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Energy adaptive response during parthanatos is enhanced by PD98059 and involves mitochondrial function but not autophagy induction

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ABSTRACT

Parthanatos is a programmed necrotic demise characteristic of ATP (adenosine triphosphate) consumption due to NAD^+ (nicotinamide adenine dinucleotide) depletion by poly(ADP-ribose) polymerase 1 (PARP1)-dependent poly(ADP-ribosyl)ation on target proteins. However, how the bioenergetics is adaptively regulated during parthanatos, especially under the condition of macroautophagy deficiency, remains poorly characterized. Here, we demonstrated that the parthanatic inducer *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) triggered ATP depletion followed by recovery in mouse embryonic fibroblasts (MEFs). Notably, $Atg5^{-/-}$ MEFs showed great susceptibility to MNNG with disabled ATP-producing capacity. Moreover, the differential energy-adaptive responses in wild-type (WT) and $Atg5^{-/-}$ MEFs were unequivocally worsened by inhibition of AMP-activated protein kinase (AMPK), sirtuin 1 (SIRT1), and mitochondrial activity. Importantly, $Atg5^{-/-}$ MEFs disclosed diminished SIRT1 and mitochondrial activity essential to the energy restoration during parthanatos. Strikingly, however, parthanatos cannot be exasperated by bafilomycin A1 and MNNG neither provokes microtubule-associated protein 1A/1B-light chain 3 (LC3) lipidation and p62 elimination, suggesting that parthanatos does not induce autophagic flux. Intriguingly, we reported unexpectedly that PD98059, even at low concentration insufficient to inhibit MEK, can promote mitochondrial activity and facilitate energy-restoring process during parthanatos, without modulating DNA damage responses as evidenced by PARP1 activity, p53 expression, and γH2AX (H2A histone family, member X (H2AX), phosphorylated on Serine 139) induction. Therefore, we propose that Atg5 deficiency confers an infirmity to overcome the energy crisis during parthanatos and further underscore the deficits in mitochondrial quality control, but not incapability of autophagy induction, that explain the vulnerability in Atg5-deficient cells. Collectively, our results provide a comprehensive energy perspective for an improved treatment to alleviate parthanatos-related tissue necrosis and disease progression and also provide a future direction for drug development on the basis of PD98059 as an efficacious compound against parthanatos.

1. Introduction

Parthanatos is a particular type of programmed necrosis characterized by the death signal poly(ADP-ribose) (PAR) polymers, poly(ADP-ribose) polymerase (PARP)-mediated PAR accumulation, ATP and NAD^+ depletion, mitochondrial perturbation, and apoptosis-inducing factor (AIF) nuclear translocation from mitochondria [1–4]. Among all PARP superfamily, PARP1 is the most abundant protein localized in the nucleus that could immediately respond to the DNA damage, such as alkylation or double-strand breaks, to promote DNA repair machinery through NAD^+-dependent PARylation on acceptor proteins, such as histones and PARP1 itself [5,6]. However, if the DNA damage is irreparable, PARP1 over-activation will culminate in PARP1-dependent parthanatic cell death. Widespread as an environmental mutagen, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) is such a commonly used DNA-alkylating agent that potently initiates parthanatic cell death in a caspase-independent manner [3]. In our previous study, we have confirmed that MNNG is able to induce AIF nuclear translocation and necrotic cell death in mouse embryonic fibroblasts (MEFs) [7]. Moreover, the cell death was prevented by PARP1 inhibitors such as 3-aminobenzamide (3AB) and 3,4-dihydro-5 [4-(1-piperindinyl) butoxy]-1(2H)-isoquinoline (DPQ), implicating MNNG-induced cell death due to PARP1 over-activation [7].

