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Diacylglycerol kinase inhibitor R59022-induced autophagy and apoptosis in the neuronal cell line NG108-15

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ABSTRACT

Phosphatidic acid (PA) is a lipid second messenger and is believed to be involved in cell proliferation and survival. PA is mainly produced by phospholipase D (PLD) and diacylglycerol kinase (DGK). Elevated PLD activity is believed to suppress apoptosis via activation of the mammalian target of rapamycin (mTOR). On the other hand, DGK inhibition has been demonstrated to induce apoptosis, but it is unclear whether DGK can regulate mTOR. Here, we investigated whether DGK inhibition can induce apoptosis and autophagy in neuronal cells, since mTOR is a key mediator of autophagy and the simultaneous activation of apoptosis and autophagy has been detected. A DGK inhibitor, R59022 induced autophagy and apoptosis without serum in NG108-15 cells. Autophagy preceded apoptosis, and apoptosis inhibition did not affect R59022-induced autophagy. R59022-induced autophagy was inhibited by exogenous PA, and protein kinase C activation and increases in intracellular Ca^{2+} levels, which are assumed to be caused by diacylglycerol accumulation, did not appear to be involved in R59022-induced autophagy. We also investigated the effects of R59022 on mTOR signaling pathway, and found that the pathway was not inhibited by R59022. These results imply that DGK plays an important role in cell survival via mTOR-independent mechanism.

Introduction

Phosphatidic acid (PA)[1] is the simplest phospholipid and a central intermediate in the synthesis of membrane and storage lipids. PA is also a lipid second messenger and is associated with many aspects of cell physiology, including cell proliferation, survival signaling, tumor progression and differentiation [1]. PA is produced by two major pathways: hydrolysis of phosphatidylcholine by phospholipase D (PLD) and phosphorylation of diacylglycerol (DG) by DG kinase (DGK). PLD activation is critical for inhibiting apoptosis in various human cancers, including mammary, gastric, renal, and colonic cancers [2–5]. On the other hand, DGK inhibition induces apoptosis in mouse thymocytes [6] and human leukemia (HL-60) cells [7], and DGK mutation causes degeneration in *Drosophila* photoreceptor cells [8,9]. Consequently, these enzymes appear to be involved in the regulation of apoptosis.

PA has been shown to activate the mammalian target of rapamycin (mTOR), which is a Ser/Thr protein kinase that regulates cell growth and proliferation. mTOR is constitutively activated in many cancers [10–12], and inhibitors of mTOR, rapamycin, and its derivatives possess potent antitumor effects [13–15]. One of the potential targets of PLD is speculated to be mTOR [1,16,17]. Although a DGK isoform has been reported as a mediator of mTOR signaling [18], the involvement of DGK in mTOR regulation remains unclear [19,20].

In this study, we examined whether DGK inhibition can induce apoptosis and autophagy in the neuronal cell line NG108-15. Autophagy is suggested to be induced through mTOR inhibition [21], and it was indicated that apoptosis and autophagy share common aspects and crosstalk between these two processes exists [22–24]. The results of this study showed that the DGK inhibitor R59022 induced apoptosis and autophagy. R59022-induced autophagy was blocked by exogenous PA, but mTOR signaling pathway did not appear to be involved in R59022-induced autophagy in NG108-15 cells.

Materials and methods

Chemicals

DGK inhibitors I (R59022), 3-methyladenine (3-MA), phorbol 12,13-dibutylate, and bafilomycin A1 were obtained from Sigma (St. Louis, MO, USA), BAPTA-AM was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA), and z-DEVD-FMK was obtained from R&D Systems, Inc. (Minneapolis, MN, USA).

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[1] *Abbreviations used*: PA, phosphatidic acid; DG, diacylglycerol; DGK, diacylglycerol kinase; PLD, phospholipase D, mTOR, mammalian target of rapamycin; 3-MA, 3-methyladenine; PKC, protein kinase C; OAG, 1-oleoyl-2-acetyl-sn-glycerol.

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1-Palmitoyl-2-oleoyl PA was obtained from Doosan Serdary Research Laboratories (Toronto, Canada), GF109203X was obtained from Tocris Bioscience (Ellisville, MO, USA). Anti-cleaved caspase-3, anti-LC3, anti-phospho-p70 S6 kinase (Thr389), and p70 S6 kinase antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-β-tubulin antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), HRP- and Alexa 594-conjugated anti-rabbit IgG secondary antibodies were obtained from Invitrogen (Carlsbad, CA, USA). Annexin V-EGFP was obtained from BioVision Research Products (Mountain View, CA, USA).

Cell culture and treatment

NG108-15 cells (mouse neuroblastoma × rat glioma hybrid cells) were obtained from the European Collection of Cell Cultures (Salisbury, UK) and maintained in DMEM containing 10% FBS and HAT at 37 °C under 5% CO_2. For all experiments, these cells were seeded in culture dishes and grown for 24–48 h. The cells were washed with serum-free DMEM and pretreated with reagents or vehicles in serum-free DMEM for 30 min. Next, the DGK inhibitor or vehicle was added and the cells were incubated for 24 h. 3-MA was dissolved in DMEM and other reagents were dissolved in DMSO. PA was suspended by vortexing in 150 mM NaCl and 10 mM Tris–Cl (pH 8.0) for 5 min, as described by Park et al. [25]. The freshly made PA solution was immediately added to the cell culture at a final concentration of 100 μM.

