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Ubiquitin-dependent lysosomal degradation of the HNE-modified proteins in lens epithelial cells

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SPECIFIC AIMS

Oxidative stress causes various forms of damage, including protein oxidation, DNA breaks, and lipid peroxidation, to most types of cells. 4-Hydroxynonenal (HNE), generated by peroxidation of polyunsaturated fatty acids, is the most reactive and cytotoxic product of lipid peroxidation. Lysine, histidine, and cysteine residues in proteins and nucleophilic groups in DNA are all potential targets for HNE. HNE-modified proteins have been identified in animal and human tissues under certain pathological conditions, suggesting an involvement of HNE modification in the pathophysiology of degenerative diseases and cellular aging. HNE modification may alter protein structures and functions, and accumulation of such modified proteins is thought to be cytotoxic. Thus, efficient removal of HNE-modified proteins is critical for the survival of cells or organisms. It is known that selective degradation of modified or damaged proteins by intracellular proteolytic enzymes is an important protein quality control mechanism. However, the proteolytic pathways that degrade the HNE-modified proteins remain to be defined. The majority of intracellular proteins are degraded by either the lysosomal or the ubiquitin-proteasome pathway. The lysosome is responsible for the degradation of membrane or extracellular proteins that enter cells by endocytosis. The ubiquitin-proteasome pathway is a major proteolytic pathway in eukaryotic cells and is responsible for conditional degradation of intracellular short-lived regulatory proteins or abnormal cytosolic and nuclear proteins. In most cases, poly-ubiquitination targets proteins for degradation by the 26S proteasome and mono-ubiquitination facilitate the internalization and degradation of membrane proteins by the endosome-lysosome pathway.

PRINCIPAL RESULTS

We evaluated the role of the proteasome and lysosome in degradation of HNE-modified proteins using α-crystallins as model substrates. αA and αB-crystallins could both be modified by HNE, but αB-crystallin was ~9-fold more susceptible than αA-crystallin to modification by HNE. In ATP and Ubc4-supplemented proteasome-free fraction II, untreated and HNE-modified αB-crystallins were mono-ubiquitinated, but levels of ubiquitinated HNE-modified αB-crystallin were ~2-fold greater than those for untreated αB-crystallin (**Fig. 1*A***, compare lane 2 with lane 1 and lane 6 with lane 5), indicating that HNE-modified αB-crystallin is a preferred substrate for ubiquitination. However, in a cell-free system ATP- and proteasome-dependent degradation rates of untreated αB-crystallin and HNE-modified αB-crystallin were comparable (Fig. 1*B*).

The increased susceptibility of HNE-modified proteins to being ubiquitinated without significant alteration in their susceptibility to degradation by the proteasome in the cell-free systems suggests that HNE-modified proteins may be degraded by a proteolytic pathway other than the proteasome pathway. To further determine how the HNE-modified proteins were removed from intact cells, ^{125}I-labeled, untreated, or HNE-modified αB-crystallins were delivered into cultured HLEC using the BioPORTER protein delivery reagent. In the absence of protease inhibitors, untreated and HNE-modified αB-crystallins were degraded rapidly, and it appears that HNE-modified αB-crystallin was degraded faster than unmodified αB-crystallin (**Fig. 2**, compare lanes 2 with 1). In the presence of MG132, which inhibits the proteasome and lysosome, untreated and HNE-modified αB-crystallins were stabilized (Fig. 2, lanes 3 and 4). Lactacystin-β-lactone, a specific inhibitor, only partially stabilized untreated αB-crystallin in the cells, but not as much for the HNE-modified αB-crystallin (Fig. 2, compare lanes

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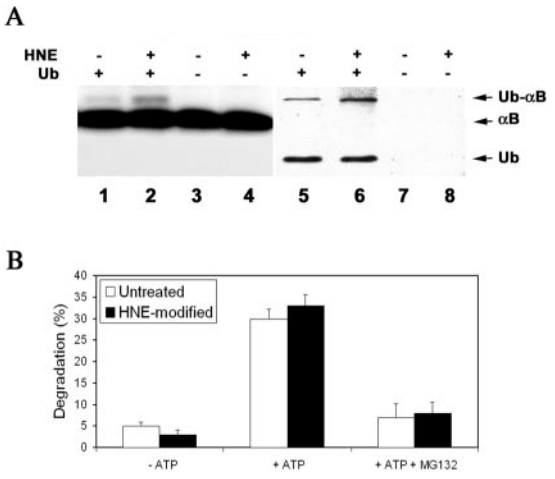

