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Noxa couples lysosomal membrane permeabilization and apoptosis during oxidative stress

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Abstract

The exact roles of lysosomal membrane permeabilization (LMP) in oxidative stress-triggered apoptosis are not completely understood. Here, we first studied the temporal relation between LMP and mitochondrial outer membrane permeabilization (MOMP) during the initial stage of apoptosis caused by the oxidative stress inducer H₂O₂. Despite its essential role in mediating apoptosis, the expression of the BH3-only Bcl-2 protein Noxa was dispensable for LMP. In contrast, MOMP was dependent on Noxa expression and occurred downstream of LMP. When lysosomal membranes were stabilized by the iron-chelating agent desferrioxamine, H₂O₂-induced increase in DNA damage, Noxa expression and subsequent apoptosis were abolished by the inhibition of LMP. Importantly, LMP-induced Noxa expression increase was mediated by p53 and seems to be a unique feature of apoptosis caused by oxidative stress. Finally, exogenous iron loading recapitulated the effects of H₂O₂ on the expression of BH3-only Bcl-2 proteins. Overall, these data reveal a Noxa-mediated signaling pathway that couples LMP with MOMP and ultimate apoptosis during oxidative stress.

INTRODUCTION

In multicellular organisms, apoptosis is a cellular suicide process critical for the maintenance of normal tissue homeostasis, which preserves a proper balance between the rate of cell proliferation and the rate of cell death (1, 2). Apoptosis can be induced by ligation of death receptors through the extrinsic pathways or by various death stimuli through the intrinsic pathways. Recent genetic and biochemical studies have revealed a conserved network that modulates the well-organized self-destruction of cells. In mammalian cells, intrinsic apoptotic signal leads to mitochondrial outer membrane permeabilization (MOMP) and the release of apoptogenic factors such as cytochrome c into the cytosol, where they activate a cascade of aspartate-directed cysteine proteases (caspases)

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subsequently leading to apoptosis (3). MOMP and activation of caspases are usually considered as the molecular hallmarks of apoptosis (4). Apoptosis signaling is regulated by the Bcl-2 family of proteins, which can be either pro-apoptotic or anti-apoptotic (5–7). Pro-apoptotic Bcl-2 proteins promote apoptosis by increasing MOMP, whereas anti-apoptotic Bcl-2 proteins inhibit MOMP and prevent or delay apoptosis (8–10).

Although mitochondria play a central role in apoptosis regulation, other organelles including the endoplasmic reticulum (ER), the golgi apparatus, and lysosomes are also involved in apoptotic signaling (11–14). Lysosomes are major intracellular organelles responsible for degrading and recycling of cellular components. Lysosomes contain at least 50 hydrolytic enzymes including nucleases, proteases, phospholipases, lipases, phosphatases, sulfatases and glycosidases which, upon release, can degrade macromolecules in the cytosol (15). The best characterized lysosomal enzymes belong to the cathepsin protease family (16). A wide variety of stressors including osmotic stress, growth factor deprivation, death receptor activation, proteasome inhibitors and oxidative stress inducers have been shown to target lysosomes and cause LMP through which lysosomal hydrolytic enzymes are released into the cytosol (17). The level of damage to the lysosome determines the fate of the cell. Massive lysosomal damage causes an excessive release of lysosomal contents into the cytosol resulting in indiscriminate degradation of cellular contents and cytoplasmic acidification, which in turn promotes cell death by necrosis. On the other hand, selective or partial lysosomal damage induces cell death by apoptosis (18–20). For instance, in tumor necrotic factor alpha (TNF α -treated cells, cathepsin B, D and L released into the cytosol trigger apoptosis by converting the inactive pro-apoptotic BH3-only Bcl-2 protein Bid into its truncated active form (tBid), promoting subsequent MOMP and caspase activation (17, 21).

Oxidative stress inducers, including hydrogen peroxide (H₂O₂), are capable of inducing cell death by both necrosis and apoptosis; mild oxidative stress causes apoptosis whereas severe oxidative stress triggers necrosis (22). The extent of oxidative stress determines the level of lysosomal membrane damage. H₂O₂ interacts with intralysosomal iron to generate highly reactive hydroxyl radicals that initiate lipid peroxidation of lysosomal membranes and subsequent LMP. In support of this model, the iron-chelating agent desferrioxamine (DFO) has been shown to abolish oxidative stress-triggered LMP and apoptosis (23). However, the involvement of LMP in oxidative stress-induced apoptosis signaling and how LMP is modulated by the complex Bcl-2 protein network are still unclear. Here, we investigated the temporal relation between LMP and MOMP during oxidative stress-induced apoptosis. In mouse embryonic fibroblasts (MEFs), H₂O₂ was able to induce LMP prior to MOMP during apoptosis. MOMP and subsequent apoptosis signaling, but not LMP, depended on Noxa expression. The iron-chelating agent DFO prevented H₂O₂-induced MOMP and apoptosis by inhibiting LMP, oxidative DNA damage and subsequent p53-dependent Noxa expression increase. Therefore, LMP-induced Noxa expression is critical for MOMP and subsequent activation of the apoptosis cascade during oxidative stress.

RESULTS

Oxidative stress-induced LMP is independent of Noxa expression

In lysosomes, the oxidative stress inducer H₂O₂ causes lysosomal membrane permeabilization (LMP) and subsequent apoptosis (24–26). Our previous studies have shown that the elevated expression of the pro-apoptotic BH3-only Bcl-2 protein Noxa mediates apoptosis triggered by H₂O₂ (27). To explore the involvement of Noxa in H₂O₂-induced LMP, we first examined lysosomal membrane stability in mouse embryonic fibroblasts (MEFs) deficient in Noxa expression (Noxa-KO) and their wild-type counterparts. The lysosomotropic base acridine orange (AO) is a metachromatic

fluorochrome exhibiting red fluorescence when it is highly concentrated in intact lysosomes, but emitting green fluorescence when its concentration is low as observed in lysosomes with disrupted membrane integrity (13). Two complementary approaches (AO relocation and AO uptake) were used to examine lysosomal membrane stability following H₂O₂ exposure. In AO relocation experiments, cells were first incubated with AO before an exposure to H₂O₂. Thirty minutes post H₂O₂ exposure, there was a significant increase in green fluorescence levels in wild-type and Noxa-KO MEFs, indicating minor damage to lysosomal membrane integrity (Figure 1A). Importantly, the green fluorescence intensity increase was similar in both cell lines, providing evidence that Noxa expression did not influence H₂O₂-induced LMP despite its critical role in mediating apoptotic signaling pathways. Furthermore, pre-incubation of cells with the highly potent iron chelator desferrioxamine (DFO) markedly reduced LMP independent of Noxa expression (Figure 1A).

