

Linköping University Postprint

Lysosome-targeted stress reveals increased stability of lipofuscin-containing lysosomes

Yuri Stroikin, Hanna Mild, Uno Johansson, Karin Roberg and Karin Öllinger

Original publication:

Yuri Stroikin, Hanna Mild, Uno Johansson, Karin Roberg and Karin Öllinger, Lysosome-targeted stress reveals increased stability of lipofuscin-containing lysosomes, 2008, AGE

<http://dx.doi.org/10.1007/S11357-007-9045-9>.

Copyright: The original publication is available at <http://www.springerlink.com>

Postprint available free at:

Linköping University E-Press:

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-11051>

Lysosome-targeted stress reveals increased stability of lipofuscin-containing lysosomes

Yuri Stroikin^{a*}, Hanna Mild^a, Uno Johansson^a, Karin Roberg^b and Karin Öllinger^a

^aDivision of Experimental Pathology, Department of Neuroscience and Locomotion,

Faculty of Health Sciences, Linköping University, 581 85 Linköping, Sweden

^bDivision of Oto-rhino-laryngology,

Faculty of Health Sciences, Linköping University, 581 85 Linköping, Sweden

*
Correspondence:

Y. Stroikin, Division of Experimental Pathology, Linköping University, 581 85
Linköping, Sweden; Phone: +46 13 221525; Fax: +46 13 221529; E-mail:
yuri.stroikin@inr.liu.se

Word counts for the text: 3815

Word counts for the abstract: 124

Number of figures: 7

Abstract

Cellular ageing is associated with accumulation of undegradable intralysosomal material, called lipofuscin. In order to accelerate the lipofuscin-accumulation, confluent, growth arrested human fibroblasts were cultured under hyperoxic conditions. To provide a better insight into the effects of lipofuscin on cellular functions, we compared lysosomal stability in control and lipofuscin-loaded human fibroblasts under conditions of lysosome-targeted stress induced by exposure to either the lysosomotropic detergent MSDH or the redox-cycling quinone naphthazarin. We show that lysosomal damage, assessed by acridine-orange relocation, translocation of cathepsin D to the cytosol, and alkalinization of lysosomes is more pronounced in control than in lipofuscin-loaded fibroblasts. Finding that lysosomal integrity was less affected or even preserved in case of lipofuscin-loaded cells enables us to suggest that lipofuscin exerts lysosome-stabilizing properties.

Keywords: *alkalinization, autophagolysosomes, bafilomycin A₁, cathepsin D, MSDH, naphthazarin quinone*

Introduction

Membrane-bound acidic organelles, generally termed lysosomes, are considered a major site for degradation of both extra- and endogenously derived material (De Duve and Wattiaux 1966). In the latter case, degradation of the cell's own constituents, so-called autophagy, not only serves nutritional purposes but also secures intracellular homeostasis through the removal of damaged and potentially hazardous biomolecules and organelles (Klionsky 2005). Along with the ability to provide for intracellular degradation and repair, the lysosomal system is also involved in different signal transduction pathways (Miaczynska et al. 2004), including that of programmed cell death (Ferri and Kroemer 2001; Yin et al. 2005). It has been shown that lysosome-targeted stress, induced by either the lysosomotropic detergent *O*-methyl-serine dodecylamide hydrochloride (MSDH; Li et al. 2000) or the redox-cycling quinone 5,8-dihydroxy-1,4-naphthoquinone (NzQ; Roberg et al. 1999), results in permeabilisation of lysosomal membrane, relocation of lysosomal constituents to the cytosol and, finally, apoptosis.

Another distinctive feature of lysosomal compartment is that it serves as a site for storage of non-degraded material. Such material is collectively called lipofuscin, when age-related, and ceroid when its accumulation is caused by pathological conditions (Porta 2002; Seehafer and Pearce 2006). According to the free-radical theory of ageing (Harman 1956), formation of oxidatively damaged intracellular structures is an inevitable side effect of aerobic life. Imperfect degradation of such damaged cellular components results in an accumulation of so-called "biological garbage" of which lipofuscin is an example (Brunk and Terman 2002a). A suitable model of induced cellular senescence is established by culturing cells under

conditions of chronic oxidative stress, which accelerates age-related changes and results in premature lipofuscin-accumulation (Grune et al. 2005; Terman and Brunk 1998). For the sake of simplicity and due to the similarity of the mechanisms behind age-related and oxidative stress-induced accumulation (Brunk and Terman 2002a), we here refer to such intralysosomal pigment as lipofuscin.

Physiological effects of lipofuscin are generally viewed as deleterious due to the suggested implication of lipofuscin in the formation of free radicals (Brunk and Terman 2002b). It has been shown that lipofuscin acts as a photosensitizer, compromising the integrity of lysosomal membrane and finally resulting in cell death (Wihlmark et al. 1997). The age-related decline in the efficacy of certain proteases can also be explained in terms of lipofuscin effects. Thus, it has been suggested that newly produced lysosomal enzymes are misplaced to the lipofuscin-loaded lysosomes in a futile attempt to degrade lipofuscin instead of performing a useful function within autophagolysosomes (Terman and Brunk 2004). On the other hand, we recently found, that moderate levels of lipofuscin are protective and increase the resistance of ageing fibroblasts to cell death (Stroikin et al. 2007). Beneficial effect of lipofuscin in this case is explained in terms of hormesis - an adaptation to low doses of otherwise harmful agents (Rattan 2004).

As has been recently shown by our group, complete starvation causes activation of programmed cell death through the destabilization of lysosomal compartment. Such a destabilization was suppressed in lipofuscin-loaded fibroblasts (Stroikin et al. 2007). The presented study is a continuation of the previous one in order to further investigate the possible hormetic effect of lipofuscin on lysosomal function. Instead of investigating the apoptotic response, which has been already found disturbed in lipofuscin-loaded cells, we focus on the stability of lysosomal compartment,

considered one of the major regulators of programmed cell death. For this purpose we compared the integrity of lysosomes in lipofuscin-loaded and control growth-arrested human fibroblasts under conditions of lysosome-targeted stress induced either by a lysosomotropic detergent (MSDH) or acute oxidative stress (NzQ). Here we demonstrate that lysosome-targeted stress results in deleterious changes of lysosomal compartment that are significantly more pronounced in control fibroblasts than in lipofuscin-loaded cells.