Western blot analysis

All proteins were extracted from the cells, and their levels were determined using the Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). Equal amounts (10 μg) of proteins were separated on 12.5% SDS–PAGE gels. The resolved proteins were then transferred to a polyvinylidene fluoride membrane that was incubated with the primary antibodies, followed by incubation with HRP-conjugated secondary antibody. The blots were developed using the Immobilon Western Chemiluminescence HRP Substrate (Millipore, Billerica, MA, USA).

Cell staining

To detect any apoptotic cells, NG108-15 cells were seeded on coverslips and labeled with annexin V-EGFP for 5 min. For immunocytochemical analyses, cells were seeded on coverslips and fixed in 4% paraformaldehyde. After blocking with normal goat serum, the cells were labeled with the anti-LC3 antibody, followed by incubation with Alexa 594-conjugated secondary antibody. Cells were analyzed by fluorescence microscopy.

Statistical analysis

Data are presented as means ± SE of at least three independent experiments. Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

DGK inhibitor-induced apoptosis and autophagy

First, we examined whether R59022 induced apoptosis in NG108-15 cells. As shown in Fig. 1A, cleaved caspase-3 levels slightly increased with serum withdrawal, although this increase was not statistically significant. R59022 (5 μM) upregulated the levels of cleaved caspase-3 in serum-free medium, but no significant effects on protein levels were observed in serum-containing medium. R59022-induced apoptosis was verified by staining the cells with annexin V. The number of cells binding annexin V significantly increased after treatment with R59022 in serum-free medium (Fig. 1B).

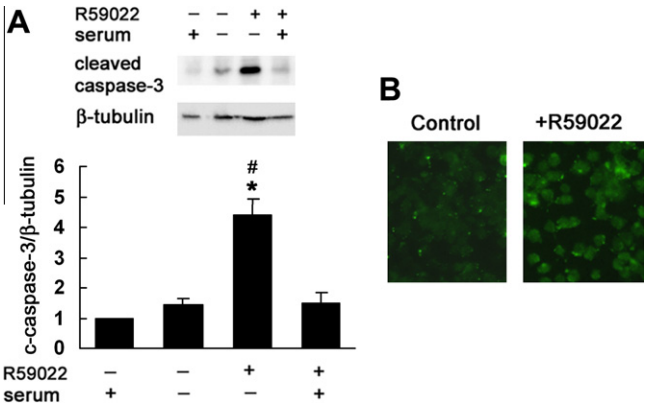

Fig. 1. R59022-induced apoptosis in NG108-15 cells. (A) Cells were treated with R59022 (5 μM) for 24 h in the presence or absence of serum. Cleaved caspase-3 levels were analyzed by Western blotting, and the band densities were normalized to those of the control cells (treated with serum alone). β-Tubulin was used as the loading control. $^{*}P < 0.05$ compared to control cells; $^{\#}P < 0.05$ compared to cells treated with vehicle in serum-free medium. (B) Cells were treated with R59022 (5 μM) for 24 h in the absence of serum and stained with annexin V.

Next, we investigated the effects of R59022 on LC3-II levels, which is a marker of autophagy. LC3-II expression in NG108-15 cells was significantly increased with serum withdrawal and augmented by the addition of R59022 (5 μM) to serum-free medium (Fig. 2A). Treatment of cells with R59022 in serum-containing medium slightly increased the levels of LC3-II, but this effect was not statistically significant. Accumulation of LC3-containing dot structures was observed when the cells were treated with R59022 in a serum-free medium (Fig. 2B). The autophagy inhibitor 3-methyladenine (3-MA) suppressed R59022-induced increases in LC3-II levels (Fig. 2C). Furthermore, upregulation of protein levels by R59022 was observed in the presence of bafilomycin A1 (0.1 μM), which inhibits autolysosomal degradation (Fig. 2D). These results indicate that R59022 enhanced autophagy in the absence of serum. We also examined whether R59022 induces apoptosis and autophagy in human neuronal cells. When SH-SY5Y neuroblastoma cells were incubated with R59022, both events were observed (data not shown).

To understand the relationship between apoptosis and autophagy in R59022-treated cells, we examined the effects of autophagy and apoptosis inhibitors in R59022-treated cells. R59022-induced increases in cleaved caspase-3 levels were almost completely inhibited by 3-MA (10 mM) (Fig. 3A). In contrast, R59022 upregulated LC3-II levels in the presence of the caspase-3 inhibitor z-DEVD-FMK (50 μM) (Fig. 3B). These results suggest that autophagy precedes apoptosis in R59022-treated cells and that apoptosis inhibition does not affect R59022-induced autophagy.

Signaling pathways involved in R59022-induced autophagy

Since it is known that DG is an activator of protein kinase C (PKC), we investigated the effects of a PKC inhibitor on R59022-induced autophagy. As shown in Fig. 4A, R59022-induced increases in LC3-II levels were observed in the presence of GF109203X (3 μM), which was shown to block DG-dependent PKC isoforms expressed in NG108-15 cells (PKCα, β, δ, ε) [26–28]. Treatment of cells with a PKC activator phorbol 12,13-dibutylate in serum-free medium had no effects on LC3-II levels in NG108-15 cells (data not shown).