Endoplasmic Reticulum Proteins are Major Targets of Oxidative Stress

Application of a Novel Fluorescent Probe

Eiwitten van het endoplasmatisch reticulum zijn de voornaamste doelwitten van oxidatieve stress

Toepassing van een nieuw ontwikkeld fluorescerend sensormolecuul
(met een samenvatting in het nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
woensdag 15 januari 2003 des middags te 2.30 uur

door

Dennis van der Vlies

Geboren op 14 april 1970 te Purmerend

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ISBN 90-393-3253-3

Het in dit proefschrift beschreven onderzoek werd mede mogelijk gemaakt met financiële steun van het Unilever Research Laboratorium in Vlaardingen en de Stichting Technische Wetenschappen, toegepaste wetenschapsdivisie van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek, en het technologie programma van het Ministerie van Economische Zaken (UBI 4443).

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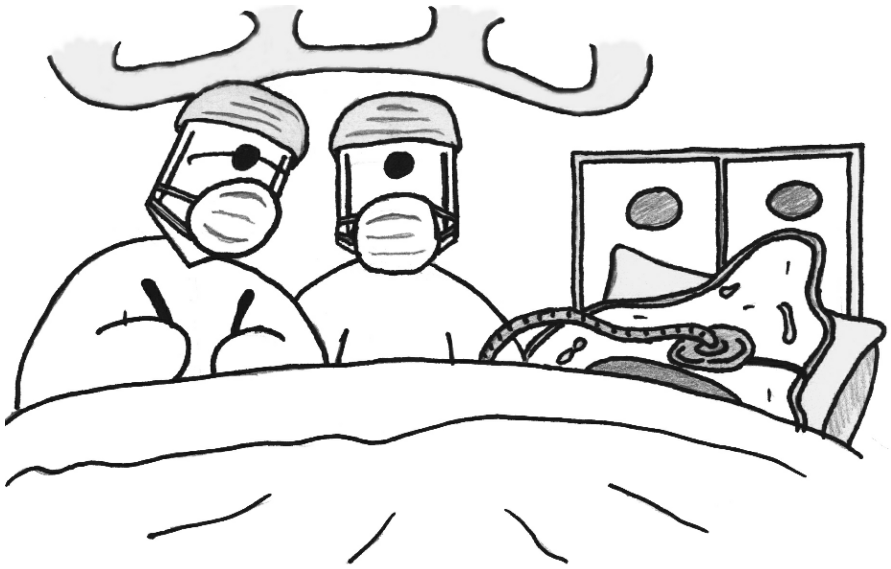
Reproductie: Cuvillier Verlag, Göttingen, Germany



" 0, 0 Dr Tyr - I think you've damaged the endoplasmic reticulum ! "

You don’t stop laughing because you grow old.
You grow old because you stop laughing.

- Michael Pritchard

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General introduction

Oxidative stress

Although oxygen is essential for life, breathing pure oxygen at atmospheric pressure for longer than 48 hours will lead to respiratory distress and death. This toxicity is not entirely due to oxygen *per se* but more to the production of highly reactive reduced products from oxygen. Even under normal conditions these reactive oxygen species (ROS) are produced by the body as part of normal metabolism or are produced by irradiation or environmental factors. Reduction of molecular oxygen (O_2) can give rise to a variety of ROS, which are generally short-lived and highly reactive and include the superoxide anion radical ($O_2^{-\cdot}$) hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·) (figure 1). Normally the body is able to defend itself against ROS by means of enzymatic reduction, antioxidants (endogenous and exogenous) and metal chelators. When the level of ROS exceeds the defence mechanisms, a cell is in a state of oxidative stress. Under these circumstances proteins, as well as DNA and lipids may be irreversibly damaged. Oxidatively modified proteins have been shown to accumulate during aging [1-5] and as a result of several degenerative diseases including inflammatory diseases [6], atherosclerosis [7, 8], neurological disorders [9], ischemia and reperfusion injury and carcinogenesis [10].

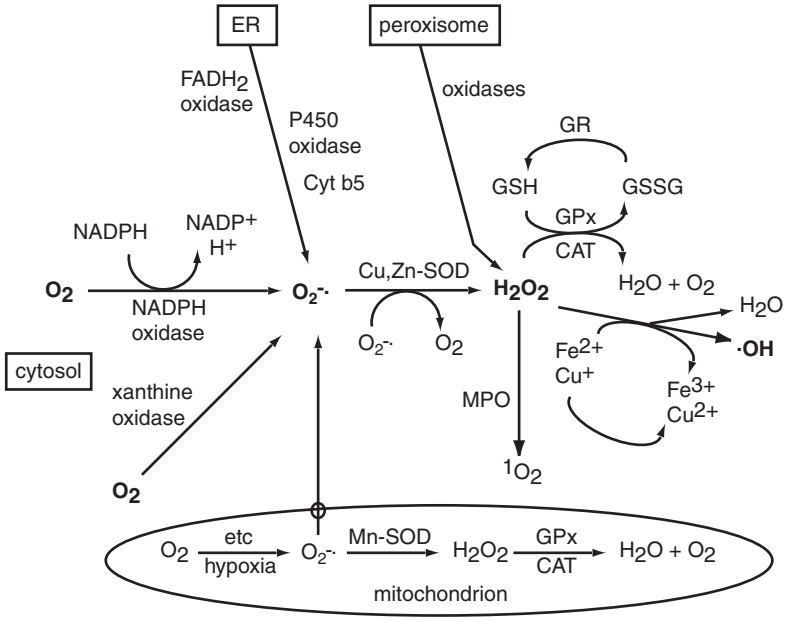

Fig 1: **Generation of reactive oxygen species and the defence mechanisms against damage by active oxygen.** During hypoxia generated $O_2^{-\cdot}$ may be degraded in the mitochondria by Mn-SOD or, if it reaches the cytosol, by Cu,Zn-SOD. In the endoplasmic reticulum (ER), NADPH-cytochrome P450 reductase can leak electrons onto O_2 generating $O_2^{-\cdot}$. $FADH_2$ and cytchrome b5 can also contribute to this system. Peroxisomes contain several enzymes that produce H_2O_2 without intermediation of $O_2^{-\cdot}$. In contrast to $O_2^{-\cdot}$, H_2O_2 is able to cross cell membranes and within the cell it can react with Fe^{2+} or Cu^{+} to form OH· via Fenton reaction. CAT = catalase, GPx = glutathione peroxidase, GR = glutathione reductase, MPO = myeloperoxidase, $^1O_2$ = singlet oxygen, etc = electron transport chain.

Reactive oxygen species and their sources

Since ROS seem to play a role in the aging process, these species and their sources have been a subject of many age-related studies. In thyroid hormone and body size studies, a positive association was found between the mitochondrial proton leak and ROS-induced oxidative damage [11]. In mitochondria, ROS are produced in large amounts as by-products of electron transport in the respiratory chain [12]. The electron transport chains of the endoplasmic reticulum and chloroplasts have also been reported as sources for ROS and other radicals [13]. These transport chains, especially the mitochondrial, have been suggested to be the main age-related source of $O_2^{-\cdot}$ and H_2O_2 [14].

Superoxide, a relative unreactive species, is in equilibrium with its protonated form the hydroxyperoxyl radical ($HO_2\cdot$). In biological systems $O_2^{-\cdot}$ can be converted into more reactive species, such as peroxyl ($ROO\cdot$), alkoxyl ($RO\cdot$), peroxynitrite ($ONOO^-$) and hydroxyl ($HO\cdot$) radicals. Superoxide is formed when molecular oxygen acquires an additional electron:

$$O_2 + e^- \rightarrow O_2^{-\cdot}$$

It is formed by subjecting oxygen to ionising radiation and is an intermediate in a number of biochemical reactions. Xanthine oxidase converts xanthine to uric acid and produces $O_2^{-\cdot}$ as a byproduct. Other flavoprotein oxidases (e.g. aldehyde oxidase) give also rise to $O_2^{-\cdot}$. Along with H_2O_2 it is the major "microbicide" produced by circulating phagocytic leukocytes [15] via NADPH oxidase. The phagocyctic cells flow around the bacterium or foreign particle and engulfs it within a phagosome. Oxygen uptake in neutrophils and macrophages is due to the action of the NADPH-complex associated with the plasma membrane, the electrons released on oxidation of NADPH reducing oxygen to superoxide radical. Therefore the particles are exposed to a high flux of $O_2^{-\cdot}$ in the phagocyte cytoplasm, some of which are also released extracellularly. Normally the $O_2^{-\cdot}$ is short-lived and is converted to H_2O_2 by the enzyme superoxide dismutase (SOD) [14], which maintains the steady-state levels of $O_2^{-\cdot}$ at less than 10^{-11}M. $O_2^{-\cdot}$ has a relatively low reactivity. Probably it is the production of H_2O_2, via SOD and its subsequent utilisation by myeloperoxidase, with chloride as a co-substrate producing hypochlorous acid (HOCl), that kills bacteria. In addition, altered SOD function has been linked to Down's syndrome (overexpression of SOD) and to amyotrophic lateral sclerosis or Lou Gehrig's disease (dysfunctional SOD) [16, 17].

Hydrogen peroxide is formed by several metabolic reactions including those catalysed by SOD and by D-amino acid oxidase. SOD catalyses the formation of H_2O_2 as follows:

$$2\ O_2^{-\cdot} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

H_2O_2 is also formed by the auto-oxidation of hydroquinones (e.g. ubiquinone), cathecholamines and thiols. H_2O_2 may also be formed from $O_2^{-\cdot}$ via the very short-lived and spontaneously disproportionating hydroperoxyl radical:

$O_2^{-\cdot} + H^+ \rightarrow HO_2^{\cdot}$
$2\ HO_2^{\cdot} \rightarrow H_2O_2 + O_2$

Moreover, H_2O_2 also takes part in $OH^{\cdot}$ formation which may account for much of the toxicity of H_2O_2. Hydroxyl radicals are very energetic, short-lived and toxic oxygen species and can be formed from either the $O_2^{-\cdot}$ (Haber- Weiss reaction) or from H_2O_2 (Fenton reaction). Both reactions require a transition metal such as iron or copper:

$O_2^{-\cdot} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$ (Haber- Weiss reaction)
$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$ (Fenton reaction)

H_2O_2 can transverse the plasma and nuclear membranes. As it enters the nucleus it contributes to many $OH^{\cdot}$-dependent DNA adduct formations, like 8-hydroxy-deoxyguanosine. Formation of 8-OHdG causes G to T and A to C substitutions [18]. Protection against H_2O_2 is achieved by degradation by catalase and by several peroxidases (e.g. glutathione peroxidase):

$H_2O_2 \rightarrow H_2O + O_2$ (catalase)
$H_2O_2 + AH_2 \rightarrow H_2O + A$ (peroxidases)

Unfortunately, in many cells the catalase concentration is very low and most of it is compartmentalised within peroxisomes, which prevents it from being an efficient scavenger of H_2O_2 resulting from SOD activity in the cytosol. So, in most tissues the water-soluble enzyme, which ensures the degradation of H_2O_2 (and other hydroperoxides), is glutathione peroxidase.

Nitric oxide ($NO^{\cdot}$) is a normal product of arginine metabolism [19]. In intact mitochondria peroxynitrite ($ONOO^-$) may be generated in yields depending on the formation rate of $NO^{\cdot}$ and $O_2^{-\cdot}$ and the levels of mitochondrial SOD. Peroxynitrite rapidly reacts with carbon dioxide (CO_2), yielding the unstable nitroso-peroxycarbonate anion ($ONOOCOO^-$), which can rearrange into highly reactive species [20]. Hydrogen peroxide and peroxynitrite are powerful oxidants that can easily cross membranes, resulting in oxidative modifications throughout the cell [14]. Peroxynitrite may possibly increase the mitochondrial proton leak by peroxidation of mitochondrial membrane lipids, leading to an increase in ROS production [11, 21]. Peroxynitrite has also been shown to inactivate glutathione peroxidase [22].

Metal-catalyzed oxidation (MCO) systems play a key role in ROS production as well [5]. Under conditions of oxidative stress or in the absence of an electron acceptor, MCO systems generate H_2O_2 and the reduced form of the transition metals iron, Fe(II), and copper, Cu(I). These reduced transition metals catalyze the production of hydroxyl radicals through Fenton or Haber Weiss reactions. Hydroxyl radicals are thought to be the main initiators of protein modification [19]. Hydrogen peroxide is also involved in the inactivation of enzymes by

sulfhydryl oxidation [23]. The activity of MCO systems is increased by enhancing the availability of iron and copper as a result of changes in the efficacy of metal binding proteins and chelating agents [24]

Cellular defence mechanisms against protein oxidation

At least three major endogenous mechanisms appear to play a role in reducing the harmful effects of oxidants in the cell (enzymatic, antioxidants and metal chelators). At normal oxygen tension these mechanisms are sufficient to maintain homeostasis. Even though free radicals are produced, their levels are easily controlled.

Cellular defence systems prevent the buildup of ROS and collectively help to protect living organisms against oxidative damage [25]. These defence mechanisms can be divided into primary and secondary systems [26].

The primary system consists of a battery of low molecular weight antioxidative compounds and enzymatic defence mechanisms. All these systems are directed towards the prevention of the oxidative interaction of free radicals with target molecules.

The reducing power of the water-soluble tripeptide glutathione (g-glutamyl-cysteinyl-glycine, GSH) plays an important role in diverse biological processes in order to maintain cellular homeostasis, like protein and DNA synthesis, cell growth/division, metabolic reducing reactions, transmembrane transport, receptor action and leukotriene synthesis [27]. Moreover, GSH is an antioxidant, antitoxin (conjugation to xenobiotics), and antioxidant-enzyme cofactor (glutathion peroxidase). The reactive nucleophilic thiol, found on the cysteine residue, plays a central role during detoxification processes. GSH can protect macromolecules (i.e., proteins, lipids, DNA) by either the formation of adducts directly with reactive electrophiles (glutathiolation) or by acting as a proton donor in the presence of reactive oxygen species (ROS) or organic free radicals, yielding GSSG. The reductant GSH forms together with its oxidized sulfur-sulfur linked form (GSSG) a very potent redox couple: $2GSH \leftrightarrow GSSG + 2H^+ + 2e^-$, and is considered to be the major (thiol-disulfide) redox buffer of the cell [28]. Therefore, generation and maintenance of reduced GSH pools, either by *de novo* synthesis (figure 2A) or via recycling by glutathione disulfide reductase (GR), using NADPH as a cofactor and electron donor, is of vital importance for the cell. GSH is synthesized in the cytosol of all mammalian cells, yet the liver is the major organ for synthesis and export of GSH into the plasma [29]. GSH synthesis is mainly determined by the availability of cysteine and the activity of the rate-limiting enzyme, g-glutamylcysteine synthetase (GCS), which activity can be inhibited by BSO (L-buthionine-SR-sulfoximine). The availability of cysteine depends on the diet, membrane transport activities of cysteine, cystine and methionine, and the conversion of methionine to cysteine via the trans-sulfuration pathway [30]. On average, the GSH concentration in the cytosol is 1-11 mM [31].

Cells undergoing oxidative stress are characterised by an alteration in the amount of reducing and oxidising equivalents (redox status); oxidising equivalents (i.e., glutathione disulfide (GSSG), cystine, $NADP^+$, $thioredoxin_{ox}$) are abnormally high or reducing

equivalents (i.e., glutathione (GSH), cysteine, NADPH, thioredoxin$_{red}$) are abnormally low. Oxidative stress results in the formation of GSSG at the expense of GSH [32]. This shift in the ratio of GSH/GSSG would change the redox status to a less negative potential. If this potential rises too much this would clearly be deleterious. 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 [33, 34]. Protein conformation is also dependent on the cell's redox status. Changes in oxidized or reduced GSH concentrations can lead to the abnormal oxidation or reduction of thiol residues involved in the formation of disulfide bridges in proteins [35]. Therefore, maintaining homeostatic concentrations of GSH (and GSSG) is necessary for normal cellular function. Indicative for this, the half-cell reduction potential (E_{hc}) of the GSSG/2GSH couple is correlated with the biological status of the cell as follows: proliferation $E_{hc} \approx$ -240 mV; differentiation $E_{hc} \approx$ -200 mV; or apoptosis $E_{hc} \approx$ -170 mV [36]. Besides export of GSH also export of GSSG has been observed when cells or tissues are subjected to oxidative stress. Export of GSSG would prevent this shift thereby maintaining the half-cell reduction potential and a favourable redox environment in the cell. It has been shown that induction of oxidative stress by perfusion of isolated heart with *tert*-butylhydroperoxide results in the rapid efflux of GSSG [37]. This efflux appears to be a part of the protection of cells and tissues from oxidative stress.

Specific enzymes for the reduction of disulfides are disulfide reductases and MetSOx reductase, the latter in the case of methionine (Met) oxidation. The cyclic oxidation of Met residues might serve as a ROS scavenger system to protect proteins from more extensive irreversible oxidative modifications [19]. In glutamine synthetase (GS) as well as in catalase surface-exposed oxidizable Met residues seem to guard the active site from oxidative insult [38]. Upon reduction of MetSOx, MetSOx reductase becomes oxidized itself. The reduced form is regenerated by thioredoxin, which oxidized form is regenerated by thioredoxin reductase, using NAD(P)H [38]. Thioredoxin and thioredoxin reductase also have alkyl hydroperoxide reductase activity and are efficient electron donors to cytoplasmic glutathione peroxidase [13]. As already mentioned before, two other enzymes involved in the primary defence system are superoxide-dismutase (SOD) and catalase. SOD catalyzes the conversion of $O_2^{-\cdot}$ into H_2O_2 while catalase catalyzes the degradation of H_2O_2. Catalase also plays a role in the inhibition of enzyme modifications by metal catalyzed oxidation (MCO) systems [24].

With regard to the essentiality of GSH for the survival of the whole organism, substantial information is available from studies on hereditary GSH depletion in the human, and from experimental depletion and repletion of GSH in animal models and cell cultures. In human siblings inherited the deficiency of the enzyme g- glutamylcysteine synthetase causes generalised GSH deficiency, hemolytic anemia, spinocerebellar degeneration, peripheral neuropathy, myopathy, and aminoaciduria. As a result severe neurological complications appeared during adulthood. GSH levels in erythrocytes were less than 3% of normal, in muscle less than 25%, and in leukocytes less than 50% [39]. The deficiency in GSH synthetase is also associated with a defective function of the central nervous system. This

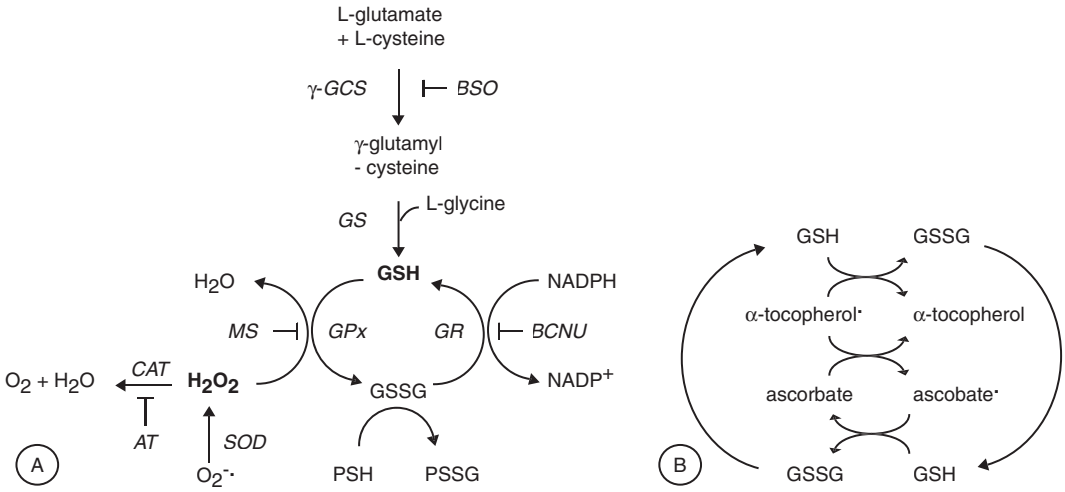

Fig.2: **Schematic relationships between antioxidant enzymes and glutathione.**
A) AT = 3-amino-1,2,4-triazole; BCNU = carmustine; BSO = L-buthionine-SR-sulfoximine; CAT = catalase; γ-GCS = γ-glutamylcysteine synthetase; GS = glutathione synthetase; GPx = glutathione peroxidase; GR = glutathione reductase; MS = mercapto-succinate; PSH = protein sulfhydryl; PSSG = mixed disulfide of a protein and GSH; SOD = superoxide dismutase. Blunt arrows show inhibition of the system.
B) Regeneration of ascorbate and tocopherol radicals by glutathione.

condition is complicated by the metabolic consequences of an excess of 5-oxoproline, formed as a "spillover" from the accumulation of γ-glutamylcysteine after its normal synthesis by the first enzyme and its lack of conversion to GSH by the second enzyme. Human hereditary GSH deficiency states are not necessarily lethal, probably because some GSH is obtained directly from the diet.

Meister found that dietary ascorbate can protect against the tissue damage that typically results from depletion of GSH [40]. In animals such as adult rats and mice, which are able to make adequate amounts of ascorbate on their own, GSH depletion was not lethal. By contrast, GSH depletion was lethal in those animals that could not make their own ascorbate (newborn rats, guinea pigs). Supplementation of the diet with ascorbate protected these animals against GSH depletion and saved their lives. Interestingly, guinea pigs placed on an ascorbate-deficient diet were also saved by dietary administration of GSH and its precursors. Thus, these two water-phase antioxidants are tightly linked: GSH can replace ascorbate in vivo, and ascorbate can replace GSH [41, 42].

A huge range of dietary constituents has been suggested to exert antioxidant effects *in vivo*, of which vitamin C is probably the best known. *In vitro* ascorbate has shown to be a strong antioxidant and scavenges hydroxyl (OH·), superoxide ($O_2^{-\cdot}$), peroxyl ($RO_2\cdot$), singlet oxygen, ($^1O_2$) oxysulfur radicals, hypochlorous acid, peroxynitrate, ozon (O_3) a.o. [43]. Plants and most animals can synthesize ascorbate from glucose, but humans, other primates, guinea-pigs

and fruit-bats have lost the enzyme gulonolactone oxidase, because of extensive mutations in the gene, to catalyse the last step in the synthesis and so require ascorbate to be present in the diet [44]. Ironically, high rates of ascorbate synthesis, in animals such as the rat, induce oxidative stress since H_2O_2 is produced as a by-product in the gulonolactone oxidase-catalysed reaction. The most striking property of ascorbate is its ability to act as a reducing agent to reduce Fe^{3+} to Fe^{2+}. Ascorbate is required *in vivo* as a cofactor for some enzymes, (e.g. proline hydroxylase and lysine hydroxylase for the biosynthesis of collagen) probably to keep iron or copper at the active site in the reduced, which is required for hydroxylation [45-47]. On the other hand, it is also well established that vitamin C can serve as a pro-oxidant. Metal ions such as Cu^{2+} and Fe^{3+} catalyse rapid oxidation of ascorbate producing the relatively unreactive ascorbyl radical and Fe^{2+}. Fe^{2+} can be used in Fenton chemistry to produce $OH\cdot$ from H_2O_2.

Glutathione seems to play a central role in the defence and regenerates tocopherol and ascorbate by the reduction of their radical form (figure 2B) [48-50]. It is also known that a tocopheryl radical (formed after reduction of oxidized lipids) is quenched by vitamin C to yield an ascorbyl radical, which, in turn is reduced by the conversion of glutathione to oxidized glutathione.

Lipophilic antioxidants (like vitamin E) protect membranes from oxygen radical attack, while hydrophylic antioxidants like vitamin C and uric acid prevent water-soluble compartments from oxidation. Vitamin C and E also terminate lipid chain reactions involving peroxyl radicals [25]. Trolox C, a vitamin E analogue, might be involved in the repair of amino acid radicals at neutral pH. Trolox C, vitamin C and E and thiols have been shown to react with protein and peptide peroxyl and alkoxyl radicals [51]. These reactions are expected to inhibit chain reactions within a protein. Vitamins A, C and E and metabolites such as uric acid are capable of scavenging free radicals directly or act by facilitating the regeneration of metabolites to scavenge [19].

Other non-enzymatic antioxidants are metal ion chelators, which can suppress the rate of ROS formation by forming complexes with iron or copper. These complexes inhibit their ability to catalyze ROS formation or alter their redox potentials and therefore their ability to undergo cyclic interconversion between oxidized and reduced states [19]. Divalent cations (like Mn^{2+}, Mg^{2+} and Zn^{2+}) may compete with Fe(II) and Cu(I) for metal binding sites on proteins and thereby prevent site-specific generation of hydroxyl radicals. Mn(II) is also able to inhibit the reduction of Fe(III) to Fe(II) [19] and thus prevents its ability to promote hydroxyl radical formation.

The secondary system of defence is directed towards the degradation of non-functional cellular components or the metabolism of toxic products. Additionally, this system prevents the accumulation of damaged and non-functional cellular components during aging [26]. The enzymatic DNA repair system can identify a DNA-oxidized adduct, remove it and incorporate an undamaged base. The protein and membrane repair systems consist of molecules, which can donate H-atoms to molecules that were attacked by a reactive species. Detoxification and regeneration systems also take part in the repair machinery [26].

Another cellular response to ROS-induced oxidation of biomolecules is the synthesis of

highly conserved stress proteins, like glucose-regulated proteins (GRPs) and heat shock proteins (HSPs) [52]. These stress proteins are able to assist in the reconstruction of tertiary protein structure [53] and protect proteins against aggregation [26]. Moreover, these chaperones are involved in the solubilization of protein aggregates and the targeting of irreversibly damaged proteins to degradation [26]. Especially HSP90 plays a role in the degradation of damaged biomaterial.

ROS-induced protein oxidation

Although ROS are involved in the oxidation of various types of biomolecules, some ROS, such as the hydroxyl and alkoxyl radical, are mainly involved in the oxidation of proteins [13, 54]. ROS can react directly with the protein or they can react with molecules like sugars and lipids, generating products that can react with the protein. These reactions are frequently influenced by redox cycling cations, especially by iron and copper bound to proteins. Subsequent exposure to H_2O_2 generates OH·, which selectively damages the amino acid residues at the binding site. For example, histidine residues on albumin, Cu/Zn SOD and apoprotein-B of low-density lipoproteins can bind copper ions to form 2-oxohistidine after exposure to H_2O_2 [55-57]. Due to these reactions, the protein may be cleaved yielding low molecular weight fragments or it may form protein-protein cross-linkages, yielding higher molecular weight proteins [58].

When amino acid radicals are generated in a protein, electrons can 'migrate' to other residues, i.e. the final products observed need not necessarily represent the initial sites of free-radical attack upon the protein. For example, methionine radicals can oxidize trypthophan, and tryptophan radicals can oxidize tyrosine. Tyrosine radicals can react with several anti-oxidants and also with $O_2^{-}\cdot$. Thus, although $O_2^{-}\cdot$ rarely reacts directly with proteins, its presence can alter the distribution of products generated by exposure to more reactive ROS, such as OH· [13]. For example, if TyrO· is generated, $O_2^{-}\cdot$ can decrease dityrosine formation, since reaction of TyrO· with $O_2^{-}\cdot$ 'diverts' tyrosine radicals and diminishes their ability to cross-link.

For assessing oxidative damage it is of great importance that a potential marker of protein side chain oxidation is a stable product. Whereas most amino acids can be oxidized (a summary is given in table 1), oxidation of only a limited number of amino acids (i.e. tyrosine, cysteine etc.) has profound effects on the biological function of proteins. ROS attack can lead to the oxidation of an amino acid side chain. Oxidation of the protein backbone may result in protein fragmentation or protein-protein crosslinking via *o,o'*-dityrosines. Being a substrate for tyrosine kinases, oxidation of a single tyrosine residue can lead to a complete down-regulation of cellular signalling. In model substrates it has been demonstrated that nitration of tyrosine residues interferes with their phosphorylation [59, 60].

