Localization of diacylglycerol kinase ζ in rat pancreatic islet cells under normal and streptozotocin-induced stress conditions

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Summary. The pancreas comprises exocrine and endocrine portions, the latter of which is a glucose-responsive tissue that secretes hormones in response to serum glucose levels. One pathway implicated in the regulatory mechanism of this gland is the phosphoinositide (PI) cycle, which generates second messengers. Diacylglycerol (DG), the major second messenger in the PI signaling cascade, is catalyzed by the diacylglycerol kinase (DGK) family. We previously described characteristic expression and localization patterns of DGKs in various organs under pathophysiological conditions. Nevertheless, little is known about the characteristics and morphological aspects of this enzyme family in the pancreas. This study was conducted to investigate the pancreas, specifically the expression and localization of the DGK family. RT-PCR analysis reveals that DGKζ is the major isozyme in the pancreas. Additionally, we show that DGKζ is expressed in pancreatic islet cells, but not in the exocrine cells. It localizes predominantly to the nuclei of α-, β-, and δ-cells. We found further that DGKζ translocates from the nucleus to the cytoplasm in β-cells in response to a β-cell-selective toxin streptozotocin (STZ) and that it disappears over time. These findings will substantiate and extend our understanding of the functional roles of DGKζ in pancreatic islet cells.

Introduction

Gq protein-coupled receptor activation triggers the phosphoinositide (PI) signal transduction cascade, in which phospholipase C (PLC) cleaves phosphatidylinositol-4,5-bisphosphate (PIP2) to yield a pair of second messengers: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP3). In this system, diacylglycerol kinase (DGK) phosphorylates DG to produce phosphatidic acid (PA). A well-known functional role of DGK is in the regulation of proteins containing DG-binding C1 domain, for which DG acts as an allosteric activator. Those proteins include protein kinase C (PKC) (Nishizuka 1992; Ron and Kazanietz 1999; Martelli et al., 2004), protein kinase D (PKD) (Baron and Malhotra 2002), chimaerin (Caloca et al., 1999), and RasGRP (Ebinu et al., 1998). In addition, a reaction product of DGK, PA, also serves as a messenger molecule to regulate various proteins such as RasGAP, mTOR, PI(3)K, and Raf-1 (Merida et al., 2008; Zhang and Du 2009). The proteins activated by DG or PA play a central role in many and diverse cell types. Therefore, DGK is thought to mediate signal transduction by...
modulating the levels of DG and PA, thereby switching off 
DG signal and/or switching on the PA signal. The importance 
of this enzymatic conversion might differ depending on the 
types of cells, stimuli, and cascades that are triggered.

Molecular cloning studies have identified 10 mammalian 
DGK isozymes to date (Topham 2006; Goto et al., 2007; 
Sakane et al., 2007; Martelli et al., 2011). A prominent 
feature of the DGK family is that most isozymes are expressed 
abundantly in the brain, suggesting the physiological 
importance of this enzyme family for proper brain function 
(Goto and Kondo 2004; Goto et al., 2006). In addition, recent 
studies have shown characteristic expressions and localization 
patterns of DGKs in other organs, including the peripheral 
nervous system, heart, vascular smooth muscle and endothelial 
cells, lung, liver, female reproductive organs, pituitary gland, 
adrenal gland, and retina under pathophysiological conditions 
(Goto et al., 2007; Nakano et al., 2009; Hozumi et al., 
2010; Hozumi and Goto 2012; Nakano et al., 2012; Hozumi 
et al., 2013; Goto et al., 2014; Hozumi et al., 2015). These 
observations suggest that the DGK family is involved in 
widely diverse functions of various tissues and organs.

The pancreas comprises exocrine cells and endocrine 
cells, the latter of which are glucose-responsive, secreting 
hormones in response to glucose levels (Jensen et al., 2008). 
Recently, DGKα and DGKγ were detected at the protein 
level in mouse pancreatic islet cells (Kurohane Kaneko et 
al., 2013), although details of the whole DGK family study 
remain to be elucidated.