Figure 1. Ubiquitination and degradation of HNE-modified αB-crystallin. ^{125}I-Labeled recombinant αB-crystallin was incubated with or without 100 μM HNE for 2 h at 37°C. After removal of unreacted HNE, the ^{125}I-labeled αB-crystallins were incubated in proteasome-free fraction II prepared from rabbit reticulocyte lysate in the presence or absence of ubiquitin. *A)* Ubiquitination of HNE-modified αB-crystallin: Lanes 1, 3, 5, and 7 are unmodified αB-crystallin and lanes 2, 4, 6, and 8 are HNE-modified αB-crystallins. Lanes 1, 2, 5, and 6 show the formation of ubiquitin conjugates in the presence of ubiquitin; lanes 3, 4, 7, and 8 are the negative control after incubation without ubiquitin. The formation of ubiquitin ^{125}I-labeled αB-crystallin conjugates was assessed by autoradiography after SDS-PAGE and transfer to nitrocellulose membrane (lanes 1–4); the ubiquitin conjugates were confirmed by Western blot with antibody to ubiquitin (lanes 5–8). *B)* Degradation of HNE-modified αB-crystallin. % degradation was calculated from acid-soluble radioactivity recovered in supernatants after 2 h of incubation with reticulocyte lysate at 37°C. Values shown are mean ± SD of 4 independent determinations, each done in duplicate.

5 and 6 with lanes 1 and 2). In the presence of lactacystin-β-lactone, levels of untreated and HNE-modified αB-crystallin in the cells were much higher than those of HNE-modified αB-crystallin (Fig. 2, compare lane 5 with 6). The greater difference in levels between untreated and HNE-modified αB-crystallins is due to stabilization of untreated αB-crystallin by the proteasome inhibitor. The inability of lactacystin-β-lactone, in contrast to MG132, to stabilize HNE-modified αB-crystallin in the cells suggests that HNE-modified αB-crystallin was degraded by the lysosome pathway, since MG132 inhibits not only the proteasome but also cathepsins in the lysosome. Indeed, treatment with chloroquine, a lysosome inhibitor, partially stabilized the HNE-modified αB-crystallins (Fig. 2, lanes 7 and 8), and levels of untreated and HNE-modified αB-crystallins were comparable under these conditions. If lactacystin-β-lactone and chloroquine were added together to the cells, untreated and HNE-modified αB-crystallins were stabilized and levels of crystallins remaining in the cells were similar to those observed in the presence of MG132 (Fig. 2, compare lanes 9 and 10 with lanes 3 and 4). Longer exposure of the film revealed a small fraction (<1%) of ^{125}I-labeled αB-crystallin migrated at a slower rate. Based on molecular weight, the slower migrating moiety appears to be mono-ubiquitinated αB-crystallin (Fig. 2, upper panel). This putative mono-ubiquitinated αB-crystallin was more readily detectable in the presence of MG132 (Fig. 2, upper panel, lanes 3 and 4) or of lactacystin-β-lactone and chloroquine (Fig. 2, lanes 9 and 10). Together, these data indicate that untreated αB-crystallin is a good substrate for the ubiquitin-proteasome pathway but that HNE-modified αB-crystallin is a preferred substrate for the lysosome pathway.

To determine whether ubiquitin-dependent lysosomal degradation of HNE-modified αB-crystallin pertains to other HNE-modified proteins, we examined the effects of proteasome and lysosome inhibitors on the removal of intracellular HNE-modified proteins. After treatment with 5 μM HNE, several cellular proteins formed adducts with HNE. The HNE-modified proteins were enriched particularly in the fraction of ubiquitin-conjugates, indicating that HNE-modified proteins are ubiquitinated in the cells. HNE-modified protein levels decreased rapidly upon removal of HNE from the medium. After 24 h of recovery in HNE-free medium, HNE-modified protein levels returned almost to the levels observed in untreated cells. When the cells were treated with MG132, the HNE-modified proteins were significantly stabilized. However, treatment with proteasome-specific inhibitors could not stabilize the HNE-modified proteins. In contrast, chloroquine inhibited removal of the HNE-modified proteins. These data indicate that the majority of HNE-modified proteins are degraded by the ubiquitin-dependent lysosomal pathway rather than by the proteasome.

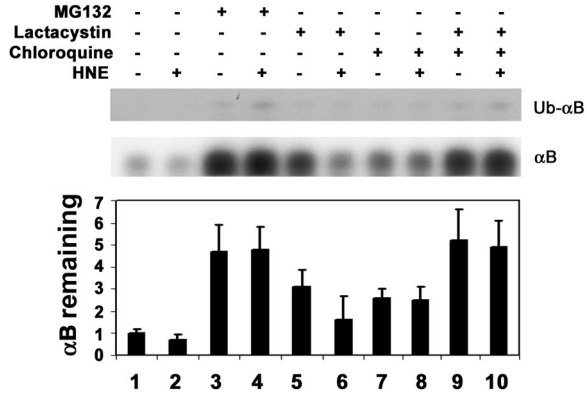

Figure 2. Effects of different protease inhibitors on the stability of HNE-modified αB-crystallin delivered into lens cells. αB-Crystallin was iodinated and modified by HNE. ^{125}I-αB-crystallins were delivered into cultured human lens epithelial cells using the BioPORTER protein delivery reagent. Levels of ^{125}I-αB-crystallins (middle panel) and the putative mono-ubiquitnated ^{125}I-αB-crystallins (upper panel) were determined after incubation in the absence or presence of the indicated inhibitors for 4 h. Autoradiograms were scanned and ^{125}I-αB-crystallin levels in the cells were quantified with a densitometer (lower panel). Data in lower panel represent the mean ± SD of 3 independent experiments.

