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Original article

The caspase-8/Bid/cytochrome *c* axis links signals from death receptors to mitochondrial reactive oxygen species production

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ABSTRACT

Ligation of the death receptors for TNF-α, FasL, and TRAIL triggers two common pathways, caspase-dependent intrinsic apoptosis and intracellular reactive oxygen species (ROS) generation. The apoptotic pathway is well characterized; however, a signaling linker between the death receptor and ROS production has not been clearly elucidated. Here, we found that death receptor-induced ROS generation was strongly inhibited by mitochondrial complex I and II inhibitors, but not by inhibitors of NADPH oxidase, lipoxygenase, cyclooxygenase or xanthine oxidase, indicating that ROS are mostly generated by the impairment of the mitochondrial respiratory chain. ROS generation was accompanied by caspase-8 activation, Bid cleavage, and cytochrome *c* release; it was blocked in FADD- and caspase-8-deficient cells, as well as by caspase-8 knockdown and inhibitor. Moreover, Bid knockdown abrogated TNF-α- or TRAIL-induced ROS generation, whereas overexpression of truncated Bid (tBid) or knockdown of cytochrome *c* spontaneously elevated ROS production. In addition, p53-overexpressing cells accumulated intracellular ROS via cytochrome *c* release mediated by the BH3-only protein Noxa induction. In a cell-free reconstitution system, caspase-8-mediated Bid cleavage and recombinant tBid induced mitochondrial cytochrome *c* release and ROS generation, which were blocked by Bcl-xL and antioxidant enzymes. These data suggest that anti-apoptotic Bcl-2 proteins play an important role in mitochondrial ROS generation by preventing cytochrome *c* release. These data provide evidence that the FADD/caspase-8/Bid/cytochrome *c* axis is a crucial linker between death receptors and mitochondria, where they play a role in ROS generation and apoptosis.

1. Introduction

Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals, are important regulators of redox-dependent signaling processes and cell homeostasis [1]. Transient fluctuations of small quantities of ROS serve as intracellular signals for cell growth, proliferation, and survival via activation of NF-κB and mitogen-activated protein kinases [2]. However, excessive ROS exert cytotoxic effects by inducing DNA damage, apoptosis, and necrosis [3,4], and are involved in the pathogenesis of various human diseases [5].

ROS are generally produced by two distinct mechanisms: enzymatic and non-enzymatic [4]. Typical enzymes are NADPH oxidase, xanthine oxidase, cyclooxygenase, and nitric oxide synthase. In the non-enzymatic mechanism, superoxide anion is generated during mitochondrial oxidative phosphorylation by leaking 2–5% of electrons to O_2 from complexes I and III of the electron transport chain [6]. Moreover, the inhibition of complexes I and III by rotenone and antimycin A leads to robust ROS generation from mitochondria by leaking electrons to O_2

Abbreviations: ROS, intracellular reactive oxygen species; mROS, mitochondrial ROS; FADD, Fas-associated death domain protein; ActD, actinomycin D; CH11, anti-Fas antibody clone 11; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; MMP, mitochondrial membrane potential; NAC, *N*-acetylcysteine

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[7]. This suggests that interference with the electron flow caused by defects in respiratory chain components increases ROS generation from mitochondria. Similarly, nitric oxide stimulates mitochondrial ROS (mROS) generation by inhibiting complex III activity [8]. Therefore, impairment of the respiratory chain is directly associated with increased mROS formation.

The production of mROS is largely increased by activation of the extrinsic and intrinsic apoptosis pathways [9–12]. The extrinsic pathway is triggered by cell death ligands, including TNF-α, FasL or TRAIL, leading to the sequential cellular events of Fas-associated death domain protein (FADD)-dependent caspase-8 activation, Bid cleavage to tBid, and mitochondrial cytochrome *c* release into the cytosol. The intrinsic pathway, triggered by cytotoxic drugs, leads to mitochondrial cytochrome *c* release by regulating the expression or activity of pro-apoptotic Bcl-2 family proteins [13]. Then, cytosolic cytochrome *c* causes sequential and rapid activation of caspase-9, caspase-3, and caspase-activated DNase, leading to DNA fragmentation. Therefore, both pathways converge upon the release of mitochondrial cytochrome *c*, resulting in defects in mitochondrial electron flow, which is coupled with superoxide anion generation [12]. This evidence suggests that death receptor ligands elicit robust increases in ROS production from the mitochondrial respiratory chain. However, the signaling cascade that links the death receptors and mitochondria to produce ROS remains unclear.

We hypothesized that the caspase-8/Bid/cytochrome *c* axis plays a crucial role in mROS generation by acting as a signaling linker between death receptors and the mitochondrial respiratory chain. Here, we found that mROS generation induced by death receptor activation was associated with the sequential induction of FADD-dependent caspase-8 activation, Bid cleavage, and cytochrome *c* release and was strongly suppressed by Bcl-xL. The generation of mROS was also increased by p53-dependent Noxa expression and cytochrome *c* release. These results provide new insight into the mechanism of death receptor-mediated mROS production and uncover how p53 and Bcl-xL exert indirect oxidant and antioxidant activities, respectively, probably by regulating mROS generation.

