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

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**Abstract**

Oxidative stress induces apoptosis or necrosis of many cell types, which can cause tissue injury. Hydrogen peroxide (H₂O₂) induced apoptotic death of Jurkat cells. This effect was inhibited by over-expression of human Bcl-2, by silencing of cytochrome c, and by ablation of Bax/Bak, indicating that H₂O₂-induced apoptosis was mediated by the mitochondrial pathway in Jurkat cells. Treatment with H₂O₂ caused an increase of Noxa protein, via activating transcription factor 4-dependent accumulation of Noxa mRNA and inhibition of Noxa protein degradation. H₂O₂-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)

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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]. H₂O₂ induces either apoptosis or necrosis, depending on the type of cell tested and H₂O₂ concentration used. It is generally believed that low levels of H₂O₂ induce apoptosis, whereas higher concentrations induce necrosis. For example, H₂O₂ 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 H₂O₂ 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 H₂O₂-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 H₂O₂-induced apoptosis of Jurkat cells and that H₂O₂ 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
(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-glyceroldehyde-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 (H2O2) 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 pU6-CAGGS expression vector or pZsProSensor-1 vector (Clontech) were used to transfect Jurkat cells with the Amaza 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′-AAGCAAUAGAGAGGAAGA-3′ (#54) and 5′-AAGGAAAGGCAGACUUA-3′ (#60). siRNAs for other human genes, including Noxa as well as negative control siRNA, were purchased from Qiagen (Hilden, Germany); Cells (1 × 10⁶) were transfected twice on alternate days with 3–13 μg of siRNA using the Amaza 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 MgCl₂, 250 mM sucrose, and 1 mM NaCl–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 pNoxa174-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 (CTAGCTCA). Cells were co-transfected with 1 μg of control Renilla luciferase pRL-SV40 (promega) and with 10 μg of Firefly luciferase reporter constructs by Amaza 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 H2O2 in Jurkat cells

H2O2 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 H2O2 induced apoptosis of Jurkat cells.

Cytochrome c (Cyt. c) was released from the mitochondria of H2O2-treated Jurkat cells (Fig. 1C). Silencing of Cyt. c (Fig. 1D) strongly suppressed H2O2-induced apoptosis (Fig. 1E). To determine whether H2O2-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 H2O2-induced apoptosis (Fig. 1G). These results indicated that the mitochondrial pathway was involved in H2O2-induced apoptosis of Jurkat cells.

3.2. Crucial role of Noxa in H2O2-induced apoptosis of Jurkat cells

BH3-only members of the Bcl-2 family either activate multidomain pro-apoptic 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 H2O2-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 H2O2-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 H2O2-induced apoptosis (Fig. 2C and Fig. S3). To investigate a role of Noxa in H2O2-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 H2O2-induced apoptosis (Fig. S4).

Silencing of either Bid or Bim also significantly protected Jurkat cells against H2O2-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
known to inhibit Mcl-1 (and Bfl-1/A1), but not Bcl-2 and Bcl-xL [14,15]. Consistent with the second model, silencing of Noxa/Bim did not significantly increase protection against apoptosis compared with silencing of Noxa alone, and that of Noxa/Bid provided no more than slight resistance (Fig. 2D). Also consistent with the second model, Mcl-1-bound Noxa increased and Mcl-1-bound Bim showed a significant decrease in response to H2O2 (Fig. 2E). These results might support the possibility that increased Noxa in response to H2O2 binds with Mcl-1 to release Bim (Bid), which activates Bax/Bak.
3.3. Accumulation of Noxa by inhibition of proteasomal degradation after exposure to H₂O₂

Noxa protein could be upregulated after exposure to H₂O₂ either by increased protein production or by inhibition of degradation. Since H₂O₂ reportedly affects the proteasomal degradation of proteins [2,17], we investigated this possibility for Noxa by using cycloheximide and the proteasome inhibitor MG132. Since we found that treatment of Jurkat cells with these inhibitors resulted in an increase of endogenous Noxa mRNA, whereas Flag-tagged...
Noxa mRNA was not, which was produced from the transiently transfected pUC-CAGGS expression plasmid, we used the Flag-tagged Noxa for this purpose. The experiments with cycloheximide demonstrated that Flag-tagged Noxa turnover was more rapid in healthy cells than in H2O2-treated cells, with the t1/2 being 9.43 in H2O2-treated cells versus 2.08 in the control (Fig. 3A). Addition of MG132 restored Noxa levels in untreated cells (Fig. 3A). These results indicated that Noxa protein was subjected to proteasome-dependent degradation in healthy cells, whereas its degradation was inhibited by H2O2.

Inhibition of Noxa degradation might result from either H2O2-induced inhibition of ubiquitin processing or inhibition of proteasome. H2O2 treatment has been reported to directly inhibit 26S-proteasome activity in K562 cells at 400 µM concentration [17]. Therefore, we measured the activity of 20S and 26S proteasomes in lysates of Jurkat cell treated with not only 90 µM of H2O2 but also up to 500 µM of H2O2 as described in Section 2. As shown in Fig. 3B and C, neither 20S-proteasome nor 26S-proteasome activity was significantly affected by H2O2, suggesting that the proteasome machinery itself was not affected by H2O2.

