Endoplasmic reticulum resident proteins of normal human dermal fibroblasts are the major targets for oxidative stress induced by hydrogen peroxide

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The membrane-permeable fluorescein-labelled tyramine conjugate (acetylTyrFluo) was used to identify the proteins of normal human dermal fibroblasts most susceptible to oxidation by hydrogen peroxide [Van der Vlies, Wirtz and Pap (2001) Biochemistry **40**, 7783–7788]. By exposing the cells to H_2O_2 (0.1 mM for 10 min), TyrFluo was covalently linked to target proteins. TyrFluo-labelled and [^{35}S]Met-labelled cell lysates were mixed and subjected to two-dimensional PAGE. After Western blotting the ^{35}S-labelled proteins were visualized by autoradiography and the TyrFluo-labelled proteins by using anti-fluorescein antibody. The TyrFluo-labelled proteins were matched with the ^{35}S-labelled proteins and identified by comparison with our mastermap of proteins. Protein disulphide isomerase (PDI), IgG-binding protein (BiP), calnexin, endoplasmin and glucose-regulated protein 58 (endoplasmic reticulum protein 57/GRP58) were identified as targets of oxidation. All these proteins reside in the endoplasmic reticulum and are part of the protein folding machinery. In agreement, confocal laser scanning microscopy showed co-localization of TyrFluo-labelled proteins and the KDEL receptor ERD-2, a marker for the endoplasmic reticulum.

Key words: fluorescent probe, oxidized protein, two-dimensional electrophoresis.

INTRODUCTION

Upon attack by reactive oxygen species (ROS) cellular proteins may become irreversibly damaged. This attack may result in amino acid side-chain oxidation, protein fragmentation or protein–protein crosslinking via *o*,*o*′-dityrosines. Oxidative damage to proteins has been postulated as a major cause of various degenerative diseases including inflammatory diseases [1], atherosclerosis [2,3], neurological disorders [4], ischaemia and reperfusion injury [5], carcinogenesis [6] and aging [7,8].

There are numerous stable markers of oxidant damage to proteins [9]. One of the targets of ROS is the aromatic amino acid tyrosine leading to the formation of a tyrosyl radical. These radicals are formed either by transition metal ion-catalysed Fenton and Haber-Weiss reactions (e.g. H_2O_2/Fe^{2+}) [10] or by peroxidase/H_2O_2 systems. Protein tyrosyl radicals may form intra- or intermolecular *o*,*o*′-dityrosine bonds [11]. Human phagocytes, neutrophils and macrophages employ the myeloperoxidase/H_2O_2 system to synthesize *o*,*o*′-dityrosine, a specific marker of protein oxidation [12,13]. In the presence of HOCl this system also gives rise to 3-chlorotyrosine [14]. Tyrosine may also react with reactive nitrogen species yielding 3-nitrotyrosine and *o*,*o*′-dityrosine [15,16]. It is known that the concentrations of *o*,*o*′-dityrosines in heart, skeletal muscle and lens proteins increase with aging [17,18]. Moreover, free radicals can convert protein-bound tyrosine into 3,4-dihydroxyphenylalanine (DOPA). DOPA can be further oxidized to DOPA-quinone, which can react with thiols to form 5-cysteinylDOPA [19].

Oxidation of tyrosine may have profound effects on the biological function of proteins. For example, it has been demonstrated that nitration of tyrosine residues interferes with their phosphorylation [20,21]. Physiological levels of peroxynitrite and H_2O_2 have been reported to oxidize critical tyrosine residues in superoxide dismutase [22], glutathione reductase [23], mitochondrial electron transport chain components and ATPase [24], and protein tyrosine phosphatases [25,26]. Cross-linking of signalling enzymes by intermolecular dityrosine bonds has also been observed in cells [27,28].

Recently, we reported on the development of a highly sensitive method for the detection of oxidized proteins in intact cells [29]. The method is based on the labelling of oxidized proteins with a membrane-permeable fluorescein-labelled tyrosine analogue, tyramine (acetylTyrFluo). Upon oxidation of the tyramine by ROS, the ensuing tyrosyl radical may form cross-links with target proteins, making them suitable for identification. In the present study, we have used this TyrFluo probe to demonstrate that proteins of normal human dermal fibroblasts, residing in the endoplasmic reticulum (ER), are most susceptible to a low oxidative challenge with H_2O_2.

