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Oxidation of ER Resident Proteins Upon Oxidative Stress: Effects of Altering Cellular Redox/Antioxidant Status and Implications for Protein Maturation

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ABSTRACT

Previous work showed that from all cellular proteins, the endoplasmic reticulum (ER) resident proteins are most sensitive to oxidative stress [hydrogen peroxide (H_2O_2)], as determined using the oxidation-sensitive, membrane-permeable, acetylTyrFluo probe. Because of the importance of these proteins in proper cellular functioning, we studied (a) whether modifying the cellular redox state/antioxidant status alters the susceptibility of those proteins toward H_2O_2 oxidative stress and (b) whether H_2O_2 affects ER function with regard to protein folding. The cellular redox and/or antioxidative capacity was modified in several ways. Lowering the capacity increased H_2O_2-induced protein oxidation, and increasing the capacity lowered H_2O_2-induced protein oxidation. The effect of H_2O_2 on ER-related protein maturation was investigated, using the maturation of the low–density lipoprotein receptor as a model. Its maturation was not affected at low concentrations of H_2O_2 ($\leq$400 μ*M*), which do result in oxidation of ER resident proteins. Maturation was slowed down or reversibly inhibited at higher concentrations of H_2O_2 (1.5–2.0 m*M*). These results might be caused by several events, including oxidation of the low-density lipoprotein receptor itself or ER resident proteins resulting in decreased folding (capacity). Alternatively, oxidation of cytosolic proteins involved in ER Golgi transport might attenuate transport and maturation. Clearly, the mechanism(s) responsible for the impairment of maturation need further investigation. *Antioxid. Redox Signal.* 5, 381–387.

INTRODUCTION

CELLS UNDERGOING OXIDATIVE STRESS are characterized by an alteration in the amount of reducing and oxidizing equivalents (redox status); oxidizing equivalents [*i.e.,* glutathione disulfide (GSSG), cystine, $NADP^+$, $thioredoxin_{ox}$] are increased and/or reducing equivalents [*i.e.,* glutathione (GSH), cysteine, NADPH, $thioredoxin_{red}$] are decreased. Oxidative stress results in the formation of GSSG at the expense of GSH (16). This shift in the ratio of GSH/GSSG would change the redox status to a less negative potential. If the potential rises too much, this will hamper proper cell functioning. Redox-sensitive cellular processes, like signal transduction pathways, transcription factor activation, calcium release, phosphorylation of macromolecules, and enzyme activation, can be stimulated or impeded as a result of shifts in redox potential (7, 16). Protein folding in the endoplasmic reticulum (ER) is also redox-dependent, and the ER must maintain a strongly oxidizing environment compared with the highly reduced environment of the cytosol. Ero1p, an ER

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resident protein, is an essential component of a specific cellular redox machinery required for disulfide-linked protein folding in the ER (16).

In a recent article, we addressed the question whether all cellular proteins are equally susceptible toward an external oxidative stress. This was studied by subjecting cells to oxidative stress [hydrogen peroxide (H_2O_2)] in the presence of the membrane-permeable acetylTyrFluo probe. The application of oxidative stress in the presence of the acetylTyrFluo in either *in vivo* (cellular) systems or an *in vitro* system of isolated proteins resulted in a covalent coupling of the probe to the proteins (20, 21). The probe consists of a fluorescein-labeled tyrosine analogue (*i.e.,* tyramine) that upon oxidation by reactive oxygen species is converted into a tyrosyl radical that can form cross-links with oxidized target proteins. As a result of the coupling reaction, these target proteins can be visualized by fluorescence microscopy or detected on a western blot by immunodetection using an anti-fluorescein antibody (20, 21). Application of oxidative stress (H_2O_2) to cells preincubated with acetylTyrFluo resulted in predominant labeling of a specific subclass of cellular proteins: ER resident proteins, specifically protein disulfide isomerase (PDI), binding protein (BiP), GRP94, ER60, and calnexin (21).

This finding prompted us to investigate (a) whether susceptibility toward oxidative stress of these proteins can be modulated by modifying the cellular redox/antioxidant status and (b) whether this affects ER function with regard to protein folding. To answer the first question, the intracellular glutathione concentration was altered by preincubation with GSH or GSH monoethyl ester or with buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl-cysteine synthetase (10), to impair the synthesis of GSH. Inhibition of two major antioxidant enzyme systems was achieved by preincubation with mercaptosuccinate (MS), an inhibitor of glutathione peroxidase (GPx) (3), or aminotriazole (AT), a catalase inhibitor (6). Finally the effect of exogenous antioxidants, vitamins C and E, was studied.