For this study, we investigated the expression and 
localization of the DGK family in the pancreas. Results 
demonstrate that DGKζ is expressed most abundantly in the 
pancreas. It localizes to the nucleus of pancreatic islet cells, 
including α-, β-, and δ-cells. Additionally, we found that 
upon stimulation with a β-cell-selective toxin streptozotocin 
(STZ), DGKζ translocates from the nucleus to the cytoplasm 
in β-cells and disappears over time. Our results suggest that 
DGKζ is intimately involved in β-cell function.

Materials and Methods

Animals

Adult male Wistar rats (Japan SLC) were used in the present 
study. Rats were housed under freely ranging circumstances 
in our animal facilities under normal lighting conditions 
(lightons 08:00–20:00) and were allowed ad libitum access 
to normal rodent chow and water. All animals were treated 
according to the guidelines for the care and use of laboratory 
animals of the Yamagata University School of Medicine.

RT-PCR analysis

Total RNAs were extracted from pancreata of 9–12-week-
old Wistar rats by TRIzol (Invitrogen, Carlsbad, CA). First-
strand cDNA was synthesized from 2 μg of RNA using 
PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara Bio, 
Shiga, Japan) following the manufacture’s instructions. 
Polymerase chain reaction (PCR) amplification was performed 
with KOD-plus polymerase (Toyobo, Tokyo, Japan) using 
gene specific oligonucleotide primers for rat DGK isozymes 
(Hozumi et al., 2010). PCR conditions were as follows: 
94°C for 5 min; 33 cycles of 94°C for 30 s, 62°C for 30 s, 
and 68°C for 40 s; and 68°C for 2 min. For control, rat 
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA 
was simultaneously amplified using forward 5'-TTAGCA 
CCCCGGCGAAGG-3' and reverse 5'-CCTACTCCT 
TGAGGCCATG-3' primers. PCR products amplified were 
separated by agarose gel electrophoresis and stained with 
ethidium bromide. The size of the expected fragment is as 
follows: DGKα, 405 bp; DGKβ, 424 bp; DGKγ, 394 bp; 
DGKε, 425 bp; DGKζ, 419 bp; DGKι, 410 bp; GAPDH, 523 bp. We repeated the RT-PCR twice for one sample 
derived from one rat (total 2 rats). Similar expression 
 patterns were obtained from these experiments, and one 
 experiment representative of four experiments is shown.

Immunoblotting

Pancreas and brain of 9-week-old rats were homogenized 
with 4 volumes of a buffer containing 10 mM Tris-HCl 
(pH 7.4), 20 mM KCl, 0.1 mM EDTA and 0.25 M sucrose, 
and centrifuged at 1000 g for 10 min at 4°C to remove 
debris. Protein concentration was determined using BCA 
Protein Assay Reagent (Thermo Scientific, Rockford, IL). 
Values were the means of triplicate determinations. The 
resulting supernatant (pancreas 40–120 μg, brain 20 μg) 
were boiled for 5 min in sodium dodecylsulfate (SDS) sample 
buffer (New England Biolabs, Inc., Beverly, MA) and
subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). After blocking the non-specific binding sites with 4% non-fat dry milk (w/v) in phosphate-buffered saline (PBS) containing 0.02% sodium azide and 0.2% Tween 20, the membrane was incubated for 1 h at room temperature with rabbit anti-DGKζ antibody (0.5 μg/ml) (Hozumi et al., 2003) or mouse anti-β-actin (1:5000; Sigma-Aldrich, St. Louis, MO) in 2% non-fat dry milk (w/v) in PBS containing 0.02% sodium azide and 0.1% Tween 20. Sites of antigen-antibody reaction were visualized using the chemiluminescent Immobilon Western blotting detection system (Millipore). We repeated the immunoblot 3 times and obtained the same results from those experiments.

Streptozotocin (STZ) treatment

Adult male Wistar rats (9~12-week-old, n=3) were intraperitoneally injected with a single bolus dose of 60 mg/kg STZ (Wako, Osaka, Japan) solution in citrate buffer and control rats (9~12-week-old, n=3) were injected with the citrate buffer alone (Liao et al., 2010). Both STZ treated and control rats were then routinely maintained for 7 days. Serum glucose levels were measured in the tail vein using a serum glucose reader (SANWA KAGAKU KENKYUSHO CO., Aichi, JAPAN). Rats with blood glucose >200 mg/dl were considered to use in this study (Yildirim et al., 2008).