To further assess the effects of Noxa expression on late lysosomal membrane rupture, we carried out AO uptake experiments to estimate the percentage of “pale” cells (cells with less than normal red fluorescence, indicating ruptured lysosomes). The fraction of cells displaying “pale” cell phenotype markedly increased in both wild-type and Noxa-KO MEFs, indicating late lysosomal membrane rupture (Figure 1B). Furthermore, the increase in late lysosomal membrane rupture was abolished by preincubation with DFO. Consistent with the results of AO relocation experiments (Figure 1A), H₂O₂ was able to induce comparable late lysosomal membrane rupture in wild-type and Noxa-KO MEFs (Figure 1C). Overall, these results indicate that H₂O₂ induces LMP independent of Noxa expression, suggesting that Noxa mediates H₂O₂-triggered apoptosis downstream of the destruction of lysosomal membrane integrity.

Noxa-dependent MOMP occurs downstream of LMP during H₂O₂-induced apoptosis

MOMP and subsequent release of apoptogenic proteins such as cytochrome c from mitochondria to the cytosol are the molecular hallmarks of apoptosis (8). To determine the temporal relation between MOMP and LMP during H₂O₂-induced apoptosis, we first examined the amounts of cytochrome c released into the cytosol as an indicator of MOMP. To this purpose, both wild-type and Noxa-KO MEF cells were treated with H₂O₂, and the presence of cytochrome c in cytosolic fractions was determined by western blot (Figure 2A). The levels of cytochrome c in cytosolic fractions of Noxa-KO MEF cells were largely unchanged upon 24-hour H₂O₂ exposure (Figure 2A), indicating very little MOMP. However, the amounts of cytochrome c were notably higher in cytosolic fractions of wild-type cells after 14-hour H₂O₂ treatment. As LMP was observed 30 minutes following H₂O₂ treatment in both wild-type and Noxa-KO MEF cells (Figure 1A), these results provide evidence that MOMP occurs following LMP during oxidative stress.

As a direct result of increased MOMP, the decrease in mitochondrial membrane potential has been commonly used as an indicator of MOMP (28). To further examine the temporal relation between MOMP and LMP, we measured mitochondrial membrane potential at different time points following an exposure to H₂O₂ using the mitochondrial potential-sensitive probe MitoTracker Red and the mitochondrial potential-independent probe MitoTracker Green. An exposure to H₂O₂ led to a time-dependent decrease in mitochondrial membrane potential in both wild-type and Noxa-KO MEFs, but mitochondrial membrane potential dissipated much faster in wild-type MEFs compared with Noxa-KO MEFs (Figure 2B). In contrast to the occurrence of LMP 30 minutes following H₂O₂ treatment (Figure 1A), no changes in mitochondrial membrane potential were detected in either MEF cell line within one hour of H₂O₂ exposure. After 6 hours of H₂O₂ treatment, mitochondrial membrane potential was reduced about 30% in wild-type MEFs, whereas mitochondria in Noxa-KO MEFs were still intact. Twenty-four hours after the initial exposure to H₂O₂, mitochondrial membrane potential was almost completely abolished in wild-type MEFs, but

there was only about 25% reduction in mitochondrial membrane potential in Noxa-KO MEFs. Thus, Noxa expression is important for MOMP induction. Importantly, stabilizing lysosomal membranes by pre-incubation with DFO significantly inhibited H₂O₂-induced mitochondrial membrane potential reduction (Figure 2C). Therefore, it is likely that mitochondrial membrane rupture occurs downstream of LMP in cells undergoing oxidative stress.

Stabilizing lysosomal membranes prevents H₂O₂-induced DNA damage

Previous studies have shown that redox-active iron released from lysosomes during LMP is capable of inducing oxidative damage to DNA (29). To investigate the importance of stabilizing lysosomal membranes in preventing DNA damage and subsequent Noxa-mediated apoptosis, DNA damage in H₂O₂-exposed cells was examined. Since H₂O₂ has been shown to induce single- and double- strand DNA breaks (30), alkaline comet assay was carried out to determine DNA damage caused by H₂O₂. As expected, H₂O₂ was able to induce DNA damage in MEFs (Figure 3A). Importantly, the degree of H₂O₂-induced DNA damage was independent of Noxa expression, despite its essential role in mediating MOMP and subsequent apoptosis (Figure 3B). Moreover, stabilization of lysosomal membranes with DFO pre-incubation prevented H₂O₂-induced DNA damage in both wild-type and Noxa-KO MEFs, providing more evidence for a critical role of LMP in inducing oxidative DNA damage.

H₂O₂-induced increase in Noxa expression is mediated by LMP

To systematically examine the effect of LMP on pro-apoptotic Bcl-2 proteins expression, we carried out microarray analysis in MEF cells. While DFO itself did not significantly affect gene expression of BH3-only Bcl-2 proteins, stabilizing lysosomal membranes by DFO was able to reduce the effects of H₂O₂ on Noxa mRNA expression levels (Figure 4A). Quantitative real-time polymerase chain reaction (qPCR) experiments further confirmed that LMP mediated the increase in Noxa expression during oxidative stress-induced apoptosis (Figure 4B). To explore the distinct roles of LMP in Noxa expression induction in different apoptosis paradigms, we also examined the effects of DFO on Noxa expression induced by H₂O₂ and the chemotherapeutic drug cisplatin respectively in human colon cancer HCT116 cells (Figure 4C). DFO abolished H₂O₂-caused Noxa mRNA induction, but failed to prevent cisplatin-induced increase in Noxa mRNA expression. The results of these experiments validate the unique role of LMP-induced Noxa expression increase in apoptotic signaling triggered by oxidative stress.