A requirement for proper oxidative protein folding and disulfide bond formation by PDI is the presence of a functional Ero1 protein (15), from which oxidizing equivalents flow to secretory proteins via PDI. As ER resident proteins, including PDI, were the predominant targets of oxidative stress (21), the functionality of ER resident proteins involved in protein folding might be affected upon the oxidative challenge. To investigate whether oxidation of ER resident proteins has an impact on the processing of proteins in the ER, including posttranslational modifications, and sorting in the Golgi complex, we studied the maturation of the low-density lipoprotein receptor (LDLr) as a model (8) under conditions as applied during the acetylTyrFluo experiments and under stronger oxidative stresses.

MATERIALS AND METHODS

Materials

AcetylTyrFluo was synthesized as described before (20, 21). GSH, GSH monoethyl ester, BSO, mercaptosuccinic acid, AT, and Hanks' balanced salt solution (HBSS) were obtained from Sigma (Zwijndrecht, The Netherlands). Vitamin C, EDTA, sucrose, 2-vinylpyridine, and HEPES were from Merck (Darmstadt, Germany). The compounds added to growth medium during the preincubation of the cells did not alter the pH of the medium and did not influence cell viability.

AcetylTyrFluo labeling experiments

Rat-1 fibroblasts were cultured for 48 h on plastic Petri dishes (20 cm^2) in Dulbecco's modified Eagle medium (DMEM) containing 7.5% (vol/vol) fetal calf serum (Life Technologies, Breda, The Netherlands) in a water-saturated, 5% CO_2 environment. The cells were grown until 80% confluency. Cells were preincubated with the compound of interest: GSH, GSH monoethyl ester, BSO, mercaptosuccinic acid, AT, or vitamin (for details, see the legends to the figures), then loaded with 5 μ*M* acetylTyrFluo (10 min in HBSS/20 m*M* Tris-HCl, pH 7.4), washed, and stressed with H_2O_2 for 10 min in HBSS. Protein samples were run on one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and acetylTyrFluo-labeled proteins were detected by western blotting, using a horseradish peroxidase-conjugated anti-fluorescein antibody (Biogenesis, Poole, U.K.) with subsequent enhanced chemiluminescence detection. Within an experiment, identical amounts of proteins were loaded in each lane, which was additionally checked by staining the blots (before immunodetection) using Ponceau S stain (Sigma) to show identical protein loading.

Pulse-chase under oxidative conditions and sample preparation

Rat-1 fibroblasts were cultured for 48 h as described above. Cells were washed twice with HBSS, and ongoing protein synthesis was strongly reduced by adding 2 ml of depletion medium [10 m*M* HEPES, pH 7.4, in methionine-free culture medium, obtained from ICN-Biomedicals (Zoetermeer, The Netherlands)]. After 20 min, dishes were placed on a rack in a water bath (37°C), depletion medium was removed, and cells were pulse-labeled for precisely 10 min with 500 μl of labeling medium containing 100 μCi of ^{35}S-labeled L-methionine (Pharmacia Amersham Biotech, Roosendaal, The Netherlands) in depletion medium. Pulse was stopped by adding 2 ml of chase medium, serum-free DMEM containing 1 m*M* cycloheximide, 5 m*M* cysteine, 5 m*M* methionine, 10 m*M* HEPES, pH 7.4. Cycloheximide in the chase medium will stop elongation of unfinished nascent peptide chains. Chase/oxidation was started by replacing the chase medium with chase medium containing 0–8.0 m*M* H_2O_2, for the desired time. After this interval, chase/oxidation was stopped by putting the dishes on ice followed by immediate washing with ice-cold stop medium, 20 m*M* *N*-ethylmaleimide (NEM) in HBSS. Cells were lysed with 600 μl of ice-cold lysis buffer (10 m*M* HEPES, pH 7.4, 200 m*M* NaCl, 2 m*M* $CaCl_2$, 2.5 m*M* $MgCl_2$, 1% Triton X-100, 2.2% dimethyl sulfoxide (DMSO), 20 m*M* NEM, 1 m*M* phenylmethylsulfonyl fluoride, and 10 μg/ml CLAP (chymostatin, leupeptin, antipain, pepstatin stock of 10 mg/ml each in DMSO), and lysates were collected by scraping and transferring to precooled microcentrifuge tubes. Nuclei were pelleted by centrifugation (10 min, 13,000 rpm, 0°C). Supernatant was transferred to precooled

microcentrifuge tubes, and immunoprecipitation was started or samples were frozen in liquid nitrogen (stored at −80°C).

