

Nuclear relocation of DGK ζ in cardiomyocytes under conditions of ischemia/reperfusion

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Summary. Diacylglycerol (DG) and phosphatidic acid (PA) are generated under various conditions, such as ligand stimulation and several stresses. They serve as second messengers to respond to pathophysiological conditions. DG kinase (DGK) catalyzes DG to produce PA. It is regarded as a regulator of these lipid messengers. Previous studies show that DGK ζ , a nuclear isozyme, translocates from the nucleus to the cytoplasm in hippocampal neurons under transient ischemia and never relocates to the nucleus after reperfusion. This study examined whether a similar phenomenon is observed in cardiomyocytes, which represent another type of postmitotic, terminally differentiated cell. We performed immunostaining on ischemic hearts induced by occlusion of the left anterior descending coronary artery and on primary cultured cardiomyocytes under oxygen-glucose deprivation (OGD). In the animal model, 10 min ischemia is sufficient to cause DGK ζ to disappear from the nucleus in cardiomyocytes. However, DGK ζ is observed again in the nucleus at 10 min following reperfusion after 10 min ischemia, which contrasts sharply with ischemic hippocampal neurons. Similar results were obtained from experiments using primary cultured cardiomyocytes under OGD conditions, except that DGK ζ relocates autonomously, if at all, to the nucleus, even under continuous OGD conditions. Results suggest that DGK ζ is involved in the acute phase of cellular response to ischemic stress in cardiomyocytes in a similar, but not identical, manner to that of neurons.

Key words: Heart, Primary culture, Acute stress, Immunostaining, Rat

Introduction

Under various conditions, such as ligand stimulation and stresses, membrane lipid metabolism, including phosphoinositide turnover, generates a well-known second messenger: diacylglycerol (DG) (Rhee and Bae, 1997; Wakelam, 1998). DG transiently activates several signalling proteins to respond to physiological or pathological conditions (Nishizuka, 1992). Levels of DG must be strictly controlled to maintain the cellular environment within a physiological range because sustained elevation of DG would otherwise promote a maladaptation, such as dysregulated proliferation or necrosis/apoptosis.

Diacylglycerol kinase (DGK) catalyzes DG to produce phosphatidic acid (PA) (Kanoh et al., 1990). Recent evidence shows that PA also serves as a messenger molecule that activates the other set of proteins (Sakane et al., 2007). Considering its position in the conversion of these molecules, DGK plays a role not just in termination of DG signal, but also in subsequent generation of PA signals, suggesting that DGK is a regulator of the balance of these successive signalling pathways in a given condition.

Actually, DGK comprises a family of isozymes, each differing in terms of structure, enzymatic activity, expression and localization in tissues and cells, and each is implicated in distinct pathophysiological processes (Evangelisti et al., 2007; Goto et al., 2007; Sakane et al., 2007; Merida et al., 2008; Topham and Epanand, 2009). Of the isozymes, DGK ζ is implicated in the nuclear events in neurons under ischemic stress (Goto and Kondo, 1996; Hozumi et al., 2003; Ali et al., 2004; Nakano et al., 2006). In fact, DGK ζ translocates from the nucleus to the cytoplasm in hippocampal neurons under transient forebrain ischemia. It never relocates to the nucleus during the time course of reperfusion (Ali et al., 2004).

Transient ischemia in the hippocampus is known to cause hyperexcitation by glutamate and subsequent massive influx of Ca²⁺ which activates various catabolic processes (Choi, 1990; Ikegaya et al., 2001). Consequently, hippocampal neurons die an apoptotic cell death in a couple of days, which is known as delayed neuronal death (Kirino, 1982). From these observations, we infer that the disappearance of DGK ζ from the nucleus is an early critical and determining event associated with ischemic stress, although the molecular mechanisms remain unclear. Additionally, it remains unclear whether this phenomenon under ischemic stress is unique to neurons or occurs in cardiomyocytes, which represent another type of postmitotic, terminally differentiated cell.