Table 1: **Products of specific amino acid side chain oxidation.** Since the oxidation of the side chains of Ala, Asp, Asn, Gln and Ser only is suggested to yield carbonyl compounds, these amino acids are not included in this table.

Amino acid substrate	**Reactive species**	**Product**
Arg	$HO^{\bullet}$ in the presence of O_2	HAVA, Glutamic semialdehyde
Cys	$HO^{\bullet}$ or other hydrogen atom abstracting species	Cystine, Disulfides, Oxy acids
Glu	$HO^{\bullet}$ Glu-hydroperoxide, Oxalic acid	4-OH-Glu, Pyruvate, a-ketoglutaric acid,
Gly	$HO^{\bullet}$ or other hydrogen atom abstracting species followed by reaction with $CO_2^{\bullet}$- radicals	Amino malonic acid
His	$HO^{\bullet}$ or one-electron oxidants	Asp, Asn, 2-oxo-His
Ile	$HO^{\bullet}$ in the presence of O_2	Ile hydroperoxides, OH-Ile
Lys	$HO^{\bullet}$ in the presence of O_2	2-aminoadipic semialdehyde, Pentosidine, OH-Lys
Leu	$HO^{\bullet}$ in the presence of O_2	OH Leu, a-keto-isocaproic acid, Isovaleric acid, Isovaleraldehyde (-oxime), Hydroperoxy-Leu
Met	HO_2 or one-electron oxidants	MetSOx, Met-sulfone
Phe	$HO^{\bullet}$, one-electron oxidants or reactive nitrogen species $HO^{\bullet}$ before or after dimerization	o- and m-tyrosine, 2,3,4-OH-Phe Dimers of OH-species
Pro	$HO^{\bullet}$ in the presence of O_2	Glutamic semialdehyde, Pyroglutamic acid, 2-pyrrolidone, Glu, Hydroperoxy-Pro, OH-Pro, 5-OH-2- aminovaleric acid (HAVA)
Thr	$HO^{\bullet}$	2-amino-3-ketobutyric acid
Trp	$HO^{\bullet}$ or one-electron oxidants ONOO-	3-OH-kynurenine, (N-formyl)kynurenine, OH-Trp Nitro-Trp
Tyr	$HO^{\bullet}$ or ONOO- HOCl $HO^{\bullet}$, other radicals followed by radical recombination	DOPA, 3-Nitro-Tyr, 3,5-dinitro-Tyr 3-Chloro-Tyr, 3,5-Dichloro-Tyr Di-Tyr, tri-Tyr, Pulcherosine, p-hydroxyphenyl –acetaldehyde
Val	$HO^{\bullet}$ in the presence of O_2	OH-Val, Hydroperoxy-Val

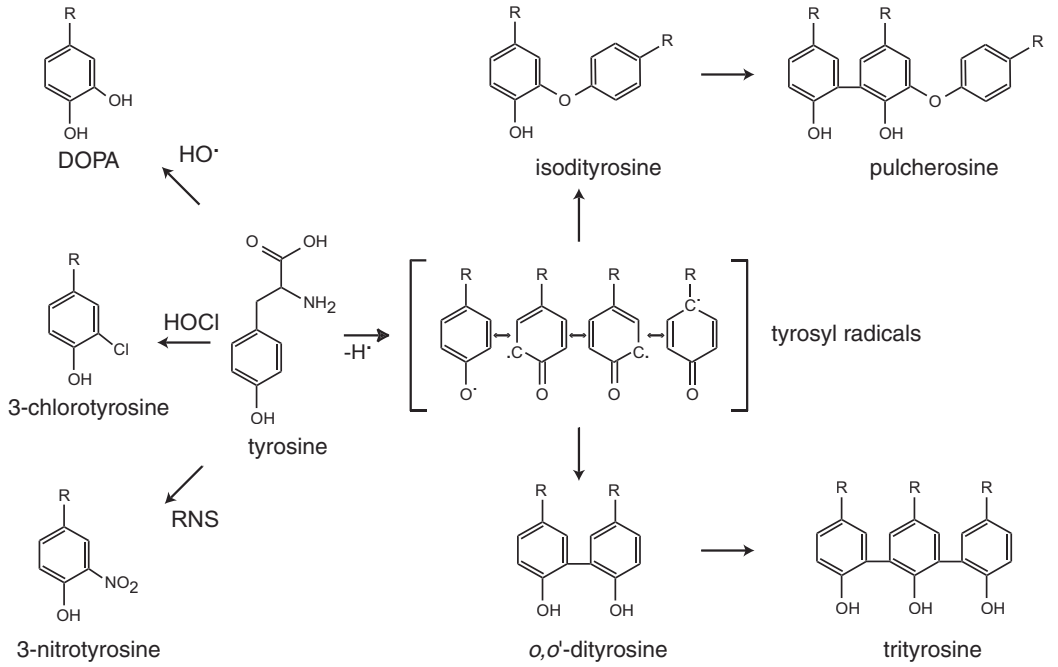

Fig 3: **Tyrosine oxidation leads to the formation of nitrotyrosine, chlorotyrosine, 3,4-dihydroxyphenylalanine (DOPA) and tyrosyl radicals.** RNS = reactive nitrogen species. For details, see text.

Tyrosine oxidation leads to the formation of (1) nitrotyrosine, (2) chlorotyrosine, (3) 3,4-dihydroxyphenylalanine (DOPA) and (4) tyrosyl radicals (figure 3). Nitrotyrosine can be introduced into proteins by reactive nitrogen species like peroxynitrite, a product of superoxide and nitric oxide [61, 62]. Chlorination is a result of attack by hypochlorite (HOCl) [63]. Attack by hydroxyl radicals can hydroxylate tyrosine to DOPA [64]. Tyrosyl radicals are formed by reaction with hypochlorite, peroxynitrite or by radicals formed in transition metal ion-catalysed Fenton and Haber-Weiss reactions (e.g. hydrogen peroxide/Fe^{2+}) [65-68]. Peroxidases, such as myeloperoxidase, which is a heme protein secreted by activated phagocytes at sites of inflammation, are also important sources of tyrosyl radicals. Protein tyrosyl radicals may form intra- or intermolecular crosslinks, so-called *o,o'*-dityrosines [69] and exhibit an enrichment in atherosclerotic lesions and tissue. In this case dityrosine cross-links are well-characterised products of myeloperoxidase-generated tyrosyl radicals [66, 70]. In search for tyrosylation products in lipoproteins by myeloperoxidase, multiple protein-bound tyrosine oxidation products were identified [71]. In addition to *o,o'*-dityrosine (3,3'-dityrosine), the compounds were identified as trityrosine (3,3',5',3"-trityrosine) and pulcherosine (5-[4"-(2-carboxy-2-aminoethyl)phenoxy]3,3'-dityrosine) by high resolution NMR spectroscopy and mass spectrometry. Additionally, dityrosine was shown to be a precursor to trityrosine, but not to pulcherosine and isodityrosine (3-[4'-(2-

carboxy-2-aminoethyl)-phenoxy]-tyrosine) as a precursor to pulcherosine.

Despite the interest in tyrosine oxidation and its effect on protein function, the identity of the major protein targets of tyrosyl radicals and the ensuing cellular consequences remain to be resolved. This is partly due to the lack of a sensitive assay that specifically detects tyrosyl radical formation. Besides measuring appropriate markers for disease- and/or age-related protein oxidation by GC/MS and HPLC [72], immunologic methods may be used as well. An alternative approach could be the introduction of probes, which will covalently label oxidatively modified proteins. Therefore, we developed a highly sensitive method for the detection of tyrosyl radical formation. The method is based on a fluorescein-labelled tyrosine analogue, tyramine (denoted as TyrFluo). Tyramine is chemically identical to tyrosine, except for the carboxyl moiety that is lacking. Upon oxidation of the tyramine by ROS, the tyramine is converted into a tyrosyl radical that can form cross-links with an oxidized target protein. By this reaction the target protein becomes fluorescently labelled, which makes them detectable (for details in detection see chapter 2 and 3).

Preferential oxidation of ER-resident proteins

The membrane-permeable fluorescein-labelled tyramine conjugate (acetylTyrFluo) has been used to identify the proteins of normal human dermal fibroblasts most susceptible to oxidation by hydrogen peroxide. By exposing the cells to H_2O_2 , TyrFluo was covalently linked to target proteins. Protein disulphide isomerase (PDI), IgG binding protein (BiP/GRP78), calnexin, endoplasmin (GRP94), and glucose regulated protein 58 (GRP58/ERp57/ER60) were identified (2D-PAGE) as targets of oxidation. All these protein chaperones reside in the lumen of the endoplasmic reticulum and are part of the protein folding machinery. In agreement, confocal laser scanning microscopy showed a co-localisation of TyrFluo-labelled proteins and the KDEL-receptor ERD-2, a marker for the endoplasmatic.

Chaperones and the ER quality control

The ER and the downstream compartments of the secretory pathway contain a quality control mechanism to ensure that only newly synthesized proteins that have passed a stringent selection process are transported to their final destination. There are several ways how a cell deals with misfolded proteins [73].

These include that proteins may be:

(1) retained inside the ER by forming aggregates with each other (trapped by GRP78) or by thiol-mediated retention via exposed free cysteines (binding to PDI and ERp72),
(2) degraded by the proteasome in the cytosol,
(3) retrieved from the cis-Golgi complex back to the ER (via COPI coated vesicles) for refolding, or
(4) re-routed from the trans-Golgi for lysosomal degradation.

The ER quality control system includes a number of chaperones and folding enzymes localised in the lumen or in the membrane of the ER [73]. It is likely that most if not all, proteins synthesized in the ER interact with chaperones at some stage of the folding and maturation pathway. The chaperones bind non-native proteins and are thought to assist folding by preventing irreversible aggregation and misfolding. Exactly how chaperones act in concert to keep non-native proteins on the productive folding pathway, and selectively retain certain proteins, is not fully understood. Although some chaperones in the ER are well studied, including 78-kDa glucose-regulated protein (GRP78 or BiP), 94-kDa glucose-regulated protein (GRP94 or endoplasmin), calnexin, calreticulin, protein disulfide isomerase (PDI), and protein disulfide isomerase ER-60 precursor (GRP58/ERp57), it is at present impossible to predict with which chaperones a specific protein will interact and to predict the consequences of the interaction.

These chaperones and folding enzymes are localised in the ER because they possess the C-terminal KDEL (Lys-Asp-Glu-Leu) retention/retrieval sequence [74]. This signal is recognized by a membrane-bound receptor that continually retrieves the proteins from a later compartment of the secretory pathway and returns them to the ER [75].

GRP78 is the ER-located Hsp70 analogue. It binds transiently to a variety of newly synthesized proteins as they are translocated into the ER and more persistently to some misfolded proteins. Furthermore, GRP78 is known to assist folding and assembly of newly synthesized proteins by recognition and binding of hydrophobic stretches of unfolded proteins. Binding of GRP78 prevents protein aggregation and maintains the proteins in a folding and oligomerisation-competent state [76]. It has been shown that GRP78 is involved in the retention of mutant proteins in the endoplasmic reticulum, for instance the LDLr [77]. Aberrant proteins destined for degradation by the proteasome in the cytosol are transported across the ER membrane with the assistance of GRP78. Under all growth conditions GRP78 is an abundant protein, but its synthesis is markedly induced under conditions that lead to the accumulation of unfolded polypeptides in the ER. This elevated expression provides a marker for those kind of disease states that result from misfolding of secretory and transmembrane proteins.

Calnexin and calreticulin are high-capacity calcium-binding proteins found in most tissues and are located at the lumenal periphery of the endoplasmic and sarcoplasmic reticulum membranes [78, 79]. They are lectin-like ER chaperones, which specifically recognise monoglucosylated *N*-linked core glycans [80]. Together with ERp57 they control glycoprotein folding and prevent misfolded protein from leaving the ER [81].

GRP94 is the ER homologue to cytosolic Hsp90. It is an abundant ER calcium-binding glycoprotein and interacts with several nascent polypeptides and presumably mediates folding of apolipoprotein B [82, 83].

Thiol oxidoreductases of the PDI family catalyse the formation and isomerisation of disulphide bonds through the transient formation of mixed disulfides with substrate proteins [84]. Moreover, PDI possesses chaperone-like activity [85]. PDI contains two redox active domains (two cystein residues each) near the N- and C-terminus that are similar to

thioredoxin. Both contribute to disulfide isomerase activity, but are functionally non-equivalent [86].

ERp57 is a member of the PDI family. In analogy with PDI it also contains two redox-active centres, which enables the protein to participate in redox reactions via the reversible oxidation of both intra- and interchain disulfide bonds [87]. In contrast to PDI, ERp57 forms complexes with calnexin and calreticulin and interacts specifically with newly synthesized glycoproteins [81].

The effect of oxidative stress on ER functionality

What does the oxidation of ER-resident proteins, which are all involved in protein folding, mean for their functionality? To investigate the influence of oxidative stress (H_2O_2) on the ER-related protein maturation, the low-density lipoprotein receptor (LDLr) was chosen as a model protein. LDLr is an endogenous protein of the rat-1 fibroblasts and its ER-related maturation involves the formation of as much as thirty disulfide bridges. The formation of these bridges inside the ER is most probably carried out by PDI, which functionality may be impaired after an oxidative challenge. In addition, disulfide bridge formation in the LDLr is a prerequisite for transportation towards the Golgi complex to complete maturation.

A requirement for oxidative protein folding and disulfide bond formation by PDI is a high redox state in the ER [35, 88]. This state is thought to be attained by an optimum concentration of oxidising and reducing equivalents in the lumen of the ER as compared to the cytosol. In the ER the relative abundance of the oxidized (GSSG) over the reduced (GSH) form of glutathione has led to the proposal that GSSG is preferentially imported from the cytosol into the ER lumen and serves as the oxidising equivalents during protein folding [35]. ($[GSSG]_{ER}/[GSH]_{ER}$ = 1:1 to 1:3 is much higher than the overall cellular ratio, which is about 1:30 to 1:100). However, it has been shown that glutathione itself is not required for oxidative protein folding in the ER [89, 90] indicating that disulfide bond formation *in vivo* relies upon different electron acceptor(s). GSH may be involved in regulating protein folding by competing with protein thiol groups for disulfide bond formation by PDI and Ero1 [89]. Redox control may be important to allow the coexistence in the ER of formation, isomerisation and reduction of disulfide bonds (reshuffling), competing reactions that are necessary for protein folding and maturation, and for the degradation of molecules that do not pass the ER quality control system [91].

The LDL-receptor

The human LDLr is an 839 amino acid transmembrane protein (type 1) that can be resolved into several structural domains (figure 4): the negatively charged cysteine-rich ligand binding repeats (class A), epidermal growth factor precursor-like repeats, cysteine-free repeats (class B), a transmembrane domain, and a cytoplasmic domain. Each structural domain serves

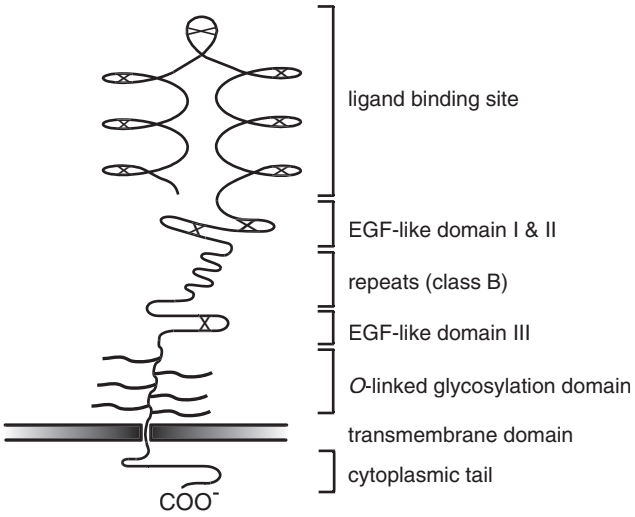

Fig. 4: **Schematic representation of the LDL receptor structure.** For details, see text.

distinct and important functions.

The extracellular amino terminal domain which is responsible for binding ligands is composed of seven imperfect repeats (~ 40 amino acids each) that are characterised by six conserved cysteine residues and a cluster of negatively charged amino acids near the carboxy terminus of each repeat [92, 93]. All the cysteines within a repeat are disulfide-linked to form a compact, stable, functionally independent motif. These acidic, cysteine-rich repeats are thought to mediate binding to apoB-100 and apoE through ionic interactions between acidic residues of the LDLr and basic residues of the ligands. Mutations in the ligand-binding domain lead to an LDLr that is defective in its interaction with apoE and apoB on LDL particles and/or in its transport to the cell surface. LDLr plays an important role in cholesterol homeostasis. Increased levels of LDL, the major carrier of cholesterol in the plasma, are considered to be an important risk factor for the development of atherosclerosis. To date, more than 600 mutations of the LDLr are reported to cause familial hypercholesterolaemia (FH) [94, 95] and premature coronary artery disease. Several different classes of mutations that disrupt synthesis, processing and transport of the LDLr have been identified in patients with the homozygous form of FH [96, 97].

A domain of about 400 amino acids that shows homology to the epidermal growth factor (EGF) precursor follows the ligand-binding domain. The three EGF like domains, each containing three disulfide bonds, are necessary for the dissociation of ligands from the receptor in endosomes [95]. Alterations to these domains can lead to impaired transport of the LDLr to the cell surface or an inability of the LDLr to bind to ligand or be recycled. The second EGF like domain is a potential calcium-binding site and calcium is required for ligand binding. The EGF-like structure is interrupted by six repeats (~ 40 amino acids each of the

class B type) between the second and third domain.

The EGF precursor homology domain is followed by a short serine and threonine rich segment (~ 70 amino acids) that becomes extensively *O*-glycosylated during maturation of the protein [98]. These glycans are not required for receptor function, but they may serve to keep the ligand binding domains away from the cell surface [99].

Furthermore, LDLr contains a single transmembrane domain (~ 20 amino acids) and a short cytoplasmic tail (~ 50 amino acids), containing an endocytosis signal at the carboxy terminus. Deletion or substitution of conserved residues in the cytoplasmic tail results in a receptor that can neither localise in clathrin-coated pits nor mediate the internalisation of ligands [100]. In addition, in contrast to signalling receptors that contain large intracellular domains all the members of the LDLr family are single transmembrane glycoproteins that contain large extracellular and comparatively shorter intracellular domains. Therefore, no enzymatic activity (like kinase) was expected for LDLr. However, a role in the activation of specific signalling pathways during the development of the mammalian brain was discovered for some family members [101].

Following synthesis, the LDL-receptor precursors are transported to the cell surface where they quickly cluster into clathrin-coated pits. When LDLr binds ApoB-100 or apoE-containing lipoproteins (like LDL) endocytosis starts with the invagination of clathrin-coated pits to form, or fuse with, endocytotic vesicles, hence called the endosome. Through the action of a proton pump in the endosomal membrane, the internal milieu acidifies and, when pH falls below 6.5, the LDLr-lipoprotein complex dissociates. By a mechanism of vesicle budding and redirection, the LDLr is recycled to the cell surface, whereas the lipoproteins are directed to lysosomes where they undergo hydrolysis. The cholesterol that is liberated triggers a number of important regulatory mechanisms including suppression of both endogenous cholesterol synthesis and the expression of the LDLr itself. Together, these series of events have been called the LDLr pathway.

Protein misfolding in aging and disease

Proteins are responsible in part for maintaining functional stability and homeostasis of cells and tissues. During aging there are many opportunities for properly transcribed peptides and proteins to become structurally altered. Accumulation of altered proteins may be correlated with a loss of function or, in some cases, a gain of inappropriate or toxic function. The ability of a protein to perform its function in the cell depends in part upon its ability to assume and retain its proper functional conformation. The proper conformation is achieved by regulated folding during synthesis, aided by chaperone proteins. However, DNA mutations, ER-stress or lack of enzyme or chaperones assistance may divert proteins from their normal folding pathways or destabilize their native state. As a consequence of misfolding a protein may be non-functional, in short supply, aggregated or unable to get to the right location. Cellular quality control machinery must recognize misfolded and/or partially folded products. Under normal circumstances defective and misfolded proteins are refolded,

aggregated or rapidly degraded. Proteins and peptides that are aggregated (for example, into amyloid plaques) or cross-linked are often resistant to degradation. The formation of these deposits, rather than the lack of native protein, may be responsible for, or contribute significantly to, cellular pathology. Misfolding may underlie several human (1) inherited diseases [102, 103], like emphysema, osteogenesis imperfecta, cystic fybrosis or familial amyloidosis (Creutzfelt-Jacob or Alzheimer's disease), (2) environmentally induced diseases, like scurvy [104], (3) old age-related diseases, like Alzheimer's disease [105] and cataract [106], and (4) infectious diseases, like prion disease [107]. Accumulation of aggregated proteins thus may play an important role in the aging process and in age-related diseases.

Scope of this thesis

This thesis starts describing the introduction of new method for the detection of protein tyrosyl radical formation (**chapter 2**). The method is based on a fluorescein-labelled tyrosine analogue, tyramine (denoted as TyrFluo). Tyramine is chemically identical to tyrosine, except for the carboxyl moiety that is lacking. Upon oxidation of the tyramine by ROS, the tyramine is converted into a tyrosyl radical that can form cross-links with an oxidized target protein. By this reaction the target protein becomes fluorescently labelled, which makes the oxidized protein detectable. In addition, immunodetection with an HRP-conjugated antibody directed to the fluorescein and subsequent ECL-detection on Western-blots shows the TyrFluo-labelling of the proteins. Treatment of TyrFluo with acetic anhydride yields an acetylated form of TyrFluo (acetylTyrFluo), which is taken up by the cells within minutes. Exposure of the cells (rat-1 fibroblasts) to oxidative stress (hydrogen peroxide) in the presence of either TyrFluo or acetylTyrFluo gave a cellular labelling characteristic for each probe.

In **Chapter 3** acetylTyrFluo is used to identify some of the major protein targets for tyrosyl radicals in normal human dermal fibroblasts (NHDF). Although previous experiments are performed on rat-1 fibroblasts it has been decided to change to NHDF for two main reasons; the greater biological relevance and the larger number of identified proteins of human as compared to rat. These protein targets show to reside in the lumen of the ER.

It was thought that (if at all) the extent of labelling of lumenal ER proteins could be prevented it would be attained by a water-soluble antioxidant. **Chapter 4** describes the experiments in which the influence of several antioxidants (GSH, GSH-ester and vitamin C), and of antioxidant enzyme inhibitors (buthionine-sulfoximine for γ-glutamylsynthetase, mercaptosuccinate for glutathione peroxidase, aminotriazole for catalase) on protein oxidation are investigated. In general, the results indicate that the degree of labelling strongly depend on the cellular GSH level.

One could wonder, "If these ER resident proteins, which are all involved in protein folding, are so sensitive to oxidation, what would that mean for the folding/maturation of proteins when a cell is in a state of oxidative stress?". Therefore, **Chapter 5** describes the pulse-chase experiments that are conducted to follow the maturation of a model protein, the low-density lipoprotein receptor (LDLr), under oxidative conditions. When cells are stressed for

10 min with 1.5 mM H_2O_2, the velocity of LDLr-maturation is strongly reduced. 2 mM completely abolishes maturation, however this can still be restored when cells are allowed to recover in culture medium. This restoration appears to depend on the ability of a cell to restore protein synthesis.

On invitation we have written a review on 'protein oxidation in aging'. In extension to the present concepts, we have hypothesized new aspects regarding the involvement of the ER in this matter (**Chapter 6**). Through the oxidation of essential cellular proteins, aging may have deleterious effects on cellular function. There are several indications that suggest the involvement of the endoplasmic reticulum (ER) in the process of aging. The age-dependent upregulation of ER stress response genes that process damaged or misfolded proteins may reflect the importance of protein oxidation for biological aging. Upregulation of these genes in aging may imply the presence of accumulated damaged and misfolded proteins in the ER caused by improper folding. Proper folding requires optimal functioning of the ER. As a consequence of oxidation of the ER Ca^{2+}-ATPases the required calcium concentration inside the lumen of the ER cannot be established, and will cause an impaired functioning of the calcium-binding chaperones. Also the cytosolic decline of glutathione (GSH) levels may have its impact in the ER thereby creating a more oxidative environment, as expressed by the low ratio of GSH/GSSG. Based on the results described in chapter 3, the contribution of oxidized sarco-/endoplasmic reticulum proteins to the accumulation of damaged and misfolded proteins in aging is proposed in this review.

Finally, the results described in this thesis are summarised and discussed in **Chapter 7**.

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Chapter 2

Detection of protein oxidation in rat-1 fibroblasts by fluorescently labelled tyramine

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Biochemistry 40 (2001), p 7783-7788

Abstract

Oxidative damage to proteins has been postulated as a major cause of various degenerative diseases including the loss of functional capacity during aging. A prominent target for oxidation by reactive oxygen species (ROS) is the tyrosine residue. Here we present a highly sensitive method for the detection of tyrosyl radical formation in cells. The method is based on the fluorescein-labelled tyrosine analogue, tyramine, which upon oxidation may couple to proteins carrying a tyrosyl radical. Coupling of the probe (denoted TyrFluo) to standard proteins could be induced by generating ROS with horse radish peroxidase/hydrogen peroxide, SIN-1 or with peroxides (cumene or hydrogen peroxide) in combination with a transition metal.

TyrFluo added to rat-1 fibroblasts remained outside the cell whereas the acetylated form (acetylTyrFluo) was membrane-permeable and accumulated in the cell. Exposure of the cells to oxidative stress in the presence of either TyrFluo or acetylTyrFluo gave a cellular labelling characteristic for each probe. Western blot analysis confirmed that each probe labelled a specific set of proteins. This new method for the detection of ROS-induced oxidation of proteins may mimic the tendency of oxidised proteins to form dityrosine bonds.

Introduction

Although oxygen is essential for life, it can give rise to a variety of reactive oxygen species (ROS)[1] like hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH\cdot$) and superoxide ($O_2^{-\cdot}$), as part of normal metabolism or produced by irradiation or environmental factors. Under normal conditions the body is able to eliminate these highly reactive compounds by means of enzymatic reduction, antioxidants (endogenous and exogenous) and metal chelators. When the level of ROS exceeds the defense mechanisms, a cell is in a state of oxidative stress. Under these circumstances proteins may become irreversibly damaged. Proteins modified by oxidation have been shown to accumulate during aging [1-5] and as a result of several degenerative diseases including inflammatory diseases [6], atherosclerosis [7, 8], neurological disorders [9], ischemia and reperfusion injury and carcinogenesis [10].

Whereas most amino acids can be oxidised, ROS-induced oxidation of, in particular, tyrosine and cysteine may have profound effects on cell function as these residues are often located in the active site of enzymes. Oxidation of essential tyrosine residues has been observed for superoxide dismutase [11] and glutathione reductase [12] resulting in their inactivation. Intermolecular cross-linking of enzymes involved in signal transduction, by dityrosine formation has been detected in cells [13, 14].

Tyrosine oxidation may lead to the formation of tyrosyl radicals. These radicals are formed by reaction with hypochlorite, peroxynitrite or by radicals formed in transition metal ion-catalysed Fenton and Haber-Weiss reactions (e.g. hydrogen peroxide/Fe^{2+}) [15-17]. Peroxidases are also important sources of tyrosyl radicals. Protein tyrosyl radicals may form intra- or intermolecular o,o'-dityrosine bonds [18]. It is known that the concentrations of o,o'-dityrosine bonds increase with aging in heart, skeletal muscle and lens proteins [19, 20]. Another oxidation product is 3-nitrotyrosine that can be introduced into proteins as a result of tyrosine nitration by peroxynitrite, a product of superoxide ($O_2^{-\cdot}$) and nitric oxide ($\cdot NO$) [21, 22], by peroxidase-catalyzed oxidation of nitrite [23] and by nitric oxide reaction with protein tyrosyl radicals [24]. Moreover, in human neutrophils the myeloperoxidase-hydrogen peroxide-chlorite system oxidizes L-tyrosine yielding 3-chlorotyrosine [25], which is also found in elevated amounts in low-density lipoprotein, isolated from human atherosclerotic intima [26].