Immunoprecipitation, SDS-PAGE, and autoradiography

The LDLr was immunoprecipitated from the postnuclear cell lysate as described before (8) using polyclonal Antiserum 121 (9). SDS-PAGE was performed on a 6% polyacrylbisacrylamide. Gels were stained for 15 min with Coomassie Brilliant Blue [0.25% (wt/vol) in destain], destained (30% methanol, 10% acetic acid, 60% water) for 45 min, neutralized (30% methanol, 70% phosphate-buffered saline) for 5 min, soaked in enhancer (1.5 *M* sodium salicylate, 30% methanol, 70% water) for 15 min, and vacuum-dried for 1 h at 80°C. Autoradiography was subsequently performed by exposing a phosphor imaging screen (Kodak) to the gel for 72 h, after which radioactivity was measured using the Personal Molecular Imager™ FX System (Bio-Rad). Autoradiograms were also obtained by exposing a BioMax MR-1 film (Kodak) to the gels for 2 weeks at −80°C.

Glutathione assay

Rat-1 fibroblasts were cultured as described above. Cells were incubated with BSO, and subsequently a modified Tietze-recycling assay (2, 18) was used as described before (5).

RESULTS

To establish the optimal conditions to study the effects of modulation of the cellular redox/antioxidant status, dose-response studies were carried out. With a fixed concentration of acetylTyrFluo present (loading 5 μ*M* for 10 min in HBSS/20 m*M* Tris, pH 7.4), cells were subjected to a concentration series of H_2O_2 (Fig. 1). Labeling steadily increased up to a maximum level at 160 μ*M* H_2O_2.

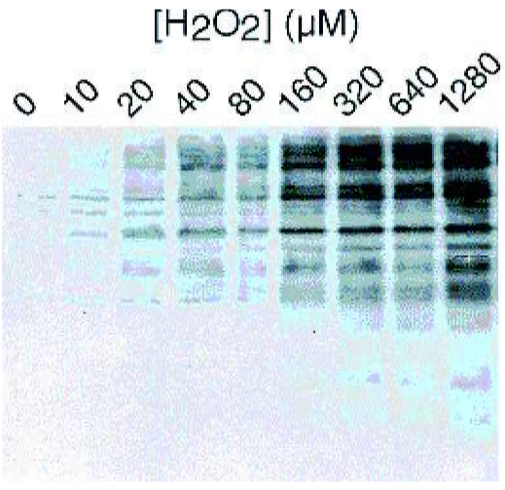

FIG. 1. Western blot of acetylTyrFluo-labeled proteins. Cells were labeled with 5 μ*M* acetylTyrFluo and subsequently subjected to a concentration series of H_2O_2. Proteins were analyzed using SDS-PAGE and western blotting.

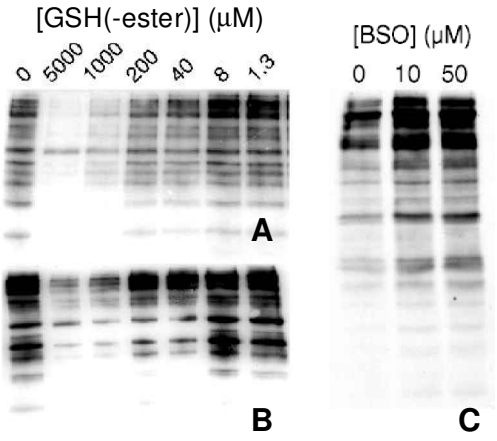

FIG. 2. Effect of modulation of cellular glutathione on protein labeling. Cells were preincubated with GSH (**A**) or GSH monoethyl ester (**B**) for 4 h in culture medium (indicated concentration in μ*M*), before loading with acetylTyrFluo and subsequent oxidation with H_2O_2. Depletion of cellular GSH was achieved by preincubation overnight with BSO. Cells were then again loaded with acetylTyrFluo and stressed with H_2O_2 (**C**).

Subsequently, the cells were preincubated overnight with GSH or GSH monoethyl ester, and after removal of the extracellular GSH or its ester, cells were labeled with acetylTyrFluo and subjected to 40 μ*M* H_2O_2. Figure 2 shows that preincubation with both GSH (A) and its ester (B) had a strong effect on the degree of oxidation, particularly when added at millimolar range.