MATERIALS AND METHODS

Materials

The polyclonal goat anti-rabbit IgG was from Sigma (St. Louis, MO, U.S.A.). Anti-fluorescein-HRP (horseradish peroxidase-conjugated polyclonal antibody against fluorescein) was purchased from Biogenesis (Poole, Dorset, U.K.). The antibody against the ER membrane receptor ERD2 was kindly provided by Dr J. Klumperman (Department of Cell Biology, Faculty of Medicine, Utrecht University, Utrecht, The Netherlands). The succinimidyl ester of Cy3-sulphoindocyanine was from Amer-

Abbreviations used: 2D-PAGE, two-dimensional PAGE; BiP, immunoglobulin heavy chain-binding protein; DOPA, 3,4-dihydroxyphenylalanine; ER, endoplasmic reticulum; Erp57, endoplasmic reticulum protein 57; GRP, glucose-regulated protein; HBSS, Hanks' balanced salt solution; LDH, lactate dehydrogenase; NHDF, normal human dermal fibroblast; PDI, protein disulphide isomerase; ROS, reactive oxygen species.

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sham Bioscience (Little Chalfont, Bucks., U.K.). Urea, thiourea, tributylphosphine, sulphobetaine 3–14 (*N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulphonate) and Hanks' balanced salt solution (HBSS) were purchased from Sigma. CHAPS was from ICN Biomedicals (Aurora, OH, U.S.A.) and the Bio-Lyte 3/10 ampholytes were from Bio-Rad (Hercules, CA, U.S.A.). Ampholytes with pH ranges 7–9 and 9–11 were from Serva (Heidelberg, Germany). Centriplus concentrators were purchased from Millipore (Bedford, MA, U.S.A.). DNase was from Promega (Leiden, The Netherlands). The acetylated tyramine-fluorescein conjugate (acetylTyrFluo) was prepared as described before [29].

Cell culture

Adult normal human dermal fibroblasts (NHDFs) were obtained from BioWhittaker (Walkersville, MD, U.S.A.) and cultured to a cell density of 80 % on plastic Petri dishes in Dulbecco's modified Eagle's medium containing 7.5 % (v/v) fetal calf serum in the presence of penicillin and streptomycin.

Oxidation of cells

For the labelling of intracellular proteins, NHDFs were preloaded with 5 μM acetylTyrFluo in HBSS/10 mM Tris/HCl (pH 7.4) for 10 min at 37 °C. AcetylTyrFluo not taken up by the cells was removed by gentle washing with HBSS. To oxidize proteins, cells were subjected to H_2O_2 (0.05–3.2 mM) in HBSS for 10 min at 37 °C. Denaturing buffer [100 mM Tris/HCl pH 7.4, 100 mM dithiothreitol, 2 % SDS, 20 % (v/v) glycerol and 0.025 % (w/v) Bromophenol Blue] was applied directly on to the cells, and proteins were collected and submitted to SDS/PAGE. In the case of two-dimensional PAGE (2D-PAGE) cells were subjected to 100 μM H_2O_2 as above. The dishes were put on ice and ice-cold lysis buffer [40 mM Tris/HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1 mM Na_3VO_4, 50 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1 % SDS and 1 % Triton X-100] was added to the cells. After scraping the proteins were collected in Eppendorf tubes and sonicated (10 s at 50 W). Cell debris was removed by centrifugation (20 min, 14000 ***g***, 4 °C) and DNA in the supernatant was digested for 1 h at 37 °C by 2 units of DNase in the presence of 6 mM $MgCl_2$. Next, the samples were desalted (final NaCl concentration less than 10 mM) using a Centriplus concentrator with a molecular-mass cut-off of 10 kDa. After lyophilization, proteins were dissolved in 2D-PAGE buffer (40 mM Tris, pH 9.5, 5 M urea, 2 M thiourea, 10 mM tributylphosphine, 2 % sulphobetaine 3-14, 2 % CHAPS and 0.5 % ampholytes 3-10).

