Expression of mRNAs for the diacylglycerol kinase family in immune cells during an inflammatory reaction

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ABSTRACT

Phosphoinositide metabolism is intimately involved in cellular signal transduction. In response to extracellular stimuli, it generates diacylglycerol (DG), which serves as a lipid second messenger molecule to activate various proteins in various organs under pathophysiological conditions. Diacylglycerol kinase (DGK) constitutes an enzyme family that catalyzes conversion of DG to phosphatidic acid. It is therefore regarded as a regulator of the DG signal. Previous studies have revealed the critical role of α and ζ types of DGK in T cell functions. Nevertheless, little is known about the expression patterns of the DGK family in immune cells of various kinds. After examination of the expression profile of DGK isozymes in immune cells that are isolated from human blood, we investigated whether their mRNA expression levels would be changed during an inflammatory reaction. Results showed that DGK isozyme mRNAs are widely expressed in immune cells, except for DGKβ and DGKι. During an inflammatory reaction, DGKε mRNA was increased transiently in the initial phase (20–40 min) of stimulation with both LPS and IL-2 in T cell-derived HUT-102 cells and macrophage-derived RAW264 cells. At the organismal level, an intraperitoneal injection of LPS also induced upregulation of DGKε mRNA in the spleen in a similar, but not identical, manner. These results suggest that DGKε is involved in inflammatory processes of the cellular immune system.

ABSTRACT

Phosphoinositide turnover is a key event of signal transduction in regulating cellular responses to widely various extracellular stimuli (6, 19). The PI-derived lipid second messengers are well recognized as carrying out specific tasks for widely various biological processes in eukaryotic cells (5). In this system, hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by an action of phospholipase C (PLC) generates diacylglycerol (DG) (30, 31), which activates various effector proteins through the DG-responsive C1 domain, such as protein kinase C (PKC), protein kinase D, Munc-13, transient receptor potential (TRP) channel, and Ras guanyl nucleotide releasing protein (RasGRP) (38). Levels of DG should be tightly controlled to maintain cellular responsiveness within a physiological range because sustained activation of the DG signaling would induce a maladaptation of cellular homeostasis, which can promote tumorigenesis or cell death (26). DG metabolism is catalyzed by its phosphorylation by diacylglycerol kinase (DGK), which subsequently generates phosphatidic acid (PA). Recent evidence has revealed that PA, the product of DGK, is also linked to the regulation of signaling molecules, including a mammalian target of rapamycin (mTOR), phosphatidylinositol 4P 5-kinase (PI4P5K), Ras guanosine triphosphatase (RasGTPase)-activating pro-

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tein (RasGAP), Ras guanine nucleotide exchanging factor (RasGEF), p47phox, and son-of-sevenless (SOS) (41). These findings indicate that DGK plays a pivotal role in PI-derived messenger signaling through the conversion of DG to PA. Consequently, DGK activity temporally regulates the strength of DG signal, which is invariably followed by a PA signal.

In fact, DGK comprises a family of isozymes that show distinct properties in terms of enzymatic activity, tissue distribution, cellular expression, subcellular localization, and binding partner (10, 23, 32, 39). It is particularly interesting that most isozymes show abundant expression in the brain and immune system, suggesting that clues exist to address a unique role played by each isozyme in these tissues. In this regard, we previously described a distinct expression pattern of each isozyme in the brain and its involvement in pathophysiological events (10). In the immune system, previous studies have disclosed that the thymus expresses high levels of DGKα (12) and DGKζ (11), each of which is proposed to exert a negative function in T cell receptor response (TCR) (33, 43). These findings were confirmed by studies using knockout mice, providing valuable information related to the functional roles of these isozymes in T lymphocytes (28, 42). Nevertheless, little is known about the nature and the expression patterns of the DGK family in immune cells of various kinds.

Therefore to elucidate the functional implications of the other DGK isozymes in immune cells, we first strove to clarify the mRNA expression patterns of the DGK family in human immune cells. Furthermore, we investigate whether their mRNA expression levels are changed during an inflammatory reaction or not.