Oxidative stress-induced increase in Noxa expression depends on p53

In response to DNA damage, transcription mediated by the tumor suppressor p53 is activated, and Noxa is a known p53-response protein (31). Therefore, the role of p53 in transcriptional response to H₂O₂-induced DNA damage was explored. Primary fibroblasts isolated from one wild-type mouse embryo and two p53^{-/-} counterparts were exposed to H₂O₂ and a microarray analysis was carried out to determine global changes in mRNA (Figure 5A). Similar to what was observed in immortalized MEFs and HCT116 cells (Figure 4), there was a significant increase in Noxa mRNA expression level in wild-type primary MEFs following treatment with H₂O₂ (Figure 5A). In contrast, Noxa expression induction was modest in p53^{-/-} primary cells, indicating that increased Noxa expression in response to H₂O₂ was mostly mediated by p53 in fibroblasts. The increase in Noxa expression could be attributed to altered p53 mRNA expression level, which was doubled upon H₂O₂ exposure (Figure 5A). The changes in Noxa and p53 mRNA levels were further validated using qPCR (Figures 5B and 5C), providing evidence that oxidative DNA damage-induced p53 activation is responsible for increased Noxa expression during oxidative stress.

LMP is essential for Noxa-mediated apoptosis in H₂O₂-treated cells

Since Noxa expression is critical for MOMP but dispensable for LMP and DNA damage, we investigated the importance of LMP for Noxa-mediated apoptosis in H₂O₂-exposed cells. As expected, wild-type MEFs were more susceptible to H₂O₂ treatment compared with their Noxa-KO counterparts (Figure 6A). Upon H₂O₂ exposure, Noxa-mediated cell death was abolished in cells pretreated with DFO, providing evidence that the impact of LMP on cellular responses to H₂O₂ depends on Noxa expression (Figure 6A). In addition, caspase 3/7 activity was measured following exposure to H₂O₂. While there was higher caspase 3/7 activity in wild-type MEFs than Noxa-KO MEFs, pre-incubation with DFO significantly reduced caspase 3/7 activity in both cell lines (Figure 6B). The correlation between caspase 3/7 activity and the level of cell death indicates that LMP exerts its influence on H₂O₂-induced apoptosis through Noxa.

To further confirm the role of Noxa expression in H₂O₂-triggered LMP and subsequent apoptosis, Noxa expression in human colon cancer HCT116 cells was stably reduced by shRNA (Figure 6C). HCT116 cells with normal or reduced Noxa expression were exposed to H₂O₂ in the presence or absence of DFO pre-incubation. Consistent with the studies with MEFs (Figure 6A), the reduction of Noxa expression rendered HCT116 cells more resistant to H₂O₂ treatment, validating the critical role of Noxa in H₂O₂-triggered apoptosis. Furthermore, DFO pre-incubation abolished H₂O₂ cytotoxicity in HCT116 cells with normal Noxa expression but not their counterparts with reduced Noxa expression (Figure 6D), providing more evidence that the pro-apoptotic effects of Noxa on H₂O₂-induced apoptosis depends on LMP.

LMP is not involved in the regulation of some apoptosis paradigms

To examine whether the induction of LMP is also critical in other apoptosis paradigms, wild-type MEFs were treated with death stimuli that have diverse mechanisms of action, including the topoisomerase inhibitor etoposide, the DNA intercalating agent doxorubicin, the transcriptional inhibitor actinomycin D, and the protein kinase inhibitor staurosporine. DFO failed to prevent apoptosis induced by any of the four death stimuli examined (Supplementary Figure 1A), indicating that LMP did not occur or was not involved in apoptosis signaling induced by any of these agents. These results demonstrate a specific role of LMP in regulating oxidative stress-induced apoptosis in MEFs. This was further confirmed by the observation that caspase 3/7 activity detected in MEFs treated with different death stimuli closely correlated with the cell viability measurements (Supplementary Figure 1B).

To further determine the relevance of LMP in apoptotic signaling pathways initiated by chemotherapeutic drugs, human HCT116 cells with different Noxa expression levels were treated with cisplatin or carboplatin, two anti-cancer agents whose cytotoxicity has also been shown to be dependent on Noxa expression (32). HCT116 cells expressing normal levels of Noxa were more sensitive to both agents compared with their Noxa knockdown counterparts (Supplementary Figure 1C). Importantly, DFO failed to antagonize the pro-apoptotic activity of both drugs, indicating that LMP is irrelevant in apoptotic signaling triggered by cisplatin or related compounds (Supplementary Figure 1C).

LMP is not involved in apoptosis induced by ectopic Noxa expression

As our studies have shown that stabilizing lysosomal membranes by DFO prevents Noxa-dependent apoptotic signaling triggered by H₂O₂ (Figure 6), it is possible that DFO directly antagonizes the pro-apoptotic activities of Noxa. To explore this possibility, we examined whether DFO could affect the pro-apoptotic activities of ectopically expressed Noxa. As Noxa expression displays much higher killing activities in MEFs deficient in Bcl-x_L

expression (Bcl-x-KO) compared with wild-type cells (27), both Bcl-x-KO and wild-type MEFs were pre-incubated with or without DFO before infection with retrovirus expressing Noxa or the empty vector control. Unlike its activity on H₂O₂-induced apoptosis, DFO failed to prevent apoptosis induced by ectopic Noxa expression in both wild-type and Bcl-x-KO MEFs (Supplementary Figure 2A). Caspase 3/7 activity measurements further validated the inability of DFO to antagonize the pro-apoptotic activities of ectopic Noxa expression (Supplementary Figure 2B), indicating that DFO inhibits H₂O₂-induced apoptosis at a step upstream of Noxa expression.