Fig. 3. H2O2-induced stabilization of Noxa protein. (A) Jurkat cells expressing Flag-tagged Noxa from the pUC-CAGGS plasmid were treated with cycloheximide (1 µg/ml) with or without H2O2 (90 µM) or with MG132 (10 µM), and Flag-tagged Noxa levels were determined by Western blotting. % values represent the Noxa signal intensity compared to the 0 h time point. The t1/2 values are shown below. (B and C) Cells were treated with H2O2 at the indicated concentrations and whole-cell lysates were analyzed for ATP-stimulated (B) and ATP-independent (C) MG132-sensitive proteolytic activity, which represented total proteasome (26S and 20S) activity and 20S-proteasome activity, respectively. A representative result of three independent experiments yielding similar findings is shown. (D) Jurkat cells were transfected with 1 µg of pZsProSensor-1 vector for 48 h. Then the cells were treated with H2O2 or MG132, and cells positive for ZsGreen were counted by FACS analysis. Data are shown as the means ± S.D. (n = 3).
We then examined whether H$_2$O$_2$-induced inhibition of protein degradation was specific for Noxa by employing the pZsProSensor-1 vector, in which mouse ornithine decarboxylase (mODC) d410 was fused to the C-terminus of a green fluorescent protein (ZsGreen). mODC d410 contains several PEST sequences [24], which render the ZsGreen-mODC d410 fusion protein highly susceptible to proteasomal degradation. As shown in Fig. 3D, ZsGreen fluorescence gradually accumulated after H$_2$O$_2$ treatment, as it did with MG132.

**Fig. 4.** Transcriptional activation of Noxa promoter by H$_2$O$_2$. (A) Noxa mRNA accumulation in Jurkat cells with H$_2$O$_2$. Jurkat cells were treated with H$_2$O$_2$/z-VAD.fmk. Noxa mRNA was quantified by RT-PCR. (B–D) Transcriptional activation of the Noxa promoter in response to H$_2$O$_2$. At 72 h after transfection of reporter constructs, cells were treated without (open bars) or with H$_2$O$_2$/z-VAD.fmk (closed bars) for 6 h, after which Firefly- and Renilla-dependent luciferase activity were determined. Firefly-dependent luciferase activity was normalized by the Renilla signals. Data are shown as the means ± S.D. (n = 3). (E and F) Jurkat cells were transfected with 13 µg of the indicated siRNA for 48 h. Then the cells were treated with H$_2$O$_2$/z-VAD.fmk. Expression of ATF4 (E) and Noxa (F) mRNA was analyzed by RT-PCR. GAPDH was used as an internal control. Data are shown as the means ± S.D. (n = 3).
These results indicated that H$_2$O$_2$-induced accumulation of Noxa was at least partly due to the general inhibition of proteasome-dependent degradation.

3.4. Transcriptional upregulation of Noxa by exposure to H$_2$O$_2$

In addition to inhibition of the proteasome-dependent degradation of Noxa, other processes (such as the accumulation of Noxa mRNA) might also contribute to the H$_2$O$_2$-induced increase of Noxa protein. In fact, the reverse transcription real-time PCR showed gradual accumulation of Noxa mRNA after H$_2$O$_2$ treatment (Fig. 4A). In general, mRNA accumulation is either due to its stabilization or to an increase of de novo synthesis. Since Noxa gene is known to be activated by different transcriptional factors in response to cellular stresses [10–13], we examined the possible contribution of transcriptional activation of Noxa promoter to H$_2$O$_2$-induced mRNA accumulation by employing the luciferase assay. The Noxa promoter contains binding sites for various transcription factors, including E2F-1, Myc, p53, and HIF, which are located at positions +130, +87, −198, and −1100, respectively, from the transcription start site [10–13]. As shown in Fig. 4B and C, pNoxa5619, the longest Noxa promoter and some shorter versions (pNoxa925, pNoxa198, pNoxa134, and pNoxa198 + 1) all exhibited a similar response to H$_2$O$_2$, indicating that transcriptional activation induced by H$_2$O$_2$ was mediated by the region of the Noxa promoter from −134 to +1 and +137 to +153, which excluded a role of p53. Consistently, a mutation of the p53-binding site (pNoxa-a198mt) did not inhibit H$_2$O$_2$-induced Noxa activation (Fig. 4B). Furthermore, silencing of p53 had no effect on the accumulation of Noxa or on cell death induced by H$_2$O$_2$ (Fig. S5), excluding a role of p53 in H$_2$O$_2$-induced Noxa transactivation.