Lactate dehydrogenase (LDH) release assay

NHDFs were grown on a 96 well plate (15000 cells/well) as described above, washed with PBS^{+++} (containing 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$ and 5 mM glucose) and stressed with H_2O_2 (25 μM–6.4 mM) in PBS^{+++} (0.1 ml/well, 37 °C). At 10, 60 or 120 min the medium was transferred to another plate and the cells were lysed by adding 0.1 ml of 1 % Triton X-100/PBS for 2 h at 37 °C. The assay was started by adding 50 μl of substrate mix (1 mg of sodium pyruvate/1 mg of NADH per ml of PBS) to the medium and the lysed cells. LDH activity was determined by measuring the oxidation of NADH (λ_{Abs} 340 nm) with a microplate reader (Ultramark; Bio-Rad) for up to 20 min. LDH release was expressed as the ratio of LDH activity in the medium to the total LDH activity (medium plus cell lysate).

[^{35}S]Methionine labelling of NHDF proteins

NHDFs were cultured for 20 h in Dulbecco's modified Eagle's medium/fetal calf serum containing 50 μCi of [^{35}S]methionine (Amersham Bioscience) per 0.1 ml of medium. At the end of the labelling period the cells were collected by trypsinization and centrifugation and resuspended in lysis solution (9.8 M urea, 2 % Nonidet P-40, 2 % ampholytes pH 7–9 and 100 mM dithiothreitol).

Immunodetection of TyrFluo-labelled proteins (2D-PAGE)

TyrFluo-labelled proteins ($\approx$ 50 μg) were mixed with [^{35}S]methionine-labelled proteins ($\approx$ 5 μg) and subjected to 2D-PAGE according to O'Farrell et al. [30] with some slight modifications [31]. Proteins were blotted on nitrocellulose (Amersham Bioscience) for 24 h at a constant current of 130 mA using a wet system (Bio-Rad Trans-Blot Cell). Blots were washed with demineralized water, air-dried and autoradiography was performed by exposing Hyperfilm MP (Amersham Bioscience) to the blot for 24 h. Blots were washed with TBST [Tris-buffered saline containing 0.05 % (v/v) Tween-20] and with TBST/0.2 % (w/v) Protifar (low-fat milk powder; Nutricia), each for 10 min at room temperature. Blots were blocked with TBST/2 % Protifar (2 $\times$ 30 min). TyrFluo-labelled proteins were detected with anti-fluorescein-HRP (1:1000 in TBST/2 % Protifar) and subsequent enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Bioscience). Labelled proteins were visualized by exposing Hyperfilm MP (Amersham Bioscience) to the blot for 0.5–2 min.

Identification of TyrFluo-labelled protein

The autoradiogram and the immunoblot were scanned with a model GS-700 Imaging Densitometer (Bio-Rad) using Molecular Analyst Software (Bio-Rad). By using PDQuest software (version 6.1.0; Bio-Rad) the spots of both images were matched with our reference map of human MRC-5 fibroblasts and keratinocytes [31]. Proteins in these maps have been identified using one or a combination of procedures that include Edman degradation, MS (Brucker Biflex III matrix-assisted laser-desorption ionization–time-of-flight) and immunoblotting with specific antibodies.

Confocal laser scanning microscopy

NHDFs were cultured on glass coverslips and labelled with acetylTyrFluo as described above. Cells were washed three times with PBS and fixed with 4 % (w/v) paraformaldehyde/0.1 % (w/v) Triton X-100 in PBS for 1 h at room temperature. After fixation, the remaining paraformaldehyde was neutralized with 50 mM NH_4Cl in PBS (3 $\times$ 5 min). Next cells were washed with PBS and blocked with 0.2 % gelatin in PBS (blocking solution) for 1 h and incubated with anti-ERD2 antibody (1:500 in blocking solution) for 1 h at room temperature. After four washes in blocking solution, the cells were incubated with the fluorescently labelled secondary antibody goat anti-rabbit Cy3 (1:400 in blocking solution) for 1 h at room temperature. Cy3 labelling of the antibody was performed as described by Wouters et al. [32]. Before mounting in Mowiol (Hoechst, Frankfurt am Main, Germany), cells were washed three times with PBS and once with demineralized water. Images were taken with a Leica TCSNT confocal laser scanning system on an inverted microscope DMIRBE (Leica Microsystems) with an argon–krypton laser as an excitation source. TyrFluo-labelled proteins were excited with the 488 nm laser line and emission was detected using a 530/30 bandpass filter. The