2. Materials and methods

2.1. Reagents and antibodies

Culture media and supplements were purchased from Life Technologies (Gaithersburg, MD, USA). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan UT, USA). Dimethyl sulfoxide was purchased from Calbiochem (La Jolla, CA, USA). All antibodies used in this study were purchased from PharMingen (San Diego, CA, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caspase inhibitors (zVAD-FMK, Ac-DEVD-CHO, Ac-IETD-CHO, and Ac-LEHD-CHO) and caspase substrates (Ac-DEVD-pNA and Ac-IETD-pNA) were obtained from Alexis Corporation (San Diego, CA, USA). Apocynin, rotenone, malonate, eicosatetraenoic acid, actinomycin D (ActD), and allopurinol were purchased from Cayman Chemical (Ann Arbor, MI, USA). MitoSOX red and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) were obtained from Molecular Probes (Eugene, OR, USA). Recombinant active caspase-8 and human TNF-α were purchased from R & D Systems (Minneapolis, MN, USA), and anti-Fas antibody (CH11) was obtained from Upstate Biotechnology (Lake Placid, NY, USA). TRAIL, Bid, Bcl-xL, and tBid were purified as previously described [14–16]. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture and transfection

HeLa cells were purchased from American Type Culture Collection and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS. FADD-deficient, caspase-8-deficient, and control Jurkat cells (JA3) were kindly provided by Dr John Blenis (Harvard Medical School, Boston, MA, USA) and cultured in RPMI 1640 medium. Expression plasmid constructs were prepared by insertion of human p53 (amino acids 1–393), human Noxa (amino acids 1–54), and murine Flag-tBid (amino acids 60–195) into pcDNA3 [15,16]. Small interfering RNAs (siRNAs) against human caspase-8, Bid, and cytochrome c were obtained from Dharmacon (Lafayette, CO, USA). Cells were transfected with or without expression plasmids (1 μg DNA/ml) and siRNA (80 nM) using Lipofectamine RNAiMAX and Lipofectamine 2000 reagent, respectively, according to the manufacturer's protocol. After 24 h, cells were treated with TRAIL (80 ng/ml), TNF-α (50 ng/ml)/ActD (100 ng/ml), anti-Fas antibody (CH11, 100 ng/ml), or staurosporine (2 μM), followed by measurement of ROS levels and caspase activation/activity.

2.3. Western blot analysis

Cells were cultured in serum-free medium for 2 h and treated with 50 ng/ml TNF-α plus 100 ng/ml ActD, 80 ng/ml TRAIL, or 2 μM staurosporine for 4 h. Cells were suspended in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor cocktail (Sigma-Aldrich) and lysed by three freeze/thaw cycles. Whole cell lysates were prepared by centrifugation at 12,000*g* for 10 min. In addition, a cytosolic fraction was prepared to measure cytochrome *c* release as follows. Cells (2.5×10^6) were suspended in 50 μl of ice-cold isolation buffer (20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10 mM phenylmethanesulfonyl fluoride, 10 M leupeptin, and 250 mM sucrose) and incubated on ice for 5 min. After homogenization using a Dounce homogenizer, cell lysates were centrifuged at 12,000*g* for 10 min at 4 °C, and the cytosolic fraction was collected. The whole cell lysates (40 μg protein) and the cytosolic fractions (40 μg protein) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were hybridised with antibodies against target proteins, and the protein bands were visualised by exposure to X-ray film.

2.4. Caspase activity and cell viability assay

Caspase activity was measured as previously described [17]. In brief, cells were cultured in serum-free medium for 2 h and treated with TNF-α/ActD, TRAIL, CH11, or staurosporine for 4 h. Cell suspensions were lysed by three freeze/thaw cycles, and the cytosolic fractions were obtained by centrifugation at 12,000*g* for 10 min at 4 °C. Caspase activities were determined in whole cell extracts (200 μg protein) by measuring the proteolytic cleavage of the chromogenic substrates (200 μM) Ac-DEVD-pNA (caspase-3-like activity), Ac-IETD-pNA (caspase-8-like activity), and Ac-LEHD-pNA (caspase-9-like activity). One unit of caspase activity was defined as an increase in absorbance of 0.1/mg protein/h at 405 nm. Cell viability were evaluated by 3-[4,5-cimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) assay after treatment with TNF-α/ActD or TRAIL for 5 h as previously described [18].

2.5. Lipid peroxidation assay

Malondialdehyde produced by lipid peroxidation was measured with the thiobarbituric acid reactive substances assay [17]. Jurkat cells were treated with TRAIL (80 ng/ml) for 6 h, harvested, and suspended in 0.5 ml of phosphate-buffered saline (PBS). Cell suspensions were mixed with 1.0 ml of freshly prepared thiobarbituric acid-trichloric acid-hydrochloric acid (30 g, 0.75 g, and 4.2 ml, respectively, in a final volume of 200 ml) reagent and 1 μl butylhydroxytoluene (0.05%). This mixture was heated for 15 min in boiling water. After it was cooled in an ice bath, we added 1.5 ml of *n*-butanol to the mixture, mixed vigorously, and centrifuged at 3000*g* for 15 min. The colour extract in the butanol layer was determined at 535 nm against a blank. A standard curve was prepared with malondialdehyde solutions (0.1–5 μM) that