In this study, we specifically examined this point and investigated the functional implications of DGK ζ in the heart under ischemic stress. We performed immunohistochemical analyses of rat heart after transient ischemia and reperfusion. Ischemic events in the heart in vivo were also simulated using primary cultured cardiomyocytes under oxygen–glucose deprivation (OGD). We demonstrate that DGK ζ disappears from the nucleus in cardiomyocytes at quite an early phase of ischemic insult, but it relocates to the nucleus after reperfusion. These results suggest that DGK ζ is also involved in the acute phase of cellular response of ischemic stress in cardiomyocytes in a similar, but not identical, manner to that of neurons.

Materials and methods

Animal model

Animals were handled according to the animal welfare regulations of Yamagata University. The study protocol was approved by the Animal Subjects Committee of Yamagata University. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health. Induction of myocardial ischemia was performed in 8-week-old male Wistar rats as described previously, with minor modifications (Takeda et al., 2001; Niizeki et al., 2007). Briefly, rats were anesthetized by intraperitoneal injection using pentobarbital (50 mg/kg body weight), after which they were intubated and ventilated through an endotracheal tube with a rodent ventilator (Harvard Apparatus, Holliston, MA, USA). An incision was made along the left sternal border. The fourth rib was cut proximal to the sternum. The left anterior descending coronary artery was identified and passed around by a 6-0 proline suture. Ischemia and reperfusion were performed, respectively, by occlusion and release of the artery. Successful ischemia was verified visually by the discoloration of the left ventricle myocardium. In sham-operated animals, the same procedure was performed, except for the coronary artery compression.

Cardiomyocyte isolation and culture

Cultured rat neonatal cardiomyocytes were prepared as described previously, with some modifications (Nitobe et al., 2003; Takahashi et al., 2005). Briefly, ventricles were obtained from 1- or 2-day-old Wistar rats. Then cardiomyocytes were isolated by digestion with collagenase. Cardiomyocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS) and 4500 mg/l glucose at 37°C and 5% CO₂ and changed to the medium without FCS 24 h before experiments. Cells were examined under conditions of normoxia (21% O₂, 5% CO₂, 74% N₂, 4500 mg/L glucose), hypoxia (5% CO₂, 95% N₂, 4500 mg/L glucose), or oxygen–glucose deprivation (OGD; 5% CO₂, 95% N₂ without glucose).

Tissue preparation and immunostaining

Animals were anaesthetized with ether and fixed with a transcardiac infusion of 4% paraformaldehyde. The heart was removed, immersed in the same solution for an additional 2 h at 4°C, and kept in 30% sucrose in 0.1 M phosphate buffer (pH 7.0) until use. Briefly, sections (20 μ m in thickness) were cut on a cryostat. Cultured cardiomyocytes were also examined after fixation with 4% paraformaldehyde. Immunostaining was performed as described previously (Goto et al., 1994; Hasegawa et al., 2008). Sections or cells were soaked with 0.3% Triton-X 100 in phosphate buffered saline (PBS) for 1 h at room temperature (RT) to facilitate antibody penetration. Endogenous peroxidase activity was inactivated with 3% H₂O₂ for 10 min at RT. Non-specific binding sites were blocked with 5% normal goat serum (NGS) in PBS for 1 h at RT. The primary antibody used was rabbit anti-DGK ζ antibody (0.1 μ g/mL) (Hozumi et al., 2003) in PBS containing 5% NGS and 0.1% Tween-20. Incubation was performed overnight at 4°C with the antibodies in a moist chamber. After washing in PBS several times, sections or cells were incubated with a biotinylated anti-rabbit IgG antibody in the same solution for 30 min at RT followed by avidin–biotin–peroxidase complex (ABC) method using a staining kit (Vector Laboratories Inc., Burlingame, CA, USA) for 30 min at RT. After rinsing, immunolabelling was performed with diaminobenzidine tetrahydrochloride (DAB). For immunofluorescence labelling, cells were incubated with anti-rabbit IgG-Alexa488 (green) (Molecular Probes Inc., Eugene, OR, USA) in PBS for 30 min at RT. The images were taken using a confocal laser scanning microscope (PASCAL; Carl Zeiss Inc., Germany) and processed with image processing software (Adobe Photoshop; Adobe Systems Inc., San Jose, CA, USA).