MATERIALS AND METHODS

Isolation of human immune cells. Human blood samples were obtained from six healthy adult volunteers (mean age 32 years, range 29–36 years) after informed consent. Blood was collected in the morning (9 AM–12 AM), transferred to sterile heparinized tubes and diluted with phosphate buffered saline (PBS) to double as the original volume. After hemolysis with subsequent treatment of 0.2% and 1.6% sodium chloride, all samples were mixed together and layered onto Ficoll-Paque (GE Healthcare, Chalfont St. Giles, UK). The gradient was obtained by centrifugation at 1000 × g for 10 min. Polymorphonuclear neutrophils were recovered from the pellet (>95% neutrophils defined by May-Giemsa method). Mononuclear cells were collected from the interface, which were washed twice in PBS/2% fetal bovine serum (FBS). Each type of cell was isolated using affinity purification with Dynabeads M-450 CD4, M-450 CD8, and M-450 CD19 following the manufacturers’ instructions (Invitrogen, Carlsbad, CA). The CD14+ cells (monocytes) were isolated directly from buffy coat in the gradient with Dynabeads M-450 CD14 to avoid activation during the washing process. The rosetted cell complexes were collected on a magnet and washed four times with PBS. The bound cells were released from the beads by incubation with DETACHaBEAD (Invitrogen) at a dilution of 1 : 10. Beads were further washed three times to collect residual cells.

Flow cytometry. Flow cytometry analysis was performed before and after cell isolation according to the standard protocols using FITC-conjugated antibodies (anti-CD14-FITC, anti-CD4-FITC, anti-CD8-FITC, and anti-CD19-FITC; Beckman Coulter, Brea, CA) (25).

Cell culture. HUT-102 cells (human T cell lymphoma with expression of surface membrane IL-2 receptor α, β, and γ) and RAW264 cells (mouse macrophage cell line) were cultured at 1 × 10^6 cells/mL in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% FBS under 5% CO2 at 37°C. In some experiments, cells were incubated with lipopolysaccharide (LPS, 200 ng/mL), human recombinant interleukin-2 (IL-2, 200 U/mL; Sigma-Aldrich) (4) or 12-O-tetradecanoylphorbol 13-acetate (TPA, 200 nM; Cell Signaling, Danvers, MA).

RT-PCR analysis. Total RNAs were extracted from human immune cells, cultured cell lines, and rat spleen using TRIZol reagent (Invitrogen). First-strand cDNA was synthesized from 2 μg of RNA using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturers’ instructions. Polymerase chain reaction (PCR) amplification was performed with KOD-plus polymerase (Toyobo, Tokyo, Japan) using gene specific oligonucleotide primers for DGK isozymes for human (22), mouse (13), and rat (17). Tumor necrosis factor α (TNFα) was also examined (forward primer 5’-TAGCCCACTGTCGTAGCAAAAC-3’, reverse primer 5’-AAGTAGACCTGCCCGGACTCT-3’). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin primer was used as internal control. PCR conditions were as follows: 94°C for 2 min; 30–35 cycles of 94°C for 30 s, 62°C for 30 s, and 68°C for 40 s; and 68°C
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Methods. Purity of the isolated cells was assessed using flow cytometry: 95% for neutrophils, 78.1% for CD14+ cells, 97.6% for CD4+ cells, 99.4% for CD8+ cells, and 98.0% for CD19+ cells. As portrayed in Figure 1, mRNAs for DGKα, DGKδ, DGKε, DGKζ, and DGKθ were expressed at various intensities in almost every type of immune cells examined. DGKγ was detected in neutrophils, CD14+ cells, and CD19+ cells, but it was undetectable in CD4+ helper and CD8+ cytotoxic T cells. However, DGKη was detected weakly in CD4+ helper and CD8+ cytotoxic T cells. DGKβ and DGKι mRNAs were below detection levels in immune cells.