Tissue and section preparation

Adult male Wistar rats at 9~12 weeks of age were used. For immunohistochemistry, they were fixed transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.2) (Hozumi et al., 2003). After fixation, pancreata were cryoprotected in a 30% sucrose/0.1 M PB and cut into 16-μm-thick sections by a cryostat (CM1900; Leica, Nussloch, Germany). To obtain adjacent slides, they were cut into 6-μm-thick sections by a cryostat (Leica).

Immunohistochemistry

All immunohistochemical incubations were performed at room temperature (~18°C). Aspecific binding sites were preadsorbed with 10% normal goat serum. Immunoperoxidase staining was performed by overnight incubation with rabbit anti-DGKζ antibody (0.5 μg/ml). Sections were further incubated with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min and avidin-biotin-peroxidase complex for 30 min using the avidin-biotine-peroxidase complex (ABC) system (Vector Laboratories). Immunoreaction was visualized with 3,3-diaminobenzidine (DAB) and photographs were taken by a light microscope (Leica). In double immunofluorescence, rabbit anti-DGKζ (1 μg/ml) antibody diluted with PBS containing 0.1% Triton was immunoreacted overnight in a mixture with one of the following antibodies: mouse anti-glucagon (1:500; G2654; Sigma-Aldrich Co., St. Louis, MO) or guinea pig anti-insulin (1 μg/ml; 18-0067; Thermo Fisher Scientific, Rockford, IL). Adjacent tissue sections were stained with rabbit anti-DGKζ or rabbit anti-somatostatin (1 μg/ml; SAB4502861; Sigma-Aldrich Co.) antibody. After thorough washing in PBS, they were visualized by 2 h incubation with Alexa Fluor 488-, Alexa Fluor 594-, or indodicarbocyanine (Cy5)-linked species-specific secondary antibodies (1:200 for each; Molecular Probes, Eugene, OR and Jackson ImmunoResearch, West Grove, PA). Images were taken with a confocal laser scanning microscope (LSM700, Carl Zeiss, Göttingen, Germany).

Results

Expression of DGK isozymes in the pancreas

We first examined the expression profile of DGK isozymes in the pancreas. RT-PCR analysis showed clearly that expression signals for DGKζ are detected intensely in this organ (Fig. 1). In addition, the signals were also detected faintly for DGKα and -ε whereas the signals for DGKβ, -γ, and -ι were below the detection level. Results show that DGKζ is the major isozyme in the pancreas. In the following experiments, we specifically examined DGKζ using detailed morphological analysis.

Localization of DGKζ in the pancreas

To examine regional and cellular localization of DGKζ, we then used immunohistochemical analysis of the pancreas
using a specific antibody (Hozumi et al., 2003). The antibody recognized a band of DGKζ at an estimated molecular mass in lysates of the pancreas and brain (Hozumi et al., 2003). We observed intense labeling for DGKζ throughout pancreatic islets, but not in the exocrine portion (Fig. 2B). In pancreatic islets, immunohistochemical signals for DGKζ were detected in most of the cells with varying degrees of intensity. Within the cells, the immunoreactivity was observed clearly in the nucleus (Fig. 2B), but it was only slightly detectable in the cytoplasm.

Next, double-labeling immunofluorescence was conducted to identify whether DGKζ is expressed in any of the types of islet cells (glucagon (α), insulin (β), and somatostatin (δ)). No co-expression was observed between insulin, glucagon, and somatostatin with the antibodies used in this study: insulin-producing β-cells are most abundant and are detected throughout the islets. However, glucagon-positive α- and somatostatin-positive δ-cells are few, and are distributed in the peripheral region (Fig. 3A). Double immunostaining revealed immunohistochemical signals for DGKζ in the nuclei of α-, β-, and δ-cells (Figs. 3B–D).

Expression and localization of DGKζ in pancreatic β-cells islets under streptozotocin-induced stress conditions

We next investigated how the expression and localization of DGKζ is changed in the course of STZ treatment, which induces DNA damage stress on pancreatic β-cells.

