion. Together with these findings, we believe that rottlerin is still accepted as a PKCδ inhibitor, although rottlerin is reported to have a number of non-PKCδ-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,
are thought to be useful to ameliorate the symptoms of STZ-induced diabetes. In fact, several reports demonstrated the anti-diabetic 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 PKCδ in HeLa cells (17). In the present study, we showed that quercetin also suppressed PMA-induced Tyr311 phosphorylation of PKCδ in isolated islets. Quercetin itself did not suppress PMA-induced insulin secretion in isolated islets. Furthermore, immunofluorescence analyses showed that PKCδ 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 PKCδ activation.

In conclusion, our data suggest that PKCδ 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 PKCδ signaling could reduce excessive glucagon secretion in diabetes and ameliorate symptoms. A detailed elucidation of the signaling pathway underlying PKCδ activation and enhanced glucagon secretion could help to development of drugs targeting PKCδ signaling, as an alternative strategy to overcome diabetes.

DISCLOSURE

The authors declare no financial conflicts of interest.

REFERENCES

PKCδ regulates glucagon secretion


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