RT-PCR

Total RNA was extracted from the heart using acid

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guanidinium thiocyanate/phenol/chloroform extraction (TRIzol; GIBCO BRL, Bethesda, MD, USA). First strand cDNA was synthesized from 2 μ g of RNA using Moloney murine leukaemia virus reverse transcriptase (Promega Corp., Madison, WI, USA) following the manufacturer's instructions. Then PCR amplification was performed using KOD-plus polymerase (Toyobo Co. Ltd., Tokyo, Japan) with gene-specific oligonucleotide primers for rat DGK isozymes and GAPDH (control), as described previously (Katagiri et al., 2005).

Results

We first examined mRNA expression patterns of DGK isozymes in adult rat heart under normal conditions using RT-PCR analysis (Fig. 1). Specific bands for the three DGK isozymes, DGK α , DGK ϵ , and DGK ζ , were detected in rat heart tissues, which is in agreement with our previous data obtained using Northern blot analysis (Takeda et al., 2001). Additionally, we observed the band for DGK β faintly. Of the isozymes, DGK ζ was apparently most abundantly expressed, followed by DGK ϵ and DGK α .

We next performed immunohistochemical examination of the subcellular localization of DGK ζ in normal heart. DGK ζ -immunoreactivity was observed

intensely in the nucleus of cardiomyocytes (Fig. 2A). The immunoreactive cells were detected in all parts of the heart-including the left ventricle, septum, and right ventricle-at equal intensity.

Our previous report described DGK ζ translocation from the nucleus to the cytoplasm in hippocampal CA1 neurons after transient forebrain ischemia (Ali et al., 2004). In the present study, we examined whether the

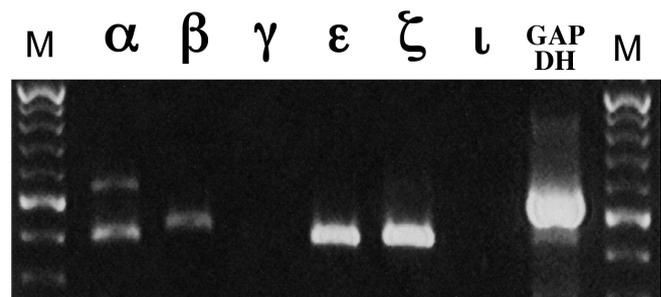


Fig. 1. Expression of DGK isozymes mRNA in normal rat heart. Reverse transcription - polymerase chain reaction (RT-PCR) analysis was performed using specific primers for each isozyme. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was also used as a control. M, size marker.