Despite the interest in tyrosine oxidation and its effect on protein function, the identity of the proteins that are major targets for ROS-induced tyrosyl radical formation, remains to be resolved. Here we present a highly sensitive method for the detection of dityrosine formation in intact cells. The method makes use of a fluorescein-labelled tyrosine analogue (i.e. tyramine) which upon oxidation by ROS is converted into a tyrosyl radical that can form cross-links with oxidised tyrosine residues in target proteins. As a result of this coupling reaction these proteins become fluorescently labelled making them suitable for identification.

[1]) Abbreviations: ROS, reactive oxygen species; HRP, horse radish peroxidase; TyrFluo, tyramine linked to 6-(fluorescein-5-(and-6)-carboxamido) hexanoic acid; acetylTyrFluo, acetylated form of TyrFluo; SIN-1, 3-morpholinosydnonimine, hydrochloride; HBSS, Hanks' balanced salt solution; TBST, Tris-buffered saline containing 0.2% tween-20; PBS, phosphate-buffered saline

We will present data to show that this probe can be used for the detection of oxidised proteins both inside and outside the cell depending on whether the acetylated or the non-acetylated form is used.

Materials and methods

Materials

6-(fluorescein-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester and SIN-1 (3-morpholinosydnonimine, hydrochloride) were purchased from Molecular Probes Europe (Leiden, The Netherlands). Tyramine and HBSS (Hanks' Balanced Salt Solution) were from Sigma-Aldrich (Bornem, Belgium). Anti-fluorescein (HRP-conjugated polyclonal antibody against fluorescein) was from Biogenesis (Poole, UK), SDS-PAGE low range molecular weight standards from Bio-Rad (Veenendaal, The Netherlands) and Protifar, a low-fat milk powder, from Nutricia (Zoetermeer, The Netherlands). Rat-liver pre-nsL-TP expressed in *Escherichia Coli* was purified according to ref 28. The mounting solution Mowiol was from Hoechst (Frankfurt am Main, Germany).

Synthesis of the Tyramine - Fluorescein conjugates

Both tyramine (1 mg) and the succinimidyl ester of 6-(fluorescein-5-(and-6)-carboxamido) hexanoic acid (1 mg) were dissolved in dimethylformamide (50 µl) and then added to 0.2 M bicine, pH 8.5 (0.2 ml). The coupling reaction was performed at room temp for 2 h. Synthesis of the conjugate was confirmed by thin layer chromatography using an alkaline solvent (chloroform / methanol / 25% ammonia / water = 90 / 54 / 5.5 / 5.5 v/v). Chloroform / methanol / 25% ammonia (2 / 1 / 0.5 v/v) was added to the reaction mixture giving phase separation. The conjugate TyrFluo (Fig.1) was present in the water phase whereas free tyramine was dissolved in the chloroform phase. The water phase was removed by vacuum evaporation in a rotary evaporator. TyrFluo was taken up either in water or in tetrahydrofurane. TyrFluo in tetrahydrofurane was acetylated by adding 4-(dimethylamino)-pyridine (0.5 mg) and acetic anhydride (3 µl) yielding acetylTyrFluo (Fig 1). As a result of acetylation the solution became colourless. Ethanol was added to react with the excess of acetic anhydride. The concentrations of both conjugates were determined spectrophotometrically (ε_{495} = 73,000 $M^{-1} \cdot cm^{-1}$) in 1 M NaOH.

Labelling of standard proteins

SDS-PAGE low range molecular weight standards (6 µg/protein) were dissolved in PBS (pH 7.4). In the presence of TyrFluo (5 µM) the mixture was oxidised for 1 h at 37°C by addition of CumOOH/hemin (2 mM), H_2O_2 /Fe^{2+} (0.1 mM), SIN-1 or HRP/H_2O_2 (10 mM) in a final volume of 20 µl in the presence or absence of 500µM tyramine. The reaction was stopped by adding 20 µl of sample buffer (100 mM Tris-HCl (pH 7.4), 100 mM DTT, 2% SDS, 20% (v/v) glycerol, bromophenolblue) and the proteins were denatured at 95°C for 5 min.

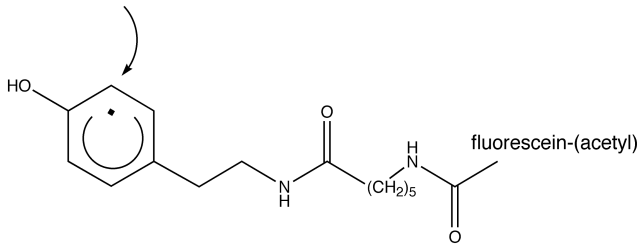

Fig 1. **Tyramine - fluorescein conjugates (TyrFluo/acetylTyrFluo)**. TyrFluo consists of tyramine linked to 6-(fluorescein-5-(and-6)-carboxamido) hexanoic acid. AcetylTyrFluo is the acetylated form of TyrFluo. The arrow indicates the site of oxidative attack by ROS. The radical may form an *o,o'*-dityrosine bond with tyrosyl radicals of proteins.

Labelling of proteins in rat-1 fibroblasts

Rat-1 fibroblasts were cultured to subconfluency (density of 80%) on plastic petri dishes (150 mm^2) in Dulbecco's Modified Eagle Medium containing 7.5% (v/v) fetal calf serum. Cells were washed twice with Hanks' balanced salt solution (HBSS). The cells were preloaded with acetylTyrFluo (5 mM) in 5 ml HBSS/10mM Tris-HCl (pH 7.4) for 10 min at 37°C and washed gently with HBSS. Then the cells were submitted to stress by adding 100 μM CumOOH/0.1 μM hemin or 100 μM H_2O_2 in 5 ml HBSS for 10 min at 37°C so as to label the intracellular proteins. Under the same conditions, the cells were submitted to stress (0.2 mU HRP/ 1 μM H_2O_2) in the presence of TyrFluo (3 mM) so as to label extracellular proteins. At the end of the labelling cells were washed with PBS and prepared for fluorescence microscopy. Cells were fixed with 4% paraformaldehyde/0.1% Triton X-100 in PBS and washed four times with PBS to remove non-covalently bound probe. After the last wash with water the fixed cells were mounted in Mowiol. For the analysis of labelled proteins by SDS-PAGE sample buffer (150 μl) was added to the cells. After transfer to Eppendorf tubes the proteins were denatured at 95°C for 5 min.

Immunodetection of labelled proteins

Aliquots of the cellular protein (15 ml) and the standard proteins (20 ml) were run on 10 % SDS-PAGE at 200 V until the dye front reached the bottom of the gel [27]. Proteins were blotted on nitrocellulose at a constant current of 54 mA (1.2 mA/cm^2) using a semidry system. Transfer of protein was checked by staining with Ponceau-S. After thorough washing with TBST (Tris-buffered saline containing 0.2% Tween-20) and TBST/0.2% Protifar, blocking was performed with TBST/2% Protifar. Immunodetection of labelled proteins was performed with an HRP-conjugated polyclonal antibody against fluorescein (1:1000 (v/v) in TBST/0.2 % Protifar) for 1 h at room temperature followed by Enhanced Chemiluminescent (Amersham) detection according to instructions. Labelled proteins were visualised by exposing Hyperfilm MP (Amersham) to the blot for 2-5 min.

Results

Labelling of standard proteins

The ability of TyrFluo to couple to proteins was established by measuring its crosslinking efficiency to a set of standard proteins under various oxidative conditions. As shown in figure 2 (panel A) each of the five proteins present were covalently linked to TyrFluo upon exposure to H_2O_2/Fe^{2+} (lane 1), to SIN-1 (lane 2) or HRP/H_2O_2 (lane 3).

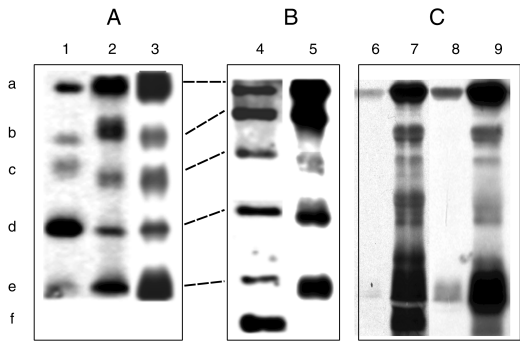

Fig 2: **Exposure of protein standards to ROS in the presence of TyrFluo (5 μM) labels all proteins**. Panel A: Proteins a to e after exposure to either 100 μM H_2O_2/100 nM Fe2+ (lane 1), 100 μM SIN-1 (lane 2) or to 16 nU HRP/10 μM H_2O_2 (lane 3). Panel B: Proteins a to f are exposed to 100 μM CumOOH/100 nM hemin. Prior to detection of TyrFluo-labelled proteins with peroxidase-coupled anti-fluorescein antibody (lane 5) proteins were stained with Ponceau-S (lane 4). Panel C: Proteins a to f after exposure to 100 μM SIN-1 (lane 6 and 7) or to 16 nU HRP/10 μM H_2O_2 (lane 8 and 9) in the presence or absence of 500 μM tyramine. a) phosphorylase B, b) bovine serum albumin, c) ovalbumin, d) carbonic anhydrase, e) trypsin inhibitor, and f) pre-nsLTP

Under non-oxidative conditions no labelling by TyrFluo of any of the proteins present was observed (data not shown). In another experiment a protein lacking tyrosine (pre-nsL-TP) [28] was added to the standard proteins and in the presence of TyrFluo exposed to CumOOH/hemin. As shown in panel B all proteins, except pre-nsL-TP, were labelled suggesting that labelling involved the formation of a dityrosine bond between the proteins and TyrFluo as a result of ROS-induced tyrosyl radical formation. In agreement with this the labelling efficiency was negligible when the experiments were carried out in the presence of an excess of free tyramine (100 fold compared to TyrFluo; panel C, lane 6 to 9). At certain conditions of oxidation labelling of pre-nsL-TP was observed (panel C lane 7). This implies that in this case TyrFluo has formed a bond with an amino acid different from tyrosine. It is known that hydroxyl radicals may convert phenylalanine into *o*-tyrosine and *m*-tyrosine [7], which may then react with oxidised TyrFluo.

The labelling efficiency differs for the various proteins and varies with the oxidant used (see Fig. 2). This has been further investigated by exposing these proteins to increasing concentrations of the oxidants (Fig. 3). Under most conditions phosphorylase B was most extensively labelled in line with this protein having the highest number of tyrosine residues

(i.e. a total of 36). For comparison, bovine serum albumin, ovalbumin, carbonic anhydrase and trypsin inhibitor have 18, 10, 7 and 4 tyrosine residues, respectively. In general, labelling of these proteins was lower. However, when H_2O_2 was present, carbonic anhydrase was a preferred target (panel A and C) (see Discussion). Labelling of the proteins increased with increasing ROS concentration. In some instances labelling reached a plateau (panel B) suggesting that the number of tyrosine residues modified was maximal. In other instances labelling decreases at high ROS concentrations (panel A and D). This may be due to dityrosine formation between probe molecules, thereby making TyrFluo unavailable for reaction with the proteins, and/or to oxidative damage of the fluorescein moiety thereby interfering with the immunodetection.

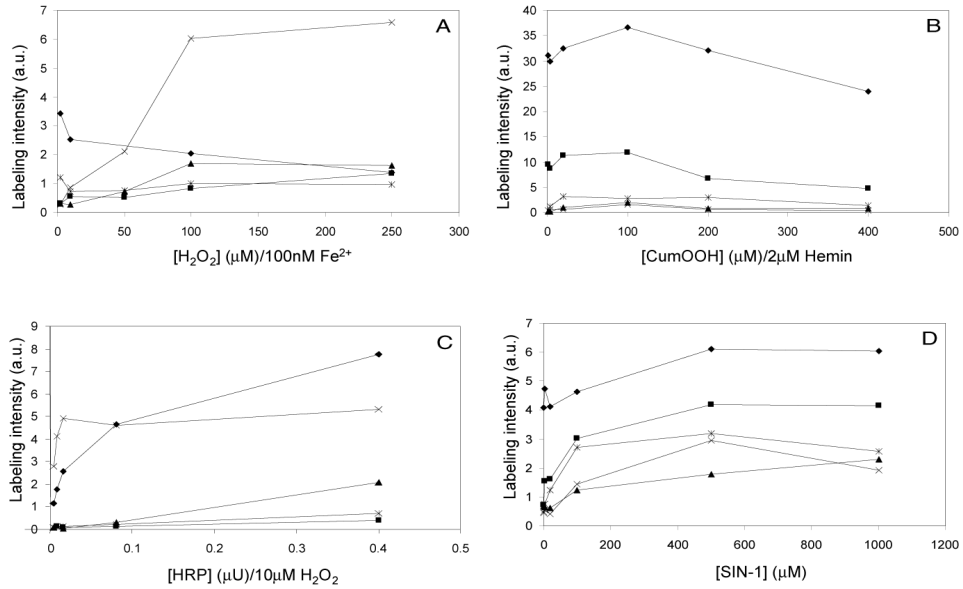

Fig 3: **Labelling of proteins as a function of ROS concentration.** In the presence of TyrFluo (5 µM) the proteins (6 mg each) were exposed to increasing concentrations of H_2O_2/100 nM Fe2+ (panel A), cumene hydroperoxide/2 µM hemin (panel B), HRP/10 µM H_2O_2 (panel C) and SIN-1 (panel D) for 1 h at 37°C. The intensity indicated was determined by densitometer. ◆ phosphorylase B; ▲ ovalbumine; ■ bovine serum albumin; × carbonic anhydrase; ✳ trypsin inhibitor

Labelling of proteins in rat-1 fibroblasts

Addition of the probes to cells shows that TyrFluo was outside the cell (Fig 4, panel A) whereas acetylTyrFluo was membrane permeable accumulating inside the cell (panel B). In the latter case the probe may be deacetylated preventing it from leaving the cell. This different localisation has been further explored by exposing cells to HRP/H_2O_2 in the presence of TyrFluo so as to generate tyrosyl radicals extracellularly. After fixation of the cells a staining pattern was observed which may represent proteins labelled at the plasma membrane (panel C). The punctated pattern may indicate that labelled protein has been internalized by

endocytosis. When cells were preincubated with acetylTyrFluo and then exposed to CumOOH/hemin so as to generate tyrosyl radicals inside the cell (panel D) a completely different labelling was observed representing the tyrosylation of intracellular proteins (panel D). Stressing the cells by addition of H_2O_2 gave a staining comparable to that of CumOOH/hemin (results not shown). Surprisingly, despite the localisation of the acetylTyrFluo in the nucleus (panel B) the labelling of nuclear proteins was very little (panel D). This suggests that the nucleus may be well protected against radical production including tyrosyl radicals.

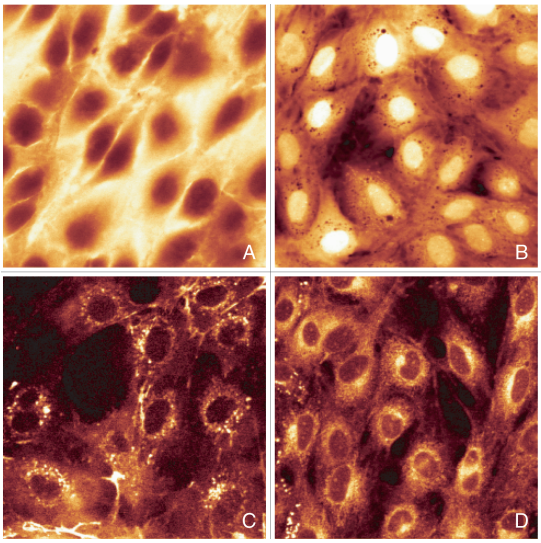

Fig 4: **Fluorescent micrographs of rat-1 fibroblasts before and after exposure to oxidative stress in the presence of TyrFluo or acetylTyrFluo.** Cells were incubated with TyrFluo (panel A) and then stressed with 0.2mU HRP/1µM H_2O_2 (panel C) or the cells were preincubated with acetylTyrFluo (panel B) and then challenged with 100 µM CumOOH/100 nM hemin (panel D). Incubations were for 15 min at 37°C. Labelling of proteins was visualized after removal of non-covalently bound probe (panel C, D). The cells were washed with PBS. Proteins were fixed with 4% paraformaldehyde/0.1% Triton X-100 in PBS. (Colour image on the backside of this thesis).

Identical experiments were carried out to obtain labelled proteins to be analyzed by Western blotting using an antibody against the fluorescein moiety. As shown in figure 5, stressing the fibroblasts preloaded with acetylTyrFluo, with both H_2O_2 (lane 2) and CumOOH/hemin (lane 3) gave rise to the labelling of a distinct set of proteins. As one would expect these proteins are different from the proteins labelled from the outside when the cells are exposed to HRP/H_2O_2 in the presence of TyrFluo (lane 5). Under both labelling conditions the tyrosylated proteins do not coincide with the overall protein staining (lane 1,

4). Some proteins that were heavily labelled (see arrows in Fig. 5) were barely detectable by protein staining.

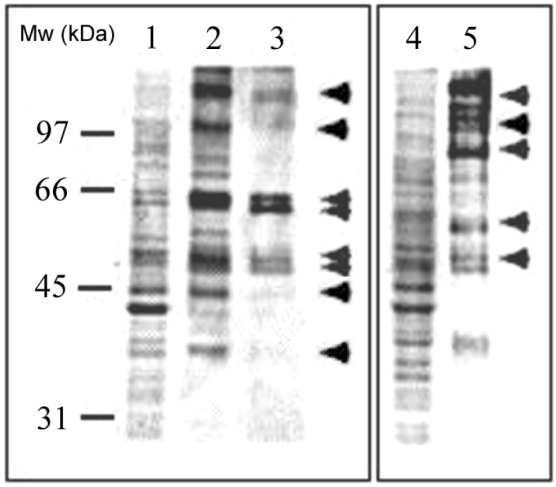

Fig 5: **Exposure of rat-1 fibroblasts to different oxidants in the presence of acetylTyrFluo or TyrFluo leads to a characteristic pattern of protein labelling.** In the presence of acetylTyrFluo cells were exposed to 100 μM H_2O_2 (lane 2) or to 100 μM CumOOH/100 nM hemin (lane 3) In the presence of TyrFluo cells were exposed to 0.2 mU HRP/ 1 μM H_2O_2 (lane 5). Overall protein staining by Ponceau-S (lane 1, 4). The arrows indicate the major proteins labelled.

Discussion

To date, the detection of dityrosine formation as a measure of protein oxidation has been carried out by HPLC [6, 29], mass spectrometry [7] and time-resolved fluorescence studies [30]. Here we present a novel sensitive method for the detection of ROS-induced protein tyrosylation in cells. In this method use is made of a probe consisting of a tyramine moiety covalently coupled to a fluorescein moiety. Tyramine resembles tyrosine except that the carboxyl moiety is lacking. In the presence of ROS both tyramine and tyrosine as part of proteins may become oxidised enabling the tyrosyl radicals generated to form an o,o'-dityrosine bond between probe and protein (here designated as protein tyrosylation). The presence of fluorescein makes that the tyrosylated proteins can be visualized in cells by fluorescence microscopy and on a blot by immunodetection using an anti-fluorescein antibody. In the present study we have used TyrFluo to label cell surface proteins. Acetylation of TyrFluo makes the probe membrane permeable so as to label intracellular proteins. Upon uptake into the cell acetylTyrFluo becomes deacetylated by esterases ensuring that the probe is present in the cell during ROS-induced oxidation [31].

To determine the sensitivity for tyrosylation a set of standard proteins (phosphorylase B,

bovine serum albumin, ovalbumin, carbonic anhydrase II, trypsin inhibitor) was exposed to various oxidative conditions (H_2O_2/Fe^{2+}, SIN-1, HRP/H_2O_2, and CumOOH/hemin) in the presence of TyrFluo. Under each of these conditions, the proteins became labelled indicating that both tyramine and tyrosine residues were converted into tyrosyl radicals and available to form an o,o'-dityrosine bond. Tyrosyl radical formation implies that each of the oxidative conditions used is suited to abstract a hydrogen atom from the aromatic ring. When these reactions were carried out in the presence of an excess of free tyramine (100 fold compared to TyrFluo) the labelling efficiency was greatly reduced showing an effective competition between free tyramine and TyrFluo for the tyrosyl radicals on the protein.

Under most conditions phosphorylase B was most extensively labelled in line with this protein having the highest number of tyrosine residues. In contrast, ovalbumin, a multimeric protein containing 40 tyrosine residues (divided over four identical subunits) was much less labelled (fig 2). A determining factor will be the susceptibility of tyrosine residues to become oxidised that may differ for each protein. The number of tyrosine residues that are solvent exposed can be estimated for each protein from its 3D-structure using the Swiss PDB Viewer. For example, if the fraction of the tyrosine surface exposed is set at 15%, phosphorylase B exposes 14 tyrosine residue as compared to 6 for bovine serum albumin, 3 for carbonic anhydrase, 1 for trypsin inhibitor and 1 for ovalbumin subunit. At this limit (set arbitrarily) the extent of labelling agrees reasonably well with the number of tyrosine residues (cf. Fig. 3). Although the exposure to the solvent must be a factor, in the end it is the ability of the TyrFluo radical to interact with the tyrosyl radical on the protein that determines whether or not labelling occurs. Moreover, the oxidation condition itself may have an effect on the extent of labelling as seen with carbonic anhydrase treated with H_2O_2 (see Fig. 3). A special feature of carbonic anhydrase is its zinc-binding domain. This domain may be involved in the formation of hydroxyl radicals from H_2O_2 by the Fenton reaction, thereby enhancing the extent of labelling [32]. It remains to be established whether the labelling by TyrFluo is restricted to o,o'-dityrosine bond formation. Multiple tyrosylations on one tyrosine residue have been reported yielding trityrosine, iso-dityrosine or pulcherosine [33], which may play a role in the observed difference in labelling efficiency of the proteins. Moreover, hydroxyl radicals may convert phenylalanine into o-tyrosine and m-tyrosine. This may explain that under certain conditions pre-nsL-TP, void of tyrosine residues, becomes labelled.

Exposure of rat-1 fibroblasts to oxidative stress in the presence of either membrane-impermeable TyrFluo or membrane-permeable acetylTyrFluo gave a cellular labelling characteristic for each probe (Fig. 4, panel C,D). Provided labelling by TyrFluo is restricted to plasma membrane proteins exposed to the medium, one may assume that in time these labelled proteins are taken up by endocytosis [34]. This may explain the punctuated labelling pattern representing labelled endosomes (panel C). Western blot analysis confirmed that upon oxidative stress each probe labelled a characteristic set of proteins (Fig. 5). And that also here the presence of an oxidant is a prerequisite for protein labelling. In agreement with what we have observed with the standard proteins the labelling efficiency of each cellular protein was different most likely reflecting the susceptibility to oxidation by ROS. In addition, the tyrosyl

radical formed must be accessible to the oxidized probe. The fact that H_2O_2 added to cells, is sufficient to cause a labelling of proteins may be explained by the presence of intracellular transition metals converting H_2O_2 into hydroxyl radicals [16]. Another possibility is that H_2O_2 may serve as a substrate for intracellular peroxidases and, when oxidized, readily utilizes tyrosine as a reducing substrate and yield a tyrosyl radical. ROS-induced labelling of proteins by TyrFluo may mimic the tendency of oxidized proteins to form intermolecular dityrosine bonds. We have to await the identification of the labelled proteins to establish which proteins are particularly sensitive to ROS-induced oxidation. This identification may give further insight in the effects of their oxidation and subsequent loss of function as it relates to cellular disorders and aging.

Acknowledgment

We thank Ginette Ploeger and Elke Pröbst-Biegelman for their contributions to this study.

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Chapter 3

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

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Biochemical Journal 366 (2002), p 825-830

Abstract

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 (D. van der Vlies *et al.* Biochemistry 40 (2001) 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 2D-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 (ER60/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 a co-localisation of TyrFluo-labelled proteins and the KDEL-receptor ERD-2, a marker for the endoplasmatic reticulum.

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], ischemia and reperfusion injury [5], carcinogenesis [6] and ageing [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. hydrogen peroxide/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-hydrogen peroxide 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 ageing [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-cysteinyl-DOPA [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 hydrogen peroxide 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 (GAR) IgG was from Sigma (St. Louis, USA). Anti-fluorescein-HRP (horseradish-peroxidase conjugated polyclonal antibody against fluorescein)

was purchased from Biogenesis (Poole, UK). The antibody against the endoplasmic reticulum membrane receptor ERD2 was kindly provided by Dr. J. Klumperman (Department of Cell Biology, Faculty of Medicine, Utrecht University). The succinimidyl ester of Cy3-sulphoindocyanine was from Amersham (Buckinghamshire, UK). Urea, thiourea, tributylphosphine, sulphobetaine 3-14 (N-tetradecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate) and HBSS (Hanks' Balanced Salt Solution) were purchased from Sigma. CHAPS was from ICN Biomedicals Inc. (Aurora, USA) and the Bio-Lyte 3/10 Ampholytes from Bio-Rad (Hercules, USA). Ampholytes with pH range 7-9 and 9-11 were from Serva (Heidelberg, Germany). Centriplus concentrators were purchased from Millipore (Bedford USA). 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 (NHDF) were obtained from Biowhittaker (Walkersville, USA) and cultured to a cell density of 80% on plastic Petri dishes in Dulbecco's Modified Eagle Medium (DMEM) containing 7.5% (v/v) fetal calf serum (FCS) in the presence of penicillin and streptomycin.

Oxidation of cells

For the labelling of intracellular proteins, NHDF were pre-loaded with 5 mM acetylTyrFluo in Hanks' Balanced Salt Solution (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 (50 μM – 3.2 mM) in HBBS for 10 min at 37°C. Denaturing buffer (100 mM Tris-HCl pH 7.4, 100 mM DTT, 2% SDS, 20% (v/v) glycerol, bromephenolblue) was applied directly onto the cells, proteins were collected and submitted to SDS-PAGE. In case of 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 mg/ml leupeptin, 1 mg/ml aprotinin, 0.1% SDS, 1% Triton-X100) was added to the cells. After scraping the proteins were collected in Eppendorf tubes and sonicated (10 seconds 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 Mw cut-off of 10 kDa. After lyophilisation, proteins were dissolved in a 2D-PAGE buffer (40 mM Tris, pH 9.5, 5 M urea, 2 M thiourea, 10 mM tributylphosphine, 2% sulphobetaine 3-14, 2% CHAPS, 0.5% ampholytes 3-10).

Lactate Dehydrogenase (LDH) release assay

NHDF were grown on 96-wells plate (15,000 cells/well) as described above, washed with PBS^{+++} (containing 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 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 Na-pyruvate, 1 mg NADH per ml 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 and the total LDH-activity (medium *plus* cell lysate).

[35S]-Methionine labelling of NHDF proteins

NHDF were cultured for 20 h in DMEM/FCS containing 50 µCi of [^{35}S]-methionine (Amersham, UK) per 0.1 ml of medium. At the end of the labelling period the cells were collected by trypsinisation and centrifugation and resuspended in lysis solution (9.8 M urea, 2% NP40, 2% ampholytes pH 7-9, 100 mM DTT).