Expression of DGK isozymes in HUT-102 cells during an inflammatory reaction

Since previous reports have described the functional implications of DGKs, especially DGKα and DGKζ, for 2 min (18). PCR products were separated by 2% agarose gels, stained with ethidium bromide, and subjected to densitometric analysis as described (24).

Induction of inflammatory reaction by LPS in rat spleen. Animal study was carried out in accordance with Guide for Animal Experimentation, Yamagata University School of Medicine. Inflammatory reaction in rats was invoked as described previously (29). Briefly, adult male Wistar rats were administered with LPS at a dose of 25 mg/kg (total volume 200 μL). Saline was injected as a control at the same volume. The rats were sacrificed by decapitation and spleens were rapidly removed at different time points after LPS administration.

Immunoblot analysis. Cells or tissues were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM NaF, 1 mM dithiothreitol, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Roche Products, Indianapolis, IN). After sonication, cell debris was removed by low-speed centrifugation (550 × g for 10 min). In immunoblot analysis, total lysate was centrifuged at 100,000 × g for 30 min to separate soluble (supernatant) from membrane (pellet) fractions (15). The pellet was resuspended in an equal volume of lysis buffer. Protein concentration was determined using BCA Protein Assay Reagent (Thermo Fisher Scientific Inc. Rockford, IL) according to the manufacturers’ protocol. Proteins samples (20 μg per lane) were subjected to 10% SDS-PAGE and electrophoretically transferred on PVDF membrane (Millipore Corporation, Billerica, MA) (11). Immunoreactive bands for anti-phospho PKCα/βII antibody (Cell Signaling Technology, Danvers, MA) were detected using a chemiluminescence ECL plus detection system (GE Healthcare).

Statistics. Values are shown as mean ± SD for each experimental group. Groups were compared using unpaired t-test. The P value < 0.05 was considered to be statistically significant.

RESULTS

Expression profile of DGK isozymes in human immune cells under normal conditions

We first examined the mRNA expression pattern of DGK isozymes in immune cells. Neutrophils, CD14+ (monocyte), CD4+ (helper T), CD8+ (cytotoxic T), and CD19+ (B) cells were isolated from the blood of healthy volunteers as described in Materials and Methods. Purity of the isolated cells was assessed using flow cytometry: 95% for neutrophils, 78.1% for CD14+ cells, 97.6% for CD4+ cells, 99.4% for CD8+ cells, and 98.0% for CD19+ cells. As portrayed in Figure 1, mRNAs for DGKα, DGKδ, DGKε, DGKζ, and DGKθ were expressed at various intensities in almost every type of immune cells examined. DGKγ was detected in neutrophils, CD14+ cells, and CD19+ cells, but it was undetectable in CD4+ helper and CD8+ cytotoxic T cells. However, DGKη was detected weakly in CD4+ helper and CD8+ cytotoxic T cells. DGKβ and DGKι mRNAs were below detection levels in immune cells.

Expression of DGK isozymes in HUT-102 cells during an inflammatory reaction

Since previous reports have described the functional implications of DGKs, especially DGKα and DGKζ,
in T cell function, we next examined how the DGK family would respond to extracellular stimuli in T cells. To this end, we stimulated HUT-102 (human T cell line) with IL-2 and LPS and investigated the time course of mRNA expression for DGKs during an immune response (Fig. 2). DGKε mRNA level was extremely faint at its basal value, but in response to IL-2 it showed a steep increase in the expression in an early phase at 20–40 min. It returned to the basal levels thereafter (left panel). However, the other DGKs including DGKα and DGKζ remained practically unresponsive. When stimulated with bacterial endotoxin LPS, a similar response was observed for DGKε mRNA expression: It increased at 20–40 min but gradually attenuated thereafter (right panel). In addition, mRNAs for DGKα and DGKδ showed a transient decrease in the early phase (20–40 min) and returned to the basal levels. These findings suggest that a transient upregulation of DGKε mRNA in the early phase is a common feature observed for T cells during an inflammatory reaction.