Materials and methods

Culture conditions and experimental design

AG-1518 human fibroblasts (obtained from Coriell Institute, Camden, NJ, USA) were cultured in Eagle's minimum essential medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin-G and 100 µg/ml streptomycin in an atmosphere of 8% O₂, 87% N₂ and 5% CO₂, at 37°C (normal conditions). The cells were sub-cultivated at a 1:2 ratio until they reached passage 22-23, and were then allowed to grow until confluency. These cultures are referred to as control. Some confluent fibroblast cultures were exposed to 40% O₂, 55% N₂ and 5% CO₂ (hyperoxia) for two months to induce lipofuscin accumulation (Terman and Brunk 1998) and are referred to as lipofuscin-loaded. The culture medium was changed twice a week. Evaluation of lipofuscin-accumulation was performed by flow cytometric estimation (Becton Dickinson Biosciences, San Jose, CA, USA) of cellular autofluorescence and was found 2-3 fold higher in lipofuscin-loaded cells compared to controls (data not shown).

Both controls and lipofuscin-loaded cells were exposed to three different agents presumably affecting lysosomal integrity: (i) MSDH at a concentration of 25 μM for 15, 30 or 60 minutes; (ii) NzQ at a concentration of 0.75 μM for 15, 30 or 60 minutes; (iii) the vacuolar ATPase inhibitor bafilomycin A₁ (Baf A1; Bowman et al. 1988) at a concentration of 20 nM for 15 or 30 minutes. Treatment with Baf A1 was used as a positive control during lysosomal pH assessment.

Lysosomal stability assessment by acridine orange (AO)

Lysosomal stability was assessed by the AO-relocation method (Olsson et al. 1989). AO is a lysosomotropic weak base with metachromatic features. Oligomeric form of highly concentrated and protonated AO (AOH⁺) exhibits red fluorescence, as is the case in intact lysosomes. Lysosomal alkalization and translocation of lysosomal content to the cytosol during lysosomal stress results in the formation of the monomeric deprotonated form of AO with green fluorescence.

Cells on cover-slips were briefly stained with 5 $\mu\text{g/ml}$ AO for 15 minutes under normal culture conditions, rinsed in complete medium and exposed to one of the lysosomal-stress-inducing agents as described in previous section. Live cultures were examined in an Axiovert S100TV microscope (Carl Zeiss, Jenna, Germany) equipped with a Hamamatsu digital camera C4742-95 (Hamamatsu, Hamamatsu City, Japan) using 60 x /1.4 oil lens, a halogen lamp and a blue-excitation filter. Emission was detected using a high pass filter above 520 nm. Phase-contrast and fluorescent images were obtained using OpenLab software (Improvision, Coventry, UK). Measurements of lysosomal red AO-fluorescence were performed using the National Institute of Health Image program (<http://rsb.info.nih.gov/nih-image/>). Fluorescence intensity was

expressed in arbitrary units (a. u.) being a product of average pixel value per lysosome and the lysosome area.

Assessment of lysosomal pH

The lysosomal pH was measured by flow cytometry as described elsewhere (Nilsson et al. 2003). Briefly, cells were exposed to 40,000 MW FITC-dextran (Molecular Probes, Eugene, OR, USA) at a concentration of 0.1 mg/ml for 3 days at 37°C. Loading with FITC-dextran was followed by incubation in complete medium for another 24 hours. After that, cells were trypsinized, centrifuged at ~300 x g for 5 minutes, re-suspended in the culture medium, filtered through a 70 µm cell strainer and analyzed by flow cytometry (Becton Dickinson Biosciences, San Jose, CA, USA). A 488 nm argon laser was used for the FITC excitation, and emission was detected in the FL1 and FL2 channels using a 530 ± 28 nm and a 610 ± 20 nm barrier filters, respectively. Data from 10,000 cells was analyzed using the CellQuest program (Becton Dickinson Immunocytometry systems, San Jose, CA, USA). Modified Britton-Robinson buffers (pH 4.0–7.0), containing sodium azide and 2-deoxyglucose at a final concentration of 50 mM each and nigericin at a final concentration of 10 µM were used for the preparation of a standard curve. The FL1/FL2 ratios were used to calculate the pH employing the standard curve.

Immunocytochemical detection of cathepsin D

Cells plated on cover-slips were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 20 minutes in 4°C, rinsed in PBS, and exposed to incubation buffer containing 0.1% saponin and 5% foetal bovine serum in PBS for another 20 minutes at room temperature. The cells were then incubated with 1:100 diluted polyclonal

rabbit anti-human antibodies to cathepsin D (DAKO, Roskilde, Denmark) for 1 hour in a humidifier at room temperature. After rinsing in the incubation buffer (2 x 5 minutes), the specimens were exposed to 1:100 diluted goat anti-rabbit IgG-Alexa 594 conjugate (Molecular Probes, Eugene, OR, USA) for 1 hour at room temperature; rinsed in PBS and distilled water, and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were obtained using a Microphot-SA fluorescence microscope equipped with an ORCA 100 Hamamatsu color digital camera (Hamamatsu, Hamamatsu City, Japan).

Extraction of cytosol

Disturbed lysosomal integrity was additionally assessed by estimation of the amount of cathepsin D translocated to the cytosol during lysosomal stress. Cytosolic extraction was performed using the cholesterol-solubilizing agent digitonin as described elsewhere (Johansson et al. 2003). Briefly, digitonin at low concentrations permeabilizes cholesterol-rich membranes such as the plasma membrane, leaving cholesterol-poor membrane of lysosomes and mitochondria more or less intact. Cells were exposed to an extraction buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA and 1 mM EGTA) containing digitonin at concentration of 25 µg/ml on ice for 12 minutes. The extraction buffer was collected and proteins were precipitated by addition of 5% trichloric acid on ice for 10 minutes and centrifuged at ~ 20,800 x g for 15 minutes to obtain the protein pellet. Immunoblotting is described below.

Western blot analysis

The pellet was dissolved in 25 μ l of lysis buffer containing 6 M urea, 63 mM Tris-HCl (pH 6.8), 10% glycerol and 2% SDS. Samples were then neutralized using 4 μ l of 1 M NaOH per sample. Consequently, 50 μ M dithiothreitol (DTT) and 0.05% bromophenol blue were added and 30 μ l aliquots of the cell lysate were fractionated by 15% SDS-PAGE. The proteins were then blotted on a nitrocellulose membrane, which was subsequently incubated in 5% skimmed milk in Tris-buffered saline (50 mM Tris, 0.15 M NaCl, pH 7.5) with 0.1% Tween-20 (TBS-T) for 90 minutes at room temperature and then washed in TBS-T. The membrane was then exposed over night at 4°C to a monoclonal mouse anti-human cathepsin D antibody (Oncogene Research Products, San Diego, CA, USA) diluted 1:1000 in 0.1% skimmed milk in TBS-T. The membrane was then washed and incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (DAKO, Roskilde, Denmark) diluted 1:1500. Bands were visualized using Western blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Equal loading was verified by reprobing the membrane with a mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biogenesis, Poole, Dorset, U.K.) antibody diluted 1:400.