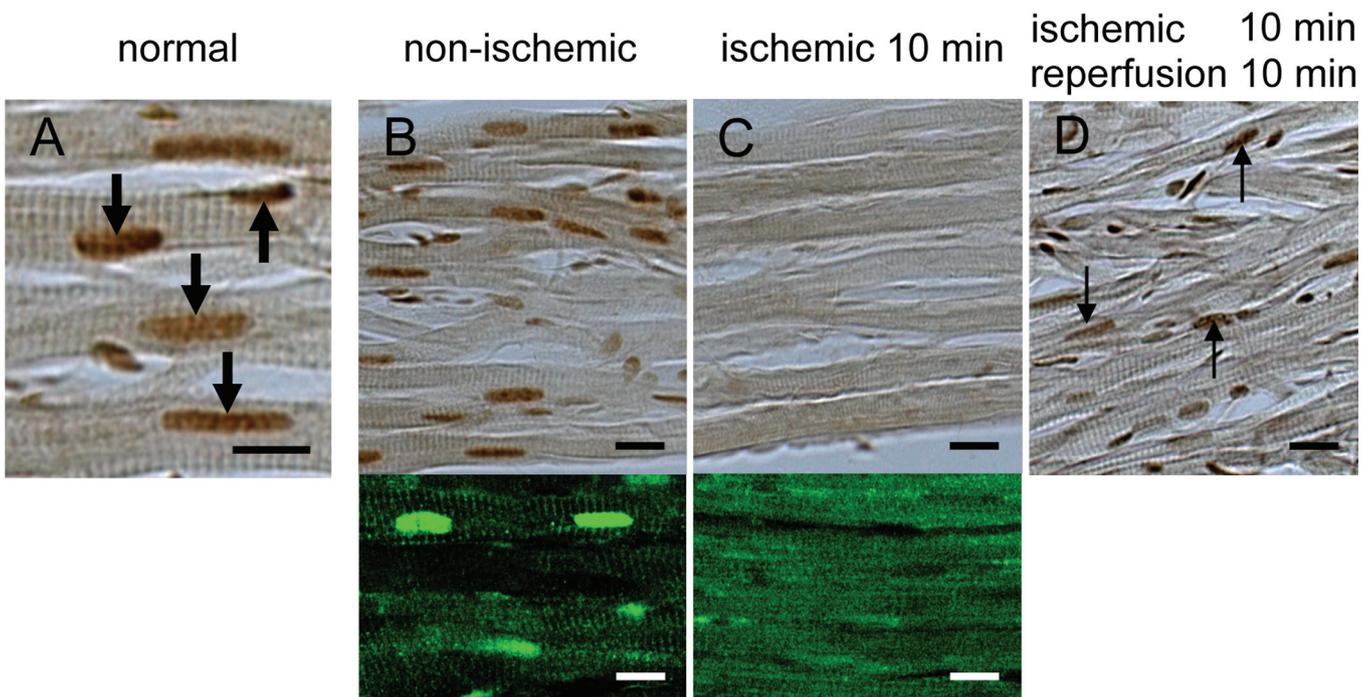


Fig. 2. Immunohistochemistry of DGK ζ in normal and ischemic hearts. DGK ζ -immunoreactivity is observed intensely in the nucleus of cardiomyocytes in all parts of a normal heart (A, arrows). Transient ischemia (10 min) was induced in rats by occlusion of the left anterior descending branch. In the non-ischemic septal region, DGK ζ -immunoreactivity is observed in the nucleus of cardiomyocytes, as in normal conditions (B). In contrast, DGK ζ -immunoreactivity almost disappears from the nucleus in the ischemic region (C). After 10 min reperfusion, DGK ζ -immunoreactivity is again observed moderately in the nucleus (D, arrows). Confocal images are also shown below. Bars: 10 μ m.

subcellular localization of DGK ζ would change after the ischemic insult in cardiomyocytes, which are postmitotic, highly differentiated cells like neurons. We first generated the 10 min-transient ischemic rat heart model by clipping the left anterior descending branch. In the non-ischemic septal region, DGK ζ -immunoreactivity was observed in the nucleus of cardiomyocytes similarly to normal conditions (Fig. 2B). However, in the ischemic region, immunoreactivity almost disappeared from the nucleus of cardiomyocytes. It was seen diffusely in the cytoplasm (Fig. 2C). In the border area of the ischemic region, DGK ζ -immunoreactivity was observed mostly in the nucleus and faintly in the cytoplasm.

To examine the extent of the severity of ischemia to cause this change in subcellular localization of DGK ζ ,

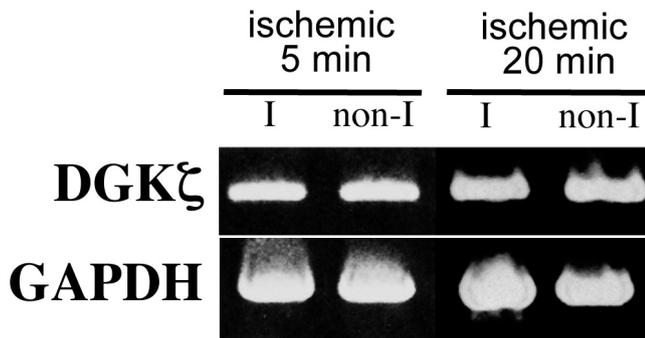


Fig. 3. RT-PCR analysis of DGK ζ in an ischemic heart. Expression levels for DGK ζ mRNA remain largely unchanged in ischemic (I) and non-ischemic (non-I) regions after 5 or 20 min of ischemia. GAPDH primer was also used as a control.

