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DIACYLGLYCEROL KINASE REGULATION OF PROTEIN KINASE D DURING OXIDATIVE STRESS-INDUCED INTESTINAL CELL INJURY

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Abstract

We recently demonstrated that protein kinase D (PKD) exerts a protective function during oxidative stress-induced intestinal epithelial cell injury; however, the exact role of DAG kinase (DGK)ζ, an isoform expressed in intestine, during this process is unknown. We sought to determine the role of DGK during oxidative stress-induced intestinal cell injury and whether DGK acts as an upstream regulator of PKD. Inhibition of DGK with R59022 compound or DGKζ siRNA transfection decreased H₂O₂-induced RIE-1 cell apoptosis as measured by DNA fragmentation and increased PKD phosphorylation. Overexpression of kinase-dead DGKζ also significantly increased PKD phosphorylation. Additionally, endogenous nuclear DGKζ rapidly translocated to the cytoplasm following H₂O₂ treatment. Our findings demonstrate that DGK is involved in the regulation of oxidative stress-induced intestinal cell injury. PKD activation is induced by DGKζ, suggesting DGK is an up-stream regulator of oxidative stress-induced activation of the PKD signaling pathway in intestinal epithelial cells.

Keywords

DGK; PKD; ROS; Intestinal epithelial cells

INTRODUCTION

Reactive oxygen species (ROS) mediate damage to cellular components and are involved in a wide range of diseases that affect tissues of many systems including the gastrointestinal (GI) tract [1]. Exposure to ROS at high concentrations or for extended periods of time damages cellular DNA, and is widely hypothesized to induce cell death via apoptosis or necrosis [2]. In particular, ROS have been implicated in the pathogenesis of necrotizing enterocolitis (NEC), a devastating disease in premature infants frequently resulting in sepsis and death [3]. However, the exact cellular signaling involved during ROS-induced intestinal cell injury is largely unknown.

Diacylglycerol (DAG) is a membrane lipid second messenger that activates protein kinase C (PKC) isoforms and PKD [4;5], which, in turn, regulate many cellular responses including growth, differentiation and apoptosis [6]. DGK phosphorylates DAG to produce phosphatidic acid (PA) and, consequently, is a potential terminator of DAG signaling. Three of DGK

isoforms (β , θ , ζ) have been identified in the intestine[8] Because DAG serves as an activator of PKC, DGK is thought to regulate PKC activity through DAG metabolism, and subsequently the signaling pathways of PKC [7]. By regulating local accumulation of DAG signaling, DGK plays a central role in many biological responses such as cell proliferation, differentiation, survival and apoptosis.

PKD is a serine/threonine protein kinase with homology to conventional PKC isoforms in the regulatory domain. However, PKD has additional protein modules not found in other PKC isoforms [9;10]. PKD is highly mobile in cells, enabling it to function as a communicator between different subcellular compartments. Importantly, PKD can be activated by oxidative stress to induce survival pathways [9]. Previous reports have demonstrated that the activity of PKD is synergistically stimulated by DAG analogs, indicating that PKD could be a novel component in the signal transduction of many growth factors, regulatory peptides, and cytokines that elevate DAG in their target cells [11]. The DAG-PKC-PKD pathway has emerged as a ubiquitous key signaling mechanism that regulates PKD localization, activation and multiple cellular functions.

We have previously shown that the survival of intestinal cells after oxidative stress may be a function of increased PKD activity [12]. However, the upstream mediator(s) in this process is/are unknown. The purpose of our current study was to determine the role of DGK during oxidative stress-induced intestinal cell injury and whether DGK acts as an upstream regulator of PKD.

MATERIALS AND METHODS

Reagents and antibodies

DGK ζ siRNA and the non-targeted control siRNA were purchased from Dharmacon (Lafayette, CO). DGK inhibitor R59022 was from Calbiochem (La Jolla, CA). The anti-PKD, DGK ζ polyclonal antibodies and the secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phospho-PKD (Ser744/748), PARP and caspase-3 antibodies were from Cell Signaling Technology (Beverly, MA). Alexa Fluor 594 antibody was from Molecular Probes (Eugene, OR). The enhanced chemiluminescence (ECL) system was from Amersham (Arlington Heights, IL). The concentrated protein assay dye reagent was from Bio-Rad (Hercules, CA). Cell death detection ELISA^{plus} kit was from Roche (Indianapolis IN). Nuclear and Cytoplasmic Extraction Reagent was from Pierce (Rockford, IL). All other reagents were purchased from Sigma (St. Louis, MO).