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

TyrFluo-labelled proteins (~50 µg) were mixed with [^{35}S]-methionine labelled proteins (~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) for 24 h at a constant current of 130 mA using a wet system (Bio-Rad Trans-Blot Cell). Blots were washed with demineralised water, air-dried and autoradiography was performed by exposing Hyperfilm MP (Amersham) 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 x 30 min). TyrFluo-labelled proteins were detected with anti-fluorescein-HRP (1:1000 in TBST/2% Protifar) and subsequent Enhanced Chemiluminescent (ECL) according to the manufacturers (Amersham) instructions. Labelled proteins were visualized by exposing Hyperfilm MP (Amersham) 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 the Molecular Analyst Software (Bio-Rad). By using the 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 (http://www.biobase.dk/cgi-bin/celis) [31]. Proteins in these maps have been identified using one or a combination of procedures that include Edman degradation, mass spectrometry (Brukker Biflex III MALDI TOF) and immunoblotting with specific antibodies.

Confocal laser scanning microscopy

NHDF were cultured on glass cover slips 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 neutralised with 50 mM ammoniumchloride

in PBS (3 x 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 washing with blocking solution (4 x), the cells were incubated with the fluorescently labelled second antibody GAR-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) cells were washed three times with PBS and once with demineralised 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 excitation source. TyrFluo-labelled proteins were excited with the 488 nm laser line and the emission was detected using a 530/30 bandpass filter. The Cy3-labelled antibody was excited with the 568 nm laser line and the emission was detected using a 560 nm longpass filter followed by a 600/30 bandpass filter.

Results

Labelling of intracellular proteins

NHDF were incubated with acetylTyrFluo and subjected to increasing concentrations of H_2O_2 for 10 min. As shown in figure 1B a distinct set of proteins (Mw 50-150 kDa) was labelled with acetylTyrFluo. Labelling was already observed at 50 µM H_2O_2 and increased with increasing concentrations of H_2O_2. It appears that even at the highest concentrations used this set of labelled proteins remained the same. Proteins that are most abundantly present (figure 1A) are different from the ones that are 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.

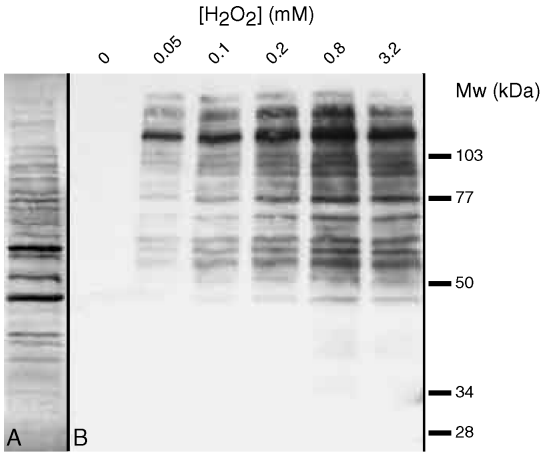

Fig 1: **Labelling of human fibroblast proteins as a function of H_2O_2.** Western blot of acetylTyrFluo-labelled proteins after exposure of NHDF to increasing concentrations of H_2O_2 (Panel B). Protein-staining by Ponceau-S (Panel A).

The sensitivity of NHDF 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 hrs 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).

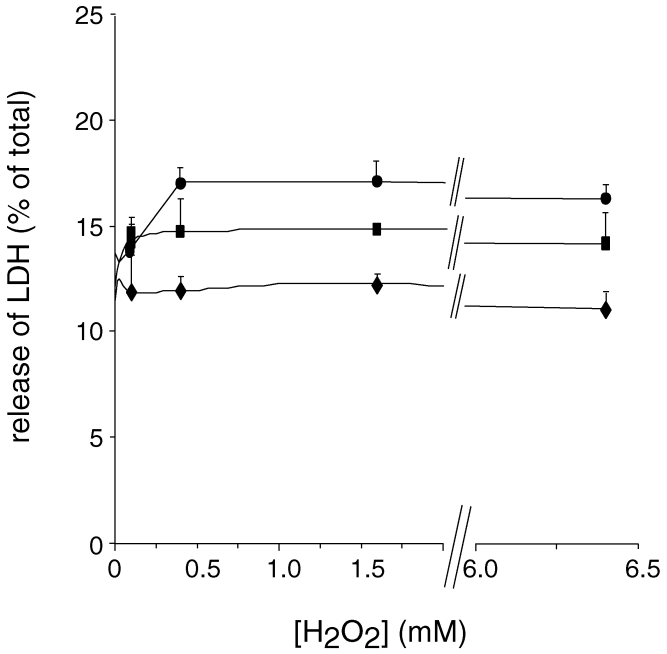

Fig 2: **Lactate dehydrogenase release of human fibroblast as a function of H_2O_2 and time of exposure.** ◆ 10 min, ■ 60 min, ●120 min; values ± SD (n = 3).

Identification of labelled proteins

The immunoblot of the TyrFluo-labelled proteins shows that at least ten 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 NHDF (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 (a), endoplasmin/GRP94 (b), BiP/GRP78 (c), PDI (f) and ER60/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 represent. 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 localisation TyrFluo-labelled cells were fixed and incubated with an antibody against the KDEL-receptor ERD-2. As shown in figure 4, a nearly perfect co-localisation (panel C) was observed between the TyrFluo-labelled proteins (panel B) and the ER marker (panel D).

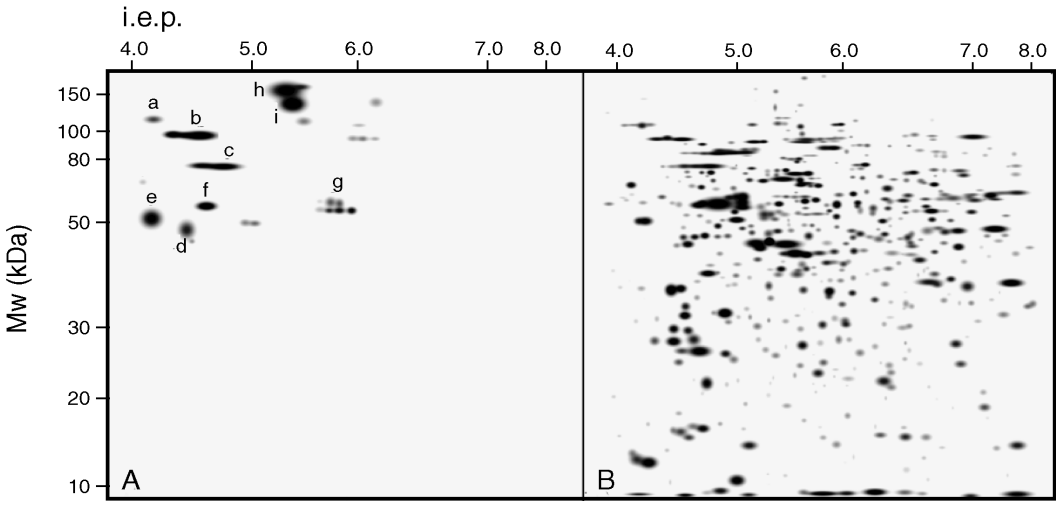

Fig 3: **Identification of TyrFluo-labelled proteins.** Immunoblot of TyrFluo-labelled proteins (A) and the autoradiogram of the [^{35}S]-methionine labeled proteins (B) were matched with our mastermap of the human MRC-5 fibroblast (see experimental procedures).
a = calnexin, b = endoplasmin/GRP94, c = BiP/GRP78, f = PDI, g = ER60/GRP58. Proteins d, e, h and i could not be matched.

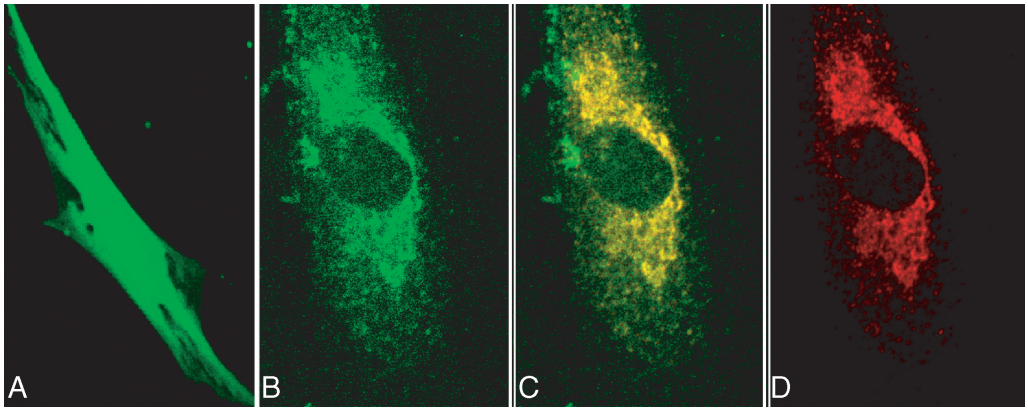

Fig 4: **Co-localisation of TyrFluo-labeled proteins with the KDEL-receptor.** Cells were loaded with acetylTyrFluo (panel A) and exposed to 100 µM H_2O_2. After exposure the cells were fixed and labeled with anti-ERD2 and GAR-Cy3 antibody. TyrFluo-labeled proteins (panel B), anti-ERD-2 staining (panel D) and the co-localisation (panel C). (Colour image on the backside of this thesis).

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 mM 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 one percent (unpublished results). 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-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 were associated with the ER. These included BiP/GRP78 (immunoglobulin heavy chain binding protein or 78 kDa glucose-regulated protein), calnexin, endoplasmin/GRP94, protein disulphide isomerase (PDI), and protein disulfide isomerase ER-60 precursor (also known as Erp60/58kDa microsomal protein/p58/GRP58/Erp57) 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 recognises 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 anti-oxidant enzymes. Thus superoxide anion produced in the respiratory chain is converted by Mn-SOD whereas H_2O_2 produced in the peroxisomal b-oxidation is degraded by catalase. Such a defence mechanism has not been described for the ER and may explain as to why proteins of the ER are susceptible to oxidation by H_2O_2. The observation that mainly the ER proteins 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· for proteins and TyrFluo to become oxidized. Given the very short lifetime of the OH· we infer that the radical formation occurs in the ER. Here H_2O_2 may be converted into OH· 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 utilising *tert-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 $OH\cdot$ 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, disulfide 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 to the reduced (GSH) form of glutathione has led to the proposal that GSSG serves as the oxidising equivalent during protein folding [41]. The ratio [GSSG]/[GSH] in the ER is 1:1 to 1:3 as compared to 1:30 to 1:100 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 ER60/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-SOD 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 an 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, an improper functioning of the ER can have large consequences for the cell. In this connection 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.

Acknowledgements

The authors wish to 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 NWO and the Technology Program of the Ministry of Economic Affairs.

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Chapter 4

Modulation of protein oxidation by altering the cellular anti-oxidative capacities

Detection with acetyl-TyrFluo

Dennis van der Vlies, Esther Kroneman, Karel W.A. Wirtz

Abstract

Subjecting cells to oxidative stress, in the presence of the membrane-permeable acetylTyrFluo probe, labelled proteins, with the endoplasmic reticulum (ER) resident proteins as the most sensitive ones. In the present study the cellular antioxidative capacity was altered so as to establish whether hydrogen peroxide-induced protein oxidation was affected. Changing the glutathione (GSH) concentration by pre-incubation of rat-1 fibroblasts with GSH or GSH-monoethylesther decreased the labelling, whereas inhibition of GSH synthesis by buthionine-sulfoximine (BSO) caused an increase (both on SDS-PAGE as well as on 2D-PAGE). This increase was also found when glutathione peroxidase was inhibited by mercaptosuccinate. Moreover, when scavenging of H_2O_2 was impaired by inhibition of catalase (by aminotriazole) the extent of protein oxidation was also positively affected. In addition, the antioxidant vitamin C (and not vitamin E) showed a protective effect on protein oxidation. On 2D-PAGE, a similar but less intense labelling pattern was observed when cells were oxidized with cumene hydroperoxide instead of hydrogen peroxide. These results indicate that GSH levels have a large influence on the degree of labelling and that lumenal ER-protein oxidation depends on the water-solubility of the oxidant and of the antioxidant used.

Introduction

Cells undergoing oxidative stress are characterised by an alteration in the amount of reducing and oxidising equivalents (redox status); oxidising equivalents (i.e., glutathione disulfide (GSSG), cystine, $NADP^+$, $thioredoxin_{ox}$) are abnormally high or reducing equivalents (i.e., glutathione (GSH), cysteine, NADPH, $thioredoxin_{red}$) are abnormally low. Oxidative stress results in the formation of GSSG at the expense of GSH [1]. 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 would clearly be deleterious. 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 [2, 3]. Protein conformation is also dependent on the cell's redox status. Changes in oxidized or reduced GSH concentrations can lead to the abnormal oxidation or reduction of thiol residues involved in the formation of disulfide bridges in proteins [4]. Therefore, maintaining homeostatic concentrations of GSH (and GSSG) is necessary for normal cellular function.

Subjecting cells to oxidative stress, in the presence of the membrane-permeable acetylTyrFluo probe, labelled proteins with the endoplasmic reticulum resident proteins as the most sensitive ones [5, 6]. In the present study the intracellular GSH concentration was altered so as to establish whether H_2O_2-induced protein oxidation was affected. This was achieved by pre-incubation of rat-1 fibroblasts with GSH or GSH-monoethylesther to increase the cellular GSH level, with buthionine-sulfoximine (BSO), an inhibitor of g-glutamyl-cysteine synthetase [7], to impair the synthesis of GSH or with mercaptosuccinate (MS), an inhibitor of glutathione peroxidase [8] to impair the break down of H_2O_2. In additon, catalase was inhibited by aminotriazole [9] to determine to what extent the scavenging of H_2O_2 affected protein oxidation. The cells were also pre-incubated with vitamin C to establish whether protein oxidation is affected by this water-soluble antioxidant.

Materials and methods

Rat-1 fibroblasts were cultured for 48 hours on plastic Petri dishes (20 cm^2) in DMEM containing 7.5% (v/v) fetal calf serum till subconfluency (± 10^6 cells ≈ 80% density). Cells were pre-incubated with the compound of interest; GSH, GSH-monoethylester, BSO, mercapto-succinic acid, amino-triazole, (all obtained from Sigma) or vitamin C (obtained from Merck) (for details see the legends to the figures), then loaded with 5 µM acetylTyrFluo (10 min in HBSS/20 mM Tris-HCl (pH 7.4) and stressed with 40 µM H_2O_2 for 10 min in HBSS. SDS-PAGE and detection were performed as described before. In addition, 2D SDS-PAGE was performed (as described before) to compare protein labelling of cells subjected to 500 µM cumene hydroperoxide (CumOOH; stock solutions of 100 mM in ethanol) or to 40 µM H_2O_2. In the latter case with or without pre-incubation of the cells with 10 µM BSO.

Results and discussion

With a fixed concentration of acTyrFluo present (loading 5 µM for 10 min in HBSS/20 mM Tris 7.4) cells were subjected to a concentration series of H_2O_2 (figure 1A). Labelling steadily increases up to a maximum level at 160 µM H_2O_2. Above 160 µM it is possible that acTyrFluo becomes the limiting factor. To be able to measure an effect on protein labelling by the compounds to be tested the H_2O_2 concentration used should be in the log phase of the sigmoidal dose-response curve (signal of labelling as a function of concentration). It was estimated at 40 µM H_2O_2 is in the correct range to obtain reliable results. The turnover of oxidized proteins labelled with TyrFluo was investigated by re-culturing cells overnight in culture medium (figure 1B lane 2). A general reduction in labelling was observed indicating that oxidized proteins are removed or degraded. It is to be noted that the labelled proteins of high molecular weight are removed to a larger extent than those of low molecular weight suggesting that the process of degradation discriminates in favour of large oxidized proteins. To find out whether acTyrFluo leaks out and/or is actively removed from the cell it was determined whether proteins could still be labelled when there was a period of 16 hours on culture medium (o/n) between the loading of the probe and the oxidation induced by H_2O_2. The complete absence of labelling (lane 3) indicates that the probe indeed has been removed from the cell during this period.

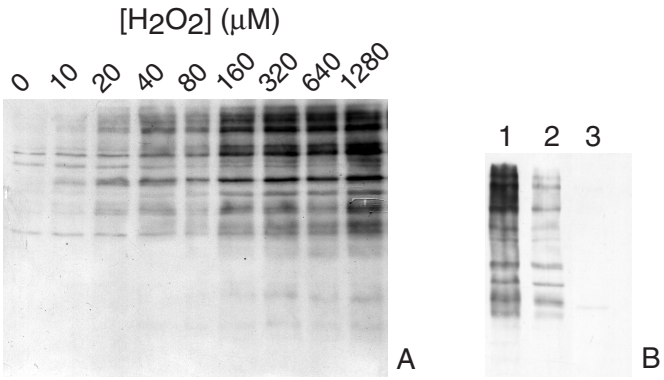

Fig 1: **Western-blots of TyrFluo labelled proteins**
A: Dose-response: 5 µM acTyrFluo → conc series H_2O_2
B: 10 µM acTyrFluo → 40 µM H_2O_2 (lane 1); 10 µM acTyrFluo → 40 µM H_2O_2 → 16h DMEM/FCS (lane 2); 10 µM acTyrFluo → o/n DMEM/FCS → 40 µM H_2O_2 (lane 3)
Arrows indicate consecutive standard procedures.

Figure 2 shows that pre-incubation with both GSH (panel A) as well as its ester (panel B) has a strong effect on the degree of oxidation, particularly when added at mmol range. The intracellular level of GSH is also in the mmol range. Hence pre-incubation of the cells with relatively low concentrations probably does not increase this level to the extent that a pronounced effect on protein oxidation is observed.

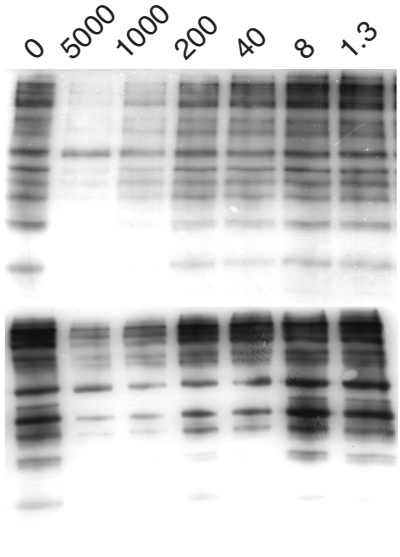

Fig 2: **Effect of glutathione and glutathione-monoethylester on protein labelling.** Cells were pre-incubated with GSH (A) or GSH-monoethylester (B) for 4 h in culture medium (indicated concentration in µM), before loading with acTyrFluo and subsequent oxidation with H_2O_2.

The synthesis of GSH was reduced by an overnight incubation of the cells with

BSO, which inhibits the rate-limiting step catalysed by g-glutamylcysteine synthetase. Figure 3 shows that a low concentration of BSO already caused an increased labelling. When the pre-incubation period was reduced to 2 h an increased labelling was not observed (result not shown). This suggests that there is an excess of GSH. Apparently, a prolonged BSO treatment is required to reduce GSH to such a concentration that is has a positive effect on protein oxidation. When this BSO-pre-incubated sample was run on 2D-PAGE (figure 4C) it appeared that the pattern of oxidized proteins showed strong similarities with its control (figure 4B) but with an increased labelling intensity. On the other hand, also some additional proteins were labelled (as indicated by the arrows in figure 4C). Unfortunately, the identity of these proteins is presently unknown. The labelled proteins in the control were identified as ER resident (chapter 3), strongly suggesting that BSO treatment increased the sensitivity of this organel to oxidative stress, most likely by reducing the GSH/GSSG ratio in the ER lumen. By reducing the GSH content, BSO will effect the anti-oxidative capacities throughout the cell. Therefore, the additionally labelled proteins (se panel C) may be from the ER as well as from other sites of the cell.

Incubating the cells with CumOOH also induced intracellular protein oxidation. Interestingly, the labelling pattern, obtained with CumOOH (figure 4A) is rather similar to that of H_2O_2 (figure 4B). This suggests that the site of oxidation of both peroxides is the same. However, the CumOOH concentration used is 12.5 times higher than that of H_2O_2, yet the same labelling intensity is not attained. This difference may be explained by the fact that CumOOH is significantly less water-soluble than H_2O_2. The octanol/water partition coefficient (log Pow) of H_2O_2 is -1.36 (solubility in water is 25 times that in octanol). The octanol/water partition coefficient of CumOOH is -0.16 (solubility in water is 1.44 times

that in octanol). Hence, both oxidants dissolve well in water, but H_2O_2 17 times better than CumOOH. For this reason CumOOH is normally used as an oxidant to study lipid peroxidation, but this experiment shows that it is also able to oxidize ER lumenal proteins. On the other hand, the encircled proteins (figure 4A & B) indicate that either oxidant also gives rise significant differences in the labelling pattern.

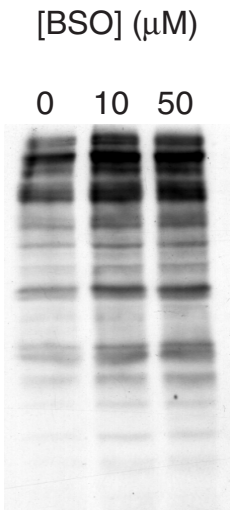

Fig 3: **Pre-treatment with BSO increases protein oxidation.** Cells were overnight pre-incubated with BSO, then loaded with acTyrFluo and stressed with H_2O_2.

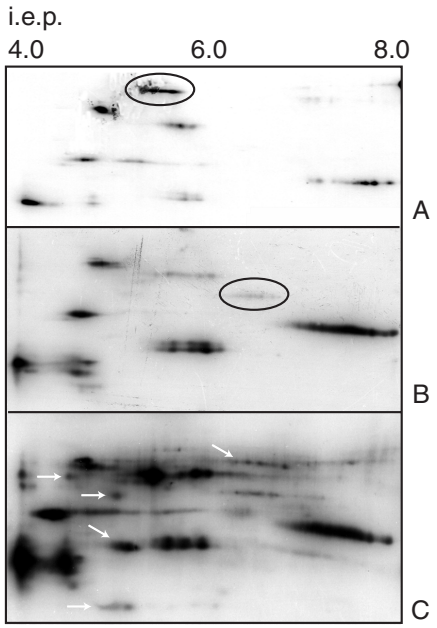

Fig 4: **2D-PAGE of TyrFluo-labelled proteins after oxidation with hydrogen peroxide, with or without pre-incubation with BSO, or cumene.**
A: 5 μM acTyrFluo → 500 μM CumOOH
B: 5 μM acTyrFluo → 40 μM H_2O_2
C: o/n 10 μM BSO → 5 μM acTyrFluo → 40 μM H_2O_2
Arrows indicate consecutive standard procedures.

Circles in A and B indicate differences in labelled proteins between CumOOH and H_2O_2. White arrows in C indicate proteins that are labelled as a consequence of pre-incubation with BSO.

GPx catalyses the reaction: 2 H_2O_2 + 2 GSH → 2 H_2O + GSSG. Mercapto-succinate (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 pre-incubation with MS and subsequent H_2O_2 treatment caused an increased labelling already at the lowest concentration used (figure 5), confirming the involvement of GSH in the defence against protein oxidation.

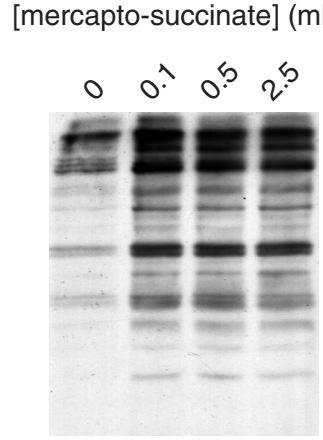

Fig 5: **Pre-treatment with mercapto-succinate increases protein oxidation.** Cells were overnight pre-incubated with MS, then loaded with acTyrFluo and stressed with H_2O_2.

H_2O_2 that was used in the labelling experiments oxidized GSH into GSSG with the aid of glutathione peroxidase (GPx). Glutathione reductase reduces GSSG back to GSH. Both enzymes are localised in the cytoplasm and the mitochodria [10] but not in the ER. A decrease of the cellular GSH concentration by pre-incubation with BSO restricts the (cytosolic) scavenging capacity of GPx and H_2O_2 has 'free play', which resulted in a higher labelling of both ER as well as cytosolic proteins. Incubation with mercapto-succinate interferes in the same scavenging mechanism and showed an increased labelling as well. In line with this, pre-incubation with GSH or GSH-ester yielded the opposite effect, i.e. a decrease in labelling. The involvement of GSH is obvious, but where and how it mediates the protection of ER proteins against oxidation is unclear. Inside the ER, but it is also possible that H_2O_2 is eliminated in the cytosol before it reaches the ER.

Masaki *et al.* showed that catalase acts as a primary defence against oxidative stress from exogenous or endogenous H_2O_2 at low concentrations in human dermal fibroblasts [11]. In contrast, GPx helps protect the cell from damage during exposure to high concentrations of H_2O_2. Figure 6 shows that an overnight incubation with amino-triazole (AT), which inhibits catalase, shows an increase in labelling, but not more efficient than MS does.

One would expect that protection of the ER proteins against oxidation is most sufficient with a water-soluble antioxidant. Therefore, cells were pre-incubated for 2 h with vitamin C, loaded with acetylTyrFluo and oxidized with H_2O_2. Indeed vitamin C showed a protective effect. The effect was already visible at a concentration as low as 1 µM (figure 7). These results show that vitamin C is a potent and fast acting antioxidant against H_2O_2. It is not known wherevitamin C scavenges H_2O_2. It may be inside the ER, but it could also be in the cytosol. In either way it prevents H_2O_2 from oxidising the ER. As expected, the lipophilic membrane-bound antioxidant vitamin E did not show an effect on labelling at any of the concentrations or incubation periods used (results not shown).

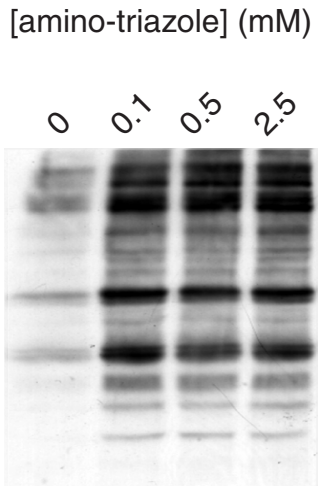

Fig 6: **Pre-treatment with amino-triazole increases protein oxidation.** Cells were overnight pre-incubated with AT, then loaded with acTyrFluo and stressed with H_2O_2.

The protective effect of vitamin C in our cellular system may be explained by the mechanism as proposed by Mukhopadhyay and Chatterjee [12]. *In vitro* the metal-ion catalysed oxidation (MCO) system [13], in which Fe^{3+} is reduced to Fe^{2+} and O_2 to H_2O_2, is accelerated by ascorbate. Subsequently, a Fenton reaction at metal binding sites on proteins produces OH·, which attack the side chains of amino acid residues. In line with this, they demonstrated that oxidation of microsomal proteins of guinea-pig liver (as measured by carbonyl groups, dityrosine formation and tryptophan loss) was accelerated by ascorbate when ADP-Fe^{3+} was present. However, *in vivo* this MCO system may be less relevant since most of the metal ions are not free and remain tightly bound to proteins. In the absence of free metal ions, NADPH initiated cytochrome P450 (cyt P450)-mediated protein oxidation, which was exclusively prevented by ascorbate. Since superoxide dismutase, catalase and glutathione had no effect, the involvement of $O_2^{-}\cdot$ and H_2O_2 was ruled out. So it was concluded that the MCO system was not responsible for the microsomal protein oxidation. The mechanism was considered to involve the initial reduction of cyt P450-Fe^{3+} to cyt P450-Fe^{2+} by NADPH-cyt P450 reductase. Subsequently cyt P450-Fe^{2+} reacts with molecular O_2 to produce cyt P450-$Fe^{2+}\cdot O_2$, the resonance form of which is cyt P450-$Fe^{3+}\cdot$-$O_2^{-}\cdot$. Cyt P450-$Fe^{3+}\cdot$-$O_2^{-}\cdot$ may oxidize

amino acid side chains. As suggested ascorbate would prevent oxidation since it provides an easily donatable hydrogen for abstraction by cyt P450-Fe^{3+}·-O_2^{-}·.