The effect of lowering cellular GSH was studied by reducing the synthesis of GSH by an overnight incubation of the cells with BSO, which inhibits the rate-limiting step catalyzed by γ-glutamyl-cysteine synthetase. Figure 2C shows that a low overnight concentration of BSO (10 or 50 μ*M*), which reduced cellular GSH to 20% and 10% of control, respectively, caused an increase in labeling intensity. When the preincubation period was reduced to 2 h, no changes in cellular GSH were detected and no increased labeling was observed, showing that the increase in labeling is not caused by BSO itself. Initial experiments with two-dimensional SDS-PAGE analysis indicated that pretreatment with BSO led not only to increased labeling of proteins, but also to labeling of new proteins.

Thus, modulation of the cellular redox/antioxidant status clearly affected the oxidative labeling of proteins during oxidative stress. Therefore, we studied the effect of inhibition of two enzymes involved in the cellular defense against reactive oxygen species: GPx and catalase. GPx catalyzes the reaction: $2H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$. MS inhibits GPx, thus preventing the scavenging of H_2O_2 by this enzyme. In line with the results obtained with GSH and BSO, overnight preincubation with MS and subsequent H_2O_2 treatment caused an increased labeling already at the lowest concentration used (Fig. 3A), confirming the involvement of the cellular antioxidant in the defense against protein oxidation.

Masaki *et al.* showed that catalase acts as a primary defense against oxidative stress from exogenous or endogenous

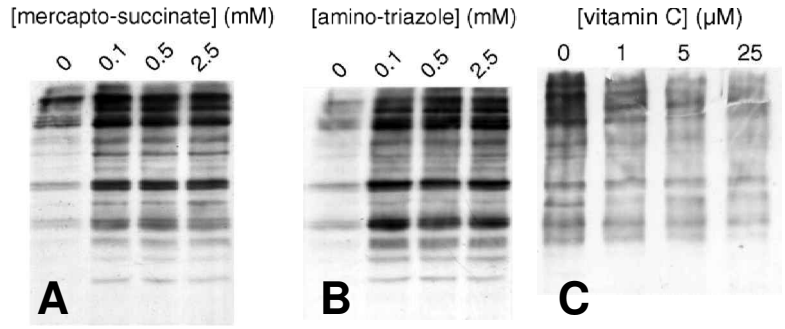

FIG. 3. Effect of modulation of cellular antioxidant systems on protein labeling. Cells were pretreated overnight with MS (**A**) or AT (**B**), then loaded with acetylTyrFluo, and stressed with H_2O_2. (**C**) shows the effect of 2h of pretreatment with vitamin C. A and B were exposed for shorter times to prevent overexposure of the pretreated lanes.

H_2O_2 at low concentrations in human dermal fibroblasts (12). Figure 3B shows that an overnight incubation with AT, which inhibits catalase, indeed resulted in an increase in acetylTyrFluo labeling upon oxidative stress to the same extent as preincubation with MS.

As protein oxidation was clearly affected by interventions that alter cellular GSH content and/or enzymatic antioxidant system, we tested whether nonenzymatic antioxidants, such as vitamins C and E, could affect this oxidation. Therefore, cells were preincubated for 2 h with vitamin C, loaded with acetylTyrFluo, and oxidized with H_2O_2. Pretreatment with vitamin C showed a protective effect and resulted in a decreased labeling of the proteins (Fig. 3C). The effect was already visible at a concentration as low as 1 μ*M*. In contrast to the protective effect of vitamin C, preincubation with the lipophilic antioxidant vitamin E (2 h or overnight, concentrations ranging from 0 to 50 μ*M*) did not show an effect on labeling at any of the concentrations or incubation periods used (data not shown).

The application of oxidative stress resulted in preferential oxidation of ER resident proteins, as detected with the acetylTyrFluo probe, and these proteins are involved in the correct folding of proteins (21). Because of this and the fact that the extent of this labeling can modulate by changing the cellular redox/antioxidant status, we subsequently studied whether the applied stress affects protein folding in the ER, using the LDLr as a model as described before (8). Maturation is here defined as the posttranslational series of ER-related events comprising folding (S-S bridge formation), vesicular transport toward the Golgi complex, and *O*-glycosylation inside the Golgi complex (4, 8, 11, 19).