Immunoblot analysis of DGKζ protein levels after STZ administration revealed that DGKζ levels are transiently upregulated on day 3, decreasing thereafter to almost undetectable levels by day 7 (Fig. 4A). Morphologically, DGKζ-immunoreactivity was detected in the nucleus of insulin-positive β-cells in control pancreatic islets (arrows in Fig. 4B). However, on day 3, when the expression levels are at the peak, DGKζ-immunoreactivity was observed mostly in the cytoplasm of β-cells (arrowheads in Fig. 4B). On the other hand, on day 3, DGKζ-immunoreactivity remained in the nucleus of α-cells (asterisk in Fig. 4B). These data suggest that DGKζ translocates from the nucleus to the cytoplasm specifically in β-cells under STZ-induced stress conditions. On day 7, no significant labeling of DGKζ was detected in α-cells and β-cells (Figs. 4C).

Discussion

This report is the first describing a study elucidating the expression and localization of DGKζ in the pancreas at the protein level. Results show that DGKζ is detected in the pancreatic islet cells, including α-, β-, and δ-cells, but only slightly in the exocrine cells. DGKζ localizes predominantly to the respective nuclei of those cells, as reported in neurons (Goto and Kondo 1996; Hozumi et al., 2003) and other types of cells (Hozumi et al., 2010; Hozumi et al., 2013;
Fig. 2. Expression of DGK isozymes in the pancreas. (A) Immunoblot analysis. Rabbit antibody to DGKζ recognizes a 104 kDa protein band in lysates of the pancreas and brain (a positive control). The position of the standard protein marker is indicated to the left (kDa).

(B) Images of immunoperoxidase for DGKζ in the pancreas are shown at a low (upper) and a high (lower) magnification. Note intense labeling for DGKζ in the nucleus of almost all of pancreatic islet cells. Scale bar, 50 μm.

Hozumi et al., 2015). These findings suggest that DGKζ plays a role in islet cell function.

Previous studies have provided evidence demonstrating that DGKζ is intimately involved in stress response (Goto et al., 2014; Tanaka et al., 2015). DGKζ translocates from the nucleus to the cytoplasm under various stress conditions such as ischemia, infarction, oxygen-glucose deprivation, and kainate-induced seizures (Ali et al., 2004; Nakano et al., 2006; Saino-Saito et al., 2011; Suzuki et al., 2012; Goto et al., 2014). Translocation occurs at the early phase of stress. Thereafter, DGKζ gradually decreases in intensity and finally disappears before cell death.

The glucose analog STZ is a DNA-alkylating agent that is taken up selectively through glucose transporter 2 (GLUT2) (Schnedl et al., 1994). STZ induces DNA damage stress and subsequent cell death of β-cells that express GLUT2 abundantly, thereby serving as a β-cell-selective toxin. We used this agent to examine how the expression and localization of DGKζ is changed in β-cells under STZ-induced stress conditions. In this regard, we show DGKζ nucleocytoplasmic translocation in rat pancreatic islet cells under STZ-induced stress conditions. Results showed that after the STZ treatment DGKζ is increased transiently at the protein level and that DGKζ-immunoreactivity is detected in the cytoplasm. Thereafter, immunoreactivity decreases gradually in intensity and almost disappears, although insulin and glucagon staining remain. These results suggest that DGKζ translocates from the nucleus to the cytoplasm in β-cells in response to STZ-induced stress and disappears before cell death. Interaction of STZ with the pancreatic β-cells via GLUT2 receptors of the cells leads to the production of ROS, thereby inducing oxidative stress (Schnedl et al., 1994). This in turn enhances...
Fig. 3. Expression and localization of DGKζ in pancreatic islet cells. Immunofluorescence for characterization of DGKζ in pancreatic islet cells. (A) No overlapped staining is observed between insulin, glucagon, and somatostatin antibodies used in the present study. Double immunostaining of DGKζ with insulin (B) and with glucagon (C). Higher magnification images are also shown in insets. Immunoreactivity for DGKζ was compared with that for somatostatin on adjacent sections (D). Note that DGKζ immunoreactivity is detected in the nuclei of insulin-positive β-cells (arrows in B), glucagon-positive α-cells (arrowheads in C), and somatostatin-positive δ-cells (asterisk in D). Scale bars, 50 μm (A, B, C), 5 μm (D).
Fig. 4. Expression and localization of DGKζ in β-cells under streptozotocin (STZ)-induced stress conditions. (A) Immunoblot analysis of DGKζ after STZ administration. Note that DGKζ levels are transiently upregulated on day 3 and decreased thereafter to become undetectable by day 7. (B) Double immunostaining of DGKζ and insulin or glucagon in pancreatic islet cells on day 3 after STZ treatment. Under normal conditions DGKζ is seen in the nucleus of insulin-positive β-cells (arrows). On day 3, DGKζ is detected to the cytoplasm of β-cells (arrowheads) whereas it remains in the nucleus of glucagon-positive α-cells (asterisk). (C) No significant labeling of DGKζ is detected in α- and β-cells on day 7. Scale bars, 10 μm (B); 20 μm (C).
the robust production of proinflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-6, IL-18, etc.), which culminates in cell death (Esposito et al., 2002; Alexandraki et al., 2008). Massive destruction of β-cells may also impinge on neighboring cells such as α- and δ-cells.