Transmission electron microscopy

Cells were fixed by adding 2% glutaraldehyde (Agar Scientific, Essex, UK) in 0.1 M sucrose-sodium cacodylate-HCl buffer (pH 7.2; Sigma, St Louis, MO, USA) and post-fixed in osmium (Johnson Matthey Chemicals, Royston, UK). Specimens were stained en bloc with 2% uranyl acetate (Sigma) in 50% ethanol, dehydrated in a graded series of ethanol, embedded in Epon-812 (Fluka AG, Buchs, Switzerland) and

polymerized at 60°C for two days. Thin sections were cut with a diamond knife (DIATOME, Bienne, Switzerland), stained with lead citrate, and then examined and photographed in a JEOL 1230-EX electron microscope (JEOL, Tokyo, Japan) at 100 kV.

Statistics

For the estimation of AO-fluorescence, 100 to 140 lysosomes from each specimen were analyzed. All experiments were repeated at least three times. Values are given as means \pm SD. Data obtained by the acridine orange relocation method were analyzed using the ANOVA and post hoc LSD test. The lysosomal pH data were analyzed using Kruskal-Wallis and post hoc Mann Whitney test. P-values < 0.05 were considered significant.

Results

Phase-contrast microscopy of MSDH- and NzQ-treated fibroblasts reveals significant morphological differences between control and lipofuscin-loaded cells (Figure 1a). Thus, MSDH-induced cytosolic vacuolization (arrows in Figure 1a), albeit present in both control and lipofuscin-loaded fibroblasts, is more pronounced in control cells. Relatively higher optical density of AO-loaded lysosomes (dark dots on phase-contrast images) enables their distinction from newly formed vacuoles. During exposure to NzQ, there is a noticeable agglomeration of lysosomes in control cells; while in lipofuscin-loaded fibroblasts this phenomenon is less obvious (arrowheads in Figure 1a).

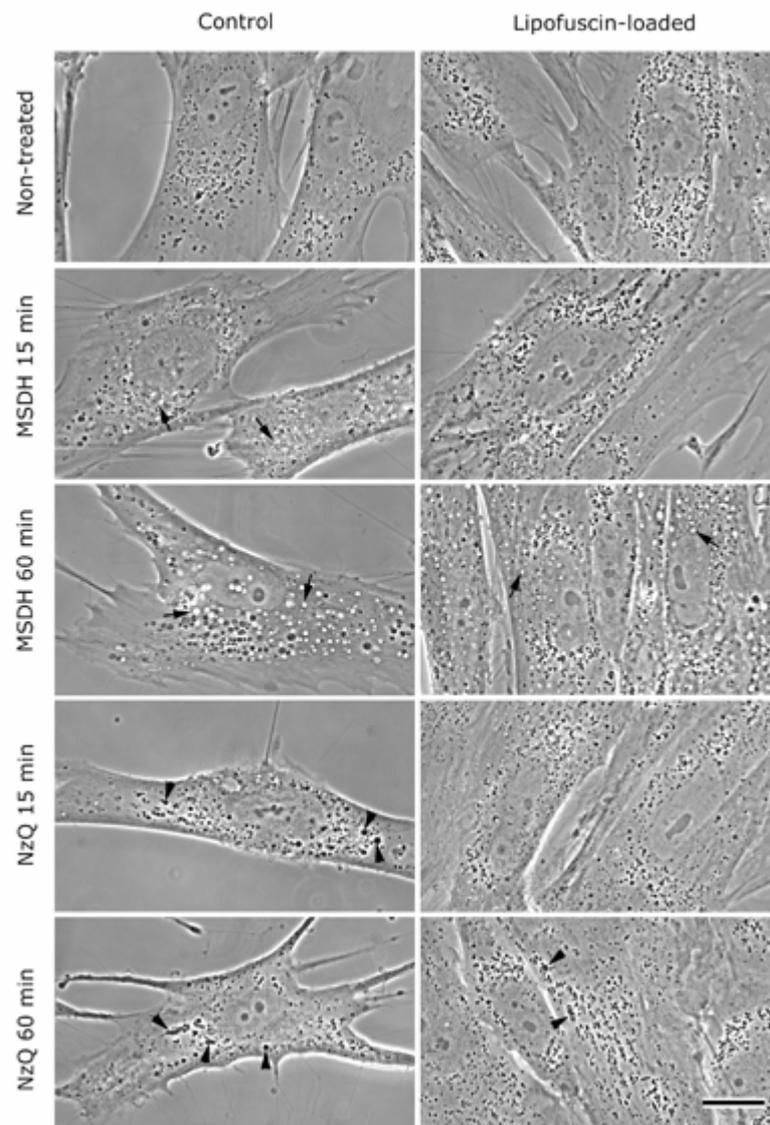


Figure 1a. Lysosomal stress causes more prominent alterations in control cells than in lipofuscin-loaded. Phase-contrast images of fibroblasts cultured under normal conditions (controls) or pre-exposed to hyperoxia in order to accelerate accumulation of lipofuscin, and then exposed to either 25 μM MSDH or 0.75 μM naphthazarin (NzQ) and stained with acridine orange. Arrows mark cytosolic vacuoles and arrowheads indicate agglomeration of acridine orange-positive lysosomes. Scale bar, 20 μm .

Non-confocal fluorescent imaging of AO-stained cells shows that the number of lysosomes does not obviously differ between non-treated control and lipofuscin-loaded fibroblasts (Figure 1b). Both MSDH- and NzQ-treatment result in decrease of the number of lysosomes in control cells, whereas neither of the treatments shows any effect on lysosomal number in lipofuscin-loaded fibroblasts (Figure 1b).