To study the effect of H_2O_2 on LDLr maturation, we first tested whether H_2O_2 affects the incorporation of [^{35}S]methionine in cellular proteins, and indeed [^{35}S]methionine incorporation is reduced already after a short exposure (10 min) to a low dose of H_2O_2 (0.1 m*M*) and at higher concentrations (0.5 and 2.0 m*M*) completely abolished. This is the result of an impairment of protein synthesis (translation) (1) and of an impaired uptake of [^{35}S]methionine as a consequence of the oxidation (data not shown). Thus, [^{35}S]methionine incorporation needed to precede oxidative stress.

Next, the influence of oxidative stress on protein folding/maturation was investigated. Oxidation of ER resident proteins (as measured by acetylTyrFluo labeling) was clearly occurring during an oxidative stress of 100 μ*M* H_2O_2 (21). Cells were first pulse-labeled with [^{35}S]methionine followed by a chase period in the presence or absence of H_2O_2 (Fig. 4). A chase/oxidation of 15 min was performed, because at this time point both the mature and the precursor forms are present. As can be seen in Fig. 4, under the stress conditions used (100 or 400 μ*M* H_2O_2) the maturation of LDLr was not affected.

Increasing concentrations of H_2O_2 had a large impact on the maturation of LDLr (Fig. 5). When cells were stressed with 2.0 m*M* H_2O_2 or higher, maturation did not occur anymore during the stress. In nonreduced samples (Fig. 5A, without recovery) the precursor (p) appears as a smear of folding intermediates, which disappears in the reduced samples (Fig. 5B, without recovery), indicating that disulfide bridges were formed as a consequence of oxidation. When cells were subsequently allowed to recover in culture medium, maturation (m = mature form) could be restored after 2.0 m*M* H_2O_2 oxidative challenge. Apparently, the defensive capacities were sufficient or quickly recovered or up-regulated to overcome this challenge, and cells resumed protein folding, vesicle transport, and glycosylation. In contrast, 8.0 m*M* H_2O_2 resulted in irreversible damage and no recovery was observed. A concentration of 1.5 m*M* H_2O_2 was used to follow LDLr maturation in time. As can be seen in Fig. 5C, the velocity of LDLr maturation is decreased under these oxidative stress conditions, but is not completely inhibited.

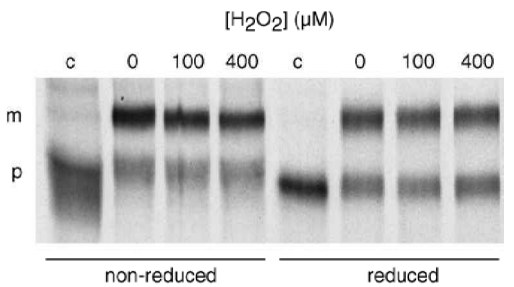

FIG. 4. Maturation of LDLr under low oxidative conditions. Cells were depleted from methionine, pulse-labeled with [^{35}S]methionine, and chased for 15 min in the presence of 0, 100, or 400 μ*M* H_2O_2. Samples were run on a nonreducing and a reducing gel c, control (no oxidation, no chase); p, 120-kDa precursor; m, 160-kDa mature form of LDLr.