we generated models of 5 min ischemia and 20 min ischemia. Surprisingly, 5 min of ischemia started to change the subcellular localization of DGK ζ in cardiomyocytes, suggesting that under ischemic conditions of the heart, DGK ζ disappears quickly from the nucleus of ischemic cardiomyocytes.

In our previous study of transient ischemic brain tissues, we demonstrated that DGK ζ never relocates from the cytoplasm to the nucleus in hippocampal CA1 neurons following reperfusion (Ali et al., 2004). Therefore we next examined whether DGK ζ would relocate to the nucleus in cardiomyocytes following reperfusion after transient ischemia. In contrast to ischemic hippocampal neurons, DGK ζ -immunoreactivity was observed again in the nucleus of cardiomyocytes at 10 min following reperfusion after 10 min ischemia (Fig. 2D), although it was weaker than the control (non-ischemic region). Taken together, the animal models of ischemic heart suggest that DGK ζ disappears from, but relocates to, the nucleus of cardiomyocytes in response to ischemic insult and reperfusion. Results of RT-PCR analysis showed no change in the levels of DGK ζ mRNA in ischemic and non-ischemic regions after 5 or 20 min of ischemia (Fig. 3).

Ischemia caused by arterial occlusion produces various changes in the cellular environment, which most influentially blocks oxygen and glucose supplies and exerts strong effects on cellular energy metabolism. To examine the factors that affect the subcellular localization of DGK ζ further, we performed experiments using primary cultured cardiomyocytes from newborn rat hearts. Under normoxic conditions (21% O₂, 5% CO₂, 74% N₂) with regular glucose (4500 mg/L),