PI metabolism is involved in LPS-induced Toll-like receptor 4 (TLR4) cascade (16), suggesting that DG-mediated PKC pathway might be operated in this cascade. Therefore we also examined whether PKC activity regulates DGKε expression. TPA is often used to investigate involvement of PKC activity because it serves as a potent activator of PKC (26). In the presence of TPA, PKCα/βII was activated at an early phase of stimulation as shown by the translocation of the phosphorylated protein from the soluble to membrane fraction (Fig. 3A). However, the DGKε mRNA level remained unchanged (Fig. 3B), demonstrating that PKC activity is not involved in the gene expression of DGKε in T cells.

**Expression of DGKε isozyme in rat spleen under inflammatory conditions**

Having shown that DGKε mRNA level is upregulated in T cells at the early phase in response to inflammatory stimuli, we focused on DGKε and investigated whether this isozyme shows a similar response at the organismal level or not. We treated rats with an intraperitoneal injection of LPS to induce an inflammatory reaction and examined DGKε mRNA level under these conditions (Fig. 3C). DGKε mRNA level increased at 20–40 min but gradually attenuated thereafter. DGKα and DGKδ mRNAs showed a transient decrease in the early phase (20–40 min) and returned to the basal levels. These findings suggest that a transient upregulation of DGKε mRNA in the early phase is a common feature observed for T cells during an inflammatory reaction.

**Fig. 2** Time course expression of DGK isozyme mRNAs in HUT-102 cells in response to IL-2 and LPS. Human T cell line HUT-102 cells were stimulated by IL-2 (200 U/mL, left panels) and LPS (200 ng/mL, right panels) and were examined for the time course expression (0, 20, 40, 60, 90, 120 min) of DGK isozyme mRNAs in RT-PCR. Note a transient increase in DGKε mRNA expression in an early phase (20–40 min) of stimulation with IL-2 and LPS. On the other hand, the expression for DGKα and DGKδ are transiently down-regulated in the early phase of LPS challenge. GAPDH primer was used as a control. PCR was performed for 30 cycles. A representative result of three independent experiments is shown.
mRNA expression in the spleen, a lymphoid organ that is rich in immune cells (Fig. 4) because the spleen, together with the liver, is shown to play a prominent role in clearing Gram-negative bacteria and their LPS endotoxin from the bloodstream (2, 3). Results revealed that DGKɛ mRNA rapidly increased in its expression level after LPS injection. That high level persisted for at least 24 h. This finding agrees with that obtained in the experiment using a T cell line, although the upregulated DGKɛ mRNA level is sustained for a longer period at the organismal level.

DGKɛ expression in macrophage cell line Raw264 cells

LPS endotoxin induces TLR4 signaling not only in T cells but also in cells of various kinds (1). Therefore we next examined the pattern of DGKɛ mRNA expression in mouse macrophage cell line Raw264 cells under the same conditions as those used for the T cell line HUT-102 cells (Fig. 5). Results showed that DGKɛ mRNA is upregulated at an early phase of LPS stimulation in a similar temporal pattern to that in HUT-102 cells. Concurrently, TNFα expression was also examined as an output for inflammatory reaction, which reveals that the temporal expression pattern of TNFα is closely correlated with that of DGKɛ.

Fig. 3 Time course expression of DGK isozyme mRNAs in HUT-102 cells upon TPA stimulation. After HUT-102 cells were stimulated by TPA (200 nM), time course study (0, 20, 40, 60, 90, 120 min) was performed for PKC activation (A) and DGK isozyme mRNA expression (B). PKC activation was evaluated by immunoblot of phospho-PKCα/βII antibody on soluble and membrane fractions using β-actin antibody as a control. DGK isozyme mRNA was evaluated in RT-PCR (32 cycles) using GAPDH primer as a control. Similar result was obtained in three independent experiments.