Cell culture, transfection and preparation of nuclear extracts

IEC-6 and HT29 cell lines were purchased from American Type Culture Collection (Rockville, MD). The RIE-1 cells were originally provided by Dr. K. Brown (Babraham Institute, Cambridge, UK) [13]. RIE-1 cells were used and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) in 5% CO₂ at 37°C. For experimental purposes, cells were plated in 60-mm dishes and grown to near confluence. Cells were starved in serum-free DMEM overnight and treated with indicated concentration of H₂O₂ at 37°C. For inhibitor treatment, cells were starved in serum-free DMEM overnight and pretreated with inhibitor for 2 h and with H₂O₂ in combination with inhibitor for another 30 min. Cells were transfected with siRNA or DGK ζ plasmid (a generous gift from Dr. Matt Topham, University of Utah, Salt Lake City, UT) by electroporation (400V, 500 μ F) using GenePulser XCell (Bio-Rad, Hercules, CA). Nuclear protein was prepared with Nuclear and Cytoplasmic Extraction Reagents (Pierce), according to the manufacturer's protocol.

Protein preparation and Western blotting

For total protein extraction, cells were washed with cold phosphate buffered saline (PBS), scraped into RIPA lysis buffer, and incubated as we have previously described [12]. The protein concentration of the supernatants was determined by a Bio-Rad assay. Equal amounts of protein were resolved on 4-12% Novex Tris-Glycine gels (Invitrogen) and electrophoretically transferred to polyvinylidene difluoride membranes. After the nonspecific binding sites were blocked with 5% dried skimmed milk in TBST for 1 h, the membranes were incubated with primary antibodies overnight at 4°C followed by secondary antibodies conjugated with horseradish peroxidase. Membranes were developed using the ECL detection system.

Immunofluorescent staining and fluorescent microscopy

Cells were grown in chamber slides. Two days later, cells were treated either with or without H₂O₂ (500 μM) over a time course. Cells were then fixed with cold methanol for 20 min at 4°C. After washes with cold PBS, the cells were blocked with 1% BSA-PBS for 20 min and then incubated with polyclonal anti-DGKζ antibody diluted 1:25 with 1% BSA-PBS for 1 h. Cells were washed with PBS and incubated with Alexa 594-conjugated anti-goat antibody diluted 1:500 in 1% BSA-PBS. The fluorescence of DGKζ immunoreactivity was observed under a fluorescent microscope as we have described [12].

Cell death assay

Cells were seeded in 24-well plates at density of 4×10⁴ per well. After 24 h, cells were transfected with siRNA and then cultured for 72 h. Cells were treated with 500 μM of H₂O₂ in growth medium for 3 h. Both adhered cells and floating cells were collected and washed using cold PBS. Cell death assay was performed using Cell Death Detection ELISA^{plus} kit following the manufacturer's instruction.

Statistical analysis

All experiments were repeated at least three times and data are represented as mean ± SEM. Data was analyzed using the Kruskal-Wallis test due to heterogeneous variability in each group. All tests were assessed at the 0.05 level of significance. All statistical computations were conducted using the SAS[®] system, Release 8.2.

RESULTS

Expression and the subcellular localization of DGKζ in intestinal cell lines

In order to investigate whether DGKζ is expressed in native intestinal epithelial cells, we utilized rat intestinal cell lines, RIE-1 and IEC-6, as well as human colon cancer cell line, HT29, and assessed for the expression of DGKζ. Because DGKζ is abundantly expressed in the brain [14], we used rat brain tissue as positive control. Western blotting using an anti-DGKζ polyclonal antibody revealed that DGKζ is expressed in all three cell lines examined (Fig. 1A). Next we explored the localization of DGKζ in RIE-1 cells. Using immunofluorescent staining, we found that the DGKζ is localized in the nucleus of RIE-1 cells (Fig. 1B). Further confirmation of nuclear localization of DGKζ was observed by Western blotting in RIE-1 cells (Fig. 1C).