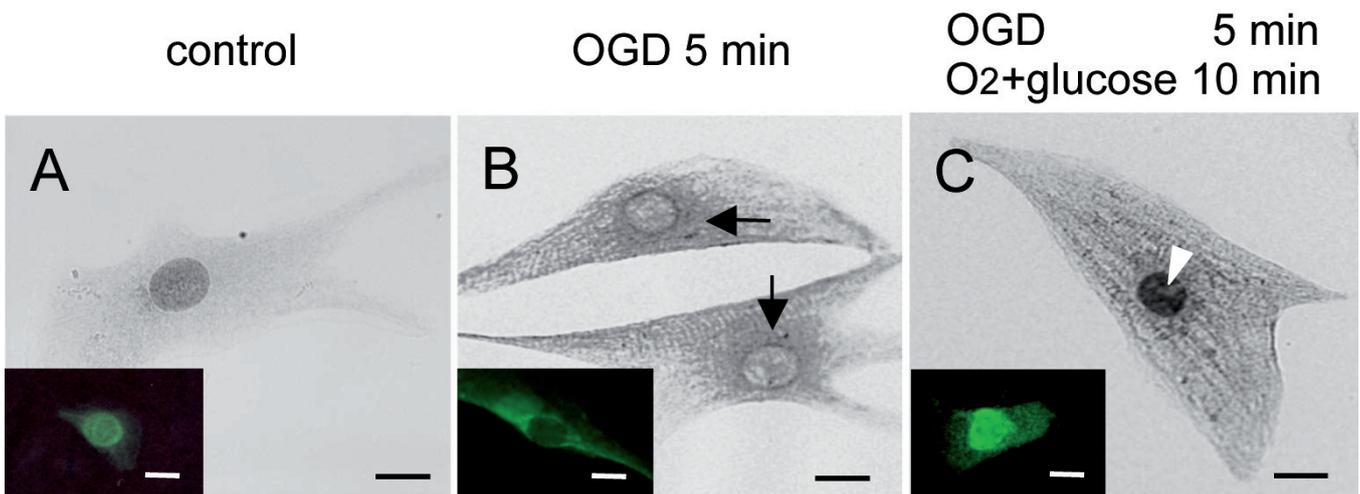


Fig. 4. Immunocytochemistry of DGK ζ in primary cultured cardiomyocytes. Under control conditions, i.e. normoxia (21% O₂, 5% CO₂, 74% N₂) and regular glucose (4500 mg/L), DGK ζ -immunoreactivity is detected in the nucleus, as in normal heart (A). At 5 min of oxygen–glucose deprivation (OGD), DGK ζ -immunoreactivity disappears from the nucleus and is observed in the cytoplasm (B, arrows). At 10 min following normoxic and regular glucose conditions after 5 min OGD, DGK ζ -immunoreactivity is observed again in the nucleus (arrowhead) (C). Insets are confocal images. Bars: 10 μ m

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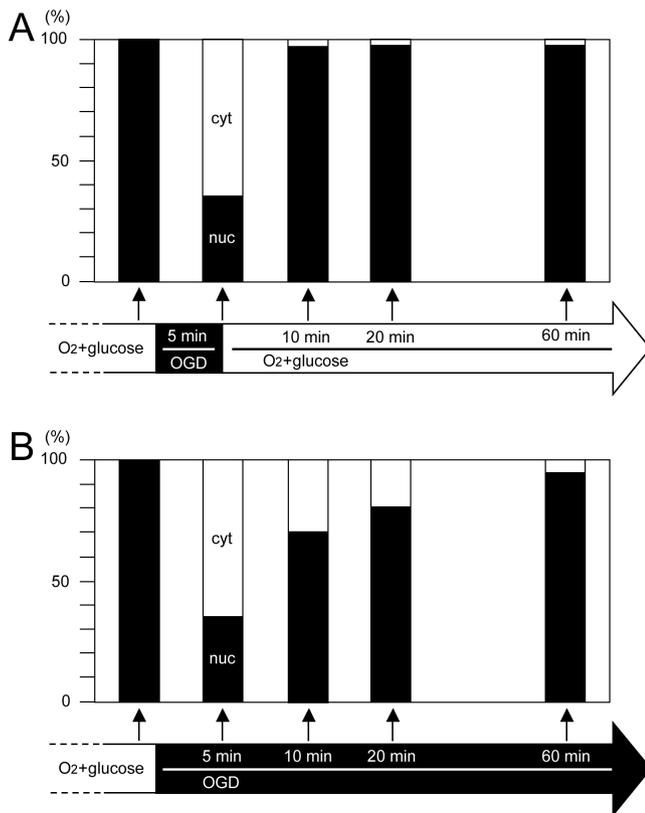


Fig. 5. Quantitative analysis of subcellular localization of DGK ζ in primary cultured cardiomyocytes. After immunostaining, 200 cells were classified into two categories, nuclear dominant staining (solid bar) and cytoplasmic dominant staining (open bar), during the time course of normoxic and regular glucose conditions (O₂ + glucose) after 5 min OGD (**A**) or of continuous OGD conditions (**B**). Similar results were obtained for two separate experiments.

DGK ζ -immunoreactivity was detected in the nucleus of cultured cardiomyocytes as in normal heart (Fig. 4A). We incubated cells under hypoxic conditions (5% CO₂, 94% N₂) with regular glucose and followed the time course. The DGK ζ -immunoreactivity did not change; it remained in the nucleus for up to 120 min (ca. 98% of the cells). Similarly, under normoxic conditions without glucose, DGK ζ -immunoreactivity was invariably detected in the nucleus (ca. 98% of the cells).