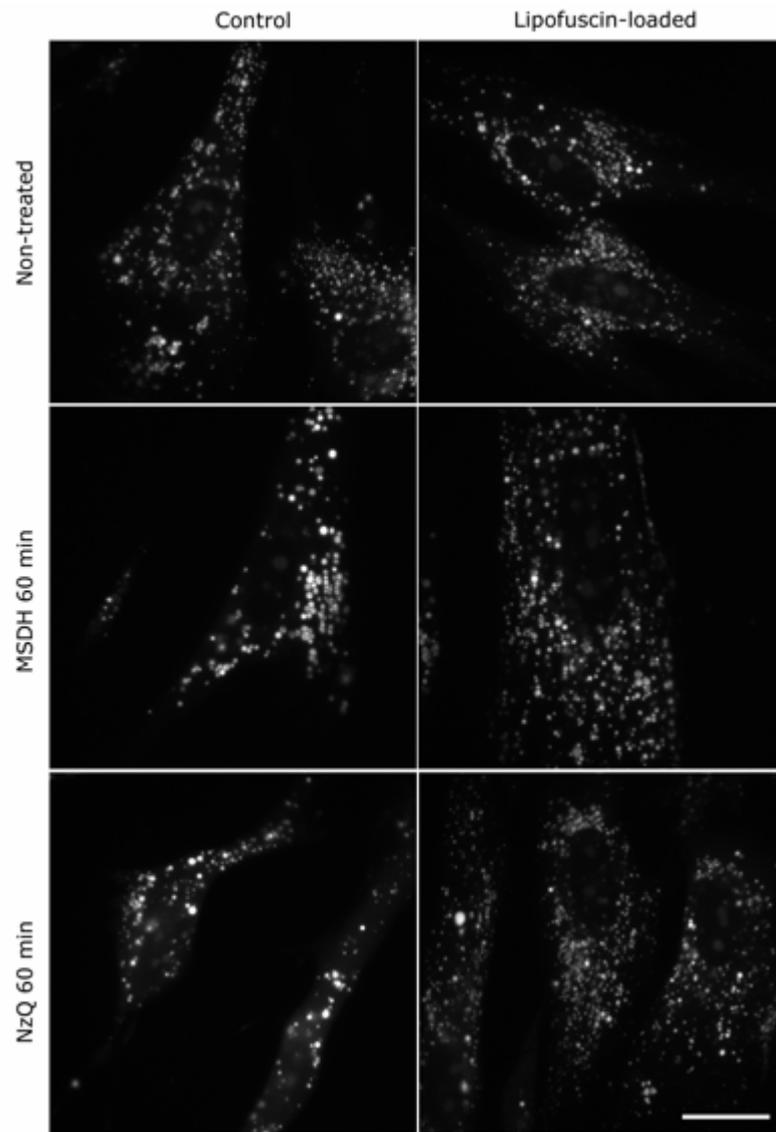


Figure 1b. *The number of lysosomes decreases during MSDH- and naphthazarin-treatment in control but not in lipofuscin-loaded fibroblasts. Non-confocal fluorescent images of fibroblasts cultured under normal conditions (controls) or pre-exposed to hyperoxia in order to accelerate accumulation of lipofuscin, and then exposed to either 25 μ M MSDH or 0.75 μ M naphthazarin (NzQ) and stained with acridine orange. Scale bar, 20 μ m.*

Ultrastructural changes of control fibroblasts exposed to 25 μ M MSDH for 60 minutes (Figure 2) are characterized by advanced autophagy represented by accumulation of autophagolysosomes (Figure 2b). Moreover, the number of organelles with typical lysosomal morphology, observed in untreated cells (Figure 2a), decreases during MSDH-treatment (Figure 2b).

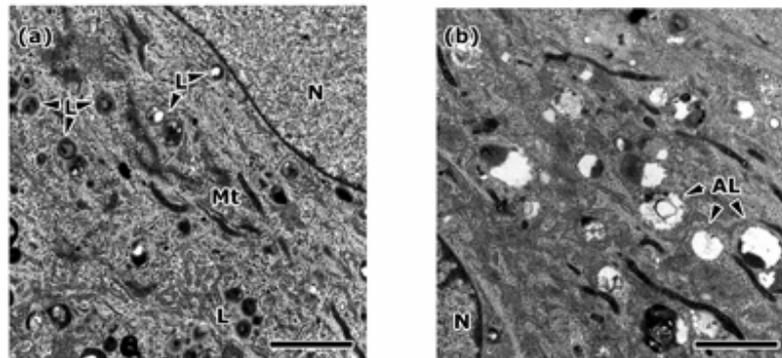


Fig. 2

Figure 2. Ultrastructural changes of MSDH-treated fibroblasts include pronounced autophagic vacuolization. (a) Untreated control fibroblast and (b) fibroblasts exposed to 25 μ M MSDH for 60 minutes. Autophagolysosomes (AL) found in MSDH-treated cells represent the advanced stage of autophagy. L: lysosome; Mt: mitochondria; N: nucleus. Scale bar, 2 μ m.

Relocation of AO to the cytosol in MSDH- and NzQ-treated fibroblasts is estimated by the decrease of red-fluorescence-intensity of lysosomes. During exposure to MSDH, the fluorescence intensity becomes significantly reduced already after 15 minutes in control fibroblasts but only after 60 minutes in lipofuscin-loaded cells (Figure 3a). NzQ-treatment results in a significant decrease (after 15 minutes) and consequent recovery (observed after 30 and 60 minutes) of AO-associated red fluorescence intensity in control cells. Lipofuscin-loaded fibroblasts, on the other hand, do not show any significant changes of AO-fluorescence during exposure to NzQ (Figure 3b).

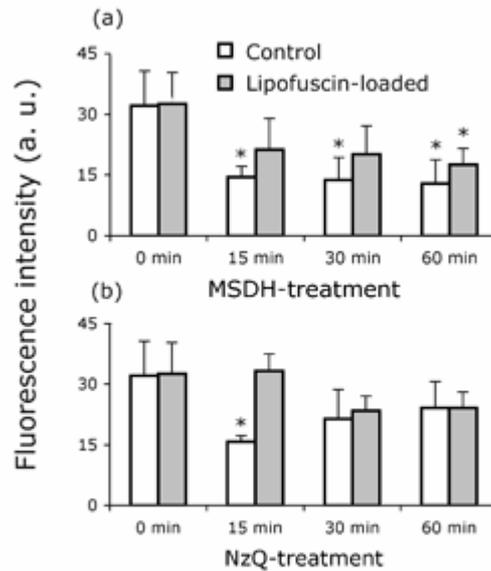


Figure 3. More pronounced acridine orange-relocation in control than in lipofuscin-loaded fibroblasts during lysosomal stress. Fibroblasts were cultured under normal conditions (control) and at hyperoxia in order to accumulate lipofuscin. Acridine orange accumulates in undamaged lysosomes and generates red fluorescence. Decrease of red fluorescence intensity denotes relocation of acridine orange to the cytosol. MSDH-treatment (a) causes significant acridine orange-relocation after 15 minutes in control cells and only after 60 minutes in lipofuscin-loaded. Exposure to naphthazarin (NzQ) for 15 minutes results in significant relocation of acridine orange in control cells with a consequent tendency toward recovery (b). Naphthazarin-treatment does not have any significant effects on lipofuscin-loaded cells. Values are mean \pm SD, $n = 3$ with 100-140 assessed lysosomes in each sample (* = $P < 0.05$ compared to corresponding non-treated cells [0 min]).

