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Illumina-microarray analysis of mycophenolic acid-induced cell death in an insulin-producing cell line and primary rat islet cells: New insights into apoptotic pathways involved

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ABSTRACT

Mycophenolic acid (MPA), widely used to prevent organ transplant rejection, may induce toxicity and impair function in β-cells. Mechanisms of MPA-induced cell death have not been fully explored. In this study, we examined gene expression patterns in INS-1E cells and isolated primary rat islets following MPA treatment using the Illumina-cDNA microarray. The MPA treatment decreases RhoGDI-α gene expression, which points to apoptosis by JNK activation through a MAPKs-dependent pathway. A strong association between RhoGDI-α and Rac1 activation during MPA-induced apoptosis is also consistent with apoptosis through JNK. Suppression of RhoGDI-α using siRNA and gene over-expression both affected the cell death rate, consistent with Rac1 activation and downstream activation of MAPKs signaling. We confirmed that Rac1 protein mediates the interaction between RhoGDI-α and JNK signaling. We conclude that MPA-induced cell death in primary β-cells and an insulin-secreting cell line proceeds through RhoGDI-α down-regulation linked to Rac1 activation, with subsequent activation of JNK. The RhoGDI-α/Rac1/JNK pathway may present a key to intervention in MPA-induced islet apoptosis.

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1. Introduction

In type 1 diabetes mellitus, an autoimmune response selectively destroys insulin-secreting β-cells in the islets of Langerhans, with potentially fatal insulin depletion [1,2]. Trials show that islet cell transplantation may cure type 1 diabetes. Although clinically more reliable and reproducible than pancreas transplantation, islet transplantation presents several problems. In particular, the transplants are susceptible to drug-induced toxicity and death, and may prove physiologically inadequate [3]. Strategies to overcome these obstacles include development of less toxic immunosuppressant drugs and agents to modulate the toxic drug response. [4].

Mycophenolic acid (MPA), a widely used immunosuppressant, inhibits inosine 5′-monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme in *de novo* guanosine nucleotide synthesis [5,6]. In pancreatic organ or islet transplants, MPA inhibits β-cell function and induces cell death through guanosine triphosphate depletion [7,8].

Rho proteins act as signaling components in cell proliferation, adhesion, motility, differentiation and apoptosis [9]. Rho proteins mediate critical steps in MPA-induced cell death, but the mechanisms for this are not clear. In mammals, the Rho family contains about 20 subfamilies, but most functional data concern Rac, Rho, and Cdc42 only [10]. Rac and Cdc42 may be dysregulated in carcinogenesis and metastasis, as well as in apoptotic cell death, in many different cell types [11]. Most Rho/Rac GTPases behave as "molecular switches" that shift between inactive and active conformations through hydrolysis of GTP [12,13]. Three types of proteins regulate the RhoGTPase activities: (i) guanine nucleotide exchange factors (GEFs) stimulate the GTP–GDP exchange reaction; (ii) GTPase-activating proteins (GAPs) mediate GTP hydrolysis; and (iii) guanine nucleotide dissociation inhibitors (GDIs) bind to Rho GTPases and block the dissociation of GDP from the GTP-binding site [14]. In addition, three types of GDI, including RhoGDI-α [15,16], RhoGDI-β (or Ly/D4GDI) [17], and RhoGDI-γ [18], regulate RhoGTPase in specific cell types. Tumor cells of ovarian, breast and hepatic origin usually over-express RhoGDI-α, and mechanisms of apoptosis induced by the etoposides and doxorubicin may involve RhoGDI-α [19,20]. RhoGDI-α may form one-to-one complexes with isoprenylated RhoA, RhoB, Rac1, Rac2, Cdc42, and express distinct functions in each association. Rac1, for example, mediates apoptosis in intestinal epithelial cells as a signaling

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component in the JNK pathway [16,21–23]. By inhibiting dissociation of Rac1 and GDP, RhoGDI-α participates in the apoptotic pathways of several cell types [21,22]. RhoGDI-α may also inhibit cleavage of Rac1 by caspases, which is required for the maximal apoptotic response to cytotoxic drugs.

Previously, we showed that MPA induces apoptosis in HIT-T15 by increasing JNK activity in a time- and dose-dependent manner [23]. We also found that MPA induces significant apoptosis in an insulin-secreting cell line, RIN-5F, through RhoGDI-α down-regulation linked with an increase in JNK expression [24].

Based on these findings, we hypothesized that genes differentially expressed during MPA-induced apoptosis may correspond to specific cellular events in insulin-secreting cells, and that *in vivo* and *in vitro* assays based on these data may be used to test the level of function in islet transplants. We focused initially on RhoGDI-α, which is differentially expressed after MPA treatment. We also observed that RhoGDI-α over-expression prevented MPA-induced cell death and decreased levels of activated JNK and MKK4/7, whereas RhoGDI-α knock-down enhanced MPA-induced cell death through MAPK activation. In this study, we found that RhoGDI-α directly affected MPA-induced apoptosis in INS-1E cells and in primary rat islet cultures via Rac1/MAPK/JNK signaling. Rac1 thus emerged as a critical intermediary between RhoGDI-α and the MAPK signaling pathway.

