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Original Contribution

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 H_2O_2. 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, H_2O_2-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 H_2O_2 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.

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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) 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 proapoptotic or antiapoptotic [5–7]. Proapoptotic Bcl-2 proteins promote apoptosis by increasing MOMP, whereas antiapoptotic 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 aparatus, 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, on 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 lysosomal membrane permeabilization (LMP) through which lysosomal hydrolytic enzymes are released into the cytosol [17]. The level of damage to the lysosome determines the fate of the

Abbreviations: MEFs, mouse embryonic fibroblasts; H_2O_2, hydrogen peroxide; DFO, desferrioxamine; MOMP, mitochondrial outer membrane permeabilization; LMP, lysosomal membrane permeabilization; AO, acridine orange

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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 (TNFα)-treated cells, cathepsin B, D, and L released into the cytosol trigger apoptosis by converting the inactive proapoptotic 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 (H_2O_2), 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. H_2O_2 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), H_2O_2 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 H_2O_2-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 H_2O_2 causes lysosomal membrane permeabilization and subsequent apoptosis [24–26]. Our previous studies have shown that the elevated expression of the proapoptotic BH3-only Bcl-2 protein Noxa mediates apoptosis triggered by H_2O_2 [27]. To explore the involvement of Noxa in H_2O_2-induced LMP, we first examined lysosomal membrane stability in mouse embryonic fibroblasts 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 H_2O_2 exposure. In AO relocation experiments, cells were first incubated with AO before an exposure to H_2O_2. Thirty minutes post H_2O_2 exposure, there was a significant increase in green fluorescence levels in wild-type and Noxa-KO MEFs, indicating minor damage to lysosomal membrane integrity (Fig. 1A). Importantly, the green fluorescence intensity increase was similar in both cell lines, providing evidence that Noxa expression did not influence H_2O_2-induced LMP despite its critical role in mediating apoptotic signaling pathways. Furthermore, preincubation of cells with the highly potent iron chelator desferrioxamine markedly reduced LMP independent of Noxa expression (Fig. 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 (Fig. 1B). Furthermore, the increase in late lysosomal membrane rupture was abolished by preincubation with DFO. Consistent with the results of AO relocation experiments (Fig. 1A), H_2O_2 was able to induce comparable late lysosomal membrane rupture in wild-type and Noxa-KO MEFs (Fig. 1C). Overall, these results indicate that H_2O_2 induces LMP independent of Noxa expression, suggesting that Noxa mediates H_2O_2-triggered apoptosis downstream of the destruction of lysosomal membrane integrity.

Noxa-dependent MOMP occurs downstream of LMP during H_2O_2-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 H_2O_2-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 H_2O_2, and the presence of cytochrome *c* in cytosolic fractions was determined by Western blot (Fig. 2A). The levels of cytochrome *c* in cytosolic fractions of Noxa-KO MEF cells were largely unchanged on 24-h H_2O_2 exposure (Fig. 2A), indicating very little MOMP. However, the amounts of cytochrome *c* were notably higher in cytosolic fractions of wild-type cells after 14-h H_2O_2 treatment. As LMP was observed 30 min following H_2O_2 treatment in both wild-type and Noxa-KO MEF cells (Fig. 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 H_2O_2 using the mitochondrial potential-sensitive probe MitoTracker Red and the mitochondrial potential-independent probe MitoTracker Green. An exposure to H_2O_2 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 (Fig. 2B). In contrast to the occurrence of LMP 30 min following H_2O_2 treatment (Fig. 1A), no changes in mitochondrial membrane potential were detected in either MEF cell line within 1 h of H_2O_2 exposure. After 6 h of H_2O_2 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 H_2O_2, 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 preincubation with DFO significantly inhibited H_2O_2-induced mitochondrial membrane potential reduction (Fig. 2C). Therefore, it is likely that mitochondrial membrane rupture occurs downstream of LMP in cells undergoing oxidative stress.

Stabilizing lysosomal membranes prevents H_2O_2-induced DNA damage

Previous studies have shown that redox-active iron released from lysosomes during LMP is capable of inducing oxidative

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