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# Noxa is necessary for hydrogen peroxide-induced caspase-dependent cell death

Tomonori Aikawa [a], Koei Shinzawa [a], Nobuyuki Tanaka [b], Yoshihide Tsujimoto [a,*]

[a] Laboratory of Molecular Genetics, Department of Medical Genetics, Osaka University Medical School, Japan
[b] Department of Molecular Oncology, Institute of Gerontology, Nippon Medical School, Japan

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ABSTRACT

**Oxidative stress induces apoptosis or necrosis of many cell types, which can cause tissue injury. Hydrogen peroxide ($H_2O_2$) induced apoptotic death of Jurkat cells. This effect was inhibited by overexpression of human Bcl-2, by silencing of cytochrome *c*, and by ablation of Bax/Bak, indicating that $H_2O_2$-induced apoptosis was mediated by the mitochondrial pathway in Jurkat cells. Treatment with $H_2O_2$ caused an increase of Noxa protein, via activating transcription factor 4-dependent accumulation of Noxa mRNA and inhibition of Noxa protein degradation. $H_2O_2$-induced apoptosis was strongly suppressed by silencing of Noxa, indicating that Noxa plays a crucial role in this form of apoptosis.**

*Structured summary:*
MINT-7543162: *Mcl-1* (uniprotkb:Q07820) *physically interacts* (MI:0914) with *Bim EL* (uniprotkb:O43521), *Bim L* (uniprotkb:O43521) and *NOXA* (uniprotkb:Q13794) by *anti bait coimmunoprecipitation* (MI:0006)



## 1. Introduction

An increase of intracellular reactive oxygen species, referred to as oxidative stress, is cytotoxic and could be the underlying cause of various diseases, including neurodegenerative diseases [1,2]. $H_2O_2$ induces either apoptosis or necrosis, depending on the type of cell tested and $H_2O_2$ concentration used. It is generally believed that low levels of $H_2O_2$ induce apoptosis, whereas higher levels induce necrosis. For example, $H_2O_2$ concentrations up to 200 μM induce the apoptosis of Jurkat cells, while higher concentrations induce necrotic death [3]. In the case of mouse embryonic fibroblasts (MEF), however, there is some controversy, since using the similar concentrations of $H_2O_2$ some authors have reported that MEFs undergo caspase-independent necrotic death [4], while others have suggested that MEFs die of apoptosis mediated by Ask1 [5]. Thus, the detailed mechanisms of $H_2O_2$-induced apoptosis and necrosis are still poorly understood.

The mitochondria play a crucial role in apoptosis by releasing several apoptogenic molecules, such as cytochrome *c* [6] via mitochondrial outer membrane permeabilization (MOMP). MOMP is directly regulated by the Bcl-2 family of proteins, which are categorized into anti-apoptotic members (such as Bcl-2, Bcl-xL, and Mcl-1) and pro-apoptotic members that consist of multidomain proteins (such as Bax and Bak) and BH3-only proteins (including Bid, Bim, Noxa) [7]. Apoptotic stimulation causes translocation of BH3-only proteins to the mitochondria and induction of MOMP by inactivating anti-apoptotic Bcl-2 family members and/or activating the multidomain pro-apoptotic members [8,9].

Noxa is a p53 transcriptional target involved in the response to DNA-damaging agents [10]. Noxa can also be activated by other transcriptional factors, including HIF-1, E2F-1, and c-Myc [11–13], suggesting its broad role in the response to cellular stresses. Noxa has a high affinity for the anti-apoptotic proteins Mcl-1 and Bfl-1/A1, but a low affinity for the other anti-apoptotic proteins (Bcl-2, Bcl-xL, and Bcl-w) [14–16].

In the present study, we showed that Noxa is involved in $H_2O_2$-induced apoptosis of Jurkat cells and that $H_2O_2$ induces accumulation of Noxa protein, through inhibition of protein degradation and the accumulation of Noxa mRNA.