H₂O₂-induced cytoplasmic translocation of endogenous nuclear DGKζ

To determine whether subcellular translocation of DGKζ occurs during oxidative stress injury, RIE-1 cells were treated with or without H₂O₂ and then observed with immunofluorescent staining. In untreated cells, DGKζ was localized predominantly in the nucleus. In contrast, DGKζ was observed in the cytoplasm after treatment with H₂O₂ for 30 sec. The majority of DGKζ translocated to the cytoplasm within 5 min of treatment (Fig. 2A).

Western blotting was again used to confirm localization. Continued exposure to H₂O₂ over time resulting in decreased levels of nuclear DGK ζ ; this change was accompanied by an increase in the cytoplasmic DGK ζ level (Fig. 2B).

Oxidative stress-mediated PKD activation is regulated by DGK

To investigate whether H₂O₂-induced PKD activation is mediated by DGK isoforms, RIE-1 cells were pretreated with various doses of R59022 for 2 h, then in combination with H₂O₂ for another 30 min. We found that PKD phosphorylation was increased by R59022 with or without H₂O₂ treatment compared with control cells (Fig. 3A). To further demonstrate the role of DGK ζ in the regulation of PKD activity in H₂O₂-induced cells, siRNA targeting DGK ζ was transfected into RIE-1 cells for 3 days. The expression of DGK ζ was significantly decreased by DGK ζ siRNA when compared with control siRNA (Fig. 3B; *top row*). PKD phosphorylation was increased by DGK ζ siRNA either in the presence or absence of H₂O₂ compared with the control siRNA (Fig. 3B; *middle row*). We also transiently transfected RIE-1 cells with either wild-type (WT) or kinase-dead (KD) DGK ζ plasmids for overexpression or knockdown of DGK ζ , respectively (Fig. 3C). Compared with control cells, a weaker PKD phosphorylation signal was detected in RIE-1 cells transfected with DGK ζ WT upon H₂O₂ treatment, suggesting negative regulation of PKD by constitutively active DGK ζ under oxidative stress. Interestingly, PKD phosphorylation was significantly increased in RIE-1 cells transfected with DGK ζ KD, even in the absence of H₂O₂ treatment (Fig. 3C; *top row*).

DGK inhibitor and DGK ζ siRNA decrease H₂O₂-induced cell death

We next examined whether DGK activation could protect intestinal epithelial cells from H₂O₂-induced cell apoptosis. RIE-1 cells were treated with H₂O₂ alone or in combination with R59022 and apoptosis was quantified via DNA fragmentation (Fig. 4A). Treatment with R59022 significantly attenuated H₂O₂-induced cell death, which is consistent with the protective function of PKD [12]. We also measured the levels of cleaved-caspase 3, an apoptotic marker [15], and found that treatment of RIE-1 cells with H₂O₂ induced caspase-3 activation. In contrast, compared to H₂O₂ treated-cells without R59022 treatment, cells treated with the inhibitor demonstrated minimal to no active caspase-3 expression (Fig. 4B).

To further elucidate the specific role of DGK ζ , we transfected DGK ζ siRNA into RIE-1 cells and after 3 days, cells were treated with H₂O₂. The cell death assay demonstrated that H₂O₂ treatment increased RIE-1 cell apoptosis compared to cells without H₂O₂ treatment in control siRNA transfected cells (Fig. 4C; *upper panel*). Western blotting demonstrated that the inhibition of DGK ζ expression by siRNA significantly decreased endogenous DGK ζ levels when compared to control siRNA (Fig. 4C; *middle panel*). More importantly, H₂O₂-induced cell death decreased in DGK ζ siRNA transfected cells compared to control cells with H₂O₂ treatment (Fig. 4C; *upper panel*), suggesting that the suppression of endogenous DGK ζ decreases H₂O₂-induced intestinal epithelial cell apoptosis. Additionally, this decrease in H₂O₂-induced apoptosis in DGK ζ siRNA transfected RIE-1 cells was confirmed by decreased protein expression of active caspase-3 (Fig. 4D).