Cy3-labelled antibody was excited with the 568 nm laser line and emission was detected using a 560 nm longpass filter followed by a 600/30 bandpass filter.

RESULTS

Labelling of intracellular proteins

NHDFs were incubated with acetylTyrFluo and subjected to increasing concentrations of H_2O_2 for 10 min. As shown in Figure 1(B) a distinct set of proteins (50–150 kDa) was labelled with acetylTyrFluo. Labelling was observed at 0.05 mM H_2O_2 and increased with increasing concentrations of H_2O_2. It appears that even at the highest concentrations this set of labelled proteins remained the same. Proteins that were most abundantly present (Figure 1A) were different from the ones that were most heavily labelled, whereas some heavily labelled proteins were barely detectable by Ponceau-S staining. Given that the probe is present throughout the cell [29], one may conclude that the labelling efficacy is determined by factors (e.g. site of radical generation, susceptibility) different from the amount of protein present.

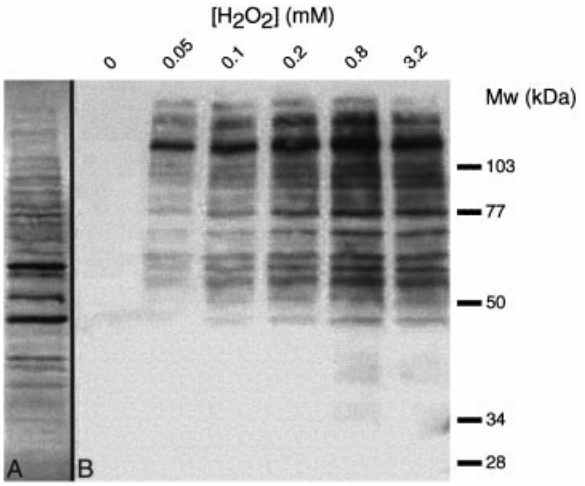

Figure 1 Labelling of human fibroblast proteins as a function of H_2O_2 concentration

Western blot of acetylTyrFluo-labelled proteins after exposure of NHDFs to increasing concentrations of H_2O_2 (**B**). Protein staining by Ponceau-S (**A**).

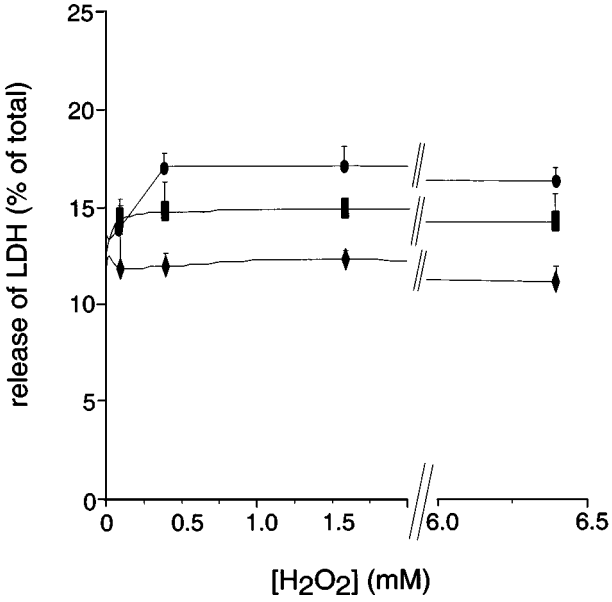

Figure 2 LDH release of human fibroblasts as a function of H_2O_2 and time of exposure

◆, 10 min; ■, 60 min; ●, 120 min; means ± S.D. are shown ($n = 3$).

