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Research paper

Mitochondrial contribution to lipofuscin formation

Jeannette König[a], Christiane Ott[a], Martín Hugo[a], Tobias Jung[a,d], Anne-Laure Bulteau[b], Tilman Grune[a,c,d,e], Annika Höhn[a,c,*]

[a] Department of Molecular Toxicology, German Institute of Human Nutrition Potsdam-Rehbruecke (DIfE), 14558 Nuthetal, Germany
[b] Institut de Génomique Fonctionnelle de Lyon (IGFL) – ENS de Lyon, 69007 Lyon, France
[c] German Center for Diabetes Research (DZD), 85764 München-Neuherberg, Germany
[d] German Center for Cardiovascular Research (DZHK), 10117 Berlin, Germany
[e] NutriAct-Competence Cluster Nutrition Research Berlin-Potsdam, Nuthetal 14458, Germany

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ABSTRACT

Mitochondria have been in the focus of oxidative stress and aging research for decades due to their permanent production of ROS during the oxidative phosphorylation. The hypothesis exists that mitochondria are involved in the formation of lipofuscin, an autofluorescent protein aggregate that accumulates progressively over time in lysosomes of post-mitotic and senescent cells. To investigate the influence and involvement of mitochondria in lipofuscinogenesis, we analyzed lipofuscin amounts as well as the mitochondrial function in young and senescent cells. In addition we used an aging model and Lon protease deficient HeLa cells to investigate the influence of mitochondrial degradation processes on lipofuscin formation.

We were able to show that mitophagy is impaired in senescent cells resulting in an increased mitochondrial mass and superoxide formation. In addition, the inhibition of mitochondrial fission leads to increased lipofuscin formation.

Moreover, we observed that Lon protease downregulation is linked to a higher lipofuscinogenesis whereas the application of the mitochondrial-targeted antioxidant mitoTEMPO is able to prevent the accumulation of this protein aggregate.

1. Introduction

The intracellular accumulation of protein aggregates is one important hallmark of the aging process [1]. Especially cells with a low mitotic rate accumulate high amounts of protein aggregation products during their lifetime [2–4]. In this context the formation of covalent cross-linked protein aggregates, such as lipofuscin, is of special importance because of its abundance in aged cells where it has detrimental effects. For instance, it is clearly established that lipofuscin is able to inhibit intracellular proteostasis mechanisms, especially proteasomal activity and, therefore, the effective degradation of oxidatively modified proteins [3, 5–8]. This in turn leads to an increased formation of protein aggregation products further promoting the formation of lipofuscin [9]. Additionally, it was shown that lipofuscin generates reactive oxygen species (ROS) due to its ability to incorporate redox-active metals such as iron, thus promoting the Fenton reaction [10]. Moreover, the accumulation of lipofuscin is of pathophysiological relevance. In this regard, it was shown that an accelerated accumulation is linked to the development of neurological diseases such as Alzheimer's disease [11] and Parkinson's disease [12] as well as age-related macular degeneration, which seems to be the most frequent reason for blindness in the Western world [13].

Abbreviations: CCCP, Carbonyl cyanide 3-chlorophenylhydrazone; COX IV, Cytochrome c oxidase subunit IV; DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethyl sulfoxide; Drp-1, Dynamin-related protein 1; DNPH, 2,4-dinitrophenylhydrazine; DNP, 2,4-dinitrophenylhydrazone; Dox, Doxycycline; EDTA, Ethylenediaminetetraacetic acid; Em, Emission wavelength; ETC, Electron transport chain; Ex, Excitation wavelength; FBS, Fetal bovine serum; FCCP, Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Fis1, Mitochondrial fission 1 protein; FITC, Fluorescein isothiocyanate; H_2DCFDA, 2',7'-Dichlorodihydrofluorescein diacetate; Ki-67, Kiel 67 protein; Mdivi-1, Mitochondrial division inhibitor 1; MitoTEMPO, (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenyl-phosphonium chloride; MT-CO1, mitochondrially encoded cytochrome c oxidase I; mtDNA, Mitochondrial DNA; MTG, MitoTracker Green^{FM}; MTT, Methylthiazolyldiphenyl-tetrazolium bromide; OCR, Oxygen consumption rate; PI, propidium iodide; PINK1, PTEN-induced putative kinase 1; PQ, Paraquat; ROS, Reactive oxygen species; RNAi, RNA interference; SA-β-Gal, Senescence associated β-galactosidase; SDHA, Succinate Dehydrogenase Complex Flavoprotein Subunit A; shRNA, Short hairpin RNA; SIPS, Stress-induced premature senescence; SDS, Sodium dodecyl sulfate; VDAC, Voltage-dependent anion channel