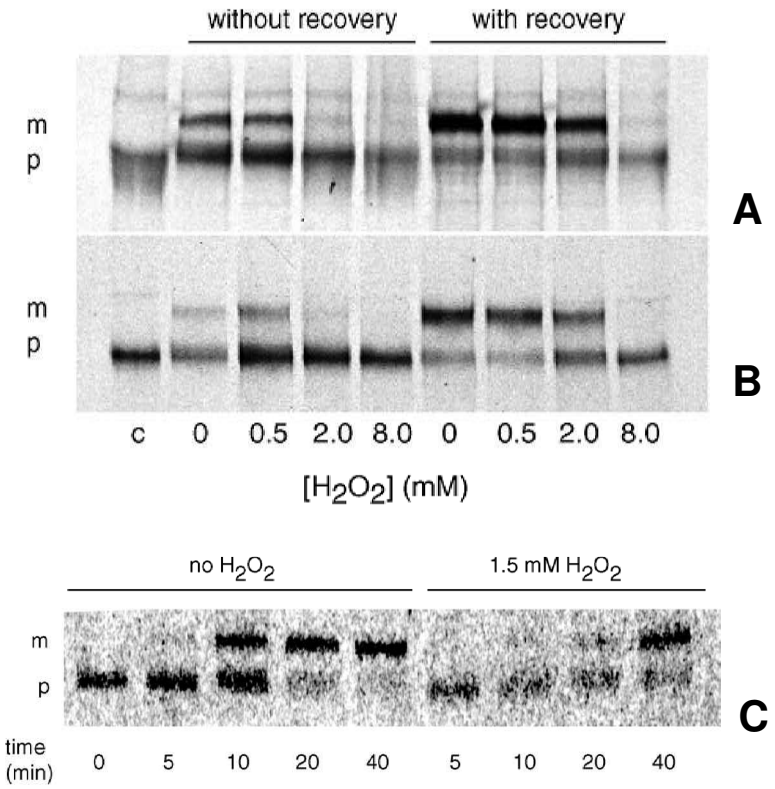

FIG. 5. Maturation of LDLr under moderate to high oxidative conditions. Cells were depleted from methionine, pulse-labeled with [^{35}S]methionine, and chased for 10 min in the presence of 0, 0.5, 2.0, or 8.0 m*M* H_2O_2 with or without subsequent recovery in culture medium for 30 min. (**A**) Nonreduced samples. (**B**) Reduced samples. p, 120-kDa precursor; m, 160-kDa mature form of LDLr; c, control (no oxidation, no chase). **C** shows the retardation of LDLr maturation in the presence of 1.5 m*M* H_2O_2.

DISCUSSION

Oxidative modification of cellular proteins, using the membrane-permeable acetylTyrFluo probe and subjecting the cells to oxidative stress (H_2O_2), revealed a predominant labeling of ER resident proteins, specifically PDI, BiP, GRP94, ER60, and calnexin (20, 21). Because of the importance of these proteins in proper functioning of the ER and thus the cell, we addressed two questions: (a) can susceptibility toward oxidative stress of these proteins be modulated by modifying the cellular redox/antioxidant status and (b) does this oxidative stress affect ER function with regard to protein folding? The latter question was studied using the maturation of the LDLr as a model, as described by Jansens *et al.* (8).

To answer the first question, we first determined the dose-response relationship between the oxidative stress and degree of labeling of the cellular proteins. Figure 1 clearly shows that a plateau is reached at ~160 μ*M* H_2O_2 under the conditions applied. This plateau might be caused by limitation of the intracellular acetylTyrFluo. To be able to measure an effect on protein labeling, the H_2O_2 concentration used should result in a label intensity in the rising part of the dose-response curve. Thus, 40 μ*M* H_2O_2 was selected as the optimal concentration to perform the subsequent studies on cellular redox/antioxidant status and protein oxidation.

Manipulation of cellular GSH levels clearly has a large influence on the degree of protein labeling with acetylTyrFluo during oxidative stress. Preincubation of the cells with glutathione or GSH monoethyl ester dose-dependently attenuated the acetylTyrFluo labeling of cellular proteins during oxidative stress. The compounds were most effective at millimolar concentrations, in the same range as the intracellular GSH concentration. Lowering the cellular GSH content was achieved by BSO. This clearly resulted in an increase in the acetylTyrFluo labeling intensity (Fig. 2). Preliminary two-dimensional gel-electrophoresis experiments (data not shown) show that also additional proteins were labeled, whose identities are presently unknown. They might represent other ER resident proteins or proteins present in other cellular compartments. The findings suggest that BSO treatment increased the sensitivity of the ER to oxidative stress, most likely by reducing the GSH/GSSG ratio in the ER lumen. By reducing the cellular GSH content, BSO will also affect the antioxidative capacities throughout the cell, possibly leading to labeling of additional proteins.

The degree of oxidative modification, as detected by the acetylTyrFluo probe, was also clearly increased by inhibiting the cellular antioxidant enzymes GPx and catalase (Fig. 3). This raises the question whether the oxidation can be attenuated by antioxidants. The water-soluble antioxidant vitamin C effectively reduced the protein oxidation, already at concentrations of 1 μ*M*. Although at this moment we do not know the mechanism(s) responsible for the preferential oxidation of ER resident proteins, the observed protection is in line with the observation that vitamin C prevented NADPH-initiated cytochrome P450-mediated, free metal ion-independent, oxidative damage of microsomal membranes (13). In contrast, the lipophilic antioxidant vitamin E did not have any effect on the degree of protein oxidation, which suggests that oxidation

of luminal ER protein might be best prevented by water-soluble antioxidants.