Exogenous Iron specifically enhances Noxa expression

As lysosomal labile iron translocates into the nucleus and causes oxidative damage to DNA during oxidative stress-induced apoptosis (Figure 3), it is possible that redox active lysosomal iron released into the cytosol might be responsible for LMP-induced Noxa expression. To examine this possibility, the effects of exogenous iron on changes in global mRNA expression were determined by microarray experiments performed in wild-type MEFs treated with ferric ammonium citrate. Upon iron exposure, the most prominent increase in gene expression in the BH3-only Bcl-2 protein family was Noxa, which was similar to the expression profile of H₂O₂ exposure (Figure 7A). DFO was able to prevent iron-induced increase in Noxa expression but had little effect on the mRNA expression levels of other BH3-only Bcl-2 proteins. qPCR analyses further confirmed that exogenous iron specifically induced Noxa mRNA expression (Figure 7B).

DISCUSSION

Oxidative stress-triggered LMP is well documented, but the detailed mechanisms through which LMP is coupled with MOMP and eventual apoptosis are still not completely understood. Here we investigated the role of lysosomal membrane rupture in regulating apoptosis caused by the oxidative stress inducer H₂O₂. Upon H₂O₂ exposure, the integrity of lysosomal membranes was disrupted independently of endogenous Noxa, despite its essential role in apoptosis signaling. Noxa-dependent MOMP appeared to occur downstream of LMP. Stabilizing lysosomal membranes specifically inhibited oxidative DNA damage, p53-dependent Noxa expression increase and Noxa-mediated apoptosis in cells under oxidative stress. Overall, our studies provide evidence that LMP modulates cellular responses to oxidative stress by regulating Noxa expression.

The mechanisms of apoptosis signaling induced by LMP have been under intensive study. It is generally believed that the rupture of lysosome membranes releases hydrolytic enzymes normally resided inside lysosomes into the cytosol, which may in turn trigger apoptotic signaling by hydrolyzing their substrates (21). However, severe lysosomal membrane damage leads to a massive exodus of lysosomal enzymes into the cytosol, indiscriminately digesting cellular components, causing deleterious cytoplasmic acidification, and ultimately inducing necrosis instead of apoptosis. The concentration of H₂O₂ examined in our studies induced maximal apoptotic cell death but minimal necrosis in MEFs (27). In this oxidative stress paradigm, the induction of MOMP and subsequent apoptosis depended on Noxa expression (Figures 2 and 6). On the contrary, both early and late lysosomal membrane rupture occurred at same rates in wild-type and Noxa-KO MEFs (Figure 1), suggesting that the hydrolytic activities of discharged lysosomal enzymes are unlikely to be responsible for initiating more prominent apoptotic cascades in Noxa-expressing cells. It is conceivable that partial lysosomal damage under the conditions in our studies only promotes limited release of lysosomal enzymes whose concentrations in the cytosol are not high enough to trigger apoptosis by themselves. Instead, the induction of Noxa expression by LMP is responsible for oxidative stress-induced apoptosis in MEFs. Thus, the strength of oxidative insults and

the cell type could determine how apoptotic signaling is activated in response to oxidative insults.

Recent studies have demonstrated that both anti- and pro-apoptotic Bcl-2 proteins are involved in apoptotic signaling initiated by LMP. Overexpressing anti-apoptotic Bcl-2 in murine histiocytic lymphoma cells suppresses oxidant-induced apoptosis by stabilizing lysosomal membranes and the phosphorylation of Bcl-2 is required for this activity (13, 33). In multiple apoptosis paradigms, Bax has been shown to translocate to lysosomes to mediate LMP, probably by oligomerizing and forming permeation channels on lysosomal membranes (34, 35). Upon TNF α treatment, tBid is generated by caspase 8 and induces Bid-dependent lysosomal membrane rupture, leading to discharge of the lysosomal protease cathepsin B into the cytosol and eventual apoptosis (36, 37). Given that Noxa expression is dispensable for LMP induction and DNA damage in response to oxidative stress, Noxa likely functions at the mitochondrial level to mediate apoptosis, which is supported by our evidence about the temporal relation of MOMP and LMP in H₂O₂-treated cells (Figures 1 and 2).

Originally identified as a primary p53-response protein, Noxa expression has been shown to increase in response to diverse apoptotic stimuli, including some chemotherapeutic agents (31). For example, the chemotherapeutic drug cisplatin has been shown to generate reactive oxygen species (ROS), which is responsible for Noxa expression upregulation (38). However, the underlying mechanisms of Noxa induction in cisplatin-mediated apoptosis paradigm and the apoptotic signaling cascade described in this study are different as LMP was critical for Noxa expression induction and subsequent apoptosis induced by H₂O₂ but not cisplatin (Figures 4 and 6). p53 is normally kept at low levels and its activities are enhanced in response to DNA damage through several mechanisms, including transcriptional upregulation and post-translational modification (39). Although it is generally believed that post-translational modification are largely responsible for the increase in p53 activities, transcriptional induction of p53 has been demonstrated in glial cells treated with H₂O₂ (40), which is in agreement with the findings in this paper (Figure 5). In primary fibroblasts undergoing oxidative stress, increased Noxa expression could be at least partially attributed to elevated p53 mRNA level. Whether post-translational modification of p53, such as phosphorylation and acetylation, contributes to Noxa expression during oxidative stress warrants further studies.