CONCLUSIONS

We and others have shown that the ubiquitin-proteasome pathway is involved in the removal of many damaged proteins, including oxidized proteins. Data from this study showed that HNE-modified proteins are also ubiquitinated, but are degraded by the lysosome. With previous observations, these results indicate that the ubiquitin-conjugating system may serve as a common mechanism to recognize various types of modified or damaged proteins, although different E2s and E3s may be used to ubiquitinate different types of damaged proteins. The ubiquitinated proteins can be degraded by either the proteasome or lysosome (**Fig. 3**). However, the mechanism for the differential destinations of the ubiquitinated proteins remains elusive. The destination of ubiquitinated proteins could be determined by the nature of the protein substrates or by structures of the ubiquitin conjugates. For example, proteins destined for the proteasome are often poly-ubiquitinated and proteins destined for the lysosome are often mono-ubiquitinated.

Available data regarding the degradation of HNE-modified proteins are limited. One study showed that HNE-modified proteins in homogenates of kidney were degraded by the ubiquitin-proteasome pathway. Other studies showed that HNE-modified proteins were resistant to proteasome-dependent degradation and that in some cases the HNE-modified proteins acted as potent noncompetitive inhibitors of the proteasome. The demonstration that HNE-modified proteins are degraded by the ubiquitin-dependent lysosomal pathway reconciles the apparent differences of opinion as to which proteolytic pathways are responsible for the degradation of HNE-modified proteins.

Selective degradation of damaged proteins is essential for cellular functions. The ubiquitin-conjugating system provides a mechanism with which to distinguish damaged or adversely modified proteins from native proteins. Identification of the enzymes required for the recognition and ubiquitination of various types of modified or damaged proteins will help us understand the molecular mechanisms of the cellular protein quality control system and will provide clues for prevention and/or treatment of diseases related to the accumulation of damaged proteins, such as cataract and other age-related degenerative diseases.

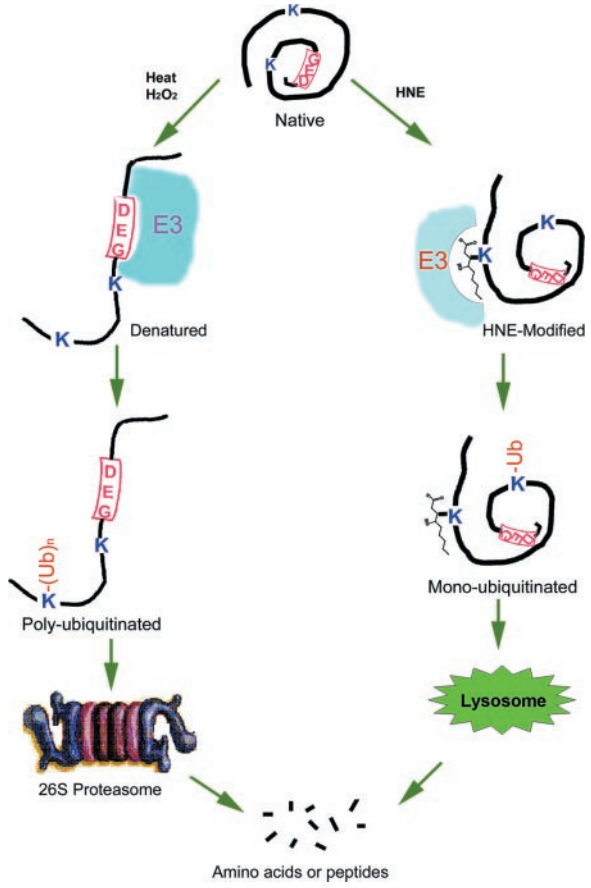

Figure 3. The hypothesis of ubiquitin-dependent protein quality control mechanism. We hypothesize that many proteins have internal signal for ubiquitination, indicated by "DEG" in the diagram. The ubiquitination signal is hidden in the native conformation; therefore, native proteins are not recognized by the ubiquitination machinery. However, various modifications and damage may cause protein unfolding and exposure of ubiquitination signal. Thus, the unfolded proteins will be recognized by a combination of specific E2s and E3s and be ubiquitinated. Specific modification, such HNE, may serve as the signal for ubiquitination. Poly-ubiquitinated proteins will be recruited to the 26S proteasome for degradation and mono-ubiquitinated proteins may enter into the lysosome for degradation.