This phenomenon in pancreatic β-cells resembles that observed in neurons under transient ischemia, as described above. Previous studies have suggested that DGKζ nucleocytoplasmic translocation represents a sort of negative feedback mechanism of p53 (Goto et al., 2014; Tanaka et al., 2015). Cytoplasmic DGKζ facilitates p53 degradation through ubiquitin-proteasome system, thereby attenuating p53-mediated cytotoxicity (Tanaka et al., 2013). In addition, decreased levels of nuclear DGKζ engender downregulation of p53 transcriptional activity. Both of these effects in the cytoplasm and nucleus synergistically repress p53-mediated cytotoxicity (Goto et al., 2014). Therefore, DGKζ nucleocytoplasmic translocation in the early phase of STZ exposure might be a protective response to attenuate DNA damage-induced p53 toxicity on β-cells. However, decreasing levels of DGKζ in the late phase of STZ exposure might weaken this protective effect against p53, which reminds us that cells have numerous protective stress responses, most of which switch into execution mode during prolonged activation (Herrup and Yang 2007; Goto et al., 2014).

In terms of the subcellular localization of DGKζ, DGKζ is shown to contain both the nuclear localization signal (NLS) and the nuclear export signal (NES) (Evangelisti et al., 2010). Under stress conditions, the dynamic balance between nuclear import and export might shift toward a predominance of NES under stress conditions, resulting in nuclear exclusion and cytoplasmic accumulation of DGKζ.

With regard to β-cell function, previous reports have described that DG plays an important role in the regulation of insulin secretion in pancreatic β-cells (Malaisse et al., 1985; Peter-Riesch et al., 1988; Corkey et al., 1989; Arkhammar et al., 1994). The primary role of DG is to activate protein kinase C (PKC), which is involved in insulin secretion (Prentki and Matschinsky 1987; Uchida et al., 2007) and β-cell apoptosis (Eitel et al., 2003; Wrede et al., 2003). In the pancreas, PKCδ activation and its nuclear translocation are required for fatty acid-induced apoptosis of insulin-secreting cells (Eitel et al., 2003). PKCδ null mice manifest glucose intolerance with impaired insulin secretion (Uchida et al., 2007), suggesting the importance of this PKC isoform in β-cell function.

In addition, PA is generated in β-cells upon glucose and muscarinic receptor activation; PA stimulates insulin secretion by increasing insulin granule traffic (Farese et al., 1986; McDonald et al., 2007). This PA is generated by the action of phospholipase D (PLD). Of the PLD family, PLD1 is necessary for the regulated secretion of insulin from pancreatic β-cells (Metz and Dunlop 1990). It modulates vesicular trafficking and exocytosis (Shen et al., 2001). Therefore, DG and PA are both involved in the physiological regulation of insulin secretion in β-cells, suggesting that DG metabolism plays a pivotal role in glucose homeostasis. Attenuating levels of DGKζ in pancreatic β-cells in response to STZ treatment is expected to engender dysregulation of DG metabolism, which might result in failure of insulin secretion and subsequent glucose homeostasis.

In conclusion, this study demonstrated that DGKζ localizes to the nucleus of pancreatic islet cells. After STZ treatment, DGKζ exhibits nucleocytoplasmic translocation in β-cells in the early phase and disappears thereafter. This cellular expression pattern and subcellular dynamics suggests that DGKζ is intimately involved in β-cell function.

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