Reportedly, oxygen utilization and glucose metabolism are closely related. Both are involved in cellular energy homeostasis (Goldberg and Choi, 1993). Therefore, we next examined the severest conditions, i.e. combined deprivation of oxygen and glucose, known as oxygen–glucose deprivation (OGD). After 5 min of OGD, DGK ζ -immunoreactivity was detected in the cytoplasm of numerous cardiomyocytes (Fig. 4B). The cells that exhibited dominant nuclear DGK ζ -immunoreactivity had greatly decreased in number by about 36% of the total cell population. When cells were put back in normoxic and regular glucose conditions

after 5 min OGD, ca. 98% of cells again exhibited nuclear localization of DGK ζ after 10 min (Figs. 4C, 5A).

However, it should be particularly noted that similar relocation was also observed under continuous OGD conditions (Fig. 5B). At 10 min of OGD, cells exhibiting nuclear DGK ζ had increased in number by ca. 86%. By 20 min of OGD, ca. 95% of cells exhibited nuclear DGK ζ -immunoreactivity.

Collectively, the experimental data using cultured cardiomyocytes can be summarized as follows: 1) Nucleo-cytoplasmic translocation of DGK ζ is not triggered by either hypoxia or glucose deprivation alone, but is induced by combined conditions, OGD. 2) DGK ζ relocates quickly to the nucleus after transferral to normoxic and regular glucose conditions. 3) Nuclear relocation of DGK ζ also occurs, even under continuous OGD conditions, in a cell-autonomous manner.

Discussion

Cardiac ischemia results from an imbalance between blood flow and the metabolic requirement to support contractile function, which induces energy depletion stress in the heart. Recent evidence has shown that myocardial metabolic responses are regulated differentially in the acute and chronic phases during ischemic stress (Rosano et al., 2008). During the acute phase of ischemia, cardiomyocytes respond to the reduction of oxygen and substrate, resulting in anaerobic glycolysis and subsequent lactate production and acidosis as they adapt to chronic hypoxic conditions by altering the cardiac metabolism, both at transcriptional and post-translational levels.

In this regard, this study has revealed that DGK ζ localizes to the nucleus of cardiomyocytes in normal heart and disappears from the nucleus at quite an early phase of ischemic insult. In addition, results show that DGK ζ relocates to the nucleus following reperfusion. These results suggest that DGK ζ is involved in the acute phase of cellular response to ischemic insult in cardiomyocytes.

During the acute phase of ischemia, the reduction in aerobic ATP formation engenders an increase in the AMP:ATP ratio and subsequent activation of AMP-activated protein kinase (AMPK) (Hardie, 2004). Upon activation, AMPK phosphorylates several downstream substrates, which switch on ATP-generating pathways to restore energy homeostasis and switch off ATP-consuming pathways (Steinberg and Kemp, 2009). One ATP-generating pathway is an increase in glucose uptake through glucose transporter and glycolysis (Xing et al., 2003).

A representative of energy-saving strategies is the inhibition of protein synthesis. Actually, AMPK inhibits protein translation by several mechanisms (Young et al., 2005), including blockade of the activation of the mammalian target of rapamycin (mTOR), a highly conserved serine/threonine kinase that plays a central

role in regulating protein synthesis (Schmelzle and Hall, 2000; Gingras et al., 2001). In active protein synthesis, mTOR sustains translation by phosphorylating the eukaryotic initiation factor (eIF) 4E-binding proteins (4EBPs) and ribosomal protein S6 kinases (S6Ks). Intriguingly, mTOR-dependent phosphorylation of p70S6 kinase, one isoform of S6Ks, reportedly requires mTOR binding to PA that is produced by DGK ζ , suggesting that DGK ζ directly controls mTOR activity (Avila-Flores et al., 2005). Therefore, one might infer that under the acute phase of ischemic stress, activation of AMPK and attenuation of DGK ζ cooperatively down-regulates mTOR signalling, which engenders an inhibition of protein synthesis. Additional studies are necessary to elucidate this point.