So in the presence of free metal ions ascorbate accelerates oxidation and in the absence of free metal ions it reduces oxidation. In the present study ascorbate reduced the H_2O_2-induced protein labelling with TyrFluo. How H_2O_2 triggered the oxidation of ER proteins is presently unknown, but a participation in a Fenton reaction with available metal ions is unlikely if the above suggested mechanism is true. However, it is still possible that protein-bound metal ions are redox-active and produce OH· at metal binding sites on the proteins.

In general, the results presented in this chapter indicate that GSH levels have a large influence on the degree of labelling and that lumenal ER-protein oxidation depends on the water-solubility of the oxidant and of the antioxidant used.

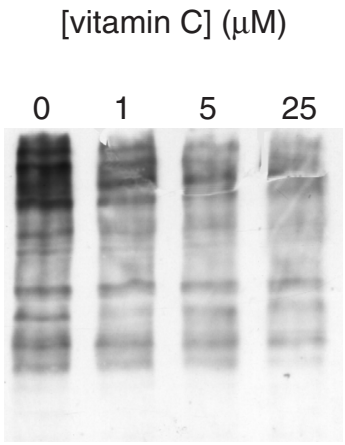

Fig 7: **Pre-treatment with vitamin C protects against protein oxidation.** Cells were pre-incubated with vitamin C for 2 hours, then loaded with acTyrFluo and stressed with H_2O_2.

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Chapter 5

Influence of oxidative stress on protein maturation

The LDL receptor as a model protein

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(Manuscript in preparation)

Abstract

Using the acetylTyrFluo probe, it has been shown that the resident proteins in the endoplasmic reticulum (ER) are the major targets for oxidation by hydrogen peroxide. To investigate the influence of oxidative stress (H_2O_2) on the ER-related protein maturation, the low–density lipoprotein receptor (LDLr) was chosen as a model protein. It has been demonstrated that LDLr is synthesized as a precursor (p) with a molecular weight of about 120,000 Da. Within 15-30 min of disulfide bond isomerisation this precursor is transported to the Golgi complex where it is rapidly converted into a mature (m) form with a mobility of about 160,000 Da as a result of extensive *O*-glycosylation. The formation of the thirty S-S bridges, inside the ER, is a prerequisite for transportation towards the Golgi complex to complete maturation. In the present study the maturation of a [^{35}S]-methionine labelled LDLr of rat-1 fibroblasts was followed in pulse-chase experiments under oxidative conditions. Under the same conditions the ratio of reduced (GSH) versus oxidized (GSSG) glutathione was measured and related to the degree of maturation of the LDLr, since changes in GSH concentration showed to have a large impact on the labelling of ER-resident proteins with TyrFluo. Comparing the signals of m and p at different time-points showed that the velocity of maturation was reduced (1.5 mM H_2O_2) or abolished (> 2.0 mM H_2O_2) as a consequence of oxidative stress. When cells were recultured in medium, after this abolishment, maturation was restored. However, when protein synthesis (translation) was blocked by cycloheximide during this recovery period, no restoration of maturation was observed. In addition, the balance of GSH/GSSG did not follow the maturation. At lower oxidative conditions, when maturation was still normal, the ratio already decreased dramatically and was not re-established after restored maturation. So, restoration of maturation requires the synthesis of new proteins, which can be performed when GSH levels are still low. These results suggest that the ER proteins may have lost their folding capacity as a consequence of the oxidation. In addition, the cytosolic proteins and the LDLr itself may have been damaged as well. Therefore, this impairment of maturation may involve other proteins besides the ER proteins. Most probably the transport towards the Golgi complex is also reduced dramatically, which will also contribute to the impaired maturation of the LDLr.

Introduction

Using the acetylTyrFluo probe [1, 2], it has been shown that the resident proteins in the endoplasmic reticulum (ER) are the major targets for oxidation by hydrogen peroxide. This oxidative damage was shown to be strongly dependent on GSH/GSSG redox status of the cell. This suggests that the functionality of PDI and other isomerases is affected. To investigate whether oxidation of ER-resident proteins has an impact on the processing of proteins in the ER, including post-translational modifications, and sorting in the Golgi complex, we have chosen the low-density lipoprotein receptor (LDLr) as a model.

The LDLr is the prototype of a family of cell-surface receptors that recognise and internalise extracellular apo-lipoprotein particles containing cholesterol and triglycerides. The other family members are very-low-density lipoprotein receptors (VLDLr), apolipoprotein E receptor 2, LDLr related protein (LRP), and megalin [3]. The LDLr is a glycoprotein that mediates the binding, internalisation, and subsequent degradation of plasma LDL [4]. The folding and maturation pathway of newly synthesized LDLr in the ER is largely unknown. Only recently, studies have shown that a 39-kDa protein known as RAP (receptor-associated protein) serves as a molecular chaperone to assist the folding of LDLr family proteins and their passage through the secretory pathway [5]. During receptor folding calcium functions, independently from RAP, by stabilising the calcium-binding conformation and allowing correct disulfide formation. Calcium depletion (by ionomycin or thapsigargin treatment) has shown to significantly disrupt the folding process of LDLr. It reversibly induced the formation of high molecular weight aggregates under non-reducing SDS-PAGE, suggesting the formation of intermolecular disulfide bonds [6].

Jansens [7] has extensively studied the folding of the LDLr and has shown that as soon as the newly formed chain enters the ER, a rapid collapse of the amino acid chain occurs. This means that in the intermediates formed during the folding the hydrophobic side chains are buried. This collapse prevents aggregation and is assisted by GRP94. Distant parts of the molecule are brought together (co-translationally), which allows the formation of non-native disulfide bonds between distant cysteines yielding loosely packed folding intermediates with very compact states (increased mobility in SDS-PAGE). As folding proceeds, isomerisation into native disulphide bonds (i.e. a process possibly catalysed by PDI) takes place, making the protein less compact. Immune-precipitation 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 weight of about 120,000 Da. Within 15-30 min of disulfide bond isomerisation this precursor is transported to the Golgi complex where it is rapidly converted into a mature form with a mobility of about 160,000 Da [8]. This shift in size during maturation has been shown to be due to addition of *O*-linked oligosaccharides [9]. The mature LDLr now contains two *N*- and a cluster of eighteen *O*-linked oligosaccharides and thirty disulfide bridges in the extracellular domain [10].

The remarkable observation that the proteins of the endoplasmic reticulum (ER), BiP/GRP78, endoplasmin/GRP94, PDI, Erp57/GRP58 and calnexin, are among the major protein-targets of hydrogen peroxide-induced oxidation [2], has raised the question: What

does the oxidation of ER-resident proteins, that are all involved in protein folding, mean for their functionality? In other words, is protein folding/maturation impaired after subjecting cells to oxidative stress? In this regard it is important to consider the involvement of the major cellular redox buffering system GSH/GSSG, since changes in GSH concentration showed a large impact on the labelling with TyrFluo (chapter 4). A requirement for oxidative protein folding and disulfide bond formation by PDI is a high redox state in the ER [11, 12]. This state is thought to be attained by an optimum concentration of oxidising and reducing equivalents in the lumen of the ER as compared to the cytosol. In the ER the relative abundance of the oxidized (GSSG) over the reduced (GSH) form of glutathione has led to the proposal that GSSG is preferentially imported from the cytosol into the ER lumen and serves as the oxidising equivalents during protein folding [12]. ($[GSSG]_{ER}/[GSH]_{ER}$ = 1:1 to 1:3 is much higher than the overall cellular ratio, which is about 1:30 to 1:100). However, it has been shown that glutathione itself is not required for oxidative protein folding in the ER [13, 14] indicating that disulfide bond formation *in vivo* relies upon different electron acceptor(s).

To investigate the influence of oxidative stress (H_2O_2) on the ER-related protein maturation, the LDLr was chosen as a model protein. LDLr is an endogenous protein of the rat-1 fibroblasts (no transformation or infection of recombinant DNA is required for sufficient expression) and its ER-related maturation involves the formation of as much as thirty disulfide bridges. The formation of these bridges inside the ER is most probably carried out by PDI, which functionality may be impaired after an oxidative challenge. In addition, disulfide bridge formation in the LDLr is a prerequisite for transportation towards the Golgi complex to complete maturation. So, a definition of maturation, as used here for the LDLr, is the series of events that have to take place for a cell to produce a fully functional, 160 kD, *O*-glycosylated protein. This post-translational series of ER-related events comprise; folding (S-S bridge formation), vesicular transport towards the Golgi complex, and *O*-glycosylation inside the Golgi complex. The maturation of a [^{35}S]-methionine labelled LDLr of rat-1 fibroblasts was followed in pulse-chase experiments under oxidative conditions. Under the same conditions the ratio of reduced (GSH) versus oxidized (GSSG) glutathione was measured and related to the degree of maturation of the LDLr, since changes in GSH concentration showed to have a large impact on the labelling of ER-resident proteins with TyrFluo (chapter 4). Moreover, the recovery of maturation after the oxidative challenge showed to depend on the ability of a cell to synthesize proteins.

Experimental procedures

Materials and Solutions

Hank's balanced salt solution (HBSS) was obtained from Sigma (Zwijndrecht, the Netherlands). Depletion medium is 10 mM Hepes (N-2-hydroxyethylpiperidine-N'-ethanesulfonic acid, pH 7.4) in methionine-free culture medium, which was from ICN-Biomedicals (Zoetermeer, the Netherlands). Dulbecco's Modified Eagle Medium (DMEM) and Fetal Calf Serum were from Gibco BRL (Life Technologies, Breda, the Netherlands).

For the pulse-chase experiments: Labelling medium is 100 µCi [^{35}S] L-methionine (from Pharmacia Amersham Biotech, Roosendaal, the Netherlands) in depletion medium. Chase medium is serum free DMEM containing 1 mM cycloheximide, 5 mM cysteine, 5 mM methionine, 10 mM Hepes (pH 7.4). Stop medium is 20 mM NEM (N-ethylmaleimide) in HBSS. Lysis buffer is 10 mM Hepes (pH 7.4), 200 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1% Triton X-100, 2.2% DMSO (dimethyl-sulfoxide), 20 mM NEM, 1mM PMSF (phenyl-methyl-sulfonyl fluoride) and 10 ug/ml CLAP (chymostatin, leupeptin, antipain, pepstatin stock of 10 mg/ml each in DMSO). Protein A-Sepharose beads (Sigma) were as a 10% (v/v) suspension in 10 mM Hepes (pH 7.4), 200 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 2.2% DMSO, 0.1% BSA. The wash buffer for the beads was 50 mM Tris-HCl (pH 8.6), 150 mM NaCl, 1% Triton X-100, 0.5% SDS (sodium dodecylsulphate). The non-reducing sample buffer contains 200 mM Tris-HCl (pH 6.8), 3% (w/v) SDS, 10% (v/v) glycerol, 1 mM EDTA, and 0.005% bromephenol-blue.

The polyclonal anti-LDLr antiserum 121 was kindly provided by Prof. I. Braakman and A. Jansens from the Department of Bio-Organic Chemistry, University of Utrecht, The Netherlands. For the glutathione assay: DTNB (5,5'-dithiobis-2-nitrobenzoic acid) also known as Ellman's reagent, triethanolamine (TEA), GSSG and glutathione reductase (GR) were obtained from Sigma. NADPH was from Boehringer. EDTA, sucrose, 2-vinylpyridine, and Hepes were from Merck and phosphoric acid (HPO_3) was from Fluka.

Pulse-chase under oxidative conditions

Rat-1 fibroblasts were cultured for 48 hours on plastic Petri dishes (20 cm^2) in DMEM containing 7.5% (v/v) fetal calf serum until subconfluency (± 10^6 cells ≈ 80% density). Cells were washed twice with HBSS and ongoing protein synthesis was strongly reduced by adding 2 ml depletion medium (incubator with 5% CO_2; 37°C) for no longer then 20 min. After 20 min, dishes were placed on a rack in a water bath (37°C), depletion medium was removed and cells were pulse-labelled for precisely 10 min with 500 µl labelling medium. Pulse was stopped by adding 2 ml chase medium. 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 mM 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, which contained NEM to prevent further formation of disulfide bonds. For the 0 min chase interval dishes were put on ice immediately after the pulse labelling. Stop medium was refreshed immediately after washing. Cells were lysed with 600 µl ice-cold lysis buffer and proteins were collected by scraping and transferred to pre-cooled micro-centrifuge tubes. Nuclei were pelleted by centrifugation (10 min; 13,000 rpm; 0°C). Supernatant was transferred to pre-cooled micro-centrifuge tubes and immuno-precipitation was started or samples were frozen in liquid nitrogen (store at –80°C).

Immunoprecipitation, SDS-PAGE and autoradiography

Antiserum 121 was raised against a mixture of native, denatured and reduced LDLr polypeptides [15], thereby increasing the chance that all conformations of the protein, including folding intermediates, are recognised by the antiserum. To 300 µl post-nuclear cell lysate 50 µl of the protein A-Sepharose beads suspension and 10 µl anti-LDLr antiserum were added. While rotating overnight at 4°C LDLr was immunoprecipitated. Beads were pelleted by centrifugation, 1 min 12,000 rpm at room temperature (rT), and washed with 1 ml washbuffer on a shaker (2 x 5 min at rT). Washbuffer was aspirated and beads were suspended in 15 µl 10 mM Tris-HCl/1 mM EDTA (pH 6.8) and 15 µl non-reducing sample buffer and heated at 95°C for 5 min. Beads were pelleted by centrifugation, 1 min 12,000 rpm at rT. Reduced samples were obtained by transferring 15 µl of each supernatant to a new tube containing 2 µl 200 mM DTT, which subsequently was heated again at 95°C for 5 min. After cooling down, 2 µl 1 M NEM was added to prevent spontaneous oxidation of the LDLr and formation of disulfide bonds. SDS-PAGE was performed on a 6% polyacryl-/bisacryl-amide. Gels were stained for 15 min with Coomassie brilliant blue (0.25% (w/v) in destain), destained (30% methanol, 10% acetic acid, 60% water) for 45 min, neutralised (30% methanol, 70% PBS) 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 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 washed twice with HBSS and subjected to H_2O_2 (0, 0.5, 2.0 or 8.0 mM) in DMEM. Then, a modified Titze-recycling assay was used [16-18]. Cells were washed twice with ice-cold SH-buffer (0.25% sucrose, 25 mM Hepes (pH 7.4)), lysed in 600 µl ice-cold buffer (3.33% HPO_3, 2.7 mM EDTA) for 5 min, scraped, and collected in micro-centrifuge tubes. After centrifugation, 8500 rpm at 4°C for 5 min, pellets were kept on ice and supernatants were transferred to a new tube, flushed with nitrogen and stored at –80°C until use (up to one week). Culture dishes were dried at 37°C, remaining proteins were dissolved in 250 µl 1 M NaOH and added to the pellets. Protein concentrations were determined with a Bradford-based protein assay (Bio-Rad protein assay). To 200 µl sample 24 µl 4 M TEA was added and immediately carefully mixed. For measuring total glutathione (GSH + GSSG), 30 µl of this mixture was mixed with 90 µl 3.33% HPO_3, 2.7 mM EDTA, 4 M TEA. For measuring GSSG only, 1.2 µl vinylpyridine was added to 120 µl sample/TEA mixture. This sample was vortexed, flushed with nitrogen and kept at rT for 1 hour. In a 96-well microplate duplo assays were started by adding 100 µl of the assay-mix (300 µM NADPH, 225 µM DTNB, 1.5 U/ml GR in 0.1 M Na_2HPO_4/10 mM EDTA (pH 7.5)) to 50 µl sample mixtures. In parallel two standard series of GSSG

(0, 0.25, 0.5, 1, 2, 3, 4 µM) in 3.33% HPO_3, 4 M TEA were assayed with and without 20 mM vinylpyridine. Absorbance of TNB was measured kinetically for 20 min at 412 nm using a Ultramark‘ microplate-reader (Bio-Rad) at 37°C. The initial slopes of the enzymatic velocities were compared.

Results and discussion

Pulse-chase under oxidative conditions

To follow the processing of LDLr a chase is always performed in the presence of cycloheximide to stop ongoing translation. By this procedure the formation of new precursors is prevented and the maturation of the precursors present can be followed in time. Newly synthesized reduced LDLr molecules are synthesized in the ER as a precursor form (p), and run at 120 kD. Correctly folded molecules pass the ER quality control and are *O*-glycosylated in the Golgi complex to a mature (m) form that runs at 160 kD. To prevent spontaneous oxidation of the LDLr during SDS-PAGE, NEM was added to each reduced and non-reduced sample before it was loaded onto the gel. When the sample is ran on a non-reducing gel it is clearly visible that multiple folding intermediates of the precursor with high mobility are formed (figure 1 A, lane 1). After reduction of the S-S bridges by DTT, the smear, formed by these intermediates, disappears (panel B, lane 1) and only one 120 kD band is visible, showing that these precursors are of the same size and that S-S bridges establish the compactness. Within 15 min (lanes 2) the mature form is produced in addition to the precursor, and after one hour all the precursors have matured (lanes 3). The mature form does not show any folding intermediates (at these time points at least). As soon as p has travelled from the ER to the Golgi complex O-glycosylation, which distinguishes m from p, takes place rapidly.

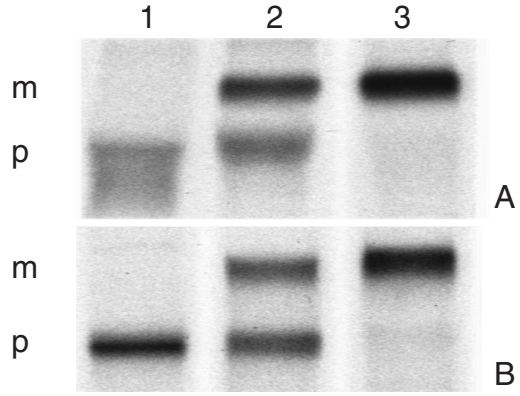

Fig 1: **Maturation of LDLr**. Cells were pulse-labelled with [^{35}S]-methionine for 10 min and chased for 0 (lane 1), 15 (lane 2) or 60 min (lane 3). Autoradiography shows the 120 kD precursor (p) and the 160 kD mature (m) form of LDLr. Panel A shows the non-reduced and panel B the reduced gel.

Figure 2 shows that protein labelling is reduced already after a short exposure (10 min) of a low dose of H_2O_2 (0.1 mM). At higher concentrations (0.5 and 2.0 mM) protein labelling is completely abolished. This may indicate a complete impairment of protein synthesis (translation) [19] or an impaired uptake of [^{35}S]-methionine as a consequence of the oxidation. To determine whether or not oxidation diminishes the uptake of the [^{35}S]-methionine, radioactivity (disintegrations per minute) was measured (Packard scintillation counter) in cell lysates obtained from cultures that were allowed to take up the radiolabel (for 10 min) after being exposed to 0.1, 0.5, 2.0 or 8.0 mM H_2O_2 for 10 min. When the uptake of control cell lysate (no H_2O_2) is set at 100% the uptake of the stressed samples were 16%, 8%, 6% and 4% respectively

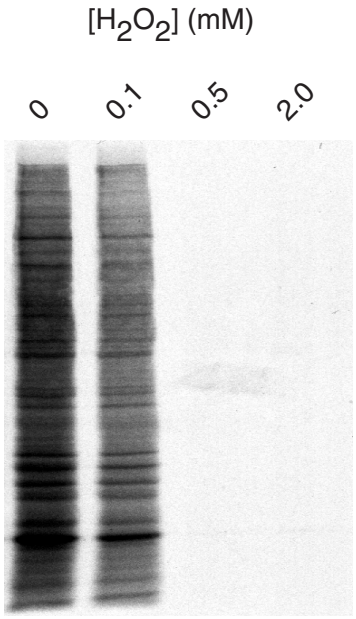

Fig 2: **Oxidative stress impairs metabolic labelling of proteins.** Cells were depleted from methionine and oxidized with 0, 0.1, 0.5 or 2.0 mM H_2O_2 for 10 min before radioactive labelling with [^{35}S]-methionine was started (6 min). No chase was performed.

Next, the influence of oxidative stress on protein folding/maturation was investigated. ER resident proteins that have to perform the folding have half-life of days [20]. So it was assumed that oxidized and thereby functionally impaired proteins would not be degraded within this period. By comparing the ratio of the signals of m versus p, at a given time-point under oxidative and non-oxidative conditions, an influence of the oxidation on the maturation of LDLr will be disclosed. Maturation has been defined as the post-translational series of ER-related events that comprise; folding (S-S bridge formation), vesicular transport from the ER towards the Golgi complex, and *O*-glycosylation inside the Golgi complex.

Oxidation of ER-resident proteins (as measured by TyrFluo-labelling) was established with 100 µM H_2O_2. From the previous experiment it was concluded that the oxidation should not precede the pulse-labelling. Therefore, cells were first pulse-labelled with [^{35}S]-methionine followed by a chase-period in the presence or absence of H_2O_2 (figure 3). A chase/oxidation of 15 min was performed, since at this time-point both the mature and the precursor forms are present. Under the stress conditions used (100 or 400 µM H_2O_2) the maturation of LDLr was not affected.

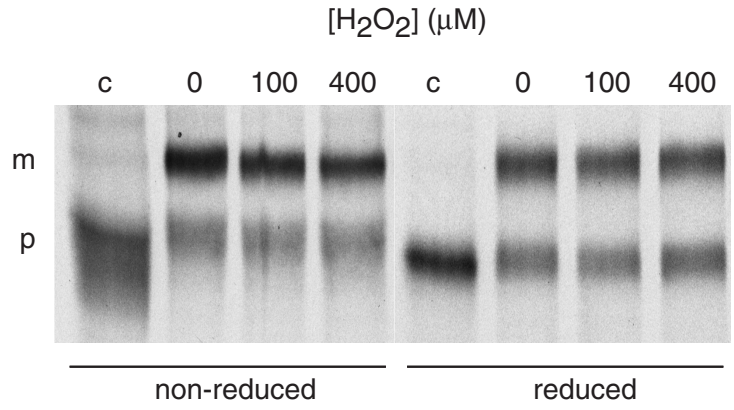

Fig 3: **Maturation of LDLr under low oxidative conditions.** Cells were depleted from methionine, pulse-labelled with [^{35}S]-methionine and chased for 15 min in the presence of 0, 100 or 400 µM H_2O_2. c = control (no oxidation, no chase). Samples were run on a non-reducing and a reducing gel. p = 120 kD precursor, m = 160 kD mature form of LDLr.

Increasing concentrations of H_2O_2 had a large influence on the maturation of LDLr (figure 4). When cells were stressed with 2.0 mM H_2O_2 or higher, maturation did not occur anymore. In non-reduced samples (panel A, w/o recovery) p appears as a smear of folding intermediates, which disappears in the reduced samples (panel B, w/o recovery) indicating that disulfide bridges were formed as a consequence of oxidation. Here the maturation of LDLr is dramatically impaired. Several explanations may be put forward: (1) the folding proteins are oxidized and are not able to fold LDLr properly (a prerequisite for transportation towards the Golgi complex for further maturation), (2) oxidation of LDLr itself may also cause improper folding, (3) oxidized LDLr is degraded by the proteasome, (resulting in lower labelling signals) (4) vesicle transport towards the Golgi complex is eradicated, or (5) glycosylating enzymes in the ER and/or Golgi lost functionality as a consequence of oxidation. When cells were allowed to recover in culture medium, after 2.0 mM H_2O_2 oxidative challenge, maturation could be restored. Apparently the defensive capacities were sufficient or quickly upregulated to overcome this challenge, and cells continued with protein synthesis, folding, vesicle transport, and glycosylation. However, 8.0 mM H_2O_2 was found to

be too high to restore maturation. Here labelling signals are also lower, because of cell death. Extreme oxidative conditions obviously cause so much damage that no recovery (at least not within 30 min) takes place.

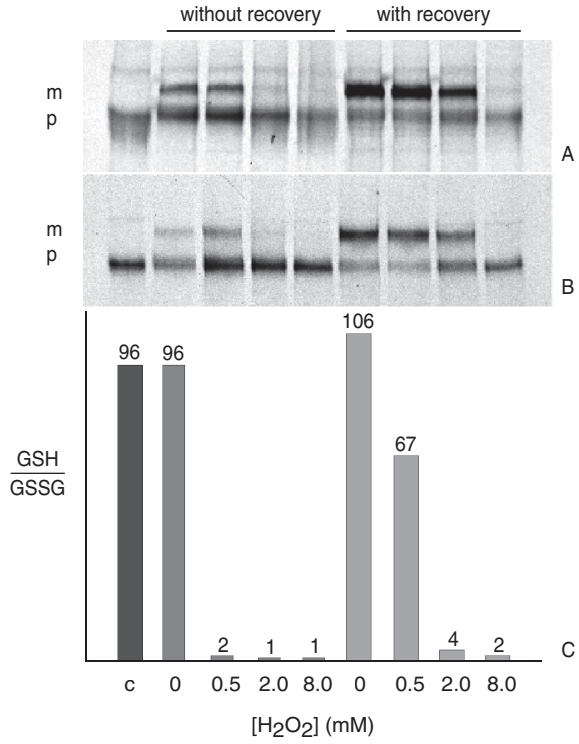

Fig 4: **Maturation of LDLr and the ratio reduced versus oxidized glutathione under moderate to high oxidative conditions.** Cells were depleted from methionine, pulse-labelled with [^{35}S]-methionine and chased for 10 min in the presence of 0, 0.5, 2.0 or 8.0 mM H_2O_2 with or without subsequent recovery in culture medium for 30 min. Non-reduced samples (panel A), reduced samples (panel B) and the ratio GSH/GSSG (panel C; n = 3). p = 120 kD precursor, m = 160 kD mature form of LDLr, c = control (no oxidation, no chase).

Glutathione assay

GSSG and total glutathione concentrations in the samples were determined with aid of the respective standard curves (in the presence or absence of vinylpyridine). Taken into account that one molecule of GSSG produces two molecules of GSH, the concentration GSH can be calculated easily: GSH = total – (GSSG / 2). In figure 4C the ratio GSH/GSSG is plotted as a function of the concentration H_2O_2 used to stress the cells. According to Hwang *et al.* [12] the ratio in untreated cells (= control) was found to be about 100:1, which did not change when cells were put on serum free culture medium for 10 min (stress = 0 mM). As a

consequence of the applied oxidative stress the ratio decreased up to 50-fold when the stress condition was 0.5 mM. This ratio could be restored for about 70% when the cells were allowed to 'recover' for 30 min in culture medium. Under more severe stress conditions the ratio dropped even further. After 2.0 mM H_2O_2 ratios were restored from 1.1 to 3.8 within 30 min of recovery. A ratio of 1:1 seemed to be hard to overcome and to restore the balance in favour of the reduced form, GSH; 8.0 mM H_2O_2 set the balance at 0.9 and could not be restored. In addittion, the figure also shows how the ratio GSH/GSSG is related to the level of maturation of the LDLr when stressed with H_2O_2. Obviously the ER and the Golgi complex were not damaged when these cells were subjected to 0.5 mM H_2O_2, since no impaired maturation of LDLr was observed. This did not coincide with the extensive oxidation of GSH that it caused (a decrease in the ratio from 96 to 2). Increasing the oxidative conditions to 2.0 mM H_2O_2 caused a further decrease of the ratio GSH/GSSG to as less as 1%. Here the maturation of LDLr is dramatically impaired. After the recovery period the cellular GSH/GSSG ratio had increased from 1 to 4 and maturation of LDLr was restored. This ratio is above the ratio for the ER (estimated to be 3). Could it be that the ER has re-acquired its relatively oxidising environment compared to the cytosol and is therefore able to perform its folding tasks again? Extreme oxidative conditions like 8.0 mM H_2O_2 obviously cause so much damage that no recovery (at least not within 30 min) takes place. In general it can be concluded that when cells are able to restore the balance between GSH and GSSG to a certain minimal level they are also able to restore protein folding and maturation. Even mitosis can take place when the ratio is still low (own observations).