Lysosomes contain abundant labile iron, and the rupture of lysosomal membranes leads to redox-active iron translocation to the nucleus during oxidative stress (41, 42). We reasoned that lysosomal redox-active iron might be responsible for subsequent transcriptional responses to oxidative responses, particularly pro-apoptotic Bcl-2 proteins. Indeed, the transcription profile of BH3-only Bcl-2 proteins in cells loaded with exogenous iron recapitulated that of cells undergoing oxidative stress (Figure 7), suggesting that LMP-triggered apoptosis signaling is transduced into the nucleus to induce Noxa transcription via released lysosomal redox-active iron. In support of this model, chelating lysosomal redox-active iron prevents oxidative damage to DNA (Figure 3). In summary, our studies reveal a novel mechanism by which lysosomal membrane stability regulates H₂O₂-induced apoptosis through a Noxa-dependent mechanism (Figure 8).

MATERIALS AND METHODS

Reagents

Desferrioxamine (DFO), etoposide, doxorubicin, actinomycin D, hydrogen peroxide (H₂O₂), acridine orange (AO), and ferric ammonium citrate were purchased from Sigma (St. Louis, MO). Staurosporine, cisplatin, and carboplatin were purchased from Enzo (Farmingdale,

NY). Propidium iodide (PI), MitoTracker Green, MitoTracker Red, and puromycin were obtained from Invitrogen (Carlsbad, CA). Unless otherwise stated, all reagents were dissolved in dimethyl sulfoxide (DMSO). Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, trypsin, and L-glutamine were obtained from Mediatech (Manassas, VA), and fetal bovine serum (FBS) was purchased from Gemini (Broderick, CA). The jetPRIME[®] transfection reagent was purchased from Polyplus-transfection (New York, NY). The SensoLyte[®] Homogeneous Rh110 caspase 3/7 assay kit was purchased from AnaSpec (San Jose, CA). Antibodies (Abs) used for western blot analysis were anti- β -actin mAb (Sigma), anti-cytochrome c mAb (Santa Cruz; Santa Cruz, CA), anti-TOM20 pAb (Santa Cruz), anti-Noxa pAb (Imgenex; San Diego, CA), peroxidase-conjugated goat anti-rabbit IgG (Thermo; Waltham, MA) and peroxidase-conjugated goat anti-mouse IgG (Thermo).

Cell lines and cell culture

Immortalized mouse embryonic fibroblasts (MEFs) deficient in the expression of the anti-apoptotic Bcl-2 protein Bcl-x_L (Bcl-x-KO) and their wild-type counterparts were generated as described previously (43). Noxa-KO MEFs and their wild-type counterparts immortalized by 3T3 protocol were provided by Professor Andreas Strasser (Walter and Eliza Hall Institute of Medical Research, Parkville, VIC Australia). All immortalized MEFs were grown and maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Primary MEFs were acquired and cultured in DMEM containing 10% FBS, 100 units/ml penicillin, 100 μ g/ml of streptomycin, 2 mM L-glutamine as described previously (44). The bulk populations of the human colon cancer cell HCT116 stably expressing Noxa shRNA or the control luciferase shRNA were provided by Dr. Onno Kranenberg (University Medical Center Utrecht; Utrecht, Netherlands). The limited dilution method was used to obtain single clones, and Noxa expression was examined by western blot. The HCT116 cells were grown and maintained in DMEM supplemented with 5% FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine and 0.5 μ g/ml puromycin. Cells were all cultured in a 5% CO₂ humidified incubator at 37°C.

Plasmid construction and retrovirus production

Murine Noxa cDNA was subcloned into the retroviral expression vector pBABE-IRES-EGFP with the marker protein enhanced green fluorescence protein (EGFP) expressed from an internal ribosomal entry site (IRES) as described previously (27). For retrovirus production, the human embryonic kidney (HEK)-293T cells were transfected with the plasmid pBABE-mNoxa-IRES-EGFP or the empty vector along with the helper plasmids pUVMC and pMDG2.0 using the jetPRIME[®] transfection reagent. Retroviral supernatant was collected 48–72 hours after transfection and supplemented with 10 g/ml polybrene to increase infection efficiency. Retrovirus expressing murine Noxa or GFP-only was used to infect MEFs as described previously (43).

Cell viability

The propidium iodide (PI) exclusion method was used to determine cell viability. Cells were plated in 48-well tissue culture plates at the density of 1×10^4 cells per well 24 hours before exposure to death stimuli. Cells were pre-incubated with or without 1 mM of the high-affinity iron chelator DFO for two hours. Cells were then washed twice with fresh medium before exposure to the respective death stimuli. At the indicated time, cells were collected in the presence of 1 g/ml PI and the percentage of live cells was measured using a flow cytometry analysis (FACScalibur, Beckon Dickinson; San Jose, CA) as described previously (45). Cell viability of treated cells was calculated as the percentage of the viability of the untreated cells.

Caspase 3/7 activity

5×10^3 MEFs were cultured in white-walled 96-well tissue culture plates for 24 hours. MEFs were pre-incubated with or without 1 mM DFO for two hours, washed twice with fresh medium before an exposure to the respective death stimuli. MEFs were treated with H_2O_2 or actinomycin D for 7 hours, exposed to etoposide, staurosporine, or doxorubicin for 12 hours, or infected with the retroviral supernatant expressing Noxa for 14 hours. Caspase 3/7 activity was determined using the Sensolyte[®] Homogeneous R110 Caspase 3/7 Assay Kit (AnaSpec) according to the manufacturer's protocol. Caspase 3/7 activity of treated or infected cells was normalized to that of untreated controls. The results were presented as relative fluorescent units per minute (RFUs/min) as described previously (46).

Western blot analysis

Equal amounts of proteins (30 μ g) were separated on a 4–12% Bis-Tris gel (Bio-Rad; Hercules, CA) and transferred onto PVDF membrane (Millipore; Billerica, MA). The membrane was incubated with appropriate primary or secondary antibodies either overnight at 4°C or at room temperature for 3 hours in 1X phosphate-buffered saline (PBS) containing 5% (w/v) nonfat dry milk (Bio-Rad) and 0.2% (v/v) Tween 20. Protein levels were detected using the enhanced chemiluminescent detection system (Pierce; Rockford, IL) as described previously (45).