A protective effect of DGK ζ on cardiac dysfunction has been demonstrated in previous model studies using transgenic (TG) mice with cardiac overexpression of DGK ζ . Under normal conditions, DGK ζ -TG mice are indistinguishable from WT mice in histological and physiological analyses. They exhibit similar hemodynamic regulations in response to angiotensin II and phenylephrine, showing no adverse effect of DGK ζ overexpression on the heart *in vivo*. However, in a cardiac hypertrophy model created by angiotensin II and phenylephrine infusion (Arimoto et al., 2006) or by thoracic transverse aortic constriction (Harada et al., 2007), increases in heart weight and interventricular thickness, dilatation of the left ventricular (LV) cavity, and decreases in LV systolic function are abolished in DGK ζ -TG mice, suggesting that DGK ζ negatively regulates the hypertrophic signalling cascade, suppresses cardiac hypertrophy and fibrosis, and prevents impaired LV systolic function caused by Gq-protein-coupled receptor activation or pressure overload. Furthermore, in a myocardial infarction model (Niizeki et al., 2007), LV chamber dilatation, reduction of LV systolic function and increases in LV weight and lung weight are attenuated in DGK ζ -TG mice, suggesting that DGK ζ suppresses LV structural remodelling and fibrosis, which improves survival after myocardial ischemia. The results of these studies imply that DGK ζ exerts its protective effects on the heart not only in the acute phase, but also in the chronic phase of cardiac stress. However, the present study specifically examines only the acute phase.

Our previous study of transient forebrain ischemia has revealed a similar, but not identical, phenomenon by which DGK ζ translocates from the nucleus to the cytoplasm in hippocampal CA1 neurons under transient ischemic insult (Ali et al., 2004). Nevertheless, it is noteworthy that DGK ζ behaves differently in hippocampal neurons and cardiomyocytes following reperfusion: DGK ζ never relocates to the nucleus in hippocampal neurons, although it relocates to the nucleus in cardiomyocytes. What are the implications of different behaviours of DGK ζ between hippocampal neurons and cardiomyocytes, both of which are postmitotic, terminally differentiated cells? Prolonged viability in the face of severe hypoxia, which

distinguishes cardiomyocytes from some other cell types, such as neurons (Bossenmeyer et al., 1998; Chihab et al., 1998), suggests the presence of intrinsic mechanisms that protect cardiomyocytes from hypoxia-related apoptosis (Malhotra and Brosius, 1999). Nuclear relocation of DGK ζ after reperfusion might reflect one of those intrinsic protective mechanisms, which maintains intracellular integrity and necessary cellular functions under stressed conditions. Autonomous relocation of DGK ζ in cultured cardiomyocytes under continuous OGD conditions might also be the case. Absence of the autonomous relocation during ischemia in the animal model is ascribed to different conditions in tissues and isolated cells. During ischemia, oxygen depletion forces the heart to switch to anaerobic glycolysis, which results in accumulation of lactic acid as a by-product and causes pH to fall in the ischemic region. Possibly, acidosis suppresses autonomous relocation of DGK ζ to the nucleus during ischemia in the heart; this blockade is released by reperfusion, which removes lactic acid and restores pH to its normal range. This possibility should be examined further.

The molecular mechanisms underlying this phenomenon are not addressed in this study, although we must consider both nuclear import and export of DGK ζ . A nuclear localization signal (NLS) family is generally known to play a pivotal role in shuttling between the nucleus and the cytoplasm through importins. DGK ζ contains a NLS sequence of bipartite type that overlaps myristoylated alanine-rich C-kinase substrate (MARCKS) domain (Bunting et al., 1996; Goto and Kondo, 1996). In addition, recent reports describe that a nuclear export signal (NES) is included in DGK ζ (362-LSTLDQLRL-370) (Evangelisti et al., 2010). Therefore, it is plausible that both the NLS and the NES are involved in reversible change in DGK ζ localization between the nucleus and the cytoplasm through importin and Crm1/exportin1 machineries. Under stressed conditions, binding partners of DGK ζ might affect the primacy of NLS over NES, or vice versa, and determine the dynamic balance between nuclear import and export, resulting in net accumulation of DGK ζ within the nucleus or to the cytoplasm.

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