DISCUSSION

DGK ζ has multiple structural features, including four ankyrin-like repeats and a nuclear localization signal [16]. DGK ζ is phosphorylated by PKC- α and - γ , resulting in its export from the nucleus and consequent elevation of nuclear levels of DAG and accelerated cell growth [7]. In this report, we showed that H₂O₂ induces translocation of endogenous DGK ζ from the nucleus to the cytoplasm and that oxidative stress-mediated PKD activation is regulated by DGK isoforms. We also found that suppression of DGK by siRNA or R59022 decreased oxidative stress-induced cell apoptosis. R59022 is known to selectively inhibit the type I DGK

isoforms [17]. Since DGK β is the only type I class of DGK in the intestine [8], we speculate that DGK isoforms other than DGK ζ may have signaling functions in intestinal cells undergoing oxidative stress. Our results also suggest that DGK acts to keep DAG concentrations relatively low in the nucleus of resting cells; however, the suppression of DAG levels is attenuated by inactivation of DGK in intestinal cells exposed to H₂O₂. During oxidative stress, with subsequent inhibition of DGK, DAG accumulates in the nucleus to synergistically activate the PKD pathway. Based on observations from the current study, DGK translocation from the nucleus to the cytoplasm may affect DAG accumulation in the nucleus of cells undergoing oxidative stress.

Several reports have noted that DGK ζ is localized to the nucleus in certain cell lines [18;19;20]. However, others have found that DGK ζ is expressed in cellular compartments other than the nucleus [21]. In this report, we found that DGK ζ is predominantly expressed in the nuclear compartment of the intestinal cell lines examined. The different subcellular locations of this enzyme may implicate multiple signaling functions. For intestinal cells, nuclear DGK ζ may be involved in the regulation of cell replication by attenuation of nuclear DAG and subsequent down-regulation of the activity of downstream proteins. Subcellular translocation of DGK ζ has also been reported in stimulated cells [22]. Interestingly, the subcellular localization of DGK ζ is reversed from the nucleus to the cytoplasm in some neurons at a very early phase of ischemic insult [14]. Other studies indicate that the regulation of DGK activity depends on the correct subcellular localization of the enzyme [7;23]. Our previous report demonstrated that PKD translocates from the cytoplasm to the nucleus upon H₂O₂ treatment [12]. Therefore, during oxidative stress, DGK ζ can regulate the levels of DAG in the nucleus, and subsequently affect DAG-dependent events, such as the activation of PKD. Under certain conditions DGK ζ might shuttle between the nucleus and cytoplasm [18]. One of the most interesting findings in our present study is that DGK ζ translocated from the nucleus to the cytoplasm in RIE-1 cells at the very early phase of H₂O₂ treatment, suggesting that DGK ζ may be involved in the process of oxidative stress in these cells. Translocation from the nucleus to the cytoplasm seems to be a general mechanism for activation of DGK ζ . Giving that DGK plays a major role in attenuating DAG, translocation into the cytoplasm may lead to a sustained increase in DAG levels and subsequent activation of PKD in the nucleus.

Exposure of cells and tissues to ROS results in cell death. The prevention of injury from ROS is critical for cellular resistance to many death stimuli. In response, cells have developed protective mechanisms in the form of pro-survival signaling pathways [24]. Here, we showed that cells treated with a DGK inhibitor or transfected with DGK ζ siRNA increased PKD phosphorylation and decreased H₂O₂-induced cell apoptosis. Phospholipase C(PLC) mediates PKD phosphorylation [25], and oxidative stress-induced PLC activation enhances cell survival [26;27] in some cell lines. In addition, PKD is an important downstream effector for DAG, whose function and subcellular localization are tightly controlled by DGK [28]. Therefore, we speculate that oxidative stress induces PLC activation and DGK inactivation; the changes of both enzyme activities result in DAG accumulation and PKD phosphorylation protecting the intestinal cells from H₂O₂ induced-cell death.

In conclusion, our results demonstrate that, following H₂O₂ treatment of RIE-1 cells, DGK ζ translocates from the nucleus to the cytoplasm, strongly suggesting that DGK ζ may be involved in the process of oxidative stress in intestinal epithelium. In addition, we found that phosphorylation of PKD is regulated by DGK isoforms. DGK ζ siRNA transfection decreased H₂O₂-induced cell death, further suggesting a novel molecular action of oxidative stress by activation of the DAG-PKD signaling pathway via an endogenous attenuator, DGKs. This molecular mechanism may be an important regulatory process in the protective function against oxidative stress-mediated intestinal epithelial cell injury. Ultimately, a better understanding of

these mechanisms is critical in developing and implementing novel clinical therapies to ameliorate intestinal injury in premature infants with NEC.