The sensitivity of NHDFs towards H_2O_2 was established by measuring LDH release at various concentrations of H_2O_2 for different periods of time. As shown in Figure 2 LDH release never exceeded 18 %, even after 2 h of severe stress (6.4 mM H_2O_2). To exclude any effect of the viability of the cells on protein oxidation the labelling experiments were carried out with 100 μM H_2O_2 for 10 min. Under these conditions LDH release was similar to the control (10 min without H_2O_2).

Identification of labelled proteins

The immunoblot of the TyrFluo-labelled proteins shows that at least 10 proteins were oxidized as a result of the H_2O_2 treatment (Figure 3A). The autoradiogram of this blot visualizes the position of each ^{35}S-labelled protein present in NHDFs (Figure 3B). The identification of TyrFluo-labelled proteins was achieved in two steps: (i) the protein spots on the immunoblot were matched with the spots on the autoradiogram and (ii) the autoradiogram was matched to our reference map of the human MRC-5 fibroblasts. This procedure provided the identity of five TyrFluo-labelled proteins, i.e. calnexin (labelled a on Figure 3A), endoplasmin/glucose-regulated protein (GRP) 94 (b), immunoglobulin heavy chain-binding protein (BiP)/GRP78 (c), protein disulphide isomerase (PDI; f) and endoplasmic reticulum protein 57 (Erp57)/GRP58 (g). Some labelled proteins (d and e) could not be unequivocally matched with the autoradiogram. Proteins representing the most heavily labelled spots (h and i) were not visible on the autoradiogram. In addition to these distinctly labelled proteins a number of minor spots are represented.

A common feature of the identified TyrFluo-labelled proteins is their connection with the ER. This cannot be explained by a preference of the probe for this subcellular organelle, as it is distributed homogeneously throughout the cell before oxidation (Figure 4A). To confirm the ER localization TyrFluo-labelled cells were fixed and incubated with an antibody against the KDEL receptor ERD-2. As shown in Figure 4(C), a nearly perfect co-localization was observed between the TyrFluo-labelled proteins (Figure 4B) and the ER marker (Figure 4D).

DISCUSSION

In this study we have used the membrane-permeable conjugate (acetyl)TyrFluo to label those intracellular proteins in living human fibroblasts that are most susceptible to oxidation by H_2O_2 added to the medium. Labelling of intracellular proteins already takes place under mild conditions (Figure 1). Under the conditions routinely used (100 μM H_2O_2 for 10 min) cell viability was not affected as measured by LDH release (Figure 2). We presume that the labelling occurs at those sites where both the target proteins as well as the probe are oxidized. The extent of labelling, however, may be less than 1 % (D. van der Vlies and J. Westerman, unpublished work). Given this low level of labelling we have not yet been able to identify the nature of the covalent bond between TyrFluo and the protein. Although the formation of a dityrosine bond is likely we cannot exclude that the TyrFluo