## 2. Materials and methods

### 2.1. Reagents

An anti-Noxa monoclonal antibody (clone 114C307) was obtained from Abcam (Cambridge, UK). An anti-Tp53 monoclonal

*Abbreviations:* RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CRE, cAMP response element; ATF, activating transcription factor; CREB, cAMP response element-binding protein

* Corresponding author. Address: 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 3369.
*E-mail address:* tsujimot@gene.med.osaka-u.ac.jp (Y. Tsujimoto).

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(DO-7), anti-Bim polyclonal, anti-Mcl-1 monoclonal, and anti-cytochrome *c* monoclonal antibodies (clone 7H8.2C12) were purchased from BD Pharmingen (San Diego). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody and anti-Mcl-1 polyclonal antibody were obtained from Chemicon International (Temecula) and from Santa Cruz Biotechnology (Santa Cruz), respectively. Anti-cAMP response element-binding protein (CREB) monoclonal antibody was from Cell Signaling Technology (Danvers). Hydrogen peroxide ($H_2O_2$) was obtained from Wako Co. (Osaka, Japan) and z-VAD.fmk was from the Peptide Institute (Osaka, Japan).

### 2.2. Cell culture and DNA transfection

Human Jurkat cells (T leukemia) and HeLa/D98 cells were grown at 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. DNA encoding FLAG-tagged human Noxa in the pUC-CAGGS expression vector or pZsProSensor-1 vector (Clontech) were used to transfect Jurkat cells with the Amaxa electroporation system (Nucleofector kit V, program A-17). The transfection efficiency was more than 50% as assessed by transfection with DNA expressing GFP. The siRNAs for cytochrome *c* were produced by Dharmacon Research (Chicago) and had the following nucleotide sequences: 5′-AAGCAUUAAGAAGAAGGAAGA-3′ (#54) and 5′-AAGGAAGAAAGGGCAGACUUA-3′ (#60). siRNAs for other human genes, including Noxa as well as negative control siRNA, were purchased from Qiagen (Hilden, Germany). Cells ($1 \times 10^6$) were transfected twice on alternate days with 3–13 μg of siRNA using the Amaxa electroporation system. At 48 h after the transfection, cells were used for the experiments.

### 2.3. Cell viability

After staining with propidium iodide (1 μM), FITC-conjugated Annexin V (1 μM), or Hoechst 33342 (1 μM) for 10 min, cell death was assessed by a flow cytometer (BD Biosciences, FACS Canto II) or by a fluorescence microscope (Olympus, BX50).

### 2.4. Preparation of whole-cell lysates and cytoplasmic fractions

For the detection of cytochrome *c* released from the mitochondria, cytoplasmic fractions were collected from Jurkat cells after incubation with 0.1 mg/ml digitonin for 5 min at 37 °C in isotonic buffer [20 mM potassium-Hepes (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 250 mM sucrose, and 1 mM $Na^{2+}$-EDTA]. After centrifugation, aliquots of the supernatant (cytoplasmic fraction) and the pellet containing the mitochondria were analyzed by Western blotting. In some experiments, cells were lysed in 50 mM Tris–HCl (pH 8.0), 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate sodium salt, and 150 mM NaCl to obtain whole-cell lysates.

### 2.5. Luciferase assay

The Firefly luciferase plasmid pGL4.18 (Promega) was used to generate plasmids carrying different regions of the Noxa promoter (−5619 to +153, −925 to +153, −198 to +153, −134 to +153, and −74 to +153): pNoxa5619-luc, pNoxa925-luc, pNoxa198-luc, pNoxa134-luc, and pNoxa74-luc, respectively. pNoxa198 + 1-luc was a derivative of pNoxa198-luc, lacking +1 to +136. pNoxa198mt-luc carried a mutation of the p53-binding site [10]. pNoxa134CB-luc had two mutations (CTACTCCA) within the cAMP response element (CRE) site (CTACGTCA). Cells were co-transfected with 1 μg of control Renilla luciferase pRL-SV40 (promega) and with 10 μg of Firefly luciferase reporter constructs by Amaxa electroporation. Luciferase activity was measured by using a luminometer and the Dual-Glo luciferase assay system (Promega).