Parthanatos has been implicated in several pathological conditions, such as neurodegenerative disorders including Parkinson's and Alzheimer's diseases, glutamate excitotoxicity, stroke, ischemia–

Abbreviations: 3AB, 3-aminobenzamide; 7AAD, 7-aminoactinomycin D; AIF, apoptosis-inducing factor; AMPK, AMP-activated protein kinase; DPI, diphenyliodonium; DXR, doxorubicin; FACS, fluorescence-activated cell sorting; FCCP, trifluorocarbonylcyanide phenylhydrazone; LC3, microtubule-associated protein 1A/1B-light chain 3; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MEFs, mouse embryonic fibroblasts; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; mtDNA, mitochondrial DNA; mTORC1, mammalian target of rapamycin complex 1; PAR, poly(ADP-ribose); PARP1, poly(ADP-ribose) polymerase 1; PGC1α, peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1 alpha; SIRT1, sirtuin 1; WT, wild-type

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reperfusion injury, and diabetes mellitus [8–12]. Many efforts have been spent to understand this distinct type of cell death to hinder from extensive tissue inflammation and disease progression [12]. Besides several parthanatic hallmarks already defined [2], mitogen-activated protein kinase (MAPK) signaling pathways such as JNK and p38 have also been implied to regulate the parthanatic responses [13,14]. Considering the energy depletion as an essential biochemical feature, enforced repletion of metabolic intermediates has been utilized to prevent from parthanatic cell death in neurons and astrocytes [15,16]. Despite the current progress in our understandings about parthanatos, the need for efficacious PARP inhibitors of better therapeutic potential is emerging [17]. Currently, the crystal structure of a DNA double-strand break in complex with human PARP1 domains obligatory for activation has been resolved [18], which may facilitate the process in designing novel PARP1 inhibitors.

Macroautophagy (hereafter termed autophagy) is highly coordinated self-digesting process involving autophagosomal engulfment and lysosomal degradation of damaged proteins or organelles for recycling to maintain intracellular energy homeostasis under normal physiological condition or in response to certain stress stimuli [19,20]. Derangement of autophagy has a pathogenic contribution to several diseases, like infection, cancer, neurodegeneration, heart diseases, and aging [21–25].

Currently, however, less is known about the regulation of energy turnover during parthanatos. Considering autophagy is a self-protective scheme in response to stress for energy preservation and parthanatos is a cell death process associated with energy depletion, we were interested in understanding the role of autophagy in energy regulation during parthanatos. Moreover, inspired by the concomitant occurrences of autophagy dysregulation and parthanatos in neurodegenerative and cardiovascular disorders, we speculated that autophagy defects may amplify the severity of parthanatic cell death. In this study, we tried to elucidate the contribution of autophagy dependency as well as the elsewhere mentioned MAPK pathways to energy homeostasis and severity during parthanatos in MEFs. Here, we demonstrate for the first time that Atg5$^{-/-}$ MEFs are extremely susceptible to MNNG and that PD98059, a traditionally known MEK inhibitor, shows an unexpected protectiveness and saves the vulnerable MEFs during parthanatos in a MEK-independent manner. On the other hand, we also highlight the indispensable roles of AMP-activated protein kinase (AMPK), sirtuin 1 (SIRT1), and mitochondrial activity in regulating energy restoration during parthanatos, which also better explain the differential response in Atg5-deficient cells. Collectively, these results provide a complete energy perspective for better treating parthanatos-related disorders and reveal unexpectedly PD98059 as an effective parthanatos-alleviating compound that should guide the future direction for drug development.