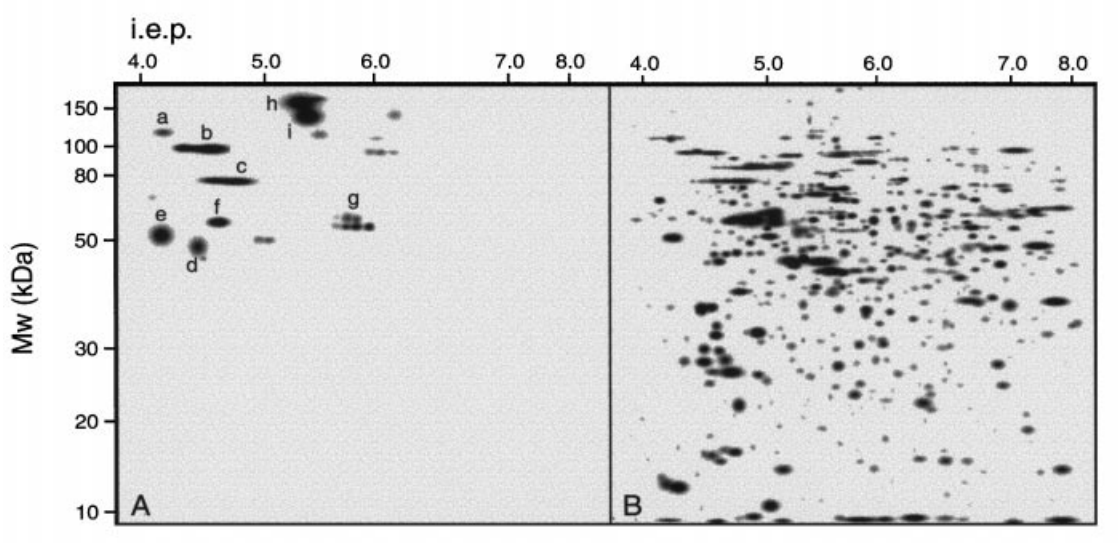

Figure 3 Identification of TyrFluo-labelled proteins

Immunoblot of TyrFluo-labelled proteins (**A**) and an autoradiogram of the [^{35}S]methionine-labelled proteins (**B**) were matched with our mastermap of the human MRC-5 fibroblast (see the Materials and methods section). Protein labels: a, calnexin; b, endoplasmin/GRP94; c, BiP/GRP78; f, PDI; g, Erp57/GRP58; proteins d, e, h and i are unknown.

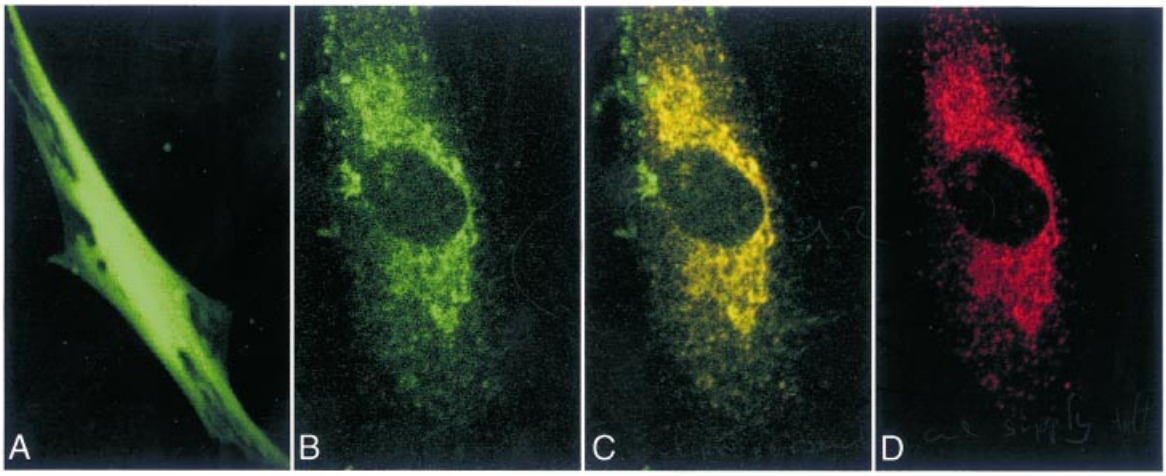

Figure 4 Co-localization of TyrFluo-labelled proteins with the KDEL receptor

Cells were loaded with acetylTyrFluo (**A**) and exposed to H_2O_2. After exposure the cells were fixed and labelled with anti-ERD2 and goat anti-rabbit Cy3 antibody. TyrFluo-labelled proteins (**B**), anti-ERD-2 staining (**D**) and co-localization (**C**) are also shown.

radical forms bonds with other amino acids. For instance, TyrFluo may be converted into DOPA-Fluo which can then react by way of DOPA-quinone-Fluo with a cysteine residue [9,19].

The identification of the labelled proteins by matching with our reference map of the human MRC-5 fibroblast revealed that the bulk of the labelled proteins was associated with the ER. These included BiP/GRP78, calnexin, endoplasmin/GRP94, PDI and PDI Erp57 precursor (also known as Erp60/58 kDa microsomal protein/p58/GRP58), which are all known to reside in the ER lumen. In agreement with this identification the TyrFluo-labelled proteins co-localized with the KDEL receptor ERD2 on the ER membrane (Figure 4). ERD2 recognizes a C-terminal KDEL motif on certain soluble ER proteins and retrieves those proteins that have leaked to the Golgi complex back to the ER [33]. Moreover, recently it was reported that PDI was selectively degraded by the proteosome, following H_2O_2 exposure of cultured rat liver epithelial cells [34].