Computer-based analysis of the Noxa promoter from −134 to +1 identified the CRE. Mutation within the CRE significantly reduced transcriptional activation of the Noxa promoter by H$_2$O$_2$ (Fig. 4D). It has been reported that CRE-binding proteins are involved in apoptotic cell death induced by various stresses [25–28]. To investigate which CRE-binding proteins were involved in the transcriptional activation of Noxa after stimulation by H$_2$O$_2$, we transfected Jurkat cells with siRNAs for five CRE-binding proteins (CREB1, c-Jun, activating transcription factor (ATF)2, ATF3, and ATF4). Silencing of ATF4 significantly inhibited H$_2$O$_2$-induced accumulation of Noxa mRNA (Fig. 4E and F), whereas silencing of the other CRE-binding proteins did not (Fig. S6 and data not shown). These results suggested that ATF4 protein was involved in the H$_2$O$_2$-induced transcriptional activation of Noxa.

4. Discussion

We showed that H$_2$O$_2$ induces apoptotic death of Jurkat cells, which is mediated by the mitochondrial pathway, because of the inhibition of H$_2$O$_2$-induced apoptosis by overexpression of Bcl-2, ablation of Bax/Bak, and by silencing of Cyt. c.

Bax/Bak is activated directly or indirectly by BH3-only proteins [14–16]. We showed that several BH3-only proteins (including Noxa) accumulated in response to H$_2$O$_2$ and that Noxa plays a major role in H$_2$O$_2$-induced apoptosis of Jurkat cells. The similar observations were made with HeLa cells. However, silencing of Noxa protein was not sufficient to completely protect cells against H$_2$O$_2$-induced apoptosis, perhaps because Noxa protein expression was not completely abolished by the siRNA. Alternatively, one or more of the other BH3-only proteins, such as Hrk, Bim, or Bid, could also contribute to H$_2$O$_2$-induced apoptosis. Certainly, we could not exclude other possibilities for roles of H$_2$O$_2$, for example, that H$_2$O$_2$ directly induces a conformational change of Bax as suggested [29].

BH3-only proteins are categorized into two groups: activators (Bid and Bim) capable of directly activating Bax/Bak, and inactivators (Bad, Noxa, etc.) that bind to and inactivate anti-apoptotic members of the Bcl-2 family to release the activators [14]. Consistent with this model, H$_2$O$_2$ treatment of Jurkat cells increased the amount of Mcl-1/Noxa complex and decreased Mcl-1/Bim complex. However, it seemed that a little Bim was released from Mcl-1 after H$_2$O$_2$ treatment, so it is not clear whether the scenario dismissed above holds true for the exact role of Noxa in H$_2$O$_2$-induced apoptosis of Jurkat cells. Further investigation will be required.

We also showed that Noxa accumulates after H$_2$O$_2$ treatment and H$_2$O$_2$ treatment triggers both transcriptional activation of the Noxa gene and stabilization of Noxa protein. H$_2$O$_2$-induced transcriptional activation of the Noxa promoter was supported by the increase of Noxa mRNA and by the results of the luciferase reporter study. The Noxa promoter contains sites for p53, E2F-1, Myc, and HIF-1 [10–13]. p53 and HIF-1 are involved in transactivation of Noxa in response to DNA damage and hypoxia, respectively [10,12]. Using luciferase-based analysis and gene silencing, we excluded the possibility that p53 plays a role in H$_2$O$_2$-induced Noxa activation. Luciferase-based analysis also revealed that the region containing the CRE is important for H$_2$O$_2$-induced transcriptional activation, suggesting a role of CRE-binding factor(s) in H$_2$O$_2$-induced transactivation of Noxa. Consistent with this result, silencing of ATF4 also suppressed H$_2$O$_2$-induced accumulation of Noxa mRNA. However, since silencing of ATF4 suppressed only partially the H$_2$O$_2$-induced Noxa mRNA accumulation, other transcriptional factors or other mechanisms, such as stabilization of Noxa mRNA might also be involved.

We showed that H$_2$O$_2$-induced accumulation of Noxa protein depends on its stabilization. It seems that inhibition of proteasomal degradation by H$_2$O$_2$ exposure was one reason for Noxa accumulation, since the proteasome inhibitor MG132 stabilized Noxa protein. Noxa was stabilized by H$_2$O$_2$ at least partly due to general inhibition of the proteasomal degradation machinery, because other proteins such as mODC carrying-PEST and c-Myc (data not shown) were also stabilized. Although it has been reported that ATP-stimulated activity of the 26S proteasome is significantly reduced by H$_2$O$_2$ [17], we observed no effect of H$_2$O$_2$ on 26S-proteasome activity in Jurkat cells. In addition, we found that the intracellular ATP content was not affected by H$_2$O$_2$ after 6 h of treatment (data not shown). More detailed studies will be necessary to understand the mechanisms by which exposure to H$_2$O$_2$ inhibits proteasomal degradation of Noxa protein.

In conclusion, we found a mechanism of H$_2$O$_2$-induced apoptosis in Jurkat cells that involved the accumulation of Noxa protein via Noxa gene transactivation and inhibition of the proteasomal degradation of Noxa protein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.01.026.
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