2. Materials and methods

2.1. Cell culture

INS-1E rat islet cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 2 mM D-glucose, 0.5 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Cells at 80% confluence were switched to serum-free medium containing 10 μM MPA in the presence or absence of various experimental reagents.

2.2. Rat islet isolation and primary cell culture

A pancreas from a Lewis rat (250–350 g) was digested using collagenase P (Roche, Indianapolis, IN, USA). The islets were purified using a discontinuous density gradient of Histopaque (Sigma Chemical Co., St Louis, MO, USA). After isolation, islets were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin, in a 5% CO_2 humidified incubator at 37 °C. The isolated islets were stabilized overnight before treatment with MPA.

2.3. MTT reduction assay

Cell survival was measured at 12 h, 24 h, 36 h and 48 h following addition of MPA. Each assay contained approximately 3×10^4 cells cultured in RPMI 1640 for 3 days, then switched to serum-free medium with or without 10 μM MPA. Cell survival was measured using the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay [25]. After MTT solution was added to cells to a final concentration of 1 mg/mL, the cells were incubated for 1 h at 37 °C, and lysed in 50% aqueous dimethylformamide (DMF) containing 20% SDS. The optical density of the dissolved formazan grains was measured at 540 nm using an ELISA plate reader (Molecular Devices, Palo Alto, CA). Measurements from each treatment were expressed as a percentage of survival in the corresponding control.

2.4. Total RNA isolation and reverse transcription

Following aspiration of medium, cells were lysed directly in the culture dish by adding TRIzol reagent. The sample was incubated for 5 min at room temperature and then mixed with chloroform in a tube by vortexing for 15 s. The tube was centrifuged for 15 min at 4 °C and the supernatant was transferred into a new microcentrifuge tube containing isopropanol and incubated at room temperature for 10 min. The sample was then centrifuged for 10 min at 4 °C and the supernatant discarded. The resultant pellet was washed in 70% ethanol and resuspended in RNase-free water. First-strand cDNA was made using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Two micrograms of total RNA was reverse transcribed using oligo dT primer (Invitrogen).

2.5. TUNEL assay and Hoechst 33342 staining

Apoptosis was identified in cultured INS-1E cells seeded on cover slips by terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL), using a commercially available kit (Roche), and by Hoechst 33342 (Molecular Probes, Eugene, OR) staining. Apoptosis was defined as TUNEL-positive cells among the INS-1E cells and the presence of nuclear condensation on Hoechst staining.

2.6. Immuno-histochemistry

Primary islets were fixed in 4% paraformaldehyde and dehydrated in sucrose. Slices of primary islets for immuno-histochemical staining were snap-frozen in a solution at optimal cutting temperature, and 4-μm sections of islets clusters were made. Slides were permeabilized and then blocked with 0.5% BSA in PBS for 4 h at room temperature. For β-cell staining, slides were incubated overnight at 4 °C with primary polyclonal antibody to insulin (Santa Cruz Biotechnology, Inc.) diluted 1:100 with blocking solution. Slides were washed and incubated with Alexa Fluor 568 goat anti-rabbit antibody (Invitrogen) for 4 h at 4 °C.

2.7. Fluorescence microscopy

Fluorescence microscopic images of fixed cells were captured using an OLYMPUS IX71 fluorescence microscope equipped with a DP71 camera and the DP-BSW Application software (Olympus Corporation, Tokyo, Japan).

2.8. Illumina-microarray gene analysis

Microarray analysis was performed according to the Macrogen Rat BeadChip technical manual (Macrogen, Seoul, Korea) used by Illumina RatRef-12 Expression BeadChip (Illumina, Inc., San Diego, CA). Biotinylated cRNAs were prepared from 0.55 μg quantities of total RNA using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX). Following fragmentation, cRNA was hybridized to the Illumina RatRef-12 Expression Beadchip in 0.75 μg quantities using protocols provided by the manufacturer. Arrays were scanned using the Illumina Bead Array Reader Confocal Scanner. Array data export processing and

Fig. 1. Effects of GTP and guanosine co-treatments on MPA-induced cell death. (A) INS-1E cells were treated with 10 μM MPA alone or co-treated with 100 μM GTP or 500 μM guanosine for the indicated time periods (12 h, 24 h, and 36 h). Cell death rates were measured with the MTT reduction assay. Cell viability was expressed as a percentage of the untreated control. Data represent the mean ± standard error from three independent experiments. *$p<0.05$ versus control. (B) MPA increased caspase-3 expression in a time-dependent manner but co-treatment with 100 μM GTP inhibited caspase-3 activation. (C) To measure MPA-induced cell death, INS-1E cells were plated on 6-well dishes and then co-treated with 100 μM GTP after 10 μM MPA. Nuclei were stained with Hoechst 33342. Morphological changes were noted and DNA fragmentation was determined using the TUNEL assay. Data represent the mean ± standard error from three independent experiments.

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