Recovery of LDLr maturation after oxidative stress.

It was investigated whether the recovery of LDLr maturation depends on the ability of a cell to perform protein synthesis. To prevent protein synthesis cells were treated with cycloheximide (chx). Figure 5A shows the efficiency of chx; in the presence of chx metabolic pulse-labelling of newly synthesized protein was abolished. It was impossible to check whether H_2O_2 had a similar effect, since the uptake of the radiolabel was strongly impaired after subjecting the cells to oxidative stress (see figure 2). Next, cells were stressed with 2.0 mM H_2O_2 during a 10 min chase (which had shown to abolish LDLr maturation; figure 4), after which they were allowed to recover in culture medium in the presence or absence of 1 mM chx for 0, 20 or 40 min (panel B). Control sample (lane 1) shows both m and p form of LDLr after a chase of 10 min. In the absence of H_2O_2 maturation occurs normally when cells are put on culture medium (lane 2 and 3) even in the presence of chx (lane 4 and 5). Here, chx has no effect on the maturation since the folding proteins are abundantly present (lane 5) and there is obviously no need to synthesise more of them.

Oxidation of the folding proteins and thereby impairing their functionality resulted in impaired folding. As expected, oxidation showed again an impaired maturation (lane 6), which could partly be restored in time (lane 7 and 8). However, when the recovery was performed in the presence of chx no restoration of maturation could be observed (lane 9

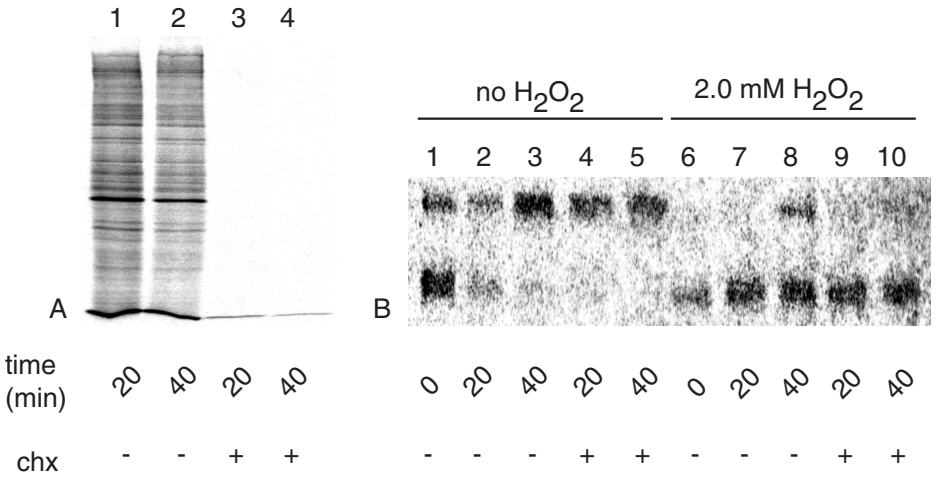

Fig 5: **Recovery of LDLr maturation after oxidative stress is blocked by cycloheximide.** A) Cells were depleted from methionine, pulse labelled with [^{35}S]-methionine in the absence (lane 1 and 2) or presence (lane 3 and 4) of 1 mM cycloheximide for 20 min (lane 1 and 3) or 40 min (lane 2 and 4). B) Cells were depleted from methionine, pulse labelled with [^{35}S]-methionine and put on chase medium with (lane 6-10) or without (lane 1-5) 2.0 mM H_2O_2, for 10 min after which they were allowed to recover in serum-containing culture medium in the presence or absence of 1 mM cycloheximide for 0, 20 or 40 min.

and 10). From this it can be concluded that during the recovery period protein synthesis must be the driving force behind maturation of LDLr. Probably this recovery requires the upregulation of stress response and/or folding chaperones, as well as proteins involved in ER to Golgi transport. In the presence of chx these proteins could not be synthesised, thereby preventing the restoration of maturation.

We have shown that 2 mM H_2O_2 abolished LDLr maturation, whereas 1 mM H_2O_2 did not (not shown). Therefore, 1.5 mM H_2O_2 was chosen to follow the maturation of LDLr in time. Figure 6 shows clearly that the velocity with which the maturation occurs is decreased as a consequence of oxidation. Under non-oxidative conditions m is detected after 10 min of chase and maturation is completed after 40 min. In contrast, when H_2O_2 is present p is detected only after 20 min and maturation is not completed within 40 min. This may imply that part of the folding chaperones were oxidized and lost their functionality, and that the remaining non-oxidized part was still functional, but logically the efficiency was restricted, since they were 'facing' an excess of unfolded proteins. Also transportation of the precursor towards the Gogli-complex may be impaired, thereby preventing the mature protein to be formed. Moreover, the free sulfydryls of LDLr may have been oxidized to sulfoxides, preventing disulfide bridge formation and subsequent maturation.

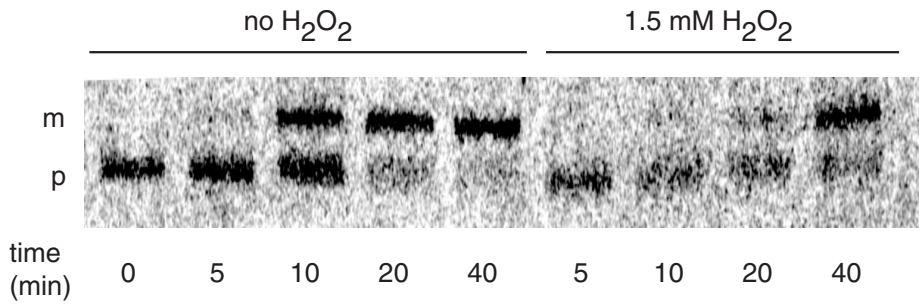

Fig 6: **Velocity of LDLr maturation is decreased under oxidative conditions.** Cells were depleted from methionine, pulse-labelled with [^{35}S]-methionine and chased for 0, 5, 10, 20 or 40 min in the absence or presence of 1.5 mM H_2O_2. p = 120 kD precursor, m = 160 kD mature form of LDLr. 0 = control (no stress, no chase).

Conclusions

The ER-resident proteins, that assist in the protein folding, were found to be labelled with TyrFluo already at a low concentration of H_2O_2 (50 μM for 10 min). Since, the detection method for the TyrFluo-labelled proteins is very sensitive it is very well possible that the number of TyrFluo-labelled proteins is so low that this concentration of H_2O_2 does not have an effect on the functionality of the machinery as a whole. Unfortunately it was not possible to measure the degree of labelling. Preliminary results indicate that it may be less than 1%.

However, at moderate to high H_2O_2 concentration (between 1.0 and 2.0 mM for 10 min) protein folding/maturation of the LDLr is impaired as shown in this chapter. 2.0 mM H_2O_2 completely abolished maturation, whereas 1.5 mM H_2O_2 greatly reduced the velocity of maturation. Under normal circumstances cells were able to recover and restore maturation. However, when protein synthesis (translation) was blocked by chx, no restoration of LDLr-maturation was observed. This indicates that the proteins involved in the maturation lost their functionality as a consequence of the oxidation and that restoration of maturation requires a newly synthesised pool of proteins. In addition, this normal functioning did not seem to depend on a complete restoration of the GSH-GSSG balance. Oxidation with 2.0 mM H_2O_2 (condition for impaired maturation) caused a decrease of the ratio GSH/GSSG to as less as 1% of control levels. After the recovery period the cellular ratio had increased only from 1 to 4.

So at this point the questions that were raised in the introduction can only partly be answered. Yes, after subjecting cells to oxidative stress the ER proteins will become oxidized, and they will lose their folding capacity. On the other hand, the cytosol and its content may be heavily damaged as well under all oxidative conditions used. Therefore, this impairment can not be put on the account of the ER solely. Most probably the transport towards the Golgi complex is also reduced dramatically, which will also contribute to the impaired maturation of the LDLr.

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Chapter 6

Protein oxidation in aging: endoplasmic reticulum as a target

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(unrevised manuscript as submitted to *Amino Acids*)

Abstract

Oxidatively modified proteins have been shown to increase as a function of age. In recent years numerous outstanding reviews have been published in which the role of protein oxidation in aging and age-related diseases has been described extensively. Most of the referred studies describe whether or not there is a correlation between the level of the modification and the age of the organism or its tissues. Increase in tissue-susceptibility to experimentally induced protein oxidation has shown to be not only dependent on the tissue type or the age of the animal, but also on the maximum life span potential of the species. A general, but also tissue dependent, decline in anti-oxidative defences during aging may very well be responsible for this difference in vulnerability. In addition, the level of protein modifications also depends on the nature and the subcellular localization of the proteins involved. Damage to the endoplasmic reticulum (ER), and its subsequent impaired functionality (e.g. in protein folding and in maintaining calcium homeostasis) may be involved in the process of aging. There are several phenomena that suggest its involvement, like (1) the upregulation of ER stress response chaperones, expression of which is known to be decreased upon caloric restriction (the only method known to extend life span), (2) the preferential oxidation of ER resident proteins that are involved in protein folding, and, (3) the disturbance of calcium homeostasis.

Therefore, after a short overview of the different aging theories and the relation between caloric restriction and protein oxidation, this review will focus on the putative involvement of the endoplasmic reticulum in the process of aging.

Introduction

During the last decades, evidence has been obtained that aging is a function of several closely interrelated parameters, such as the metabolic rate, caloric intake, genetics, lifestyle and environmental factors [1]. But what is aging? Biological aging could be defined as, "A complex process in which diverse deleterious changes in cells and tissues accumulate with advancing age. These changes cause a loss of cellular function that results in an increased mortality and represents a major risk factor for age-related diseases like cancer, diabetes mellitus, rheumatoid arthritis, neurodegenerative, and cardiovascular diseases." Due to its complexity, a very large number of theories have been proposed to explain the process of aging [2]. For example, the aging process has been attributed to molecular cross-linking [3], changes in immunologic function [4], free radical damage [5], cellular senescence [6], telomere shortening [7], mitochondrial DNA damage (reviewed in [8]) and most recently to the Maillard reactions of DNA [9]. No single theory is generally accepted, but it is now beyond doubt that reactive oxygen species (ROS) generated in vivo, play a role in aging, as already proposed in 1956 [10, 11]. This free radical theory of aging has evolved since then (reviewed [11]). Due to the high reactivity of several oxygen intermediates, many lipids, proteins and DNA are substrates for oxygen-mediated alterations. Since cellular reactive by-products of normal metabolism also lead to damage, this theory has recently been extended to the oxidative "garbage catastrophe theory" where ROS or reactive oxygen intermediates are responsible for the accumulation of age-related cellular damage of biomolecules [12]. According to this theory, aging may derive from imperfect clearance of oxidatively damaged, relatively indigestible material, the accumulation of which further hinders cellular catabolic and anabolic functions and mainly affects postmitotic non-proliferating cells. Further support is provided by the finding that overexpression of enzymes that prevent the generation of radicals, like superoxide dismutase (SOD) and catalase [13, 14], or that reduce oxidized proteins, like methionine sulfoxide reductase A (MSRA) [15], extend the lifespan of the fruit fly *Drosophila melanogaster* markedly. SOD dismutates superoxide ($O^{2-\cdot}$) to form hydrogen peroxide (H_2O_2), which is subsequently broken down by catalase to water and oxygen. MSRA catalyzes the repair of oxidized methionine in proteins by reducing methionine sulfoxide back to methionine. In agreement with this, mutations in the age-1 gene of *Caenorhabditis elegans* result in an age-specific increase in the activity of catalase and Cu/Zn-SOD and double the life span [16]. And a mutation of the mouse p66shc gene (a cytoplasmic signal transducer involved in the transmission of mitogenic signals from activated receptors to Ras) induces stress-resistance and prolongs life span [17].

Moreover, it has been shown that, in fibroblasts from individuals with the premature aging diseases progeria or Werner's syndrome, the levels of oxidatively modified proteins are significantly higher than age-matched controls [18]. In addition, the basal levels of the primary antioxidant enzymes Mn-SOD, catalase and glutathion peroxidase were found to be decreased in progeria fibroblasts [19]. These findings also suggest a contribution of ROS-mediated damage to the accelerated aging process characteristic for these diseases.

So, whether or not radicals determine life span, it is becoming increasingly apparent that

they play an important role in the (patho)-physiology of aging [20]. After a short overview of the different aging theories and the relation between caloric restriction and protein oxidation, this review will focus on the putative involvement of the endoplasmic reticulum in the process of aging.

Age-related protein oxidation

It is now well established that biological aging correlates with the accumulation of chemically modified biomolecules in tissue such as oxidized proteins, lipids, DNA bases, advanced glycation end-products and lipofuscin. Studies on oxidatively modified proteins have revealed an age-related increase in the level of protein carbonyl content [21], oxidized methionine [22], protein hydrophobicity [23], cross-linked proteins [24] and glycated proteins [25] as well as the accumulation of catalytically less active enzymes [18, 26-28] that are more susceptible to heat inactivation and to proteolytic degradation [29]. In order to find out about the role of protein oxidation in the aging process, stable markers of ROS-induced oxidation are required. Several possibilities may be the cause of an increase in the steady-state level of oxidatively modified proteins. These include (1) an increase in the formation of oxidizing species [1], (2) a decreased capacity to scavenge those species [30], (3) an increased susceptibility of the proteins to become oxidized as a consequence of transcriptional and translational errors [31], and (4) a decrease in the levels or activities of the proteasome [32] or proteases that selectively degrade oxidized proteins [33].

Although remarks can be put forward about the accuracy of the assays used or the stability of the formed products several protein oxidation markers have been shown to relate with aging. In table 1 age-related protein oxidation markers are presented of both extracellular and intracellular proteins as well as the tissues they originate from; these modifications are further discussed in the text.

One of the best-known markers of age-related protein oxidation is the carbonyl group. The carbonyl content of proteins has been observed to increase with age [21, 34-36]. While an increase in carbonyl content was measured in most regions of the mouse brain between the age of 8 to 27 months, age-associated loss of protein sulfhydryls was more uniform across brain regions. Dietary restriction resulted in reversal of age-associated carbonyl and sulfhydryl concentration and was found to retard age-associated decline in learning and coordination problems [37]. A comparison of ad libitum fed and dietary restricted mice at 9, 17 and 23 months of age indicated that the protein carbonyl content in the brain, heart and kidney increased with age and was significantly greater in the ad libitum fed group in each organ and at each of the three ages [38]. However, the accuracy of the assays used to determine the carbonyl content has been seriously questioned [39], since carbonyls may also be derived from other sources besides oxidation of amino acid residues, such as collagen crosslinking, or glycosylation of proteins [40]. In addition, nucleic acids also contain carbonyl groups. The main carbonyl products of metal-catalyzed oxidation of proteins in vitro have been shown to be glutamic and aminoadipic semialdehydes [41].

Table 1: **Specific protein oxidation markers, in extra- (1-3) and intracellular (4-9) proteins from human tissue, correlating with aging.**

Tissue	Marker
1. Skin collagen	N^{ε}-(carboxymethyl)-lysine (CML) N^{ε}-(carboxymethyl)-hydroxylysine (CMHL) pentosidine o-tyrosine methionine sulphoxide (MetSOx)
2. Articular cartilage	CML N^{ε}-(carboxyethyl)-lysine (CEL) pentosidine
3. Lens protein	CML L-3,4-dihydroxyphenylalanine (L-DOPA) *o,o*'-dityrosine 3-hydroxy valine
4. Brain protein	protein carbonyl content
5. Kidney	protein carbonyl content
6. Liver	MetSOx *o,o*'-dityrosine
7. Cardiac muscle	*o,o*'-dityrosine o-tyrosine * protein carbonyl content
8. Skeletal muscle	*o,o*'-dityrosine

*) The correlation of o-Tyr with aging in cardiac muscle is rather uncertain.

Other markers may be derived from ROS-induced protein oxidation, but may be susceptible to further reactions. For this reason, a marker like L-DOPA (L-3,4-dihydroxyphenylalanine), a product of tyrosine oxidation, is not an ideal marker for protein oxidation [42, 43]. Protein-bound DOPA may be further oxidized to dopaquinone or directly degraded by proteinases, releasing free DOPA. Subsequently, DOPA may be re-incorporated into proteins by protein synthesis, form Michael adducts with free amino acids such as cysteine or may be excreted via the urine as free DOPA or as cysteinyl-DOPA.

Tyrosine residues may be oxidized by hypochlorite, peroxynitrite or by radicals formed in transition metal ion-catalyzed Fenton and Haber-Weiss reactions (e.g. hydrogen peroxide/Fe^{2+}) [44-46]. The ensuing tyrosyl radicals may form intra- or intermolecular *o,o*'-dityrosine bonds [47]. Levels of *o,o*'-dityrosine have been shown to

increase with age in human lens [48], and cardiac and skeletal muscle of ad libitum fed mice [49]. Caloric restriction prevented the increase in *o,o*'-dityrosine levels in these animals. Moreover, the oxidation-dependent increase of hydrophobicity of rat liver proteins was even correlated with an increase in the levels of methionine sulfoxide (MetSOx) and of *o,o*'-dityrosine [50].

Several other oxidized residues, like hydroperoxides of amino acid side chains are highly unstable. Some oxidation products, like cystine, are inappropriate markers being a natural product as well as a ROS-induced oxidation product, while N-formylkynurenine (an oxidation product of Trp) can be generated enzymatically. MetSOx and disulfides may be enzymatically reduced, resulting in an underestimation of the oxidation levels, whereas other species, like 4-hydroxyproline, coelute with their stereoisomer, making it a poor marker. Besides the accumulation of glycated proteins in lens (see below), extensive hydroxylation of protein-bound amino acid residues has been shown to associate with the development of age related cataract [51]. The relative abundance of the oxidized amino acids in these lens protein (assessed per parent amino acid) is L-3,4-dihydroxyphenylalanine (DOPA) > o- and m-tyrosine > 3-hydroxyvaline, 5- hydroxyleucine > dityrosine.

In the study of age-related increases in levels of oxidized material, disparities have been observed between intracellular and extracellular proteins. In extracellular proteins, the levels of oxidative markers were found to increase more with age than in intracellular proteins [36]. This disparity might be explained by a difference in turnover between extracellular (hours-days) and intracellular proteins (minutes-hours). The difference in homeostatic control between extra- and intracellular proteins might also play a role. Upon extracellular radical damage, a lack of availability of antioxidants, reductants and repair mechanisms might explain the higher extracellular levels of oxidative modifications. Furthermore, disparities were observed in levels of oxidized proteins in the organs liver, skeletal muscle and eye lens. These differences may be based on different metabolic rates in these organs. Higher metabolic rates in mice may also account for disparities in rates of ROS production and protein oxidation between mice and rats. In mice, higher levels of oxidized protein have been observed as compared to rats [36]. In the cardiac muscle of aging mice, an increase in the levels of o-tyrosine has been observed, in contrast with the cardiac muscle of rats [36]. So the correlation of o-tyrosine with aging in cardiac muscle is rather uncertain. In several studies on the eye lens [34, 52], increases in protein carbonyls, MetSOx, L-DOPA, o- and m- tyrosine, Val and Leu hydroxides and the oxidation of Cys residues to sulfinic acids and cysteic acid have been reported with aging as well as a loss of Met and Cys. Together with the markers, presented in table 1, the accumulation of these markers gives growing evidence for the age-related accumulation of oxidized proteins in extracellular tissue. As for intracellular proteins, an age-related accumulation of oxidized proteins still requires further proof, although a decrease in cellular antioxidant defence as well as an increase in intracellular protein oxidation has been reported several times [35, 53].

Oxidation of long-lived proteins

For the study of aging and age-associated phenomena, long-lived biomolecules are of fundamental interest. Some of the longest-lived proteins of the body, the crystallins, can be found in the ocular lens. Their low turnover has made crystallins a subject of widespread research activity in determining various posttranslational modifications of aged proteins [2]. Next to crystallins, extracellular proteins like collagen and elastin have a longer lifetime, making them also suitable for studying age-associated phenomena [54]. Perhaps the most important changes in collagen and elastin with age involve the formation of intermolecular cross-links. These cross-links are initially formed (through lysyl oxidase) to provide an optimum function during development and maturation, but can subsequently over-stiffen and compromise the structure and function of the fibers throughout the body when present in excess [55]. A second process of cross-linking is based on the reaction of proteins with glucose or its metabolites that occurs with age as the turnover of the proteins is reduced to a minimum for that particular tissue [25]. The end-stage products of Maillard reactions in biological systems is the result of rearrangement, dehydration, oxidation and fragmentation reactions of glucose or its adducts to protein and are known as advanced glycation end-products (AGE) [25]. AGEs play an important role in the pathogenesis of angiopathy in diabetic patients and in the aging process. About a dozen different AGEs have been identified of which several are known to accumulate with age, particularly in long-lived proteins such as collagens and crystallins. N^{ε}-(carboxymethyl)lysine (CML in table 1) is formed on oxidative cleavage of carbohydrate adducts to lysine residues in glycated proteins in vitro [56] and has shown to accumulated in aged human lens [57]. Another AGE that has shown to accumulate with aging is pentosidine [58, 59]. Pentosidine, named after the reaction of pentoses with proteins, forms crosslinks between arginine, lysine, and a pentose [60]. In concert with CML and pentosidine, formed during glycoxidation of collagen in aged human skin [61], ortho-tyrosine and methionine sulfoxide are formed [22, 58, 62]. An accelerated rate of this age-dependent chemical modification of collagen was observed in diabetes [63], but could not be subscribed to a general increase in oxidative stress in this disease. A marked increase of CML and pentosidine, and N^{ε}-(carboxyethyl)lysine to a lesser extend were also found in articular cartilage collagen, which may very well contribute to the more rigid and fragile nature of cartilage with advancing age [59].

In view of all the above mentioned complications only a few side chain oxidation products are appropriate markers for measuring age-related increases in protein oxidation. For measuring these increases, immunologic methods may be used as well as GC/MS and HPLC [42]. An alternative approach might be the introduction of probes which will covalently label oxidatively modified proteins. We recently introduced of a probe, denoted as TyrFluo, which only labels oxidized proteins and which has made it possible to detect intracellular and extracellular oxidized proteins [64]. The probe consists of a fluorescein-labelled tyrosine analogue (i.e. tyramine) that upon oxidation by ROS is converted into a tyrosyl radical that can form cross-links with oxidized target proteins. As a result of this coupling reaction these proteins become fluorescently labelled and thus can be visualized by fluorescence microscopy.

Further more the covalently modified proteins can be detected on a Western-blot by immunodetection using an anti-fluorescein antibody. The non-membrane permeant TyrFluo has been used to label cell surface proteins. Acetylation of TyrFluo (AcetylTyrFluo) makes the probe membrane-permeable so that intracellular proteins can be labelled. (The nature of the detected proteins will be discussed later)

Caloric restriction, oxidative stress and aging

Caloric restriction, the only method known for extending life span, delays most age-related physiologic changes and is the most effective means known for reducing cancer incidence and increasing the mean age of onset of age-related diseases and tumors [65]. The process of aging has shown to be associated with specific gene expression profiles, indicative of a marked stress response and lower expression of metabolic and biosynthetic genes of individual organs [66]. When compared at the same chronological age, strains of short-lived mouse contain higher levels of oxidized proteins than the longer-lived strain [67].

The beneficial age-associated effects of caloric restriction are reflected in, (1) an inhibition of accumulation of oxidatively damaged proteins [68, 69], (2) a reduction of enzyme activity decline in old rats [70], (3) a significant decrease of H_2O_2 production of rat liver mitochondria correlating to a significant reduction of oxidative damage to mtDNA [71], and, (4) alterations of gene expression, which suggests that caloric restriction retards the aging process by causing a metabolic shift toward increased protein turnover and decreased macromolecular damage [72, 73].

Aging and the Endoplasmic Reticulum

As referred to above, there are numerous (oxidative) theories of aging. As we will discuss here damage to the endoplasmic reticulum (ER), and its subsequent impaired functionality may be involved in the process of aging.

There are several phenomena that suggest its involvement in the aging process, like (1) the upregulation of ER stress response chaperones, the expression of which is decreased upon caloric restriction, (2) the susceptibility towards oxidation of ER resident proteins that are involved protein folding and, (3) impairment of maintaining the calcium homeostasis.

In this perspective it is noteworthy that in age-related neuronal diseases ER dysfunction has been reported [74]. Whereas the ER-associated degradation of misfolded proteins is affected in Parkinson's disease, it is the unfolded protein response that is down-regulated in Alzheimer's disease and the ER calcium homeostasis that is disturbed in ischemia.

Caloric restriction and stress response proteins

By binding to other proteins, chaperones (1) protect against aggregation, (2) solubilize protein aggregates, (3) assist in protein (re)-folding, (4) target ultimately damaged proteins to

degradation and (5) sequester overloaded damaged proteins to larger aggregates [75].

The glucose regulated proteins (GRP) are a family of stress-induced molecular chaperones [76]. Expression of these proteins is induced in cultured cells by agents that interfere with the normal glycosylation, folding or assembly of proteins in the endoplasmic reticulum thereby increasing the level of misfolded proteins [77]. The importance of protein oxidation in biological aging is reflected in the age-dependent upregulation of stress response genes that process damaged or misfolded proteins [73]. Therefor, the influence of dietary energy restriction on expression of the ER associated chaperone RNA was investigated in mice liver [78, 79]. Although extreme glucose deprivation increases GRP mRNA levels in cultured cell lines, physiologically relevant reductions in blood glucose, as a consequence of long-term energy restriction, had the opposite effect in the liver, in vivo. It was suggested that life-prolonging energy restriction might act to reduce misfolded proteins in the endoplasmic reticulum of hepatic cells since the expression of nearly all endoplasmic reticulum chaperones responded rapidly and specifically to dietary energy in mice. A reduction of hepatic expression of the mRNA's of GRP78 (BiP), GRP94 (endoplasmin), GRP170, ERp72, ERp57, calreticulin and calnexin but no change in protein disulfide isomerase (PDI) was observed. PDI is responsible for isomerisation of protein disulfide bonds during or shortly after synthesis to yield proteins with native disulfide bonds [80]. ERp72 and ERp57 are also involved in the rearrangement of both intrachain and interchain disulfide bonds in proteins to form the native structures since they possess the thiol-dependent reductase activity of PDI [81] and cysteine protease activity [82].

Oxidation of ER proteins that are involved protein folding

The mitochondrion and the peroxisome as major sites of ROS production in the cell are well protected against oxidative damage by anti-oxidant enzymes. Thus superoxide anion produced in the respiratory chain is converted by Mn-SOD whereas H_2O_2 produced in the peroxisomal b-oxidation is degraded by catalase. Such a defence mechanism has not been described for the ER and may play a role in the recent observation that particularly proteins of the ER are susceptible to oxidation by H_2O_2 [83]. Upon incubation of fibroblasts with 10 μM H_2O_2 we identified, by use of the earlier discussed membrane-permeable probe (acetylTyrFluo) [64], proteins of the folding/quality control system in the ER of normal human dermal fibroblasts as the major targets for this oxidative stress. These proteins included BiP/GRP78 (Immunoglobulin heavy chain binding protein or 78 kDa glucose-regulated protein), calnexin, GRP94/endoplasmin, protein disulfide isomerase (PDI), and protein disulfide isomerase ER-60 precursor (also known as ERp60/58kDa microsomal protein/p58/GRP58/ERp57) that are all known to reside in the ER lumen. Since H_2O_2 itself is a weak oxidant it must be converted into highly reactive hydroxyl radical (OH·) for proteins and TyrFluo to become oxidized. Given the very short lifetime of the OH· ($< 10^{-6}$ sec) we infer that the radical formation most likely occurs in the ER. Here H_2O_2 may be converted into OH· 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 [84]. Anari *et al.* [85] demonstrated that cytochrome P450 in intact rat hepatocytes could function as a peroxygenase utilizing *tert-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 [86]. Another possibility is that H_2O_2 is converted into OH· by the Fenton reaction using transition metal ions available in the ER [87].