Detection of cytochrome c release

At the indicated time points following H_2O_2 treatment, MEF cells were collected and rinsed with 1X PBS, and resuspended in MS buffer (5 mM Tris-HCl, pH7.5, 210 mM mannitol, 70 mM sucrose, and 1 mM EDTA) containing 0.5 g/l BSA and protease inhibitors (Complete, Roche Diagnostics, Indianapolis, IN). The cells were broken open by a homogenizer dounce. The whole cell lysate was centrifuged at $800 \times g$ using a centrifuge (Sorvall Legend RT, Thermo Scientific) for 10 minutes at 4°C to remove cellular debris and nuclei. The supernatants were centrifuged at 4°C in a TLA 100.2 rotor using a TL-100 tabletop ultracentrifuge (Beckman; Fullerton, CA) at $10,000 \times g$ for 10 minutes and subsequently at $350,000 \times g$ for 30 minutes at 4°C to obtain the cytosolic supernatant fractions. Equivalent amounts of cytosolic fractions (6 g) were loaded on a 4–12% Bis-Tris gel (Bio-Rad). The presence of cytochrome c, TOM20, and actin was detected by western blot.

Microarray analysis

Primary MEF cells and immortalized MEF cells were pre-incubated with or without 1 mM DFO for 2 hours, washed twice with fresh medium before a treatment with either 0.4 mM H_2O_2 for 6 hours or 0.3 mM ferric ammonium citrate for 24 hours. Total RNA was acquired using the RNeasy kit (Qiagen; Germantown, MD) according to the manufacturer's instructions. RNA concentrations were determined with a NanoDrop 8000 Spectrophotometer (Thermo), and the quality of total RNA was evaluated by a 2100 Bioanalyzer (Agilent; Santa Clara, CA). Global mRNA level alterations were determined using the GeneChip Mouse Gene 1.0 ST Array for the experiments described in Figure 4 or GeneChip Mouse Genome 430 2.0 Array for the experiments described in Figures 5 and 7 (Affymetrix; Santa Clara, CA) as described previously (27, 43).

Quantitative real time Polymerase Chain Reaction (qPCR)

Total RNA was extracted using the RNeasy kit (Qiagen) and its concentration was measured using a NanoDrop 8000 Spectrophotometer (Thermo). Equal amounts (1 g) of total RNA were reverse transcribed as described previously (27). The TaqMan 20 $\times$ probes were used to determine mouse Noxa and p53 mRNA expression levels in MEFs and human Noxa mRNA levels (Applied Biosystems; Carlsbad, California) in HCT116 cells. In MEFs tubulin was

used as an endogenous control, whereas actin served as an endogenous control in HCT116 cells.

Lysosomal membrane stability

Two complementary experiments using the lysosomotropic base acridine orange (AO) were carried out to measure lysosomal membrane stability by assessing changes in either green fluorescence (AO relocation method) or red fluorescence (AO uptake method) upon an exposure to H₂O₂ (Zhao et al., 2000). In both cases, wild-type and Noxa-KO MEFs were plated in 48-well tissue culture plates at a density of 2×10^4 per well and cultured for 24 hours. For AO relocation experiments, the increase in green fluorescence was evaluated to detect early or minor changes in lysosomal membrane stability. MEFs were pre-incubated with or without 1 mM DFO for 2 hours, washed twice with fresh medium, before an exposure to 5 g/ml AO for 30 minutes. MEFs were then treated with 0.4 mM H₂O₂ for the indicated time, and changes in green fluorescence were determined by flow cytometry (FACScalibur, FL1 channel). AO uptake experiments measured changes in red fluorescence to detect severe or late lysosomal membrane damage. Here, MEFs were pre-treated with or without 1 mM DFO for 2 hours, rinsed twice with fresh medium, followed by an exposure to 0.4 mM H₂O₂ for one hour. MEFs were then returned to normal culture conditions by washing and replacing with fresh medium. At the indicated time, cells were treated with 5 g/ml AO for 15 minutes, and flow cytometry analysis was used to measure changes in red fluorescence (FACScalibur, FL3 channel).

Mitochondrial membrane potential

The mitochondrion-selective probes MitoTracker Green and MitoTracker Red were used to determine changes in mitochondrial membrane potential. Wild-type and Noxa-KO MEFs were plated in 48-well tissue culture plates at a density of 2×10^4 and cultured for 24 hours. Following a treatment with 0.4 mM H₂O₂ for the indicated time, MEFs were then incubated with 200 nM MitoTracker Green or 200 nM MitoTracker Red for 1 hour. Green and red fluorescence of collected MEFs were analyzed using a flow cytometer (FACScalibur). Mitochondrial membrane potential was shown as the ratio of fluorescence intensity of mitochondrial potential-sensitive MitoTracker Red (FL3 channel) and mitochondrial potential-independent MitoTracker Green (FL1 channel).

DNA damage analysis (comet assay)

1×10^6 wild-type and Noxa-KO MEFs were plated in 6-well tissue culture plates for 24 hours. MEFs were pre-incubated with or without 1mM DFO for 2 hours, washed twice with fresh medium before an exposure to 0.4 mM H₂O₂ for 1 hour. MEFs were washed in ice-cold Ca²⁺/Mg²⁺-free 1X PBS and 2×10^5 cells were mixed with 100 l of pre-warmed low melting agarose (1:10, v/v) and plated onto a slide (cometslide™, Trevigen; Gaithersburg, MD). After agarose solidified and attached to the slides, the slides were immersed in pre-chilled Lysis solution (Trevigen) for 1 hour on ice, then in alkaline unwinding solution (300 mM NaOH, 1 mM EDTA) for 1 hour at room temperature. Electrophoresis was performed in pre-chilled alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA) at 4°C for 45 minutes at 1 volt/cm. The slides were then washed twice with distilled H₂O for 5 minute each time, followed by a five minute-incubation with 70% ethanol. The slides were air-dried at 40°C for 15 minutes in the dark and the agarose gels were stained with SYBR Green (Trevigen) for 5 minutes at 4°C. After SYBR Green was removed and slides were air-dried, images were acquired using a TE300 epifluorescence microscope (Nikon; Melville, NY) with a CoolSNAP digital camera (Photometrics; Tucson, AZ) and the imaging software MetaMorph (Molecular Devices; Sunnyvale, CA). The levels of DNA damage were determined using CometScore software (TriTek, Sumerduck, VA).