The mitochondrion and the peroxisome as major sites of ROS production in the cell are protected against oxidative damage by antioxidant enzymes. Thus superoxide anion produced in the respiratory chain is converted by Mn superoxide dismutase whereas H_2O_2 produced in the peroxisomal β-oxidation is degraded by catalase. Such a defence mechanism has not been described for the ER and may explain why proteins of the ER are susceptible to oxidation by H_2O_2. The observation that it is the

ER proteins that mainly become oxidized cannot be explained by the selectivity of acetylTyrFluo for this organelle as prior to oxidation the probe is homogeneously distributed throughout the cell [29]. Hence, the preferential labelling of the ER proteins must be due to specific features of this organelle. Since H_2O_2 is a weak oxidant we propose that it must be converted first into highly reactive $OH^{\bullet}$ for proteins and TyrFluo to become oxidized. Given the very short lifetime of the $OH^{\bullet}$ we infer that the radical formation occurs in the ER. Here H_2O_2 may be converted into $OH^{\bullet}$ by cytochrome P450 enzymes. It has been shown that cytochrome P450 can operate *in vitro* as a peroxygenase using peroxy compounds as the oxygen donor [35]. Anari et al. [36] demonstrated that cytochrome P450 in intact rat hepatocytes could function as a peroxygenase utilizing t-butyl-hydroperoxide. They also showed the involvement of cytochrome P450 in the metabolic bio-activation of cumene hydroperoxide and suggested the formation of reactive radical metabolites in this reaction [37]. Another possibility is that H_2O_2 is converted into $OH^{\bullet}$ by the Fenton reaction using transition metal ions available in the ER [38].

The ER plays a central role in the synthesis and distribution of many cellular proteins. Before proteins can be transported towards their final destination, disulphide bonds essential for a proper folding have to be formed [39]. A requirement for this oxidative protein folding is a high redox state [40]. In the ER lumen, the relative abundance of the oxidized (GSSG) compared with the reduced (GSH) form of glutathione has led to the proposal that GSSG serves as the oxidizing equivalent during protein folding [41]. The ratio [GSSG]/[GSH] in the ER is 1:1–3 compared with 1:30–100 for the overall cellular ratio. It is remarkable that all the labelled proteins revealed in this study (i.e. PDI, BiP, calnexin, endoplasmin and PDI precursor Erp57/GRP58) reside in the lumen. An explanation for the finding that particularly these proteins are highly susceptible to oxidation by H_2O_2 may be found in a combination of the high redox state and the peroxygenase activity of cytochrome P450, Cu/Zn superoxide dismutase activity or an ER-associated Fenton reaction.

In the present study we show that ER proteins, associated with protein folding, are among the most susceptible to oxidation induced by H_2O_2 treatment. This may be due to the fact that these proteins become partly unfolded ([42] and references therein) while assisting in the folding of other proteins. This raises the important question whether oxidation of the protein-folding machinery may lead to improper folding and/or accumulation of proteins to be secreted. A quality control mechanism ensures that only correctly folded proteins exit the ER. Incorrectly folded proteins are retained and will be degraded. Therefore, improper functioning of the ER can have serious consequences for the cell. In connection with this it is also important to realise that the activation of enzymes and signalling pathways by H_2O_2 may occur under conditions of extensive protein oxidation.

We thank Jette Laurdisen from the Institute of Medical Biochemistry and Danish Center for Human Genome Research, Aarhus University, Denmark for her assistance in performing the 2D-PAGE experiments. This research was supported by Unilever, Vlaardingen, The Netherlands, and the Technology Foundation STW (grant no. UBI 4443), Applied Science Division of NOW and the Technology Program of the Ministry of Economic Affairs.

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Received 18 April 2002/10 June 2002; accepted 19 June 2002
Published as BJ Immediate Publication 19 June 2002, DOI 10.1042/BJ20020618