Intact lysosomes of non-treated cells are characterized by a grainy pattern of cathepsin D immunostaining (Figure 4). During MSDH-treatment, staining pattern becomes diffuse, as observed in control cells after 15 minutes and even more pronounced after 60 minutes. In lipofuscin-loaded fibroblasts, diffuse pattern of cathepsin D staining becomes evident after 60 minutes of MSDH-treatment and is less prominent than in control cells. NzQ-treated control fibroblast cultures initially show diffuse lysosomal staining (observed after 15 minutes of exposure), which later returns to the grainy one (observed after 60 minutes of exposure to NzQ). In

lipofuscin-loaded fibroblasts cathepsin D staining remains lysosomal during NzQ-treatment (Figure 4).

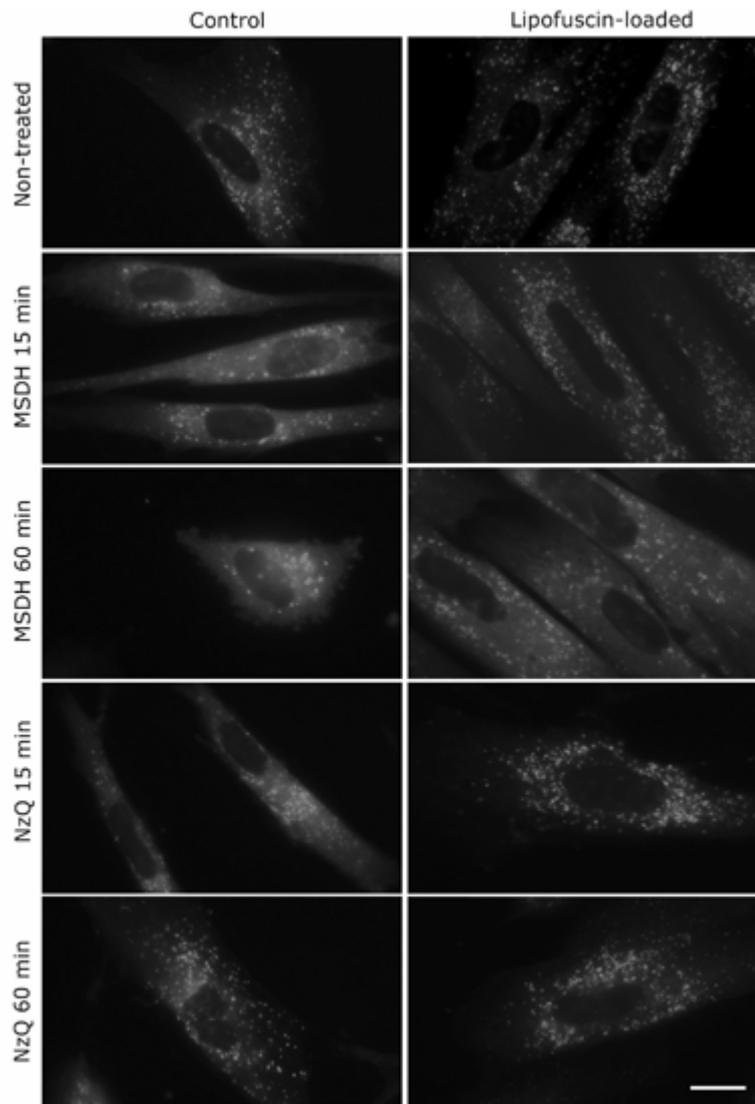


Fig. 4

Figure 4. Lysosomal stress is associated with cathepsin D translocation, which is more obvious in control than in lipofuscin-loaded fibroblasts. Fibroblasts cultured under normal conditions (control) or pre-exposed to hyperoxia in order to accumulate lipofuscin were treated with either 25 μM MSDH or 0.75 μM naphthazarin (NzQ). Cells were fixed and immunostained for cathepsin D and an Alexa 594-conjugated secondary antibody was used. The change from a grainy (lysosome-like) to a diffuse (cytosolic) pattern of cathepsin D immunostaining during MSDH-treatment is more evident in control (after 15 minutes) than lipofuscin-loaded fibroblasts (after 60 minutes). Lipofuscin-loaded cells do not show any changes in cathepsin D staining during naphthazarin-treatment, while in control cells diffuse staining appeared after 15 minutes of treatment with a consequent tendency toward recovery and acquisition of a grainy pattern of staining after 60 minutes. Scale bar, 20 μm .

Digitonin-extraction of cytosol enables the estimation of the amount of cathepsin D released from the lysosomes during lysosomal stress. Western blot analysis

revealed that during MSDH-treatment the amount of cytosolic cathepsin D increases gradually in both control and lipofuscin-loaded fibroblasts, but is apparently higher in control cells (Figure 5). During exposure to NzQ, the amount of cytosolic cathepsin D in control cells initially increases (observed after 15 minutes) but eventually (observed after 60 minutes) decreases to an even lower level than that of non-treated cells. NzQ-treatment does not affect the amount of cytosolic cathepsin D in lipofuscin-loaded cells (Figure 5).