Statistical analysis

All experiments were performed in triplicate at least three times. Results are presented as mean $\pm$ standard deviation. Statistical analysis was performed using Student's two tail t-test. A P value < 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS LIST

MEFs	mouse embryonic fibroblasts
H₂O₂	hydrogen peroxide
DFO	desferrioxamine
MOMP	mitochondrial outer membrane permeabilization
LMP	lysosomal membrane permeabilization
AO	acridine orange

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Highlights

H₂O₂ induced lysosomal membrane permeabilization (LMP) independently of Noxa.

Noxa-dependent cytochrome c release occurred downstream of LMP.

Preventing LMP blocked oxidative DNA damage and p53-dependent Noxa induction.

Stabilizing lysosomal membranes inhibited Noxa-mediated apoptosis.

Figure 1. H_2O_2 induced Noxa-independent LMP

(A) Wild-type and Noxa-KO MEFs were pre-incubated with or without 1 mM DFO for 2 hours, followed by an exposure to 5 g/ml acridine orange (AO) for 15 minutes. MEFs were then treated with 0.4 mM H_2O_2 for the indicated time. Green fluorescence emitted from AO inside cells was determined using flow cytometry. Data represent mean $\pm$ standard deviation of three independent experiments performed in triplicates. (B) Following pre-incubation with or without 1 mM DFO for 2 hours, wild-type and Noxa-KO MEFs were exposed to 0.4 mM H_2O_2 for 1 hour. MEFs were then cultured in normal growth medium for 24 hours. MEFs deficient in intact lysosomes ('pale' cells) were analyzed by flow cytometry. (C) The

summary data of AO uptake experiments. The data are shown as mean $\pm$ standard deviation of three independent experiments performed in triplicates.

Figure 2. Mitochondrial membrane permeabilization depends on Noxa expression

(A) Wild-type and Noxa-KO MEF cells were treated with 0.4 mM H₂O₂. The amounts of cytochrome c and TOM20 in cytosolic fractions at the indicated time points were detected by western blot. (B) Following an exposure with 0.4 mM H₂O₂ for the indicated time, wild-type and Noxa-KO MEFs were incubated with 200 nM Mitotracker Red or 200 nM Mitotracker Green for 1 hour. Flow cytometer was used to measure red and green fluorescence intensity of MEFs. Mitochondrial membrane potential was calculated as the ratio of red to green fluorescence intensities and normalized. Experiments were performed in triplicates and mean $\pm$ standard deviation of three independent experiments is shown. (C)

Wild-type and Noxa-KO MEFs were pre-incubated with or without 1 mM DFO for two hours before a treatment with 0.4 mM H₂O₂ for 24 hours. Changes in red and green fluorescence intensities were determined and mitochondrial membrane potential was calculated as shown in (A). Data are presented as mean $\pm$ standard deviation of three independent experiments performed in triplicates.

Figure 3. Stabilizing lysosomal membranes inhibits H₂O₂-induced DNA damage

(A) Wild-type and Noxa-KO MEFs were first pre-incubated with or without 1 mM DFO for 2 hours before being exposed to initially 0.4 mM H₂O₂ for 1 hour. The alkaline comet assay was carried out to assess DNA damage induced by H₂O₂. Representative images of cells under the indicated conditions are shown. Scale bar, 100 m. (B) The tail moments of the indicated MEF cells were measured and the mean values of tail moments of 50 randomly selected cells were calculated. The data show mean ± standard deviation of three independent experiments.

A

Gene Description	Fold changes in mRNA expression (MEFs)			
	Control	DFO-only	H ₂ O ₂ -only	DFO/H ₂ O ₂
Phorbol-12-myristate-13-acetate-induced protein 1(Pmaip1, Noxa)	1.00	1.02	16.96	6.55
Bcl2-interacting killer(Bik)	1.00	1.08	1.19	0.68
Bcl-2-like 11 (Bim)	1.00	1.18	2.20	1.25
BH3 interacting death agonist (Bid)	1.00	0.97	1.28	1.03
Bcl-2 modifying factor (Bmf)	1.00	1.14	1.16	0.99
Harakiri, Bcl2 interacting protein (Hrk)	1.00	1.19	1.14	1.12
Bcl-2 binding component 3 (Bbc3, Puma)	1.00	0.61	0.77	0.75
Bcl-2/adenovirus E1B interacting protein 3-like (Bnip3l, Nix)	1.00	0.99	1.02	0.95
Bcl-2 associated agonist of cell death (Bad)	1.00	0.83	1.03	1.04
Bcl-2/adenovirus E1B interacting protein 3 (Bnip3)	1.00	1.06	0.77	0.75

B

C

Figure 4. H₂O₂-induced increase in Noxa expression is specifically mediated by LMP

(A) Wild-type MEFs were pre-incubated with or without DFO for 2 hours, then treated with 0.4 mM H₂O₂ for 6 hours. Total RNA was extracted and microarray analysis was carried out to determine global changes in mRNA expression levels upon H₂O₂ treatment. (B) A qPCR analysis was performed using the total RNA generated in (A) to validate the results generated by microarray analysis. Data are presented as mean $\pm$ standard deviation of three independent experiments performed in triplicates. (C) DFO prevented Noxa expression increase induced by H₂O₂ but not cisplatin. After pre-incubation with or without DFO for 2 hours, HCT116 cells (shLuc) were treated with either 0.4 mM H₂O₂ for 6 hours or 50 M

cisplatin for 14 hours. Total RNA was collected and a qPCR analysis was performed to determine Noxa mRNA levels. Mean $\pm$ standard deviation of three independent experiments is presented. Asterisks indicate $P < 0.01$ (**) or $P < 0.001$ (***), Student's unpaired t test. ns, no significance.