The ER plays a central role in the synthesis and distribution of many cellular proteins. Before proteins can be transported towards their final destination, disulfide bonds essential for a proper folding have to be formed [88]. A requirement for this oxidative protein folding is a high redox-state [89], which implies that there must be an optimum ratio of thiol and disulfide (< 10). The typical redox-state of the cytosol is too reducing for formation of protein disulfide bonds. In the ER lumen, the relative abundance of the oxidized (GSSG) compared to the reduced (GSH) form of glutathione has led to the proposal that GSSG serves as the oxidizing equivalent during protein folding [90]. The ratio [GSH]/[GSSG] in the ER is 1:1 to 3:1 as compared to 30:1 to 100:1 for the overall cellular ratio.

It is remarkable that all the Tyrfluo labeled proteins identified (i.e. PDI, BiP, calnexin, endoplasmin and PDI precursor ER60/GRP58) reside in the lumen of the ER. So, an explanation that particularly these proteins are highly susceptible to oxidation by H_2O_2 may be found in the peroxygenase activity of cytochrome P450, or an ER associated Fenton reaction in combination with the high redox-state of the ER. Normally, a quality control mechanism ensures that only correctly folded proteins exit the ER. Incorrectly folded proteins are retained and will be degraded. But what if oxidation of the protein folding machinery occurs? May this lead to an improper folding and/or accumulation of proteins meant to be secreted? Will less proteins be secreted and/or be present at the surface of cells? This will not only hamper proper cellular function, but might also result in increased oxidative stress. The latter because impaired secretion and accumulation of proteins in the ER resulted in a large increase in carbonylated proteins in Saccharomyces cerevisiae [91].

The ER quality control system includes a number of chaperones and folding enzymes localized in the lumen or in the membrane of the ER [92]. It is likely that most, if not all, proteins synthesized in the ER interact with chaperones at some stage of the folding and maturation pathway. The chaperones bind non-mature proteins and are thought to assist folding by preventing irreversible aggregation and misfolding. Exactly how chaperones act in concert to guide immature proteins through the folding pathway, and selectively retain improperly folded proteins, is not fully understood. Although some chaperones in the ER are well studied, including PDI, GRP58, GRP78, GRP94, calnexin, and calreticulin, it is at present impossible to predict with which chaperones a specific protein will interact and to predict the consequences of the interaction.

Oxidation of calcium regulatory proteins

A nonselective oxidation of many cellular proteins has been suggested during aging since approximately, one-half of intracellular proteins are oxidized in senescent animals [93]. However, in the majority of cases the oxidation of one or two amino acids has a minimal effect on protein function, altering neither the stability nor the function of the protein [94]. In contrast, some proteins are selectively oxidized at critical sites that regulate their function. The structural and functional consequences associated with the oxidative modification of unique sites of calcium regulatory proteins have been identified [95-97]. Methionines in calmodulin are oxidized to their corresponding methionine sulfoxides. It seems that sarco-/endoplasmic reticulum calcium ATPase pump (SERCA) can be inhibited both by oxidation of its sylfhydryl groups [98] and nitration of specific tyrosines on the ATP-binding site [95]. In addition, oxidation by direct attack of hydroxyl radicals on the ATP-binding site [99], which could be prevented by antioxidant treatment [100], leads to decreases in ATP-dependent calcium fluxes across membranes and hence decreased ATP-consumption. Subsequent depletion of calcium in the ER inhibits protein-synthesis [101] and -processing, causing partially folded proteins to accumulate. This activates the transcription of ER chaperone genes, such as GRP78/BiP and GRP94 and calreticulin [102] to increases the capacity of intracellular calcium stores and to prevent cellular calcium toxicity. This strongly suggests that oxidative modifications contribute to the age-related decline of function of the SERCA [103] and the observed increases in intracellular calcium levels [104].

On the other hand, a decreased mitochondrial ATP-production, as a result of lower ATP-consumption by the ER, might result in a decrease of ROS production. By reducing ATP synthesis, reducing equivalents generated in the mitochondrial matrix can be used in the cytosol to enhance antioxidant defence mechanisms and cellular repair processes that minimize the accumulation of oxidized biomolecules [24].

Prolonged retention of misfolded and incompletely folded proteins in the ER leads to their degradation. ER-associated degradation (ERAD) is mainly carried out by the 26*S* proteasome located in the cytosol [105]. The process occurs in several steps: terminally misfolded or unassembled proteins are recognized by ER chaperones such as calnexin, GRP78/BiP, or by other factors such as specific mannose lectins. They are then retranslocated through the Sec61 channel into the cytosol [106], deglycosylated (in the case of glycoproteins), and polyubiquitinated before proteasomal degradation. How proteins that are destined to be degraded are identified and targeted for retrotranslocation has not been established, but it is likely that the machinery responsible for protein folding again plays a role in the selection and preparation of aberrant products for disposal.

Glutathione involvement in aging

Glutathione (GSH) is a tripeptide of glutamate-cysteine-glycine and is involved in a wide variety of biological reactions such as the maintenance of protein thiol groups in the reduced state, removal of hydrogen peroxide and detoxification of xenobiotics. GSH is converted to

oxidized glutathione (GSSG) by seleno-dependent glutathione peroxidase (GPx). GSSG is subsequently reduced back to GSH by glutathione reductase (GSSGR) on the expense of NADPH. These two enzymes maintain the cellular balance between GSH and GSSG. The relative oxidizing environment of the ER compared to the cytosol, as expressed by the ratio of GSH and GSSG, was suggested as being a factor contributing to the preferred oxidation of ER resident proteins. The imbalance of this ratio creates oxidative stress and logically, the lower amount of GSH available would give less protection upon oxidative attack. Until now it is not known whether the ER glutathione balance or concentration changes during aging. More and more evidence is obtained that the systemic GSH concentration decreases with advancing age. In healthy aging human adults low blood glutathione levels are observed [107]. Erden-Inal et al. [108] found a positive correlation between age and GPx and a negative with GSSGR resulting in a low ratio GSH/GSSG in human erythrocytes. Studies performed on houseflies showed that H_2O_2 concentration steadily increases with age and that the intracellular redox potential becomes progressively more pro-oxidizing, or less reducing, during the aging process, since the ratios of reduced/oxidized forms of glutathione, NAD and NADP decline with age [109]. At the subcellular level both cytosolic and mitochondrial GSH concentration have shown to be lower in old than in adult ad libitum fed rats [110]. Life prolonging food restriction did not prevent this decrease, but its extent was attenuated considering the cytosolic GSH. As regards the mitochondrial GSH, its content was higher in adult food restricted animals than in the age-matched ad libitum fed ones

Conclusions

During biological aging, a progressive accumulation of errors at the genetic level might lead to a shift in the balance between protein oxidation and oxidized protein degradation in favor of oxidized protein accumulation and attendant loss of biological function [35]. ROS-induced protein oxidation may lead to protein fragmentation, through the oxidation of the protein backbone, yielding protein carbonyls, and to the oxidation of amino acid side chains. In order to determine the levels of oxidative damage stable oxidation markers are required. As discussed above, in aged extracellular proteins an increase in the levels of these markers has been shown, while this increase is still a matter of debate for intracellular proteins. This relative lack of intracellular accumulation may well reflect the relative efficiency, with which cells can control their intracellular milieu and prevent, or repair, oxidant-mediated events. Another reason for this discrepancy between intracellular and extracellular proteins might be the slow turnover rate of extracellular proteins [111]. To date, limited data are available from both human and animal studies, which may be caused by the rapid excretion of oxidized amino acids via urine [111].

Through the oxidation of essential cellular proteins, aging may have deleterious effects on cellular function. The age-dependent upregulation of ER stress response genes that process damaged or misfolded proteins may reflect the importance of protein oxidation for biological aging [73] and a common defence mechanism against these effects in diverse species [112].

Upregulation of these genes in aging may imply the presence of accumulated damaged and misfolded proteins in the endoplasmic reticulum caused by improper folding (see figure 1). Proper folding requires optimal functioning of folding proteins. As a consequence of oxidation of the ER Ca^{2+}-ATPases the required calcium concentration inside the lumen of the ER cannot be established, and will cause an impaired functioning of the calcium-binding chaperones. The cytosolic decline of GSH levels may have its impact in the ER thereby creating a more oxidative environment, as expressed by the low ratio of GSH/GSSG. Oxidation of ER resident proteins will cause an even further functional decline and will contribute to an impaired folding as well as a hampered retranslocation of proteins destined for cytosolic degradation. This accompanied by an age-related decrease in the levels or activities of the proteasome [32] may contribute to the "garbage catastrophe theory". In addition, dietary energy restriction showed a reduction in the expression of these stress response genes. This suggests that life-prolonging energy restriction might act to reduce damaged and misfolded proteins in the endoplasmic reticulum.

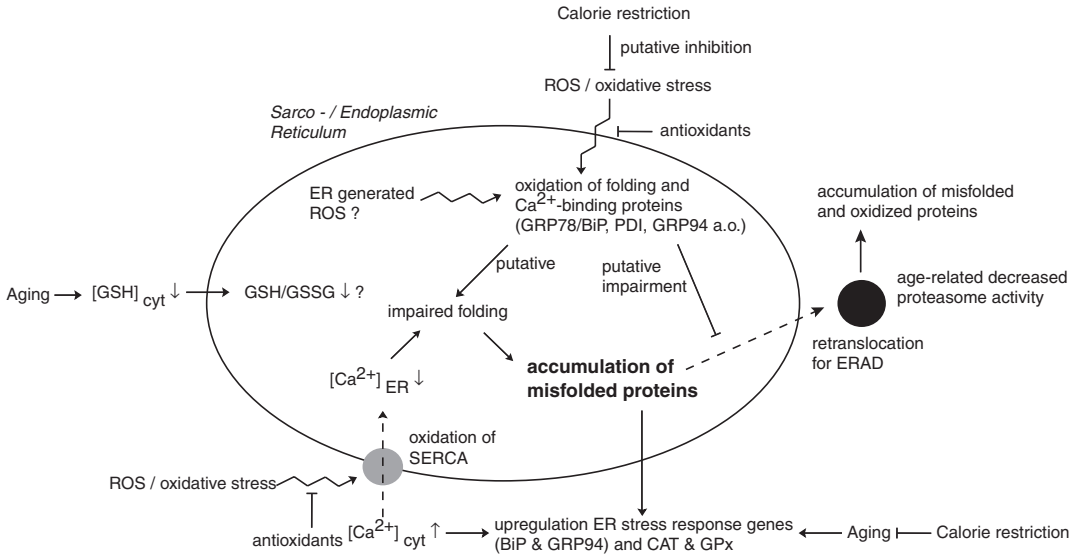

Fig 1: **Proposed mechanism for the contribution of oxidized sarco-/endoplasmic reticulum proteins to the accumulation of damaged and misfolded proteins in aging.** SERCA, Sarco-/Endoplasmic Reticulum Calcium ATPase; ERAD, ER-associated degradation; CAT, catalase; GPx, glutathione peroxidase

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Chapter 7

Summarising discussion

Development of a new probe to detect protein oxidation in living cells

Biomarkers of protein oxidation include amino acid oxidation products (methionine sulfoxide, ortho-tyrosine (o-tyr) and dityrosine, chlorotyrosine and nitrotyrosine), as well as chemical modifications of protein following carbohydrate or lipid oxidation, such as N-(carboxymethyl)lysine and N-(carboxyethyl)lysine, and malondialdehyde and 4-hydroxynonenal adducts to amino acids. These are normally measured by sensitive high-performance liquid chromatography [1, 2], or gas chromatography-mass spectrometry methods [3], requiring both complex analytical instrumentation and derivatization procedures. It was already recognized that immunochemical assays should facilitate studies on the role of oxidative stress in aging and chronic disease and simplify the evaluation of therapeutic approaches for limiting oxidative damage in tissues and treating pathologies associated with aging and disease [4, 5].

A prominent target for oxidation by reactive oxygen species (ROS) is the tyrosine residue. This thesis describes a contribution to the field of measuring protein oxidation by introducing and utilising a novel oxidation sensitive probe (chapter 2). The method is based on the fluorescein-labelled tyrosine analogue, tyramine, which upon oxidation may couple to proteins carrying a tyrosyl radical. Coupling of the probe (denoted TyrFluo) to standard proteins could be induced by generating ROS with horse radish peroxidase/hydrogen peroxide, SIN-1 or with peroxides (cumene or hydrogen peroxide) in combination with a transition metal. Immunodetection of labelled proteins was performed with an HRP-conjugated polyclonal antibody against the fluorescein moiety. The labelling efficiency differed for the various proteins and varies with the oxidant used. The efficiency of labelling of a protein depends on the type of ROS used, the ability of a target protein to bind metal ions, and the number and the accessibility (solvent exposure) of tyrosine residues. In general, labelling increased with increasing ROS concentration. In some instances labelling reached a plateau suggesting that the number of tyrosine residues modified was maximal. In other instances labelling decreases at high ROS concentrations, which may be due to diTyrFluo formation, thereby making TyrFluo unavailable for reaction with the proteins, or to oxidative damage of the fluorescein moiety, thereby interfering with the immunodetection.

Fluorescein is principally non-toxic which makes TyrFluo ideally suited to probe tyrosylation reactions in living cells. Since the fluorescein contains a charged carboxyl moiety, TyrFluo is barely membrane-permeable. Treatment of TyrFluo with acetic anhydride yielded an acetylated form of TyrFluo (acetylTyrFluo), which is taken up by the cells within minutes. After uptake, deacetylation of the conjugate by intracellular esterases yields the charged and relative membrane impermeable TyrFluo again. Exposure of the cells (rat-1 fibroblasts) to oxidative stress (hydrogen peroxide) in the presence of either TyrFluo or acetylTyrFluo gave a cellular labelling characteristic for each probe. So, besides the applicability in immunochemical assays the probe provides an extra dimension by visualising protein oxidation in living cells. Western blot analysis confirmed that each probe labelled a characteristic set of proteins. Both probes labelled cellular proteins with different efficiencies independent of the abundance of a particular protein [6].

By using a fluorescence microscope one can literally see the oxidation occurring since the probe is fluorescent. TyrFluo added to rat-1 fibroblasts remained outside the cell whereas the acetylated form (acetylTyrFluo) was membrane-permeable and accumulated in the cell. Exposure of the cells to oxidative stress in the presence of either TyrFluo or acetylTyrFluo gave a cellular labelling characteristic for each probe. Western blot analysis confirmed that each probe labelled a specific set of proteins. Although the formation of a dityrosine bond is likely we cannot exclude that the TyrFluo-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 to form 5-S-cysteinyl-DOPA-Fluo [7, 8]. The nature of the bond that has been formed between the target protein and TyrFluo may therefore not necessarily be dityrosine. However, whatever the nature of the bond, the covalent coupling of TyrFluo to proteins can be taken as a measure of oxidation.

In the search for those intracellular proteins that are most susceptible to oxidation by H_2O_2 we have used the membrane-permeable conjugate acetylTyrFluo to label proteins in living normal human dermal fibroblasts (NHDF). The switch from rat to human cells was made because of the greater importance and the larger amount of identified proteins in 2D-PAGE databases. TyrFluo-labelled and ^{35}S-methionine-labelled proteins of NHDF were mixed and ran on the same first dimension IEF-gel, second dimension SDS-PAGE and blotted on nitrocellulose. 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 identification revealed that the bulk of the labelled proteins were associated with the endoplasmic reticulum (ER). These included BiP/GRP78 (immunoglobulin heavy chain binding protein or 78 kDa glucose-regulated protein), calnexin, endoplasmin/GRP94, protein disulphide isomerase (PDI), and protein disulfide isomerase ER-60 precursor (also known as Erp60/58kDa microsomal protein/p58/GRP58/Erp57) which are all known to reside in the ER lumen. Unfortunately some labelled could not be unequivocally matched with the autoradiogram. In agreement with this identification, fluorescence microscopy showed that the TyrFluo-labelled proteins co-localized with the KDEL-receptor ERD2 on the ER membrane.

Why is the endoplasmic reticulum so sensitive to oxidative stress?

The mitochondrion and the peroxisome as major sites of ROS production in the cell are protected against oxidative damage by anti-oxidant enzymes. Thus superoxide anion produced in the respiratory chain is converted by Mn-SOD whereas H_2O_2 produced in the peroxisomal b-oxidation is degraded by catalase. Such a defence mechanism has not been described for the ER and may explain as to why proteins of the ER are susceptible to oxidation by H_2O_2. The observation that mainly the ER proteins 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 [9]. 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 proposed that it must be converted first into highly reactive OH· for proteins and TyrFluo to become oxidized. Given the very short lifetime of the OH· we inferred that the radical formation occurs in the ER. A possibility is that H_2O_2 is converted into OH· by the Fenton reaction using transition metal ions available in the ER [10]. It has been shown that cytochrome P450 can operate in vitro as a peroxygenase using peroxy compounds as the oxygen donor [11]. Here H_2O_2 may be converted into OH· by cytochrome P450 enzymes. Anari *et al.* [12] demonstrated that cytochrome P450 in intact rat hepatocytes could function as a peroxygenase utilising *tert-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 [13]. Rashba-Step and Cederbaum suggested the possibility that human liver microsomes are an important source of reactive oxygen intermediates, especially under conditions of increased NADH or NADPH availability and elevated iron concentration. [14]. Later, Puntarulo and Cederbaum showed that microsomes enriched in specific human cytochrome P450 enzyme complexes, especially CYP3A4, produce reactive oxygen species [15]. Next to the possibility to produce them, the amount of produced ROS must also exceed the defence capacities, since we are speaking here of 'a state of oxidative stress'. And evidently this state was first reached inside the ER. The oxidizing environment of the ER, that is believed to be established by the balance between the reduced (GSH) and oxidized glutathione (GSSG) levels, and which is a prerequisite for proper disulfide-bridge formation in oxidative folding of newly synthesized proteins, may also provide the right milieu for its residing proteins the to be particularly sensitive for oxidation. In spite of all these speculations it is at present unclear why the ER is the major target and it remains to be unequivocally proven that the OH· are formed in the ER.

Can the rate of oxidation be regulated?

The fact that some of the ER resident proteins were identified as being TyrFluo-labelled does not mean that other proteins cannot be labelled. In principal all proteins that contain an accessible tyrosine residue (assuming that dityrosine formation is the major bond that is formed) may be labelled. The ER proteins only turned out to be the most sensitive to oxidation and were the first ones that appeared after the western-blot-exposed film was developed. A few experiments were performed to influence the TyrFluo-labelling of proteins. The most straightforward attempt to change the GSH levels was done by the addition of GSH or its monoethylester to the culture medium. Both compounds indeed caused a decrease in labelling at comparable effective concentrations. The role of GSH in the protection against oxidation was further investigated by either inhibiting its synthesis with buthionine-sulfoximine (BSO), or by impairing glutathione peroxidase, the enzyme that utilises GSH as a substrate, with mercapto-succinate (MS). Both ways caused an increased labelling when the pre-incubation period was performed overnight. When the pre-incubation period was reduced to 2 hours this effect was not observed. Probably, there is an abundance of GSH

available to resist the first oxidative challenges and the inhibition of synthesis is not an instantaneous process. 2D-PAGE of the BSO-pre-incubated sample showed the same oxidized protein pattern but with an increased labelling intensity. This suggests that BSO increased the sensitivity of the ER to oxidative stress by reducing the ratio between GSH and GSSG, creating an environment that is even more oxidising. In addition, also some additional proteins were labelled. Unfortunately, the identity of these proteins is presently unknown. BSO treatment will reduce the GSH content, and thereby the anti-oxidative capacities, throughout the cell. Therefore, these additionally labelled proteins may be of any origin, ER as well as from other sites of the cell. The effect of MS can be subscribed to a less efficient scavenging of H_2O_2. The same is true is for amino-triazole that inhibits catalase activity.

Pre-incubation with vitamin C protected against labelling. At what sites in the cell vitamin C scavenged H_2O_2 is not known, it may be inside the ER, but it may also be in the cytosol. In either way it prevents H_2O_2 from oxidising the ER. As expected, the lipophilic membrane-bound antioxidant vitamin E did not show any effect on labelling with any of the concentrations or incubation periods used.

What happens with the functionality of the oxidized ER?

In chapter 5 experiments were conducted to get a clue in answering the question "What does the oxidation of the identified ER-resident proteins, which are all involved in protein folding mean for their functionality? In other words, is protein folding/maturation impaired after subjecting cells to oxidative stress?". In this regard the involvement of the major cellular redox buffering system GSH/GSSG was considered to be important. To answer these questions the maturation of a [^{35}S]-methionine labelled model protein, the low-density lipoprotein receptor (LDLr) of rat-1 fibroblasts was followed in pulse-chase experiments under oxidative conditions. Under the same conditions the ratio of GSH versus GSSG was measured and related to the degree of maturation of the LDLr. Maturation of the LDLr, as defined here, is the series of post-translational events that have to take place for a cell to produce a fully functional, 160 kD, *O*-glycosylated protein. This series of ER-related events comprise: (1) folding and re-folding (shuffling) of the 120 kD precursor into a more compact form (formation of 30 S-S bridges, most likely performed by isomerases like PDI), which is a prerequisite for (2) vesicular transport towards the Golgi complex, and (3) *O*-glycosylation (at 18 sites) inside the Golgi complex.

When cells were allowed to produce mature proteins in the presence or absence of 1.5 mM H_2O_2 the velocity of LDLr-maturation was reduced. This reduction was measured by determining the intensity of the ^{35}S-radioactivity signals of the precursor and the mature form at different time points. When cells were stressed with 2.0 mM H_2O_2 or higher maturation did not occur anymore. Several explanations may be put forward: (1) the folding proteins are oxidized and are not able to fold LDLr properly (a prerequisite for transportation towards the Golgi complex for further maturation), (2) the free sulfydryls of LDLr may have been oxidized to sulfoxides, preventing disulfide bridge, (3) oxidized LDLr is degraded by the

proteasome, (resulting in lower labelling signals) (4) vesicular transport towards the Golgi complex is eradicated, or (5) glycosylating enzymes in the ER and/or Golgi lost functionality as a consequence of oxidation. However, when cells were allowed to recover after 2.0 mM H_2O_2 oxidative challenge, maturation could be restored. Apparently the defensive capacities were sufficient or quickly upregulated to overcome this challenge, and cells continued with protein synthesis, proper folding, vesicular transport, and glycosylation. It was investigated whether the recovery depended on protein synthesis (translation). Therefore cycloheximide was added during the recovery period to stop protein synthesis. Under normal circumstances cells were able to recover and restore maturation. However, when cycloheximide was present no restoration of maturation was observed. This indicates that the proteins involved in the maturation lost their functionality as a consequence of the oxidation and that restoration of maturation requires the synthesis of a new pool of normal functioning proteins. In addition, this normal functioning seemed not to depend on a complete restoration of the GSH-GSSG balance. Oxidation with 2.0 mM H_2O_2 (condition for impaired maturation) caused a decrease of the ratio GSH/GSSG to as less as 1% of control levels. After the recovery period the cellular ratio had increased only from 1 to 4.

The sensitivity of the ER to oxidation was already shown before [16]. A combination of quantitative radiometry and Western blotting in 2-D PAGE revealed that PDI was selectively degraded and then new PDI was synthesized, following H_2O_2 exposure. In addition, PDI degradation was blocked by inhibitors of the proteasome, and by cell treatment with proteasome C2 subunit antisense oligonucleotides.

At this point the questions that were raised in chapter 5 can only partly be answered. Yes, after subjecting cells to oxidative stress the ER proteins will become oxidized. On the other hand, under all oxidative conditions used, the cytosol and its content will be heavily damaged as well. Therefore, this impairment can not be put on the account of the ER solely. The transport towards the Golgi complex may also be reduced dramatically, which will also contribute to the impaired maturation of the LDLr.

The possible involvement of the malfunctioning ER in aging.

The request of the editor of *Amino Acids* was to contribute to a special issue of the journal entitled 'Amino acid and protein modification by oxygen and nitrogen reactive species', on the subject 'Protein oxidation in aging'. We used this opportunity to combine this subject with our findings of the identified protein targets in the ER. The title of the unrevised manuscript as presented in chapter 6 is 'Protein oxidation in aging: endoplasmic reticulum as a target."

Biological aging is a function of several closely inter-related parameters, such as the metabolic rate, caloric intake, genetics, lifestyle and environmental factors and could be defined as, "A complex process in which diverse deleterious changes in cells and tissues accumulate with advancing age. These changes cause a loss of cellular function that results in an increased mortality and represents a major risk factor for age-related diseases like cancer, diabetes mellitus, rheumatoid arthritis, neurodegenerative, and cardiovascular diseases". Due

to its complexity, a very large number of theories have been proposed to explain the process of aging. The 'free radical theory of aging' describes the age-related accumulation of radical-induced damage to biomolecules and has been extended to the oxidative "garbage catastrophe theory". According to this theory, aging may derive from imperfect clearance of oxidatively damaged, relatively indigestible material, the accumulation of which further hinders cellular catabolic and anabolic functions and mainly affects postmitotic non-proliferating cells. An increase in the steady-state level of oxidatively modified proteins may be caused by (1) an increase in the formation of oxidizing species [17], (2) a decreased capacity to scavenge those species [18], (3) an increased susceptibility of the proteins to become oxidized as a consequence of transcriptional and translational errors [19], and (4) a decrease in the levels or activities of the proteasome [20] or proteases that selectively degrade oxidized proteins [21].

Damage to the endoplasmic reticulum (ER), and its subsequent impaired functionality (e.g. in protein folding and in maintaining calcium homeostasis) may be involved in the process of aging. There are several phenomena that suggest its involvement, like (1) the upregulation of ER stress response chaperones [22], expression of which is known to be decreased upon caloric restriction [23, 24] (the only method known to extend life span), (2) the preferential oxidation of ER resident proteins that are involved in protein folding [9, 16], and, (3) the disturbance of calcium homeostasis [25, 26].

It was suggested that life-prolonging energy restriction might act to reduce misfolded proteins in the endoplasmic reticulum since a reduction of the expression of nearly all endoplasmic reticulum chaperones (GRP78 (BiP), GRP94 (endoplasmin), GRP170, ERp72, ERp57, calreticulin and calnexin) was observed in hepatic cells of mice in response to dietary energy restriction. Upregulation of these genes in aging may imply the presence of accumulated damaged and misfolded proteins in the endoplasmic reticulum caused by improper folding (see figure 1 of chapter 6). Proper folding requires optimal functioning of folding proteins. As a consequence of oxidation of the ER Ca^{2+}-ATPases the required calcium concentration inside the lumen of the ER cannot be established, and will cause an impaired functioning of the calcium-binding chaperones. The cytosolic decline of GSH levels may have its impact in the ER thereby creating a more oxidative environment, as expressed by the low ratio of GSH/GSSG. Oxidation of ER resident proteins will cause an even further functional decline and will contribute to an impaired folding as well as a hampered retranslocation of proteins destined for cytosolic degradation. This accompanied by an age-related decrease in the levels or activities of the proteasome [20] may contribute to the "garbage catastrophe theory".

Future research

There are still many questions unanswered which should be addressed in future research, like:
*) What is the identity of the other oxidized proteins? Are they also of ER origin?
*) What other proteins were labelled after the BSO treatment?
*) Which proteins need to be synthesised to allow a cell to restore the maturation?
*) Does oxidative stress cause an accumulation of malfolded proteins in the ER?