Fig. 4 Time course of DGKɛ mRNA expression in the spleen upon LPS injection. (A) Expression profile of DGK isozyme mRNAs in rat spleen under normal conditions. DGKα and DGKɛ mRNAs are abundantly expressed in the spleen and DGKγ mRNA moderately. Note a faint expression of DGKɛ mRNA. (B) Rats were administered with LPS (25 mg/kg weight) systemically by the intraperitoneal route. The time course of DGKɛ mRNA expression (0, 0.5, 6, 24 h) was examined in the spleen by RT-PCR. Control rats received with the same amount of vehicle (saline). Band intensity was quantified by densitometry and normalized to the value obtained from the control sample. Data shown are the means ± SD of 3 samples (each data obtained in triplicate determination). Similar results were obtained in two separate experiments. *P < 0.05.
adapter capable of mediating NFκB activation and was responsible for signaling selectively via TLR4 (7) and TLR2 signaling (40). In this process, PIP2 is also known to mediate plasma membrane localization of the TIRAP, which is required for the MyD88-dependent TLR4 signaling pathway (16). These data suggest that DGKe-mediated PI metabolism is involved in the LPS-TLR4 signaling cascade because DGKe is intimately involved in PI turnover through its substrate specificity toward 1-stearoyl-2-arachidonoyl DG, a major constituent of inositol phospholipids (37). This possibility warrants further investigation, including that of a cause-and-effect linkage between the induction of DGKe and TNFα mRNAs.

Regarding other DGKs, previous reports have described that T cells express high levels of DGKα and DGKζ, both of which are proposed to exert a negative function in TCR (33, 43). DGKα-deficient T cells elicit increased RasGTP levels and mitogen-activated protein kinase (MAPK) activation. In addition, DGKα-null T cells produce more IL-2 and show increased proliferation in response to TCR (28). These studies indicate that DGKα regulates the magnitude of the TCR, thereby serving as a brake at the initial phase of TCR-dependent signaling. Moreover, DGKα protein is expressed abundantly in unstimulated cells, but its mRNA level is attenuated sharply after TCR activation (34), indicating that DGKα is down-regulated in cycling T cells. It is therefore inferred that DGKα is an anergy-induced gene (21) because anergic cells show G1 arrest that renders those cells unable to proliferate unless rescued by exogenous IL-2 (35). However, T cell proliferation correlates with the elevation of cellular PA as a result of IL-2-dependent DGKα activation (8). DGKα-dependent PA generation is shown to be required for IL-2-dependent proliferation (9). These findings show that DGKα mRNA is transiently down-regulated in the early phase of LPS challenge, but it is stably expressed during IL-2 signaling, which is compatible with earlier reports. Collectively, these findings suggest that DGKα is differentially regulated in response to distinct routes of stimulation in T cells.

Related to the functional role of DGKζ in T cells, its negative modulation is revealed by the phenotype of DGKζ-deficient mice, which show Ras/MAPK activation, together with enhanced expression of the CD69 and CD25 activation markers (42). In addition, DGKζ-null mice show deficient IL-12 and TNFα production after TLR stimulation, refractoriness to endotoxic shock, and susceptibility to Toxoplasma.
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gondii infection (20). Remarkably, DGKζ-deficient mice show a more robust immune response to choriomeningitis virus infection, which is also mediated through TLR signaling (42). In this regard, these data suggest that DGKζ is not regulated at the transcriptional level upon stimulation with LPS and IL-2.

In summary, we present a distinct expression profile of DGKs in human immune cells. Notably, DGKε mRNA is transiently upregulated in the early phase of IL-2 and LPS stimulation in T cells and macrophages. Previous studies show that DGKα and DGKζ exert a negative function in TCR. Taken together, these findings suggest that the DGK family might be involved intimately in immune reactions in a distinct manner. Knowledge of how DGKε is specialized in inflammatory processes can enhance our understanding of fundamental mechanisms of the cellular immune system.

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