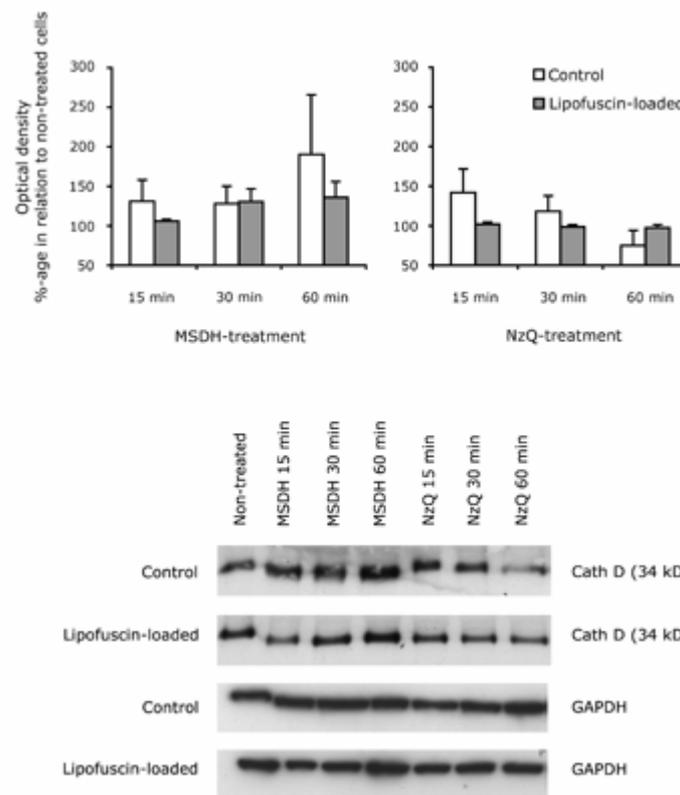


Figure 5. Lysosomal stress causes higher release of cathepsin D to the cytosol in control than in lipofuscin-loaded fibroblasts. Cytosolic fractions were isolated by digitonin extraction, and the cathepsin D content was analyzed by immunoblotting. Western blot analysis shows a gradual increase of the amount of cytosolic cathepsin D in both control and lipofuscin-loaded cells during MSDH-treatment, but is higher in controls. Naphthazarin (NzQ)-treatment does not affect the amount of cytosolic cathepsin D in lipofuscin-loaded fibroblasts, while in control cells there is some initial increase of cytosolic cathepsin D content, which after 60 minutes decreases becoming even lower than that of non-treated cells. GAPDH-staining is used as a protein loading control. One representative blot out of three is shown.

MSDH-treatment is characterized by a gradual increase of lysosomal pH in both control and lipofuscin-loaded cells (Figure 6a). Control fibroblasts show earlier and more pronounced lysosomal alkalization than lipofuscin-loaded cells (after 15 and 60 minutes in controls and lipofuscin-loaded cells, respectively). NzQ-treatment does not have any apparent effect on lysosomal pH of lipofuscin-loaded fibroblasts, while in control cells the increase in lysosomal pH is observed after 15 minutes, becomes significant after 30 minutes, and is followed by lysosomal acidification, observed after 60 minutes of exposure to NzQ (Figure 6b). Treatment with Baf A1, used as a positive control for lysosomal alkalization, results in a significant increase of lysosomal pH in both control and lipofuscin-loaded cells, but is considerably higher in controls (Figure 6c). Regardless of treatment used, the difference in pH between controls and lipofuscin-loaded cells remains significant at all time points (Figure 6a-c).

Discussion

First suggested by de Duve and Wattiaux (1966), the idea of lysosomal involvement in cell death has been gaining more evidence. During lysosome-targeted stress, the type of cell death depends on the degree of lysosomal damage. Thus, extensive release of lysosomal content results in necrosis, while partial lysosomal rupture mediates apoptosis (Brunk et al. 2001; Zhao et al. 2003). Lysosomal destabilization is an initial event in programmed cell death induced by oxidative stress, radiation, and exposure to MSDH and oxidized low-density lipoprotein (Brunk and Svensson 1999; Li et al. 2000; Persson et al. 2005; Yuan et al. 1997). It has been shown that permeabilisation of lysosomal membrane leading to translocation of

lysosomal enzymes to the cytosol precedes mitochondrial release of cytochrome c and subsequent caspase-activation (Roberg et al. 1999).

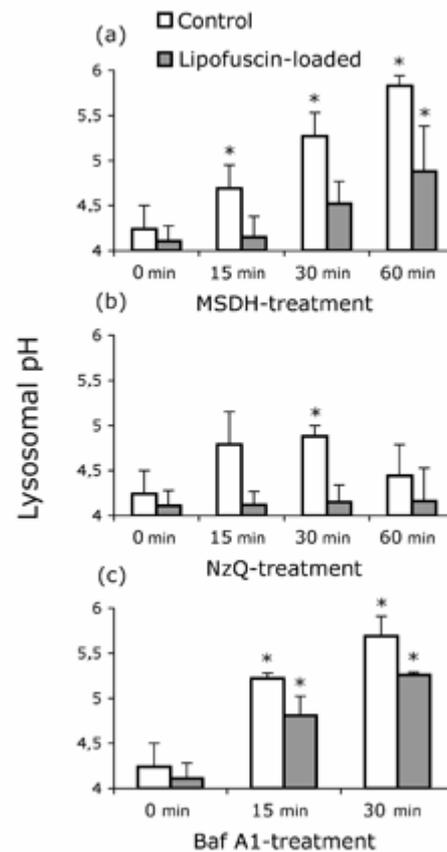


Figure 6. Treatments of fibroblasts with MSDH, naphthazarin or bafilomycin A₁ result in lysosomal alkalinization that is more pronounced in control than in lipofuscin-loaded cells. Fibroblasts were allowed to endocytose FITC-dextran for three days and the lysosomal pH was assessed by ratiometric calculation. MSDH-treatment (a) causes significant lysosomal alkalinization observed in control fibroblasts already after 15 minutes of exposure. Alkalinization of lipofuscin-loaded lysosomes becomes significant only after 60 minutes. Increase in lysosomal pH during naphthazarin (NzQ)-treatment (b) becomes significant after 30 minutes in control cells with a consequent tendency toward acidification. No effects of naphthazarin-treatment on pH of lipofuscin-loaded lysosomes are detected. Inhibition of the lysosomal proton pump using bafilomycin A₁ (Baf A1) causes significant lysosomal alkalinization in both control and lipofuscin-loaded cells (c). Values are mean \pm SD, n = 3 (* = P < 0.05 compared to corresponding non-treated cells [0 min]). Difference between control and lipofuscin-loaded cells during all three types of treatment remains significant at all time-points.

The current study is a continuation of the recently presented work of our group, showing that lipofuscin-loaded cells are more resistant to cell death (Stroikin et al. 2007). Here, instead of studying the apoptotic response, we focus on the effects of

lipofuscin on lysosomal stability, proven to be crucial for the initiation of apoptosis (Brunk et al. 2001).