A complete new direction would be to see whether oxidative stress of the ER causes an unfolded protein response and/or an ER-overload response? As a reaction to most stress situations, a cell changes the production of protein to alleviate the stress [27]. The upregulation of transcription of these proteins is achieved by activating transcription factors. Upon different stimuli (stress) the ER signals through several protein kinases to up-regulate the protein-folding capacity of the ER [28, 29, and references therein]. These stresses include glucose deprivation, calcium depletion from the ER lumen (thapsigargin), inhibition of asparagine (N)-linked glycosylation (tunicamycin, 2-deoxyglucose), reduction of disulfide bonds (dithiothreitol), expression of mutant proteins or protein subunits, overexpression of some wild-type proteins or inhibition of vesicle transport from the ER to the Golgi (brefeldin A). When protein misfolding occurs and unfolded proteins accumulate and aggregate in the ER, there is a signal that selectively activates transcription of all the genes encoding GRPs as well as other ER-localized proteins such as PDI [30]. This signal response was appropriately termed the unfolded protein response (UPR). A stress triggered by too many (properly folded) proteins (viral infection), hence called ER-overload response (EOR) activates nuclear factor kappa B (NFκB). NFκB induces transcription of genes involved in inflammatory and immune responses (cytokines). The extension of the 'garbage theory of aging' with the involvement of oxidized ER-resident proteins as proposed in chapter 6 has some tangents with the UPR and EOR that would be worthwhile to investigate. Both oxidation (H_2O_2) and other forms of ER-stress lead to release of Ca^{2+} from the ER, and to activation of NFκB (see fig 1). NFκB is also activated by a misbalance of GSH and GSSG (redox sensitive). Both NFκB activation and protein oxidation can be prevented by antioxidants. NFκB induces upregulation of 1) catalase and glutathione peroxidase, implying a response to oxidative stress, 2) BiP/GRP78 and GRP94, implying the presence of unfolded proteins. (Remember that the transcription of these GRPs was decreased upon caloric restriction = life extension.) The question would now be whether the activation of stress response genes goes via the route: H_2O_2 → oxidation of ER proteins → UPR/EOR → calcium release from the ER → NFκB → induction genes. Secondly, does H_2O_2 cause phosphorylation of ERK? Signalling in response to UPR goes via phosphorylation of the ER-membrane bound kinase ERK, which causes phosphorylation of eukaryotic translation initiation factor 2 (eIF-2a) and subsequent immediate inhibition of translation.

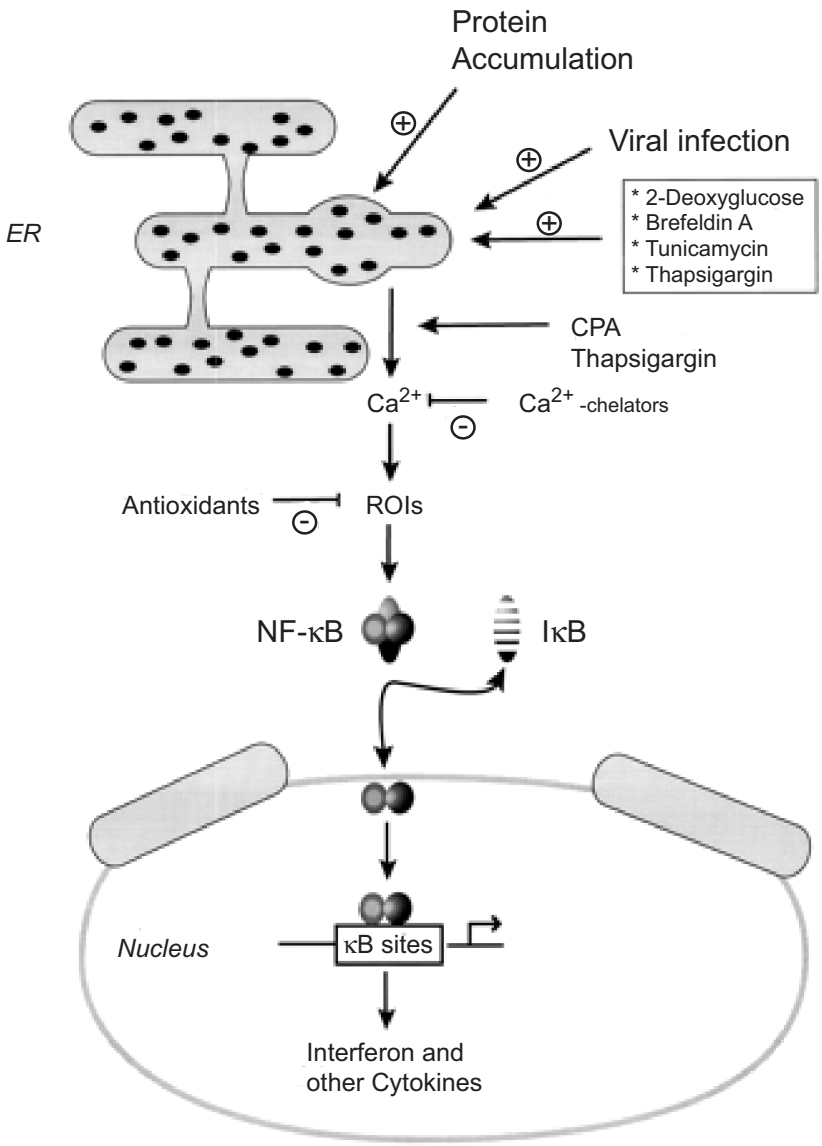

Fig 1: **ER-overload response.** Accumulation of wild-type or misfolded proteins in ER leads to a release of Ca^{2+} from the organelle. This causes or is caused by the production of reactive oxygen intermediates (ROI), which activate transcription factor NFκB. Activation can be blocked by antioxidants and by Ca^{2+} chelators, or it can be activated by drugs that cause an active release of Ca^{2+} form the ER. NFκB induced transcription of genes involved in inflammatory and immune responses and antioxidant enzymes, and some chaperones. CPA = cyclopiazonic acid. Figure taken from reference number [28].

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Chapter 8

Nederlandse Samenvatting

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Een roestige fiets, een ranzig stukje boter, zomaar twee voorbeelden uit het dagelijks leven die de 'negatieve bijwerkingen' van zuurstof demonstreren. De paradox van het leven op aarde wordt het wel genoemd: hoewel zuurstof essentieel is om te leven, is het ademen van puur zuurstof gedurende twee dagen al zeer schadelijk en zal het leiden tot de dood. Deze giftigheid komt niet zo zeer van zuurstof zelf maar meer van de zeer reactieve producten die uit zuurstof gevormd kunnen worden (ROS genaamd; een afkorting van het engelse 'reactive oxygen species' = reactieve zuurstof deeltjes). Zelfs onder dagelijkse omstandigheden worden deze ROS overal in het lichaam geproduceerd, bijvoorbeeld tijdens de voedselvertering en de energie productie, maar ook ten gevolge van luchtverontreininging (inclusief roken!) en UV-straling. De bekendste ROS zijn waterstofperoxide, ozon, superoxide anion en het hydroxyl radicaal.

Normaal gesproken is het lichaam goed in staat om zich te beschermen tegen ROS door middel van enzymatische afbraak van ROS, het wegvangen van ROS door antioxidanten (bijv. vitamine C en E) of door het wegvangen van metaal ionen (zoals ijzer en koper) om te voorkómen dat ROS geproduceerd worden. Echter, wanneer de ROS-productie hoger is dan het beschermingsmechanisme aan kan spreken we van oxidatieve of zuurstof stress. Onder deze omstandigheden kan het lichaam schade oplopen, doordat ROS reageren (oxideren) met eiwitten, vetten en/of DNA. Zelfs nu kan het lichaam zich nog beschermen door deze beschadigde moleculen ('afval') op te ruimen en te vervangen door nieuwe. Echter als deze balans, tussen aan de ene kant de productie beschadigde moleculen, en aan de andere kant de opruiming, uitslaat in het voordeel van de eerstgenoemde zal er een ophoping van onherstelbare schade plaatsvinden. Deze ophoping kan uiteindelijk bijdragen tot het ontstaan van bepaalde ziektes zoals diabetes (suikerziekte), auto-immuun ziektes (bijv. reuma), hart- en vaat-ziektes (bijv. aderverkalking), neurologische afwijkingen (bijv. Alzheimer's disease), kanker en zelfs het normale verouderingsproces.

Verouderen is aan de ene kant een heel normaal fenomeen, iedereen overkomt het en het is niet tegen te gaan (misschien alleen te vertragen door een gezonde leefwijze!). Aan de andere kant is het een heel raar fenomeen, want er zijn geen twee mensen die op dezelfde manier verouderen. Waarom kan bijvoorbeeld de ene persoon nog een marathon lopen op zijn 80-ste en is een ander al afgetakeld op zijn 50-ste? Verouderen is een zeer complex proces waarbij vele beschadigingen in cellen en weefsels optreden die zich kunnen ophopen als ze niet efficiënt opgeruimd worden. Deze beschadigingen veroorzaken uiteindelijk een functieverlies en zijn daarom de voornaamste risico factor voor, de al eerder genoemde, leeftijd-gerelateerde ziektes, met als gevolg een verhoogde sterftekans. In eerste instantie zou het voorkómen van deze (oxidatie)- schade de kwaliteit van het leven dus gunstig kunnen beïnvloeden en uiteindelijk misschien zelfs kunnen verlengen.

Om onderzoek te doen naar de relatie tussen de hoeveelheid schade en

veroudering (en ouderdom-gerelateerde ziektes) zijn er dus 'tastbare' kenmerken nodig om de 'oxidatie-status' te bepalen. Een van die kenmerken is het eiwit-oxidatie product *o,o'*-dityrosine. waarvan is aangetoond, bijvoorbeeld in urine en skelet spieren van gezonde ouderen en bij patienten met aderverkalking of Alzheimer, dat de hoeveelheid is gerelateerd aan de leeftijd van de donor. Het aminozuur tyrosine is een normale bouwsteen van lichaamseiwitten. Op het moment dat twee tyrosines geoxideerd worden, worden ze zeer reactief en kunnen ze met elkaar reageren om zo een stabiele verbinding te vormen. Dit kan gebeuren tussen tyrosines van hetzelfde eiwit maar ook tussen verschillende eiwitten. Het gevolg hiervan is dat de betrokken eiwitten beperkt worden bij het normaal functioneren of zelfs helemaal inactief kunnen worden.

Het doel van het onderzoek, zoals beschreven in dit proefschrift, was om te achterhalen welke eiwitten in gekweekte cellen het meest gevoelig zijn voor oxidatie. Hierbij wordt verondersteld dat dit ook wel eens de eiwitten kunnen zijn die in het lichaam het eerst beschadigd raken en zo bijdragen aan het verouderingsproces. Het onderzoek is gebaseerd op de genoemde dityrosine vorming. In hoofdstuk 2 is een nieuw ontwikkeld sensor-molecuul (TyrFluo genoemd) geïntroduceerd. TyrFluo bestaat uit twee gedeeltes. Tyr komt van tyramine en Fluo komt van fluoresceine, een fluorescent molecuul. Tyramine lijkt zeer sterk op tyrosine en heeft dezelfde neiging tot dityrosine vorming na oxidatie. Op het moment dat TyrFluo en een tyrosine in een eiwit geoxideerd worden kunnen ze met elkaar reageren en dezelfde stabiele dityrosine binding vormen. Op deze manier worden de eiwitten die gevoelig zijn voor tyrosine-oxidatie dus voorzien van een fluorescent label. Door dit label is het, eerder genoemde, 'tastbare' kenmerk gecreëerd en zijn deze eiwitten identificeerbaar geworden. In tegenstelling tot TyrFluo kan zijn geacetyleerde variant (acTyrFluo) opgenomen worden in de cel en is hierdoor in staat om binnen in een cel te koppelen aan eiwitten waarvan tyrosine geoxideerd wordt door ROS. Cellen van zowel de rat als van de mens zijn voor deze experimenten gebruikt. De gekweekte cellen werden 'opgeladen' met acTyrFluo (die in de cel overigens weer wordt omgezet in TyrFluo en dus niet of nauwelijks naar buiten kan lekken) en vervolgens, gedurende 10 minuten, blootgesteld aan een lage concentratie van waterstofperoxide. Na deze stress omstandigheden werden alle eiwitten uit de celkweek verzameld en met behulp van biochemische technieken van elkaar gescheiden[1]. De TyrFluo-gelabelde eiwitten werden gedetecteerd met een antilichaam dat het Fluo-gedeelte van TyrFluo herkent. In hoofdstuk 2 is laten zien dat TyrFluo en acTyrFluo inderdaad gebruikt kunnen worden om eiwitten op deze manier te labelen. In hoofdstuk 3 is acTyrFluo gebruikt

[1] Scheiding op basis van massa (electroforese op een polyacrylamide gel) gevolgd door binding op een nitrocellulose membraan (een soort eiwitbindend 'papier').

om de identiteit van deze gelabelde eiwitten te achterhalen[2]. De verkregen (2-dimensionale) labelingspatronen werden vergeleken met databanken op het internet waar de identiteit van veel (maar nog lang niet alle) eiwitten is beschreven. Op deze manier werd het duidelijk dat de TyrFluo-gelabelde eiwitten, en dus de eiwitten die het meest gevoelig zijn voor oxidatie, allemaal in eenzelfde compartiment van de cel aanwezig zijn, namelijk het endoplasmatisch reticulum (ER).

Het ER is het onderdeel van de cel (een zogenaamd organel) waar nieuwe eiwitten worden geproduceerd. Al de TyrFluo-gelabelde eiwitten zijn betrokken bij deze eiwit productie. Tijdens deze productie moeten niet alleen de aminozuren in de juiste volgorde aan elkaar worden gezet, maar ook moet de drie-dimensionale structuur, die van groot is belang om te kunnen functioneren, aangenomen worden. Om deze 3D structuur te krijgen moet het nieuw geproduceerde eiwit worden gevouwen en worden voorzien van de juiste suiker-groepen. Bij deze belangrijke processen zijn de geïdentificeerde eiwitten nauw betrokken. Op dit moment is het nog onbekend of deze eiwitten, als ze geoxideerd zijn, een rol spelen bij een ziekte of veroudering (Nog even in het midden gelaten of de schade hier de oorzaak of het gevolg van de ziekte zou zijn.). Het is in dit verband interessant dat de hoeveelheid gebonden TyrFluo be?nvloed kan worden als de antioxidatieve capaciteit van de cellen veranderd. Meer bescherming door toevoeging van bijv. vitamine C resulteerde in een afname van koppeling van TyrFluo aan de eiwitten (hoofdstuk 4).

Om vast te stellen of, ten gevolge van oxidatieve stress, de betrokken ER eiwitten hun functie verliezen is onderzocht of cellen nog in staat zijn om een goed gevouwen en functioneel eiwit te produceren (hoofdstuk 6). Hiervoor is een model-eiwit (LDLr) gebruikt. Tijdens de synthese van LDLr worden een aantal tussenvormen geproduceerd. Om de verschillende tussenvormen van de LDLr te kunnen meten is LDLr eerst voorzien van een label in de vorm van een radioactief methionine (een ander aminozuur). Om de invloed van oxidatieve stress op de ontwikkeling van LDLr te achterhalen zijn op verschillende tijdpunten de tussenvormen van LDLr, in cellen die zijn blootgesteld aan waterstofperoxide, vergeleken met cellen die niet zijn blootgesteld aan waterstofperoxide. Door het meten van de radioactiviteit van de tussenvormen bleek waterstofperoxide inderdaad de snelheid van de LDLr-ontwikkeling te verminderen of zelfs helemaal te voorkomen als de concentratie te hoog was. De conclusie was dat de ER eiwitten, die betrokken zijn bij de ontwikkeling van nieuw te produceren eiwitten, zodanig beschadigd waren, ten gevolge van de waterstofperoxide behandeling, dat de ontwikkeling van LDLr niet efficiënt verliep. Bij de vertragende omstandigheden bleek echter dat de cellen zich na

[2] Scheiding in twee dimensies; eerst op basis van iso-electrisch punt (= pH) en vervolgens op basis van massa gevolgd door binding op nitrocellulose.

enige tijd konden herstellen en in staat waren weer een volledige LDLr te produceren. Dit herstel bleek bovendien afhankelijk te zijn van de aanmaak van nieuwe eiwitten, hoogst waarschijnlijk van nieuwe ER eiwitten die de beschadigde eiwitten vervingen en hun taken overnamen.

Het is natuurlijk praktisch onmogelijk om in een laboratorium, in een celkweek, een zo complex proces als veroudering (van een mens bijv.) na te bootsen. In plaats van een zeer lange blootstelling aan kleine hoeveelheden waterstofperoxide (zoals dat gebeurt gedurende het leven) wordt in de experimenten gebruik gemaakt van een korte, hogere dosis om een meetbaar effect te vinden. Dit kan echter niet al te lang worden uitgevoerd, omdat de cellen het dan niet overleven. Hoewel de experimenten dus niet helemaal de werkelijke situatie weergeven, geven de resultaten wel aan dat oxidatieve stress schade kan veroorzaken aan het ER. Zoals eerder gezegd, één van de theoriën die veroudering probeert te verklaren, zegt dat ten gevolge van oxidatie, de opgelopen schade in een cel, op een gegeven moment een soort drempel heeft bereikt waarna een cel (en dus uiteindelijk het organisme) niet meer in staat is zichzelf afdoende te beschermen en het 'afval' niet meer kan opruimen. Uiteindelijk ontstaat er dan een situatie waarbij de overproductie van dit 'afval' te veel functies in de cel belemmerd waardoor overleven niet meer mogelijk is. In hoofdstuk 6 wordt gespeculeerd over de betrokkenheid van de oxidatie van de ER eiwitten bij het verouderingsproces. Als deze ER eiwitten niet meer functioneren, en niet voldoende vervangen kunnen worden door nieuwe, onstaat er een ophoping van onvolledig of verkeerd ontwikkelde eiwitten in het ER. De aanvoer van nieuw te maken eiwitten blijft, maar, omdat niet volledig ontwikkelde eiwitten de 'kwaliteitscontrole van het ER' niet passeren, zullen deze het ER niet verlaten. Verouderen is dus eigenlijk het net zo lang 'afval' verzamelen totdat je erbij neervalt.

Dankwoord

Zo, en waarom begin je eigenlijk hier te lezen?

Even kijken of je naam misschien genoemd wordt in het dankwoord?

Was de rest van het boekje soms niet interessant genoeg?

Als je klaar bent met het lezen van dit dankwoord zal het boekje zich binnen 5 seconden vernietigen! Ga dus heel snel terug naar hoofdstuk 1 en begin daar met lezen voordat het te laat is, want je krijgt geen nieuwe !

……

Viel best mee hè, al die hoofdstukken doorworstelen? Nu kan je met een gerust hart verder lezen.

Het is meestal een voordeel om meer dan 1 promotor, op verschillende afdelingen, te hebben. Ja, zo ook in mijn geval. Is het niet om een bredere kijk op de zaak te krijgen, dan is het toch zeker dat je van alle borrels en labuitjes kan meegenieten. Daarom wil ik Karel Wirtz en Arie Verkleij bedanken voor hun steun bij de chemische kant van de zaak en de biologische relevantie van het geheel. Echter, zonder de zeer creatieve en inspirerende geest van mijn copromotor Eward Pap zou TyrFluo waarschijnlijk nooit het (fluorescerende) levenslicht gezien hebben en zou dit proefschrift er dus heel anders uit gezien hebben. Eward bedankt.

Wat er aan vooraf ging. Het is eigenlijk begonnen met een sollicitatie gesprek in de kantine van het Kruytgebouw voor een stageplaats bij het URL met Philip Rijken. Philip bedankt dat je me hebt aangenomen, me de beginselen van het oxidatieve stress onderzoek hebt bijgebracht en er ook voor hebt gezorgd dat ik dat heb kunnen voortzetten in Utrecht. Toch nog even terug naar Vlaardingen, naar gebouw G van het URL en de mensen daar. Ik wil jullie bedanken voor alle zin en vooral voor alle onzin die er is uitgekraamd tijdens de lunches en koffiepauzes: Frans (met de grootste navel en het schoonste gereedschap), Arno (Rhodos, toffe vakantie), Jolanda (moeder de gans, het wordt waarschijnlijk niets meer met MVV), Karin (deerntje), Richard (daar heb je Flipper), Gerard, Wil (hoe is't met je tennisarm), Rinus (doseren!), e.v.a.. Jan Smelt, bedankt voor het verhuren van je studiekamer waarmee je mij zo uit de brand hebt geholpen toen ik 'dakloos' was, top!

En toen naar Utrecht.

Ik wil iedereen bij BvL bedanken voor de fijne tijd en collegialiteit: Ben, Gerry, Dmitri (спасибо), Dick, Ton, Fred, Fridolin, Claudia, Tobias (hé huisgenoot, collega, reisgenoot, fiets je nog wel eens?), Arjan, Bahram, Diana, Greg (bèèèèh), Rineke, Floortje, Albert, Maria, Grzegorz (my Polish friend dziękuję), Martijn (neem een wijze raad van me aan: kijk nou uit met je knie jongen), Yvette en mijn studenten Ilse, Ginette, Stan, Esther, Jannes. Jan (W630), jou wil ik even appart noemen, omdat ik vind dat we altijd perfect hebben samengewerkt. Bedankt voor je tomeloze inzet (er is een limiet aan het aantal 'noodzakelijke' controles), relativiteitszin en gesprekken over van alles en nog wat.

Verder wil ik de mensen van MCB bedanken en in het bijzonder (nogmaals) Arie, Paul en Johannes, omdat dankzij jullie Irina hier kon komen, eerst als summer-fellow en toen als AiO, en natuurlijk het anti-oxidantenteam, voor alle nuttige werkbesprekingen, tips, hulp en samenwerking; Jan Andries, Johannes, Sujata, Renate, Miriam, Inge, Bart, Victor, Cristina, en uit Vlaardingen Anton en Theo.

En de volgende mensen hebben ook, direct of indirect, meegeholpen bij het tot stand komen van dit boekje, bedankt allemaal: De mensen van 'de 7e': Ineke, Aafke, Eelco, Annemieke en Marije bedankt voor jullie hulp bij de pulse-chase experimenten. The people in Denmark for helping me to identify the spots, Julio Celis (the eye of the 2D master), Jette (thanks for the ins and outs in 2D PAGE), Hildur (thanks for borrowing me your tv). De sterren van Cees-1, ons zaalvoetbalteam, Cees eerst twee duveltjes en dan onder de lat, Martin, Robin, Thijn, Willie, José, Dennis F, Siebe, Ruben en alle tijdelijke krachten met twee chocolade benen. Het heeft me wel me knie gekost maar we hebben wel twee maal de cup (zonder oren) gewonnen.

David, my brother, Dr Jones, (who was the big dipper?) it has been a long way so we need a big can, cheers! Henny, alias Decibel Bouterse (dB), amigo, tijden veranderen meestal ten goede ookal mis ik die vakanties in Spanje wel, (Jaōnes met z'n sombrero in Blaōnes, Lloret of Benidorm op het strand). Ben, Giet Pietmans, succes in de States, maar onthoud een ding, høken blijft altijd normaal. Erwin, alles weer onder controle, alles is nu zoals het zou moeten, "alle vogels vliegen". Ik herinner me dat je ooit zei dat we altijd vrienden zouden blijven. We zijn al een eind op weg, zo'n 28-29 jaar, zins de kleuterschool.

Van je familie moet je het hebben. Pa en ma hartstikke bedankt voor jullie steun en voor het feit dat jullie altijd voor me klaar staan om me met wat dan ook te helpen. Jullie zijn de beste! Cindy, mijn 'kleine' zusje, vroeger deden we veel samen, nu is het iets minder maar onze band blijft bestaan. Igor, misschien moeten we eens op een slof en een oude voetbalschoen gaan tennissen, is misschien ook beter voor onze knieën. Mitchel wanneer kom je eens bij tante Irina en oom Dennis logeren in Utrecht? Nog even volhouden, en dan wordt de familie weer uitgebreid. Gena en Sveta (Servetka), ik vind het heel jammer dat we zo moeilijk met elkaar kunnen communiceren. Ik zal proberen mijn russisch te verbeteren. Я плохо говорю по-русски. Robert, alias Pièrre (ik ben bang dat je nooit van die bijnaam af zal komen), bedankt dat je mijn paranimf wil zijn, en Cecile, ik vind het fantastisch dat je met Irina mee bent gegaan om haar trouwjurk uit te zoeken. Het is altijd fijn om goede vrienden te hebben.

Een verrassing voor de mensen die het nog niet wisten? Ik ga met mijn andere paranimfje trouwen. Irina, mijn kippetje, ik ben zo gelukkig dat je mijn paranimfje wil zijn voor de rest van mijn leven. Любимая моя, я так люблю тебя. Давай поженимся.

Dennis

List of Publications

Van der Vlies, D., Pröbstl-Biegelman, E., Ploeger, G., Wirtz, K.W.A., Pap, E.H.W.
Fluorescent detection of Dityrosine Formation in Rat-1 fibroblasts.
NATO-ASI / FEBS series on Molecular Mechanisms of Signal Transduction (1999) 81-84

D. van der Vlies, K.W.A. Wirtz and E.H.W. Pap
Detection of protein oxidation in rat-1 fibroblasts by fluorescently labeled tyramine.
Biochemistry 40 (2001), 7783-7788

Dennis van der Vlies, Eward H.W. Pap, Jan Andries Post, Julio E. Celis, Karel W.A.Wirtz
Endoplasmic reticulum resident proteins of normal human dermal fibroblasts are the major targets for oxidative stress induced by hydrogen peroxide.
Biochemical Journal 366;3 (2002), 825-830

Dennis van der Vlies, Jannes Woudenberg, Jan Andries Post
Protein oxidation in aging: endoplasmic reticulum as a target.
Amino Acids (submitted for publication)

Dennis van der Vlies, Esther Kroneman, and Karel W.A.Wirtz
Modulation of protein oxidation by altering the cellular anti-oxidative capacities; Detection with acetylTyrFluo.
Manuscript in preparation

Dennis van der Vlies, Annemieke Jansens, Arie J. Verkleij, Karel W.A.Wirtz, and
Ineke Braakman
Influence of oxidative stress on protein maturation; the LDL-receptor as a model protein.
Manuscript in preparation

Curriculum Vitæ

De auteur van dit proefschrift werd geboren op 14 april 1970 te Purmerend en was sinds 1972 woonachtig te Bunschoten-Spakenburg. Na het behalen van het VWO diploma op het Baarnsch Lyceum te Baarn, in 1989, werd een jaar natuurkunde gestudeerd om in 1990 te kunnen beginnen met de studie Medische Biologie aan de Universiteit Utrecht. Tijdens de studie werd praktische ervaring opgedaan bij de vakgroepen Experimentele Neurologie van Prof. Dr. P.R. Bär (bijvakstage) en Biochemie van Metabole Stoornissen van Prof. Dr. R. Berger (hoofdvakstage), beide in het Utrechts Medisch Centrum. Er werd een extra stage gevolgd bij het Unilever Health Institute van het Unilever Research Centre in Vlaardingen. Na het behalen van het doctoraal examen in 1996, met als specialisatierichting biochemie, bleef hij werkzaam als junior onderzoeker bij het UHI. Vanaf 1998 werd het, in deze dissertatie beschreven promotieonderzoek, uitgevoerd in het Instituut voor Biomembranen van de Universiteit Utrecht, onder de supervisie van Prof. Dr. K.W.A. Wirtz en Dr. E.H.W. Pap van de vakgroep Biochemie van Lipiden van de faculteit Scheikunde en Prof. Dr. A.J. Verkleij van de vakgroep Moleculaire Celbiologie van de faculteit Biologie.