2. Materials and methods

2.1. Reagents and antibodies

MNNG (O-2392) was purchased from Chem Service. 7-Aminoactinomycin D (7AAD, 559925) was obtained from BD Pharmingen. zVAD-fmk (219007), doxorubicin (324380), SB203580 (559389), SP600125 (420119), U0126 (662005), PD98059 (513000), wortmannin (681675), and compound C (171260) were ordered from Calbiochem. Gallotannin (403040), rapamycin (R8781), bafilomycin A1 (B1793), rotenone (R8875), diphenyliodonium (DPI, D2926), trifluorocarbonylcyanide phenylhydrazone (FCCP, C2920), MTT (M2128) and Dulbecco's PBS (D5652) were obtained from Sigma-Aldrich. JC-1 (T3168), Dulbecco's Modified Eagle Medium (DMEM, 12100-046), trypsin-EDTA (25200-072), fetal bovine serum (FBS, 04001-1A), and penicillin/streptomycin (03031-1B) were purchased from Invitrogen. The FastStart Universal SYBR Green Master (14526100) was ordered from Roche Applied Science. Commercial antibodies against p-ERK1/2 (Thr202/Tyr204, 9101), p-JNK (Thr183/Tyr185, 9251), p-p38 (Thr180/Tyr182, 9211), p53 (9282), p-AMPKα1 (Thr172, 2531), AMPKα1 (2532), p-GSK3β (Ser9, 9336), p-Akt (Ser473, 9271), Akt (9272), and SQSTM1/p62 (5114) were purchased from Cell Signaling Technology (Beverly, MA, USA); GSK3β (sc-9166), ERK1/2 (sc-292838), JNK1 (sc-474), and p38 (sc-7149) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Atg5 antibody (ab54033-100) was purchased from Abcam; β-actin antibody (MAB1501) was purchased from Upstate Biotechnology; PAR antibody (551813) was purchased from BD Pharmigen; γH2AX antibody (05-636) was ordered from Merck Millipore; LC3 antibody (PM036) was purchased from MBL. Normal rabbit IgG and horseradish peroxidase-conjugated antibodies (sc-2004) were purchased from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL) reagent was ordered from Perkin Elmer.

2.2. Cell culture

Atg5$^{+/+}$ and Atg5$^{-/-}$ MEFs were kindly provided by Dr. Noburu Mizushima (Tokyo Medical and Dental University). Primary WT and PARP1$^{-/-}$ MEFs were isolated from fresh 129S WT and 129S PARP1$^{-/-}$ mice, respectively. HeLa cells were purchased from the American Type Culture Collection (Manassas, VA, USA). MEFs and HeLa cells were cultured in high-glucose DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Intracellular ATP assay

Intracellular ATP was determined by CellTiter-Glo® Luminescent Kit (G7571) from Promega according to the manufacturer's protocol. In brief, 5×10^4 cells were seeded per well in a 24-well plate for 24 h followed by the treatment indicated. Medium was then replaced with the new one, and the reagent was added to lyze the cells. The plate was gently shaken for 2 min, placed stationary in the dark for 10 min, and then 150 μl solution was transferred to a 96-well plate for luminescence detection using LB96V MicroLumat Plus (American Laboratory Trading). With background subtraction, the values were normalized to individual control group as 100%.

2.4. Determination for 7AAD$^+$ cells

MEFs were seeded in a 24-well plate (5×10^4 cells per well) for 24 h followed by the treatment indicated. The supernatant was collected, and the cells were trypsinized and then mixed with the respective supernatant, followed by centrifugation at 2500 rpm for 10 min at room temperature. The pelleted cells were washed with 1× PBS once and then centrifuged in a similar manner. Then, the cells were resuspended with PBS containing 7AAD (5 μM) and then submitted to FACS analysis (FACScan flow cytometer, Becton Dickinson) using the CellQuest program. The data were obtained based on the FL3 channel.

2.5. Lactate dehydrogenase (LDH) release assay

LDH release assay was performed using CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (G1780) from Promega according to the manufacturer's protocol. In brief, cells were seeded in a 24-well plate (5×10^4 cells per well) for 24 h followed by the treatment as indicated. The readout for the maximum LDH release was achieved by Lysis Solution (10×) 45 min before the endpoint. The supernatant from each well was collected and centrifuged for 5 min at 4 °C, and then transferred in 50 μl into a 96-well plate, followed by 50 μl reconstituted substrate mix, for a desired incubation period (protected from light). A final 50 μl stop solution was added into each well, and the plate was