The presented MSDH-induced vacuolization (Figures 1a, b; 2b) can be considered an adaptive cellular response as an attempt to limit the damage (Henics and Wheatly 1999), by means of autophagic sequestration. Since lysosomal enzymes translocated to the cytosol jeopardize survival of the cell, autophagy is primarily focused on sequestration of the released lysosomal content. It has also been suggested that damaged entire lysosomes can be autophagocytosed (Stroikin et al. 2004; Kiffin et al. 2006). Regardless of the mechanism of autophagic sequestration, it does not prevent apoptosis of MSDH-treated cells (Li et al. 2000), since the acidic interior of autophagolysosomes is attracting MSDH, which at low pH becomes protonated and acquires properties of a detergent (Firestone et al. 1979).

During MSDH-treatment, the number of vacuoles (Figure 1a), corresponding to autophagolysosomes (Figure 2b), becomes extensive, occupying the entire cell. This suggests that activated autophagy is no longer a repairing mechanism, but rather an executioner of cellular demise (Gozuacik and Kimchi 2004), which is in agreement with both the decrease of the amount of AO-positive lysosomes (Figure 1b) and ultrastructural findings of the decreased number of morphologically typical lysosomes (Figure 2a, b). These morphological features of lysosomal deterioration during MSDH-exposure are consistent with findings of gradual (i) decrease of lysosomal-associated AO-fluorescence; (ii) increase of diffuse cathepsin D immunostaining; (iii) increase of cathepsin D in the cytosolic fraction and, (iv) lysosomal alkalization. Although these changes are present in both control and lipofuscin-loaded fibroblasts, the deleterious effect of MSDH-treatment is significantly less pronounced in lipofuscin-loaded cells, which indicates reduced lysosomal sensitivity. The difference

of susceptibility toward MSDH-induced lysosomal stress between control and lipofuscin-loaded fibroblasts cannot be explained in terms of decreased tropism of MSDH to lipofuscin containing lysosomes, since lysosomal pH, responsible for both tropism and protonation of MSDH, is practically equally low in both control (4.24 ± 0.26) and lipofuscin-loaded (4.11 ± 0.17) cells. On the other hand, lipofuscin-containing lysosomes, when extensively overloaded, are excluded from physiological functioning as sites of degradation (Terman and Brunk 2004), causing formation of new, lipofuscin-free lysosomes. Such an increase of the overall volume of lysosomal compartment can be considered a possible modulator of reactivity towards lysosomotropic detergents. In this light, lipofuscin could be viewed as an indirect factor of increasing lysosomal stability. But since the number of lysosomes in non-treated cells does not markedly differ between control and lipofuscin-loaded fibroblasts (Figure 1b), the volume of the lysosomal compartment cannot be rendered as a factor responsible for the increased resistance to MSDH-treatment in the presented study.

During NzQ-treatment of control cells, the agglomeration of lysosomes without apparent cytosolic vacuolization (Figure 1a) might represent successful reparative autophagy. Lysosomal damage revealed by the initial decrease of lysosomal fluorescence (Figure 3b), translocation of cathepsin D to the cytosol (Figures 4, 5) and lysosomal alkalization (Figure 6b) triggers the mechanisms leading to the accomplished control over the damage. The idea of effective autophagic repair is consistent with the results showing the eventual (i) regaining of lysosomal-associated AO-fluorescence (Figure 3b); (ii) recovery of the grainy pattern of cathepsin D immunostaining (Figure 4); (iii) decrease of the cytosolic fraction of cathepsin D (Figure 5) and (iv) lysosomal acidification (Figure 6b) of control fibroblasts after 60

minutes of exposure to NzQ. Logically, not all cells manage to accomplish a successful damage-control. Some cells die due to the extensive lysosomal damage. Considering that one fraction of cells die, measurements performed on NzQ-exposed cells might represent cellular resistance rather than recovery from cell damage. On the other hand, during continuous exposure to NzQ, lysosomal agglomeration (Figure 1a), coexisting with a decrease of the number of lysosomes (Figure 1b) suggests an ongoing intracellular process by which cells cope with unfavorable conditions. Even if some cells are lost during NzQ-treatment, conclusions drawn from the measurements made on the surviving population of cells still emphasize the mechanism of cell survival.

The absence of changes in lysosomal compartment of lipofuscin-loaded cells suggests that NzQ-treatment does not cause significant lysosomal damage in these cells. According to recent theories, high content of iron associated with lipofuscin should have increased the lysosomal sensitivity to oxidative stress (Yu et al. 2003). Alternatively, the resistance of lipofuscin-loaded cells can be due to their pre-exposure to hyperoxic conditions. The possibility of up-regulation of anti-oxidative defense during chronic oxidative stress still remains to be investigated.

Physiological effects of lipofuscin on cellular functions in general and on lysosomal integrity in particular, have been a matter of controversial opinions. The increased sensitivity of lipofuscin-accumulating cells to lysosomal breach and apoptosis has been explained in terms of redox-active iron content, which promotes formation of free radicals under conditions of oxidative stress (Terman and Brunk 2004). In opposition, some researchers doubt that a convincing evidence of deleterious effects of lipofuscin has ever been demonstrated (Porta et al. 2002). Moreover, we recently showed that moderate levels of lipofuscin have protective

effects on cell survival during nutritional deprivation (Stroikin et al. 2007). The previously suggested idea, that lipofuscin permanently occupies active sites of lysosomal enzymes, preventing their translocation to the cytosol and engagement in programmed cell death during lysosomal stress, is consistent with the present findings of decreased cytosolic translocation of cathepsin D in lipofuscin-loaded cells. In addition, lipofuscin exhibits some proton-trapping properties, considering that decrease of the proton gradient upon treatment with Baf A1 is significantly lower in lipofuscin-loaded cells (Figure 6c). Positive correlation between cellular lipofuscin content and resistance to oxidative stress can also be indicative of lipofuscin functioning as a trap for free radicals, explaining the high resistance of lipofuscin-loaded cells to NzQ-treatment. While the exact mechanism of lipofuscin influence on cellular functions remains to be elucidated, we suggest that lipofuscin has lysosome-stabilizing properties, making these organelles less sensitive and diminishing their influence on cellular functioning.

In conclusion, increased autophagy following MSDH-treatment can be viewed as an adaptive cellular response in order to limit the damage caused by the leakage of protons and proteolytic enzymes into the cytosol because of impairment of the lysosomal membrane. Relocation of lysosomal content to the cytosol and decrease of intralysosomal pH are indicators of lysosome-targeted stress induced either by exposure to the lysosomotropic detergent MSDH or the redox-cycling quinone naphthazarin. Such a destabilization is significantly hampered in lysosomes, which contain the ageing-associated pigment lipofuscin. Increased stability of lipofuscin-containing lysosomes can be related to the decreased inducibility of apoptosis in ageing cells.