In the experiments described above, we analyzed the effect of modulating cellular redox/antioxidant status on oxidation, but we did not determine the exact nature of the proteins oxidatively damaged. However, in a preceding study, we have proven that the application of this oxidative stress resulted in preferential oxidation of ER resident proteins, as detected with the acetylTyrFluo probe (21). As these proteins are involved in the correct folding of proteins within the ER and their oxidation can be modulated by altering the cellular redox/antioxidant status, we addressed the question whether the observed oxidation of these ER resident proteins had an effect on protein folding and maturation. The ER resident proteins assisting in protein folding were found to be labeled with acetylTyrFluo already at relatively low concentrations of H_2O_2 (50–100 μ*M* for 10 min) (21). The result presented in Fig. 4 clearly shows that these levels of oxidative stress do not attenuate maturation of the LDLr and the formation of the mature form of the protein (m, 160 kDa), which indicates both correct formation of S-S bridges and folding of the LDLr within the ER and subsequent transport to the Golgi and *O*-glycosylation inside the Golgi complex (4, 8, 11, 19). Thus, it seems that these levels of oxidative stress do not affect the ER folding machinery, at least with respect to folding of the marker we chose for this process. As the detection method for the acetylTyrFluo-labeled proteins is very sensitive, it is very well possible that the number of acetylTyrFluo-labeled proteins is relatively low and does not have an effect on the functionality of the machinery as a whole.

Clearly, the maturation of LDLr is dramatically impaired by the higher oxidative stresses. Pulse-chase experiments showed that at moderate to high H_2O_2 concentration (between 1.0 and 2.0 m*M*), protein folding/maturation of the LDLr is slowed down (Fig. 5C). In the absence of oxidative stress, the mature form is detected after 10 min of chase, and maturation is complete after 40 min. In contrast, in the presence of 1.5 m*M* H_2O_2, the mature form is detected only after 20 min and maturation clearly is not completed after 40 min. H_2O_2 at 2 m*M* during the 15-min chase completely abolished maturation. However, this inhibition is not due to a complete damage of the machinery and was shown to be reversible: recovery of cells in culture medium restored LDLr maturation (Fig. 5A and B).

Maturation of the LDLr occurs in several steps, each of which might be susceptible to oxidative stress. Jansens *et al.* (8) showed that as a first step distant parts of the LDLr molecule are brought together, allowing the formation of nonnative disulfide bonds between distant cysteines, which yields folding intermediates with very compact states. As folding proceeds, isomerization into native disulfide bonds (*i.e.,* a process catalyzed by disulfide isomerases, such as PDI) takes place, making the protein less compact. Immunoprecipitation and SDS-PAGE studies of the receptor from human fibroblasts grown in the presence of [^{35}S]methionine demonstrated that LDLr is synthesized as a precursor with a molecular mass of ~120 kDa. This precursor is transported to the Golgi complex, where it is rapidly converted into a mature form with a mobility of ~160 kDa due to addition of O-linked oligosaccharides (3, 11, 19).

Several explanations can be put forward to explain the observed effect of oxidative stress on maturation of the LDLr. Inhibition of proper folding of the LDLr might be caused by oxidation of LDLr itself. As proper folding is a prerequisite for transportation toward the Golgi complex for further maturation, less or no mature form of the LDLr is observed. This will also lead to an attenuation of transport of the premature form to the Golgi. Proteins of the folding machinery are oxidized, as positively proven using the acetylTyrFluo probe (21). This might lead to a decrease in the capacity of the protein folding machinery and inhibition of proper LDLr folding. As the maturation of the LDLr depends on vesicle transport toward the Golgi complex (19), an alternative explanation might be that proteins involved in this vesicular transport are oxidized, resulting in the impairment of the transport and subsequent maturation of the LDLr. Finally, inhibition of glycosylating enzymes in the ER and/or Golgi as a consequence of oxidation might play a role in the delayed or inhibited maturation of the LDLr during oxidative stress.

In conclusion, oxidative modification of ER resident proteins (as detected using the acetylTyrFluo probe) can be manipulated by altering the cellular redox/antioxidant status. Low levels of oxidative stress, although capable of oxidizing a fraction of the ER resident proteins, do not attenuate the folding of the LDLr in the ER and the subsequent further maturation of the protein. Increased oxidative stress does attenuate this maturation reversibly; however, at this moment, the exact mechanism of this is not clear yet and needs further investigation.

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ABBREVIATIONS

AT, aminotriazole; BiP, binding protein; BSO, buthionine sulfoximine; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; HBSS, Hanks' buffered saline solution; H_2O_2, hydrogen peroxide; LDLr, low-density lipoprotein receptor; MS, mercaptosuccinate; NEM, *N*-ethylmaleimide; PDI, protein disulfide isomerase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

REFERENCES

1. Ayala A, Parrado J, Bougria M, and Machado A. Effect of oxidative stress, produced by cumene hydroperoxide, on the various steps of protein synthesis. Modifications of elongation factor-2. *J Biol Chem* 271: 23105–23110, 1996.