### 2.6. Other methods

Immunoprecipitation was done with anti-Mcl-1 antibody as described [14] and proteolytic activities were measured as described [17].

## 3. Results

### 3.1. Induction of mitochondria-dependent apoptosis by $H_2O_2$ in Jurkat cells

$H_2O_2$ induced death of Jurkat cells as assessed by Annexin V staining (Fig. 1A and Fig. S1), which was inhibited by the pan-caspase inhibitor z-VAD.fmk or by Bcl-2 overexpression (Fig. 1A and Fig. S1). Staining with Hoechst 33342 revealed typical apoptotic nuclear morphological changes (chromatin condensation and fragmentation), which were also inhibited by z-VAD.fmk (Fig. 1B). These results demonstrated that $H_2O_2$ induced apoptosis of Jurkat cells.

Cytochrome *c* (Cyt. *c*) was released from the mitochondria of $H_2O_2$-treated Jurkat cells (Fig. 1C). Silencing of Cyt. *c* (Fig. 1D) strongly suppressed $H_2O_2$-induced apoptosis (Fig. 1E). To determine whether $H_2O_2$-induced apoptosis was dependent on Bax/Bak, Jurkat cells which lacks Bax expression [18] were transfected with Bak siRNA (Fig. 1F). Silencing of Bak markedly reduced $H_2O_2$-induced apoptosis (Fig. 1G). These results indicated that the mitochondrial pathway was involved in $H_2O_2$-induced apoptosis of Jurkat cells.

### 3.2. Crucial role of Noxa in $H_2O_2$-induced apoptosis of Jurkat cell

BH3-only members of the Bcl-2 family either activate multidomain pro-apoptotic Bax/Bak or inhibit anti-apoptotic members, such as Bcl-2 [19,20]. In response to apoptotic stimuli, BH3-only proteins are activated by either transcriptional upregulation or by posttranslational modifications [10,21–23]. Toward determining which BH3-only proteins were involved in $H_2O_2$-induced apoptosis of Jurkat cells, we analyzed levels of various BH3-only proteins. As shown in Fig. 2A and Fig. S2, there was an increase of Noxa, Bim, Hrk, Bnip3, and Bnip3L protein levels, whereas Bmf, Bad, and Bik proteins were decreased.

To determine which BH3-only proteins were involved in $H_2O_2$-induced apoptosis, Jurkat cells were transfected with siRNAs for various BH3-only proteins, and the influence on apoptosis was assessed. Silencing of the respective BH3-only proteins was verified by Western blot analysis or reverse transcription polymerase chain reaction (RT-PCR) (Fig. 2B and D and data not shown). Among the proteins tested, silencing of Noxa was found to most significantly protect Jurkat cells against $H_2O_2$-induced apoptosis (Fig. 2C and Fig. S3). To investigate a role of Noxa in $H_2O_2$-induced apoptosis in other cell line, we examined with HeLa/D98 cells and found silencing of Noxa provided cells a significant level of resistance to $H_2O_2$-induced apoptosis (Fig. S4).

Silencing of either Bid or Bim also significantly protected Jurkat cells against $H_2O_2$-induced apoptosis, although to lesser extents (Fig. 2D). Two models have been proposed with regard to BH3-only protein function. According to one model, all BH3-only proteins act by inhibiting anti-apoptotic members of the Bcl-2 family [16]. According to the other model, Bim and Bid (or possibly Puma) directly activate Bax and Bak, while other members (including Noxa) inactivate the anti-apoptotic Bcl-2 family members, suggesting Noxa's role upstream of Bid/Bim [14,15]. Noxa is