Acknowledgements

We thank Linda Vainikka for technical assistance. This study was financially supported by Lions Research Foundation and by grant from the Medical Branch of the Swedish Research Council (Vetenskapsrådet).

References

- Bowman EJ, Siebers A and Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc Natl Acad Sci USA* 85:7972-7976
- Brunk UT and Svensson I (1999) Oxidative stress, growth factor starvation and Fas activation may all cause apoptosis through lysosomal leak. *Redox Rep* 4:3-11
- Brunk UT, Neuzil J and Eaton JW (2001) Lysosomal involvement in apoptosis. *Redox Rep* 6:91-97
- Brunk UT and Terman A (2002a) Lipofuscin: Mechanisms of age-related accumulation and influence on cell functions. *Free Radic Biol Med* 33:611-619
- Brunk UT and Terman A (2002b) The mitochondrial-lysosomal axis theory of aging: Accumulation of damaged mitochondria as a result of imperfect autophagocytosis. *Eur J Biochem* 269:1996-2002
- De Duve C and Wattiaux R (1966) Functions of lysosomes. *Annu Rev Physiol* 28:435-492
- Ferri KF and Kroemer G (2001) Organelle-specific initiation of cell death pathways. *Nat Cell Biol* 3:255-263
- Firestone RA, Pisano JM and Bonney RJ (1979) Lysosomotropic agents. 1. Synthesis and cytotoxic action of lysosomotropic detergents. *J Med Chem* 22:1130-1133
- Gozuacik D and Kimchi A (2004) Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* 23:2891-2906

- Grune T, Merker K, Jung T, Sitte N and Davies K.J (2005) Protein oxidation and degradation during postmitotic senescence. *Free Radic Biol Med* 39:1208-1215
- Harman D (1956) Aging: a theory based on free radical and radiation chemistry. *J Gerontol* 211:298-300
- Henics T and Wheatley DN (1999) Cytoplasmic vacuolation, adaptation and cell death: a view on new perspectives and features. *Biol Cell* 91:485-498
- Johansson AC, Steen H, Öllinger K and Roberg K (2003) Cathepsin D mediates cytochrome c release and caspase activation in human fibroblast apoptosis induced by staurosporine. *Cell Death Differ* 10:1253-1259
- Kiffin R, Bandyopadhyay U and Cuervo AM (2006) Oxidative stress and autophagy. *Antioxid Redox Signal* 8:152-162
- Klionsky DJ (2005) The molecular machinery of autophagy: unanswered questions. *J Cell Sci* 118:7-18
- Li W; Yuan X, Nordgren G, Dalen H, Dubowchik GM, Firestone RA and Brunk UT (2000) Induction of cell death by the lysosomotropic detergent MSDH. *FEBS Lett* 470:35-39
- Miaczynska M, Pelkmans L and Zerial M (2004) Not just a sink: endosomes in control of signal transduction. *Curr Opin Cell Biol* 16:400-406
- Nilsson C, Johansson U and Öllinger K (2003) Analysis of cytosolic and lysosomal pH in apoptotic cells by flow cytometry. *Methods Cell Sci* 25:185-194
- Olsson GM, Rungby J, Rundquist I and Brunk UT (1989) Evaluation of lysosomal stability in living cultured macrophages by cytofluorometry. Effect of silver lactate and hypotonic conditions. *Virchows Arch B Cell Pathol Incl Mol Pathol* 56:263-269

- Persson HL, Kurz T, Eaton JW and Brunk UT (2005) Radiation-induced cell death: importance of lysosomal destabilization. *Biochem J* 389:877-884
- Porta EA (2002) Pigments in aging: an overview. *Ann N Y Acad Sci* 959:57-65
- Porta EA, Berra A, Monserrat AJ and Benavides SH (2002) Differential lectin histochemical studies on lipofuscin (age-pigment) and on selected ceroid pigments. *Arch Gerontol Geriatr* 34:193-203
- Rattan SI (2004) Aging, anti-aging, and hormesis. *Mech Ageing Dev* 125:285-289
- Roberg K, Johansson U and Öllinger K (1999) Lysosomal release of cathepsin D precedes relocation of cytochrome c and loss of mitochondrial transmembrane potential during apoptosis induced by oxidative stress. *Free Radic Biol Med* 27:1228-1237
- Seehafer SS and Pearce DA (2006) You say lipofuscin, we say ceroid: defining autofluorescent storage material. *Neurobiol Aging* 27:576-588
- Stroikin Y, Dalen H, Lööf S and Terman A (2004) Inhibition of autophagy with 3-methyladenine results in impaired turnover of lysosomes and accumulation of lipofuscin-like material. *Eur J Cell Biol* 83:583-590
- Stroikin Y, Johansson U, Asplund S and Öllinger K (2007) Increased resistance of lipofuscin-loaded prematurely senescent fibroblasts to starvation-induced programmed cell death. *Biogerontology* 8:43-53
- Terman A and Brunk UT (1998) Ceroid/lipofuscin formation in cultured human fibroblasts: The role of oxidative stress and lysosomal proteolysis. *Mech Ageing Dev* 104:277-291
- Terman A and Brunk UT (2004) Lipofuscin. *Int J Biochem Cell Biol* 36:1400-1404

- Wihlmark U, Wrigstad A, Roberg K, Nilsson SE and Brunk UT (1997) Lipofuscin accumulation in cultured retinal pigment epithelial cells causes enhanced sensitivity to blue light irradiation. *Free Radic Biol Med* 22:1229-1234
- Yin L, Stearns R and Gonzalez-Flecha B (2005) Lysosomal and mitochondrial pathways in H₂O₂-induced apoptosis of alveolar type II cells. *J Cell Biochem* 94:433-445
- Yu Z, Persson HL, Eaton JW and Brunk UT (2003) Intralysosomal iron: a major determinant of oxidant-induced cell death. *Free Radic Biol Med* 34:1243-1245
- Yuan XM, Li W, Olsson AG and Brunk UT (1997) The toxicity to macrophages of oxidized low-density lipoprotein is mediated through lysosomal damage. *Atherosclerosis* 133:153-161
- Zhao M, Antunes F, Eaton JW and Brunk UT (2003) Lysosomal enzymes promote mitochondrial oxidant production, cytochrome c release and apoptosis. *Eur J Biochem* 270:3778-3786