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Figure 1. Expression and subcellular localization of DGK ζ in the intestinal cells

(A) Cells were lysed and Western blot analysis was performed to determine the expression of DGK ζ . Rat brain tissue was used as a positive control. (B) RIE-1 cells were grown on chamber slides for 2 days. Cells were fixed and incubated with anti-DGK ζ antibody, followed by incubation with Alexa 594-conjugated anti-goat antibody. Images were recorded by fluorescent microscopy where intense red staining represents DGK ζ . (C) Cells were fractionated into nuclear and cytoplasmic portions. The proteins (20 μ g/lane) were analyzed with anti-DGK ζ antibody by Western blotting. I κ B α was used for confirming the cytoplasmic portion and Lamin B for confirming the nuclear portion.

Figure 2. H₂O₂-induced cytoplasmic translocation of DGK ζ in RIE-1 cells

(A) RIE-1 cells were grown on glass chamberslips for 2 days. Following treatment with H₂O₂ (500 μ M) for 0.5 and 5 min, they were washed, fixed and incubated with anti-DGK ζ antibody (1:25) for 1 h followed by Alexa 594-conjugated secondary antibody (1:500) in the dark. Images recorded by fluorescent microscopy. (B) RIE-1 cells were stimulated with H₂O₂ (500 μ M) over a time course. The cytoplasm and nuclear fractions were prepared and the expression of DGK ζ in each fraction was determined by Western blotting. I κ B α was used for confirming the cytoplasm portion and Lamin B for confirming the nuclear portion.

Figure 3. Regulation of PKD activation by DGK inhibitor, DGK ζ siRNA or DGK ζ plasmids

(A) RIE-1 cells were pretreated with or without R59022 for 2 h and then treated with H₂O₂ (500 μ M) in combination with R59022 for another 30 min. PKD phosphorylation was determined by Western blot analysis. (B) RIE-1 cells were transfected with DGK ζ siRNA or control siRNA. After 3 days, cells were treated with H₂O₂ (500 μ M) for 30 min and protein was extracted and analyzed by Western blotting. DGK ζ siRNA decreased expression of DGK ζ (*first row*). PKD phosphorylation was determined using anti-phospho-PKD (Ser744/748) antibody (*second row*). (C) RIE-1 cells were transiently transfected with either DGK ζ WT or DGK ζ KD or their empty vector (pcDNA) and recovered for 48 h. Cells were then serum-starved overnight and treated with H₂O₂ (500 μ M) for 30 min. PKD phosphorylation was determined by Western blot analysis (*first row*). Effects of DGK ζ plasmid transfection were detected using anti-flag antibody (*third row*).

Figure 4. DGK inhibitor or DGK ζ siRNA decreased H₂O₂-induced cell death

(A) RIE-1 cells were pretreated with or without R59022 for 2 h and then treated with H₂O₂ (500 μ M) for another 3 h prior to cell death assays. Results from 3 experiments are expressed (mean $\pm$ SEM; * $p < 0.05$ vs. H₂O₂ (-); † $p < 0.05$ vs. H₂O₂ only). (B) RIE-1 cells were pretreated with or without R59022 for 2 h and then treated with H₂O₂ (500 μ M) for another 3 h. Both attached and floating cells were collected, lysed and analyzed by Western blot analysis for the expression of caspase-3. (C) RIE-1 cells were transfected with DGK ζ or control siRNA. After 3 days, cells were treated with 500 μ M of H₂O₂ for 3 h and cell death assays performed. Results from 3 experiments are expressed (mean $\pm$ SEM; * $p < 0.05$ vs. control siRNA (-); † $p < 0.05$ vs. control siRNA + H₂O₂) (upper panel). Inhibition of DGK ζ expression by DGK ζ siRNA using anti-DGK ζ antibody (middle panel). (D) RIE-1 cells were transfected with DGK ζ or control siRNA for 72 h, and then treated with H₂O₂ (500 μ M) for 3 h. Both attached and floating cells were collected, lysed and analyzed by Western blot analysis for the expression of caspase-3.