A

Gene Description	Fold changes in mRNA expression (primary MEFs)					
	Wild-type		p53 ^{-/-} #1		p53 ^{-/-} #2	
	0 hr	6 hr	0 hr	6 hr	0 hr	6 hr
Phorbol-12-myristate-13-acetate-induced protein 1 (Pmaip1, Noxa)	1.00	7.20	1.00	1.41	1.00	1.07
Bcl2-interacting killer (Bik)	1.00	1.01	1.00	1.11	1.00	0.93
Bcl-2-like 11 (Bim)	1.00	1.26	1.00	1.50	1.00	1.61
BH3 interacting death agonist (Bid)	1.00	1.45	1.00	1.14	1.00	1.01
Bcl-2 modifying factor (Bmf)	1.00	1.01	1.00	1.06	1.00	1.08
Harakiri, Bcl2 interacting protein (Hrk)	1.00	1.02	1.00	0.97	1.00	0.92
Bcl-2 binding component 3 (Bbc3, Puma)	1.00	1.33	1.00	1.04	1.00	1.28
Bcl-2/adenovirus E1B interacting protein 3-like (Bnip3l, Nix)	1.00	0.86	1.00	1.11	1.00	1.04
Bcl-2 associated agonist of cell death (Bad)	1.00	0.97	1.00	1.07	1.00	1.10
Bcl-2/adenovirus E1B interacting protein 3 (Bnip3)	1.00	0.79	1.00	1.11	1.00	0.94
Transformation related protein 53 (Trp53)	1.00	2.09	N/A	N/A	N/A	N/A

B **C** **Figure 5. H₂O₂-induced Noxa expression increase depends on p53**

Primary fibroblasts isolated from one wild-type mouse embryo and two p53-deficient mouse embryos were treated with 0.4 mM H₂O₂ for 6 hours, and total RNA was extracted. **(A)** Microarray analysis was performed to determine global mRNA levels. Changes in mRNA expression levels of ten pro-apoptotic BH3-only Bcl-2 proteins and p53 are shown. **(B)** A qPCR analysis was carried out to evaluate the expression levels of Noxa mRNA of the indicated primary MEFs. Mean $\pm$ standard deviation of three independent experiments is presented. **(C)** p53 mRNA expression levels of wild-type primary MEF cells with or without H₂O₂ exposure were determined by qPCR experiments. Data are presented as mean $\pm$ standard deviation of three independent experiments. Asterisks indicate $P < 0.01$ (**) or $P < 0.001$ (***), ns, no significance, Student's unpaired t test.

Figure 6. Stabilizing lysosomal membranes by DFO inhibits Noxa-dependent apoptosis induced by H₂O₂

(A) Wild-type and Noxa-KO MEFs were pre-incubated with or without 1 mM DFO for 2 hours. MEFs were then treated with 0.4 mM H₂O₂ for the indicated time, and cell viability was determined using a flow cytometry analysis. Data are presented as mean $\pm$ standard deviation of three different experiments that were carried out in triplicates. (B) Caspase 3/7 activity was measured after 2 hour-incubation with or without DFO followed by a treatment with 0.4 mM H₂O₂ for 6 hours. Data shown are mean $\pm$ standard deviation of three independent experiments performed in triplicates. (C) Noxa expression was reduced in HCT116 cells by a small hairpin RNA (shRNA). The expression levels of Noxa were

determined by western blot. **(D)** The HCT116 cells with normal (shLuc) or reduced (shNoxa) Noxa expression were incubated with or without DFO for 2 hours before an exposure with 0.4 mM H₂O₂ for the indicated time, and cell viability was measured. Mean ± standard deviation of three independent experiments is presented.

A

Gene Description	Fold changes in mRNA expression (MEFs)			
	Control	DFO-only	Fe ³⁺ -only	DFO/Fe ³⁺
Phorbol-12-myristate-13-acetate-induced protein 1(Pmaip1, Noxa)	1.00	1.10	1.78	1.27
Bcl2-interacting killer(Bik)	1.00	0.83	1.01	0.99
Bcl-2-like 11 (Bim)	1.00	0.98	0.79	0.95
BH3 interacting death agonist (Bid)	1.00	0.99	0.93	0.98
Bcl-2 modifying factor (Bmf)	1.00	0.99	0.99	1.02
Harakiri, Bcl2 interacting protein (Hrk)	1.00	0.87	0.93	0.91
Bcl-2 binding component 3 (Bbc3, Puma)	1.00	1.09	1.13	0.94
Bcl-2/adenovirus E1B interacting protein 3-like (Bnip3l, Nix)	1.00	0.98	0.97	1.96
Bcl-2 associated agonist of cell death (Bad)	1.00	0.95	1.08	1.02
Bcl-2/adenovirus E1B interacting protein 3 (Bnip3)	1.00	1.09	0.93	1.04

B

Figure 7. Iron overload specifically increases Noxa expression

(A) Wild-type MEFs were pre-incubated with DFO before a treatment with 0.3 mM iron ammonium citrate for 24 hours. Total RNA was extracted and used for microarray experiments to determine changes in mRNA expression levels. (B) Total RNA described in (A) was used for a qPCR analysis to determine Noxa mRNA expression levels. Mean $\pm$ standard deviation of three independent experiments is presented. Asterisk indicates $P < 0.05$ (*), Student's unpaired t test.

Figure 8. Oxidative stress-induced LMP promotes Noxa-dependent apoptosis

In lysosomes, the oxidative stress inducer H_2O_2 interacts with intralysosomal iron to generate highly reactive hydroxyl radicals and induce subsequent LMP, which can be inhibited by the iron-chelating agent DFO. LMP leads to an increase in oxidative DNA damage and p53-dependent Noxa expression, which in turn induces MOMP and eventual activation of apoptosis signaling.