2. Baker MA, Cerniglia GJ, and Zaman A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Anal Biochem* 190: 360–365, 1990.
3. Chaudiere J, Wilhelmsen EC, and Tappel AL. Mechanism of selenium-glutathione peroxidase and its inhibition by mercaptocarboxylic acids and other mercaptans. *J Biol Chem* 259: 1043–1050, 1984.
4. Cummings R, Kornfeld S, Schneider W, Hobgood K, Tolleshaug H, Brown M, and Goldstein J. Biosynthesis of N- and O-linked oligosaccharides of the low density lipoprotein receptor. *J Biol Chem* 258: 15261–15273, 1983.
5. de Wit R, Makkinje M, Boonstra J, Verkleij AJ, and Post JA. Hydrogen peroxide reversibly inhibits epidermal growth factor (EGF) receptor internalization and coincident ubiquitination of the EGF receptor and Eps15. *FASEB J* 15: 306–308, 2001.
6. Feinstein RN and Braun JT. Effect of compounds related to aminotriazole on blood and liver catalase. 3. ANL-7409. *ANL Rep* 123, 1967.
7. Forman HJ, Torres M, and Fukuto J. Redox signaling. *Mol Cell Biochem* 234–235: 49–62, 2002.
8. Jansens A, van Duijn E, and Braakman I, Coordinated nonvectorial folding in a newly synthesized multidomain protein. *Science* 298: 2401–2403, 2002.
9. Jensen HK, Holst H, Jensen LG, Jorgensen MM, Andreasen PH, Jensen TG, Andresen BS, Heath F, Hansen PS, and Neve SA. Common W556S mutation in the LDL receptor gene of Danish patients with familial hypercholesterolemia encodes a transport-defective protein. *Atherosclerosis* 131: 67–72, 1997.
10. Keogh BP, Allen RG, Pignolo R, Horton J, Tresini M, and Cristofalo VJ. Expression of hydrogen peroxide and glutathione metabolizing enzymes in human skin fibroblasts derived from donors of different ages. *J Cell Physiol* 167: 512–522, 1996.
11. Lee LY, Mohler WA, Schafer BL, Freudenberger JS, Byrne-Connolly N, Eager KB, Mosley ST, Leighton JK, Thrift RN, and Davis AR. Nucleotide sequence of the rat low density lipoprotein receptor cDNA. *Nucleic Acids Res* 17: 1259–1260, 1989.
12. Masaki H, Okano Y, and Sakurai H. Differential role of catalase and glutathione peroxidase in cultured human fibroblasts under exposure of H_2O_2 or ultraviolet B light. *Arch Dermatol Res* 290: 113–118, 1998.
13. Mukhopadhyay CK and Chatterjee IB. NADPH-initiated cytochrome P450-mediated free metal ion-independent oxidative damage of microsomal proteins. Exclusive prevention by ascorbic acid. *J Biol Chem* 269: 13390–13397, 1994.
14. Ohba H, Harano T, and Omura T. Biosynthesis and turnover of a microsomal protein disulfide isomerase in rat liver. *J Biochem (Tokyo)* 89: 901–907, 1981.
15. Pollard MG, Travers KJ, and Weissman JS. Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. *Mol Cell* 1: 171–182, 1998.
16. Sen CK. Cellular thiols and redox-regulated signal transduction. *Curr Top Cell Regul* 36: 1–30, 2000.
17. Sies H and Akerboom TPM. Glutathione disulfide (GSSG) efflux from cells and tissues. *Methods Enzymol* 105: 445–451, 1984.
18. Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 27: 502–522, 1969.
19. Tolleshaug H, Goldstein JL, Schneider WJ, and Brown MS. Posttranslational processing of the LDL receptor and its genetic disruption in familial hypercholesterolemia. *Cell* 30: 715–724, 1982.
20. Van der Vlies D, Wirtz KWA, and Pap EHW. Detection of protein oxidation in Rat-1 fibroblasts by fluorescently labeled tyramine. *Biochemistry* 40: 7783–7788, 2001.
21. Van der Vlies D, Pap EHW, Post JA, Celis JE, and Wirtz KWA. Endoplasmic reticulum resident proteins of normal human dermal fibroblasts are the major targets for oxidative stress induced by hydrogen peroxide. *Biochem J* 366: 825–830, 2002.

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