* Correspondence to: German Institute of Human Nutrition Potsdam-Rehbruecke, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany.
E-mail addresses: jeannette.koenig@dife.de (J. König), christiane.ott@dife.de (C. Ott), martin.hugo@dife.de (M. Hugo), tobias.jung@dife.de (T. Jung), anne-laure.bulteau@ens-lyon.fr (A.-L. Bulteau), scientific.director@dife.de (T. Grune), annika.hoehn@dife.de (A. Höhn).

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The cellular consequences of lipofuscin accumulation are well studied. However, no data are available on its protein sources from different protein pools such as cytosolic or organelle proteins in the formation of lipofuscin. Nevertheless, it can be hypothesized that the composition of lipofuscin is strongly dependent on the intracellular site of ROS attack and, therefore, different oxidatively modified proteins and lipids might contribute to the formation of lipofuscin. However, it is postulated that especially mitochondria are involved in the development of lipofuscin. The hypothesis of Terman and Brunk, referred to as the "mitochondrial-lysosomal axis theory of aging" assumes the incomplete degradation of damaged mitochondria mediated by autophagy as the cause for lipofuscin accumulation [14]. Although this hypothesis has not clearly been experimentally proven, some results found in the literature support this hypothesis.

This includes for example the finding of the mitochondrial ATP synthase subunit c as a major component in lipofuscin formed under pathological conditions during the progression of neuronal ceroid lipofuscinosis (NCL) [15]. Furthermore, it is generally accepted that mitochondria are the primary site of ROS formation in normal metabolism [16,17]. Moreover, it was shown that mitochondrial DNA mutations which accumulate during aging lead to further mitochondrial malfunction [18,19], and that the mitochondrial protease Lon which is able to degrade oxidatively modified mitochondrial proteins declines during aging [20–22]. Also these findings strengthen the hypothesis of a potential mitochondrial involvement in lipofuscin formation.

The system to remove damaged mitochondria effectively is generally believed to be mitophagy [23,24]. Recently we were able to demonstrate a reduction of macroautophagic processes in senescent cells and murine brain tissue [25,26]. Before mitophagy occurs, the mitochondrial network frequently undergoes fusion and fission processes, as a part of the mitochondrial quality control and to ensure mitochondrial integrity [27]. Interestingly, fission occurs mainly as the initial step of mitochondria-selective autophagy and ensures, thereby, the adequate degradation of damaged or dysfunctional mitochondria by the lysosomal system [28].

The aim of this study was to investigate the role of mitochondrial proteins and mitochondrial protein oxidation on lipofuscinogenesis; especially to reveal whether mitophagy or the Lon protease are effective in the protecting of cells from lipofuscin formation.

2. Materials and methods

2.1. Materials

Chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany) except Mdivi-1 (Enzo Life Science, Lörrach, Germany) as well as doxycycline, blasticidin and zeocin (Invivogen, San Diego, CA, USA). Cell culture materials and media were obtained from Biochrom (Berlin, Germany). MitoTracker Green^{FM} (MTG) and MitoSOX™ Red superoxide indicator (MitoSOX) were purchased from Molecular Probes (Eugene, USA).

The following primary antibodies were used: rabbit anti-DNP (Sigma-Aldrich, Deisenhofen, Germany), rabbit anti-PINK1 (Cell Signaling, Boston, MA, USA), mouse anti-β-actin (Cell Signaling, Boston, USA), rabbit anti-COX IV (Cell Signaling, Boston, MA, USA), rabbit anti-Lon protease (Abcam, Cambridge, UK), mouse anti-GAPDH (Abcam, Cambridge, UK), rabbit anti-Ki-67 (Abcam, Cambridge, UK), mouse anti-CDKN2A/p16INK4α (Abcam, Cambridge, UK), rabbit anti-p21 Waf1/Cip1 (Cell Signaling, Boston, MA, USA), mouse anti-MT-CO1 (Abcam, Cambridge, UK), mouse anti-SDHA (Abcam, Cambridge, UK), mouse anti-VDAC (Abcam, Cambridge, UK) and rabbit anti-Fis1 (Cell Signaling, Boston, MA, USA). Secondary antibodies used for immunoblotting were purchased from LI-COR Biosciences (Lincoln, AL, USA). The FITC-labeled antibody for immunofluorescence was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture

Experiments were performed by using primary human dermal fibroblasts as well as Lon protease deficient HeLa cells. Human dermal fibroblasts were divided in two categories: "young" and "senescent". Young fibroblasts were obtained from human skin tissue of a 1-year old donor, kindly provided by Prof. Scharffetter-Kochanek (University of Ulm, Germany). Senescent fibroblasts originated from human skin tissue of a 81-year old donor and were kindly provided by Beiersdorf AG (Hamburg, Germany) [29]. Fibroblasts were grown in high-glucose (4.5 g/l) Dulbecco's Modified Eagle's Medium (DMEM) with 5% glutamine and 10% fetal bovine serum (FBS) at 7% CO_2, 95% humidity and 37 °C.

Lon protease deficient HeLa cells were kindly provided by Dr. Anne-Laure Bulteau (Institute of Functional Genomics of Lyon, France). Experimental design and establishment of these cells was described previously [30,31]. In brief, Lon protease deficient HeLa cells were generated using HeLa T-Rex™ cells (Invitrogen Life Technology, Carlsbad, CA, USA) which were stably transfected with the short hairpin RNA (shRNA) expressing vector pENTR/H1/T0+(Invitrogen, BLOCK-IT™). For the realization of Lon protease knockdown a Lon-specific 68-base sequence complementary oligonucleotide was cloned into the pENTR/H1/T0+ vector. Furthermore, the expression of the Lon-specific shRNA underlies a doxycyline (Dox)-regulated promotor (tet-on system). Therefore, the addition of Dox to the medium resulted in the expression of shRNA against Lon protease leading subsequntly to the downregulation of this enzyme. Clones of HeLa-tet-on-shLon cells were selected in low-glucose (1 g/l) DMEM with 10% FBS, 5% glutamine, 5 μg/ml blasticidin and 200 μg/ml zeocin. Cultivation was carried out at 7% CO_2, 95% humidity at 37 °C. Expression of Lon-specific shRNA was induced by the addition of 2 μg/ml doxycycline to the medium for 10 days. During this period medium was replaced every second day with fresh Dox. Efficacy of Lon protease knockdown was verified by immunoblotting.

2.3. Stress-induced premature senescence (SIPS)

For the induction of intracellular lipofuscin formation cells were chronically treated with paraquat (PQ) for 10 days. This procedure is known as stress-induced premature senescence (SIPS) and is described elsewhere in detail [32]. To induce premature senescence in fibroblasts, confluent cells were incubated daily with 40 μM PQ for a period of 10 days. In contrast to fibroblasts, HeLa Lon cells were treated in a subconfluent state with 20 μM PQ for 10 days. SIPS of HeLa Lon cells started after the ten-day Dox treatment for initial Lon protease downregulation. However, Lon protease downregulation was also continued during SIPS by adding Dox to the medium. Mdivi-1 (mitochondrial division inhibitor-1) as well as mitoTEMPO was applied by co-incubation with PQ. Fibroblasts received 50 μM Mdivi-1 or 2.5 μM mitoTEMPO. HeLa Lon cells were treated with 5 μM Mdivi-1 or 10 μM mitoTEMPO.

2.4. Senescence-associated β-galactosidase staining

β-Galactosidase activity was determined at pH 6 using "Senescence-associated β-galactosidase staining Kit" from Cell Signaling (Boston, USA) according to manufacturer's instructions.

2.5. Lipofuscin detection

Detection and quantification of lipofuscin was performed by measurement of its autofluorescence [33] either by confocal microscopy (Laser scanning microscope 700, Carl Zeiss, Jena, Germany) (Ex: 405 nm; Em: 498 nm) or by flow cytometry with the Cell Lab Quanta™ SC MPL (Beckman Coulter GmbH, Krefeld, Germany) or the MACSQuant® Analyzer 10; (Miltenyi Biotec, Bergisch Gladbach,